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Project leader:	Professor Adrian C Newton, The James Hutton Institute
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Key staff:	Daniel De Vega Perez – JHI PhD student Nicola Holden – JHI co-supervisor Ingo Hein – JHI second co-supervisor Nicola Stanley-Wall – University of Dundee supervisor
Location of project:	The James Hutton Institute (JHI), Invergowrie, Dundee, DD2 5DA
Industry Representative:	Neal Ward, Cantelo Nurseries Ltd (Left post 2016)
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The results and conclusions in this report are based on an investigation conducted over a three-year period but comprise separate individual experiments. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

[Name] Professor Adrian C Newton

[Position] Senior Research Scientist

[Organisation] James Hutton Institute

Signature  Date 16th March 2017

[Name]

[Position]

[Organisation]

Signature ..... Date .....

Report authorised by:

[Name]

[Position]

[Organisation]

Signature ..... Date .....

[Name]

[Position]

[Organisation]

Signature ..... Date .....

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GROWER SUMMARY

Headline

Resistance elicitors are compounds that can protect commercial crop varieties (tomato cv. Money-maker and aubergine cv. Black Beauty) against fungal pathogens such as *Botrytis cinerea* (gray mold disease) by foliar spraying the crop.

Resistance elicitors could offer the potential to reduce fungicide use by including resistance elicitors, such as the natural polysaccharide chitosan and/or the phytohormone methyl-jasmonate, into crop protection strategies. Chitosan in particular can prime tomatoes for a more efficient and fine-tuned defence response against fungal pathogen *Botrytis cinerea*.

Background

Conventional crop protectants (pesticides) can lose their efficacy due to selection pressure for pathogen resistance caused by their widespread use. To date, there is a lack of genetic resistance in commercial crop varieties against fungal aggressive pathogens such as *Botrytis cinerea*, *Alternaria Solani* and *Sclerotinia sclerotium*. The aggressive fungal pathogen *Botrytis cinerea* infects almost all vegetable and fruit crops (>1400 plant species), including most of the crops of the *Solanaceae* family, trees and ornamentals. Botrytis kills the plant by inducing necrosis with degradation enzymes and manipulating its host defences. Fungal resistance to benzimidazoles in the 1970's, due to extensive use of some newer fungicides such as dicarboximides, has subsequently led to the appearance of benzimidazoles-resistant *Botrytis cinerea* strains. Pesticide availability and use is also limited by European regulations due to human health and environmental issues. The recent European Directives "Plant Protection Products Regulation" 1107/2009 and the "Sustainable Use Directive" 2009/128/EC are the latest in a series of legislative changes that aim to reduce pesticide use in Europe. Besides the above challenges to crop protection, market/consumer requirements sometimes require crop varieties to be grown that are susceptible. However, even susceptible plants have inducible defence mechanisms that, if triggered in a focussed, specifically-targeted way, can prevent disease and reduce the need for conventional fungicide use.

Resistance elicitors are compounds that can help plants to defend themselves. Elicitors can stimulate pathogen-induced defence mechanisms in the plant resulting in a broad-spectrum and more efficient resistance (Induced Resistance-priming) against pathogens such as *Botrytis cinerea.* Their effective application requires understanding of their associated defence responses (gene expression) and mode of action in the plant and the agronomy of the crop. Priming is based on a fine-tuned and enhanced resistance to biotic/abiotic stress that results in a faster and stronger expression of resistance after pathogen attack.

The aim of this PhD project was to determine the mode of action of specific resistance elicitors, to characterise their molecular function and to investigate their role in priming crops against *Botrytis cinerea.* Ultimately this project set out to identify novel alternatives in crop protection to reduce fungicide usage in the horticultural sector. This project used the *Botrytis cinerea* – tomato pathosystem as a model.

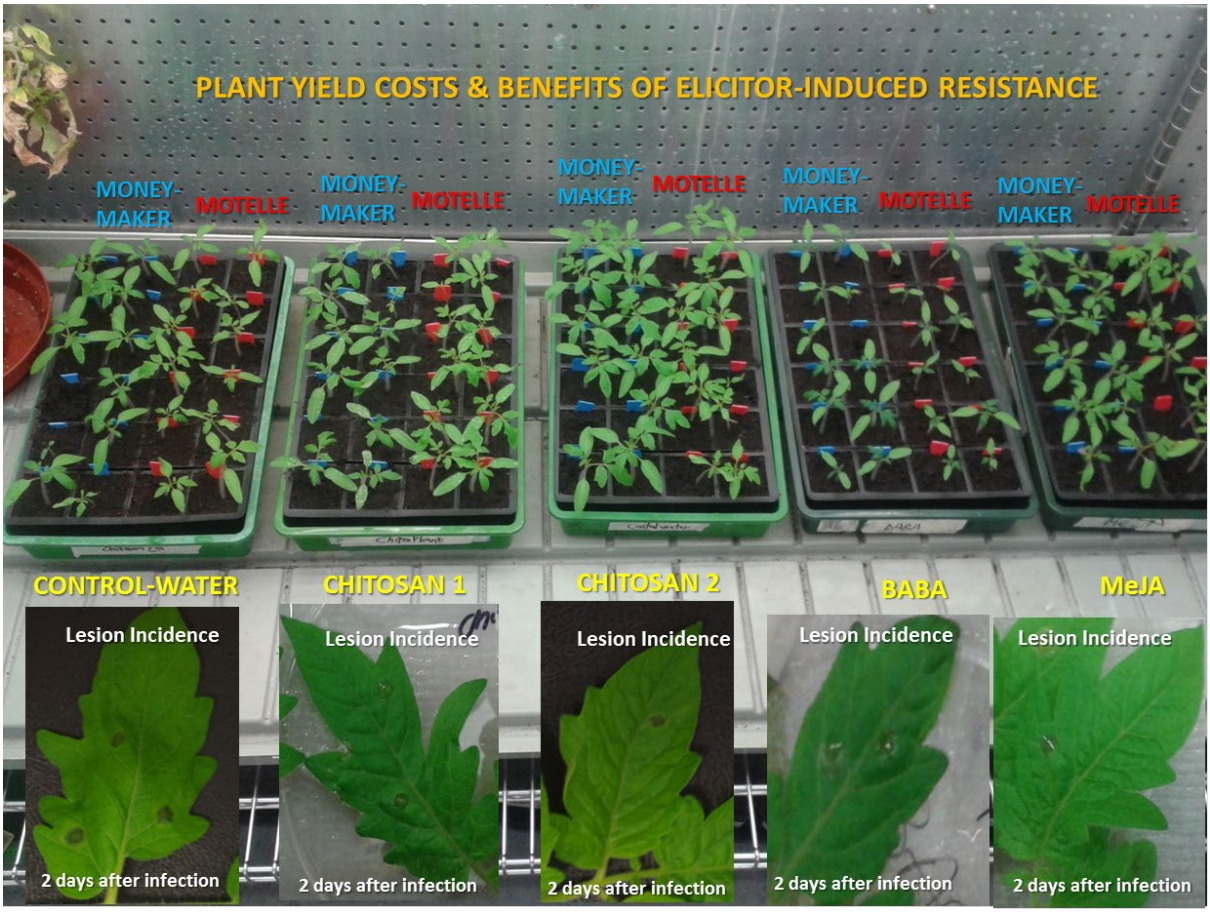
Summary

A selection of resistance elicitors were evaluated for their ability to induce resistance in the *Botrytis cinerea* – tomato pathosystem as follows; methyl-jasmonate (MeJA, a plant phytohormone), Benzothiadiazole (BTH, A chemical analogue of Salicylic Acid marketed by Syngenta under the tradename 'BION'), beta-aminobutyric acid (BABA, an amino acid compound) and chitosan (a low molecular weight and water soluble formulation marketed under the trade name 'ChitoPlant'). All four resistance elicitors were shown to induce resistance by significantly reducing gray mould disease (*Botrytis cinerea*) on two 4-weeks old tomato varieties (tomato cv. Moneymaker and cv. Motelle).

MeJA and ChitoPlant also induced resistance in 4-week-old *Arabidopsis thaliana* (thale cress, a model plant) cv. Columbia0 and Aubergine cv. Black Beauty plants and significantly decreased *Botrytis cinerea* infection in comparison to water treated control plants. Treatments were applied by foliar spraying the solutions once on to the shoot 4 to 17 days before Botrytis infection demonstrating long-lasting induced resistance.

Resistance elicitors can induce "early acting" defence mechanisms in the plant by reinforcing plant cell-wall. Experiments showed that the phytohormone elicitors MeJA and chitosan were able to reinforce the cell-walls of tomato plant cells by inducing callose deposition on tomato epidermal cells. Callose is a plant polysaccharide that can delay pathogen penetration in the epidermis. Furthermore, MeJA and chitosan were able to induce plant defences such as peroxidases and H_2O_2 production, involved in the fight against fungal and bacterial toxic compounds upon attack.

Elicitors can reduce plant growth when applied at medium-high concentrations. To help understand more about the balance between the value of priming crop defences and elicitor interaction with plant development, the costs of the elicitor-induced resistance were measured in relation to crop yield. Treatment of tomato seedlings (1-2 wks old) with the resistance elicitors revealed that BABA had a strong growth repression effect (GS Figure 1). BABA reduced relative growth rate by an average of 41% across the different cultivars tested. The other elicitors tested did not lead to reduced growth at the concentrations tested.



GS Figure 1. Two-week-old tomato cv. Motelle (red) and Moneymaker (blue) seedlings one week after elicitor treatment (Chitosan2, ChitoPlant/Chitosan1, BABA, MeJA and water as a non-treated control). BABA-treated plants were smaller than the rest of the treatments and Motelle-BABA treated plants were even smaller than Moneymaker BABA-treated seedlings. Seventeen days after elicitor-treatment, tomato plants were infected with Botrytis spores and 2 days after infection necrotic lesions were measured. In all elicitor-treated plants lesion incidence was lower than water-treated plants and gray mould disease expansion was significantly slower compared to water-treated control plants.

To determine the duration of elicitor-induced resistance in the crop tomato seedlings were foliar sprayed with the different resistance elicitors or distilled water (as a control). Seventeen days after elicitor treatments plants were infected with spore solution of *Botrytis cinerea* and necrotic lesion expansion was measured in order to look for an induced resistance phenotype. We saw that all elicitors were able to significantly reduce Botrytis lesion expansion (GS Figure 1 inset) up to 4 days after infection in both tomato varieties tested. Therefore, we can conclude that elicitors can prime tomato plants for a stronger defence response for over two weeks from application.

Of the four types of Resistance Elicitor tested, Chitosan was chosen for more in depth study as it was the most effective at reducing disease lesion expansion following inoculation of Botrytis across a range of host species tested (tomato, thale cress and aubergine).

In vitro tests, in which chitin was exposed to Botrytis in petri dishes, demonstrated that Chitosan has a direct fungicide effect; either stopping or reducing fungal spore germination and fungal hypha growth in a concentration dependent manner (>0.1% w/v).

Chitosan foliar treatment was shown to have a phytotoxic effect on young (<4 week) tomato and aubergine plants, in a concentration-dependent manner (>0.1% w/v).

The mode of action of the resistance eliciting properties of Chitosan was revealed using a large scale gene expression analysis approach called Microarray analysis. This approach enabled the comparison of gene expression between chitosan-treated and Botrytis-infected tomato plants verses water-treated and Botrytis-infected tomato plants. The main findings were:

- Chitosan-treated plants displayed stronger and faster defence mechanisms by differentially expressing more than 2,100 defence-related genes after Botrytis infection compared to water treated plants which only differentially expressed 363 genes.
- Chitosan-treated tomatoes were able to repress Botrytis virulence genes expression (used by the pathogen to facilitate infection) while water-treated plants couldn't avoid Botrytis gene expression.
- Analysis on the 2,100 genes induced by chitosan revealed key pathways involved in tomato defences against Botrytis, such as cell-wall modification genes (lignin and cellulose synthesis), regulatory and signalling genes, jasmonate and ethylene-dependent genes, redox state (glutaredoxins, involved in the cell oxidative stress) and secondary metabolites (phenylpropanoids).

These results unveiled potential molecular pathways involved in chitosan-induced priming for resistance in tomato against the aggressive fungal pathogen *Botrytis cinerea*, which may also be applicable to other crops (e.g. aubergine). This information may assist breeders to develop commercial crop varieties with increased expression of genes in the key pathways identified in this study.

Finally, the effects of a resistance elicitor combination treatment were evaluated. Low-dose combination of chitosan (0.01% w/v) + methyl-jasmonate (0.1mM) applied to the foliage of tomato plants showed increased protection against Botrytis compared to the each treatment alone. This result suggests a potential synergistic effect of chitosan in combination with other elicitors (i.e. methyl-jasmonate).

Financial Benefits

Botrytis cinerea is a fungal pathogen that infects almost all vegetable and fruit crops and annually causes $10 billion to $100 billion in losses worldwide (Weiberg et al., 2013). Understanding how elicitors, such as chitosan, can prime horticulture crops against Botrytis

infection may facilitate novel antifungal strategies and therefore reduce crop losses due to pre- and post-harvest fungal infection.

Outcomes of this project are in the form of preliminary knowledge which may lead to novel crop protection products. This PhD project has relevance to a number of different sectors because the nature of the research is to investigate common mechanisms of plant defence. This research begins to explore the principles and potential for using resistance elicitors in integrated crop protection strategies of the future.

Action Points

- There are few resistance elicitors currently licenced for use on horticultural crops (Harpin, SiTKO-SA, Amistar). AHDB project FV 417 demonstrated that elicitors can interact with fungicides and they may have beneficial effects on controlling bacterial diseases on red onion, cabbage and broccoli, depending on the elicitor and the crop
- Chitosan was approved as a *basic substance* in 2014 by the EU, thus it may be used on fruit and vegetables in the field or in the greenhouse
- Resistance elicitors, such as chitosan, can lead to phytotoxic effects in a concentration dependent manner
- Key defence pathways identified in this work may lead to future breeding targets for plant breeders

SCIENCE SECTION

Introduction

Horticultural crops are challenged, before and after harvest, by many microbes such as fungi, bacteria, oomycetes, viruses and nematodes. Some of these are actual pathogens and can cause disease in susceptible hosts. Failure of induction of resistance in plants can lead to infection, disease, heavy reduction of crop yield and premature death. Hence, crop diseases result in important economic losses worldwide.

Necrotrophic fungi are the largest class of fungal phytopathogens and cause serious crop losses worldwide (Lawrence & Kononowicz, 2010). Fungal pathogen *Botrytis cinerea* has a broad host range (B. Williamson, B. Tudzynski, P.Tudzynski, 2007; Staats, van Baarlen, & van Kan, 2005) being able to infect more than 200 crop species including most of the crops of the *Solanaceae* family, trees and flowers. *Botrytis cinerea* is an aggressive and destructive pathogen that can infect wounded and undamaged plant tissue directly by penetration of the cuticle (J. a van Kan, van't Klooster, Wagemakers, Dees, & van der Vlugt-Bergmans, 1997) and can cause disease pre- and post-harvest.

Plants are capable of defending themselves and fight off pathogen attack through constitutive and inducible defence mechanisms. Non-specific defence inducers, called resistance elicitors, are able to mimic pathogen-induced defence mechanisms in the plant (Aranega-Bou et al., 2014). Activation of plant endogenous defences by elicitors can result in a broad-spectrum resistance against a wide range of pathogens, called 'Induced resistance'. Induced resistance leads to two general systemic defence mechanisms in the plant: direct activation of defence responses in systemic tissue after local stimuli and priming, which implies activation of systemic responses only when the pathogen reaches these sites (Aranega-Bou *et al*., 2014). Priming can be related to a faster, more efficient and robust defence response and enhanced resistance to biotic/abiotic stress (Conrath, 2009) as well as it can intensify the perception of the defence-inducing signals in the plant.

There is currently a challenge in the fight against pathogen attack to crops worldwide, as there is evidence of the ineffectiveness of conventional crop protectants due to pathogen resistance. Conventional crop protectants (i.e. fungicides) can lose their efficacy due to pathogen resistance from their widespread use (Pappas, 1997). This is not a new phenomenon, after fungal resistance to benzimidazoles in the 1970's, an extensive use of some newer fungicides, such as dicarboximides, has subsequently led to the appearance of resistant *B. cinerea* strains (Pappas, 1997).

For these reasons, present crop protection strategies are aimed at reducing usage of toxic active ingredients. In the last decades, research on more benign alternatives to cope with pathogens has become in a priority. One potential replacement for pesticides can be plant's endogenous defence mechanisms.

Non-host inducing agents, called resistance elicitors (RE), are able to mimic pathogen-induced defence mechanisms in the plant. This activation of plant defence results in a broad-spectrum resistance called induced resistance (IR). Induced resistance elicitors are often not toxic to pathogens themselves and therefore can be a benign alternative strategy in crop protection to reduce pesticide usage.

To date, systemic resistance, which is induced in a spatially different part of the plant from the induction point, has been divided in two types.

One form is called systemic acquired resistance (SAR) and occurs in distal plant parts following localized infection by a necrotizing pathogen (Walters & Heil, 2007). As studied in the model plant *Arabidopsis thaliana*, SAR depends on the activation of the salicylic acid (SA) signalling pathway and requires the action of the regulatory protein NPR1. SAR has also been associated with the systemic expression of a family of genes encoding pathogenesis-related proteins (Sticher, Mauch-Mani, & Métraux, 1997). Unlike the gene-for-gene resistance, SAR is able to provide resistance against a broad spectrum of would-be pathogens, such as fungi, viruses, bacteria and oomycetes.

The second systemic defence is called induced systemic resistance (ISR) and is induced by certain strains of plant growth promoting rhizobacteria (PGPR) that are present in large numbers on the root surface (Loon, Bakker, & Pieterse, 1998). Unlike SAR, ISR is not associated with local necrotic formation nor with changes in the expression of PR genes and it is known that, in *Arabidopsis thaliana*, the ISR pathway functions independently of salicylic acid (SA) but requires responsiveness of specific ethylene and jasmonate-responsive genes (van Wees, Luijendijk, Smoorenburg, van Loon, & Pieterse, 1999). Several strains of the species *Bacillus amyloliquefaciens* and *Bacillus subtilis* among others are well-characterised PGPR capable to induce ISR in multiple crops and other plants; including tomato, pepper, muskmelon, watermelon, sugar beet, tobacco, *Arabidopsis sp*., cucumber and loblolly pine; against various viral, fungal, nematodes and bacterial pathogens (Akram, Anjum, & Ali, 2014).

The plant hormones salicylic acid (SA), abscisic acid (ABA) and jasmonic acid (JA) are involved in systemic resistance against biotrophic and necrotrophic fungal pathogens. SA- and JA-signalling pathways can be induced in tomato throughout the use of specific RE. However, these REs also interfere with JA/SA/ABA pathway cross-talk and they can be used to understand resistance in tomato against the necrotrophic fungus *Botrytis cinerea*.

Furthermore, production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), and cell-wall defence mechanisms, such as callose, can be potent instruments to combat pathogen attack in the early stages of the infection

PhD Year 1

Botrytis cinerea is the fungal pathogen causative of gray mould disease in tomato and other crops. As previously mentioned its infection strategy has being unveiled; *Botrytis cinerea* is an opportunistic pathogen that is able to infect all parts of the plant (stem, leaves, flowers and fruits), it can initiate infection at wounded sites, sites previously infected by other pathogens and even healthy tissue (J. A. L. Van Kan, 2005). During the first year of my PhD project we screened for resistance elicitors that had a significant effect on tomato protection against *Botrytis cinerea*. After specific elicitors revealed to induce resistance in tomato, analysis on elicitor cell-wall reinforcement properties, defence gene expression and costs on plant development were evaluated.

Material and Methods

Long-lasting Elicitor-induced resistance in tomato against *Botrytis cinerea*: Screening for a resistance phenotype

Seeds of two tomato varieties (Moneymaker and Motelle) were placed in petri-dishes containing wetted tissue paper, and maintained at 28°C in the dark for 4 and 3 days, respectively, to stimulate germination. Germinated seeds of each cultivar were planted in plant cell propagators containing Scott's M3 soil and cultivated under tomato controlled standard growth conditions (16h- 8h/ day- night cycle; 23°C/ 20°C) for one week. Each propagator contained 12 seedlings of each cultivar. Seedlings were then soil drenched or foliar sprayed with Elicitors, to the following final concentrations.

- Control (ddH_2O) + 0.02% Silwet L-77 (adjuvant/surfactant)+ 0.05% ethanol + Control 2 soil-drench of DDW (150ml water per tray) foliar sprayed
- DL-β-aminobutyric acid (BABA) soil drenched (5mM stock solution= 0.5mM final concentration)
- Benzo (1,2,3)-thiadiazole-7-carbothioic acid S- methyl ester (BTH, BION; Syngenta, Basel, Switzerland) (1mM) + 0.02% Silwet L-77 (surfactant) + 0.05% ethanol foliar sprayed
- Methyl-jasmonate (MeJA) (0.1 mM) + 0.02% Silwet L-77 (surfactant) foliar sprayed

- Combination of BTH+MeJA (0.25mM+0.1 mM)+ 0.02% Silwet L-77 (surfactant) foliar sprayed
- Chitosan1 (ChitoPlant) 1% w/v + 0.02% Silwet L-77 (surfactant) foliar sprayed
- Chitosan 2 (1:100) + 0.02% Silwet L-77 (adjuvant) foliar sprayed

1-week-old plantlets were treated with the different elicitors, 7 days post treatment, plants were transplanted to larger pots and 17 days after elicitor treatments, leaves were excised for a detached leaf assay and subsequently, leaves were challenged with *B. cinerea* by drop inoculation ($5*10^4$ spores/ml) after detached leaves assay.

One week after elicitor treatment, roots were washed to remove elicitors, and seedlings were transplanted to ~200ml individual pots. Eight seedlings were selected and used for each treatment.

17 days after elicitor treatments (long-lasting defence induction), leaves of every plant were excised and prepared for infection/detached leaves assay. Detached leaves were infected with *Botrytis cinerea* as described in the Lancaster protocol with major modifications (Infection/Pathogenicity Assay):

4-5 weeks-old active *Botrytis cinerea* hypha growing into potato dextrose agar (PDA) plates were kept in the dark at room temperature. Once *Botrytis cinerea* was sporulating, 20ml of ddwater (dd for distilled) with 0.01% Tween 20 (adjuvant) was added to the plate and it was subsequently scratched with a spatula to release and harvest spores. Spore concentration was then counted with a cell counter Haemocytometer and adjusted to $5*10^4$ spores/ml (or $2*10^4$ spores/ml for the rest of the infection assays). As a final inoculum solution, 3.3 ml of 1M glucose (freshly prepared/autoclaved) + 2.2 ml of 0.1M KH_2PO_4 (pH 5) (freshly prepared/autoclaved) were added and the incubation time was reduced to 10-15 min in order to decrease the virulence of the fungal strain. Detached tomato leaves were settled into trays with moistened paper and challenged by droplet inoculation with *Botrytis cinerea* spores. Detached infected leaves were incubated under high humidity, dark and high temperature (22°C) conditions for 5 days. Infection was scored at 3 and 4 days after inoculation by measuring the diameter (mm) of the lesions with an electronic ruler.

Basal callose deposition induced by BABA, MeJA, BTH+MeJA and BTH, Chitosan1 (ChitoPlant) and Chitosan2-IR in tomato cv. Moneymaker and tomato cv. Motelle.

Tomato cv. Money-maker and tomato cv. Motelle plants were grown under controlled standard conditions in trays containing 12 seedlings of each cultivar. 1-2 weeks-old seedlings of tomato cv. Money-maker and tomato cv. Motelle were foliar sprayed with ddH_2O + 0.02% Silwet (surfactant) + 0.05% ethanol (control1) + soil-drench of DDW (150ml water per tray) (control 2); BTH (1mM) + 0.02% Silwet (surfactant) + 0.05% ethanol; Chitosan 2 (1:100) + 0.02% Silwet (surfactant) + 0.05% ethanol; Chitosan1 (ChitoPlant) 1% w/v + 0.02% Silwet (surfactant) + 0.05% ethanol; MeJA (0.1 mM) (which was dissolved in ddH_2O + 0.05 % ethanol) + 0.02% Silwet (surfactant); a combination of BTH+MeJA (0.25mM+0.1 mM) + 0.02% Silwet (surfactant); and soil drenched with BABA (5mM stock solution= 0.5mM final concentration).

Cotyledons of every treatment were excised one week after treatment and store in 100% ethanol before Aniline Blue staining as described previously (Luna et al., 2011). Briefly, cotyledons were incubated for at least 24 h in 95 to 100% ethanol until all tissues were transparent, were washed in 0.07 M phosphate buffer (pH =9), and were incubated for 1 to 2 h in 0.07 M phosphate buffer containing 0.01% aniline-blue (Sigma), prior to microscopic analysis. Observations were performed with an epifluorescence microscope with UV filter (BP 340 to 380 nm, LP 425 nm). Callose was quantified from digital photographs by the number of white pixels (callose intensity) or the number of depositions relative to the total number of pixels covering plant material, using ImageJ software.

For double staining (Aniline Blue + Calcofluor):

Another similar experiment was conducted in order to determine the effectiveness of chitosan treatment priming cell wall defences against fungal pathogen attack. 1-2 weeks-old tomato cv. Moneymaker and tomato cv. Motelle seedlings (12 per cultivar) were foliar sprayed with ddH_2O + 0.02% Silwet (surfactant) + 0.05% ethanol (control1) + soil-drench of DDW (150ml water per tray) (control 2); Chitosan 2 (1:100) + 0.02% Silwet (surfactant) + 0.05% ethanol; Chitosan1 (ChitoPlant) 1% w/v + 0.02% Silwet (surfactant) + 0.05% ethanol; MeJA (0.1 mM) + 0.02% Silwet (surfactant) and soil drenched with BABA (5mM stock solution= 0.5mM final concentration).

17 days after elicitor treatments (long-lasting defence induction) leaves of every plant were excised and prepared for infection/detached leaves assay. Leaves were settled into trays and challenged by droplet inoculation with *Botrytis cinerea* spores. Finally, double staining (Aniline Blue + calcofluor) was performed at 2 dpi in order to see pathogen-induced callose deposition in all treatments. Briefly, leaf discs surrounding infected tissue were harvested and fixed into 96% ethanol and left overnight prior to staining. Leaves were set on 0.05% aniline solution and 0.001% calcofluor for approximately 15 minutes, staining solution was replaced with fresh

aniline (without calcofluor) and incubate at room temperature overnight in the dark. Slides were prepared in fresh aniline solution and view under 365nm excitation light with DM 400 LP. To get a less aggressive and slower infection, measurements were done at 2 dpi and the spore inoculum concentration was previously reduced ($2*10^4$ spores*ml^{-1}).

Bacillus spp. Induced Systemic Resistance assay on tomato vs. *Botrytis cinerea*

To evaluate the capacity of two Bacillus spp. strains to colonise tomato rhizosphere and to induce systemic resistance (ISR) against *Botrytis cinerea*, 3 weeks-old tomato plants (20 plants per Bacillus strain), growing in pesticide-free compost, were soaked with 2.5 L of bacterial culture at $1*10^7$ cells for one hour with a solution of a wild type strain (WT) of *Bacillus subtilis* and the *Bacillus* spp. GB03 biocontrol strain. 4 days after inoculation, leaves of tomato plants were excised for infection/pathogenicity assay (see above, spore inoculum concentration at $2*10^4$ spores/ml) and lesion diameters were measured to look for resistance phenotype at 3 and 4 dpi.

Elicitor-induced resistance effect on growth on tomato cv. Moneymaker and Motelle growth

To evaluate the costs of Chitosan 1 (Chitoplant, ChiPro), Chitosan 2 (Naturcrop formulation), methyl-jasmonate (MeJA), Benzo (1,2,3)-thiadiazole-7-carbothioic acid S- methyl ester (BTH, ASM, BION; Syngenta, Basel, Switzerland), the combination of MeJA + BTH and β-amino-butyric acid (BABA)-induced resistance in plant development, seeds of 2 tomato cultivars (Moneymaker and Motelle) were placed in petri-dishes containing wetted tissue paper, and maintained at 28°C in the dark for 4 and 3 days, respectively, to stimulate germination. Germinated seeds of each cultivar were planted in plant cell propagators containing Scott's M3 soil and cultivated under tomato standard growth conditions (16h- 8h/ day- night cycle; 23°C/ 20°C) for one week.

Each propagator contained 12 seedlings of each cultivar. Seedlings were then soil drenched/sprayed with elicitors, to the following final concentrations.

- Control-spray DDW + 0.02% Silwet (surfactant)
- Chitosan 2 (Naturcrop formulation) (1:100) + 0.02% Silwet (surfactant)
- Chitosan1 (ChitoPlant, ChiPro) 1% w/v + 0.02% Silwet (surfactant)
- MeJA (Positive control) foliar sprayed (0.1 mM) + 0.02% Silwet (surfactant)
- BABA soil drenched (5mM stock solution= 0.5mM final concentration)

- Benzo (1,2,3)-thiadiazole-7-carbothioic acid S- methyl ester (BTH, BION; Syngenta, Basel, Switzerland) (1mM) + 0.02% Silwet (surfactant) + 0.05% ethanol foliar sprayed
- Combination of BTH+MeJA (0.25mM+0.1 mM) + 0.02% Silwet (surfactant) foliar sprayed

Plant height was measured every two days during seven days after elicitor treatment to determine elicitor-induced growth reduction. After measuring seedlings height, the relative growth rate was calculated.

MeJA-priming of defence marker genes in tomato against *Botrytis cinerea*

To determine whether the phytohormone derivative methyl-jasmonate (MeJA) act through priming of gene expression of marker genes, 3 weeks-old tomato cv. Moneymaker seedlings were treated with ddH_2O + 0.01% Tween 20 (surfactant) and MeJA (0.01 mM) (Wang, Liao, Kan, Han, & Zheng, 2015) + 0.01% Tween 20. Five days after elicitor treatment, tomato leaves (3 seedlings per treatment) were excised and they were infected with *B. cinerea* spore inoculum ($2*10^4$spores/ml) and mock inoculated (ddH_2O) as a non-infected control. Sample collection was done by harvesting leaf discs with a cork borer surrounding infection area at 8, 24 and 48 hour post infection (hpi). Samples were then stored in 2mL tubes at -80ºC until RNA extraction with Rneasy Plant MiniKit (Qiagen), Dnased with TurboDnase (ThermoFisher) and complementary DNA was synthesized from 5µg of total RNA using Superscript III reverse transcriptase (Invitrogen) and random hexamer/Oligo DT primers.

Elicitor direct induction of SA & JA-defence genes in tomato

To determine whether methyl-jasmonate (MeJA, the jasmonic acid derivative), BTH (Salicylic acid functional analogue), Softguard (chitin + chitosan commercial elicitor) (and water as non-treatment control) can induce direct defence gene expression of PR-1 and Pin2, salicylic acid and jasmonic acid marker genes respectively. 4-weeks old tomato cv. Money-maker plants were foliar sprayed with ddH_2O (control) + 0.01% Tween 20 (surfactant); Softguard (chitin+chitosan, Travena) 1:600 + 0.01% Tween 20 (surfactant), MeJA (0.1 mM) (this time ethanol wasn't added to dissolve MeJA) + 0.01% Tween 20 ; BTH (1mM) + 0.01% Tween 20 (surfactant) and BABA (foliar sprayed) (250 µg ml–1;(Cohen, 2002)) + 0.01% Tween 20 (surfactant); leaves of 3 plants per treatment (3 biological replicates) were harvested at 3, 9 and 24 hours after treatment (hat) for total RNA extraction. Samples were then stored in 2mL tubes at -80ºC until RNA extraction with Rneasy Plant MiniKit (Qiagen), Dnased with TurboDnase (ThermoFisher) and complementary DNA was synthesized from 5µg of total

RNA using Superscript III reverse transcriptase (Invitrogen) and random hexamer/Oligo DT primers.

RESULTS

Long-lasting Elicitor-induced resistance in tomato against *Botrytis cinerea*: Screening for a resistance phenotype

Tomato cv. Moneymaker and Motelle are two susceptible genotypes to the aggressive necrotrophic fungus *Botrytis cinerea*. To determine whether BABA, BTH, MeJA, the combination of MeJA + BTH and 2 formulations of chitosan are able to induced long-lasting resistance (17 days after treatment) against *Botrytis cinerea*, tomato cv. Moneymaker and tomato cv. Motelle, tomato were treated with the different elicitor and 17 days later excised leaves were challenged with *Botrytis cinerea* by drop inoculation (pathogenicity/infection assay).

Two experiments were conducted separately (1st experiment belongs to figure 1 and 2nd experiment belongs to figure 2) in order to test more resistance elicitors. Due to the high virulence of *Botrytis cinerea* R16 strain, in the 2nd experiment (with the 2 formulations of chitosan) the spore inoculum used for the infection was reduced to $2*10^4$ spores/ml.

Results indicate that the level of resistance induced by the Salicylic acid (SA) functional analogue BTH was comparable to MeJA-IR, with both treatments resulting in a statistically significant reduction of lesion size at 3 days post-inoculation (Figure 1a). In contrast, BABA-induced resistance provided no protection against *Botrytis cinerea*. There were no significant differences in lesion size between the two tomato cultivars (P=0.086) (data not shown) at 3 days post inoculation (dpi), while these differences were significant at 4 dpi (P=0.003) (Figure 1b). However, there were elicitor-dependent differences (P=0.05) between the two varieties but not plant variety-dependent differences at 3 dpi (Figure 1, not shown).

In the 2nd experiment, the level of all elicitors-induced resistance at both time points resulted in a statistically significant reduction of lesion size compared to the water-treated control plants in both varieties (Figure 2). In tomato cv. Moneymaker, all elicitor treatments behaved similarly in significantly reducing disease expansion, in comparison with the water-treated control plants at both time points (Figure 2a). In contrast, there were more differences among treatments in Motelle at both time points (figure 2b). In tomato cv. Motelle, BABA-IR and ChitoPlant-IR were more effective against *B. cinerea* than the other treatments, at 3 dpi. In addition, at 4 dpi Motelle MeJA-IR and both chitosans-IR were slightly more effective than BABA-IR in the fight against Botrytis. After 4 days post-inoculation, all elicitors still significantly reduced *B. cinerea* lesion expansion, in comparison with the control in both cultivars and some differences among treatments were still seen in Motelle (Figure 6b).

BABA and the two chitosan formulations significantly reduced necrotic lesion size at 3 dpi in comparison with water-treated control plants. At 4 dpi, MeJA and again both chitosan formulations behaved similarly in significantly reducing disease expansion compared to control treatment (Figure 2b). There were also significant differences among treatments and the two tomato cultivars at 3 dpi (Figure 3), although these significant differences were no longer expressed after 4 dpi.

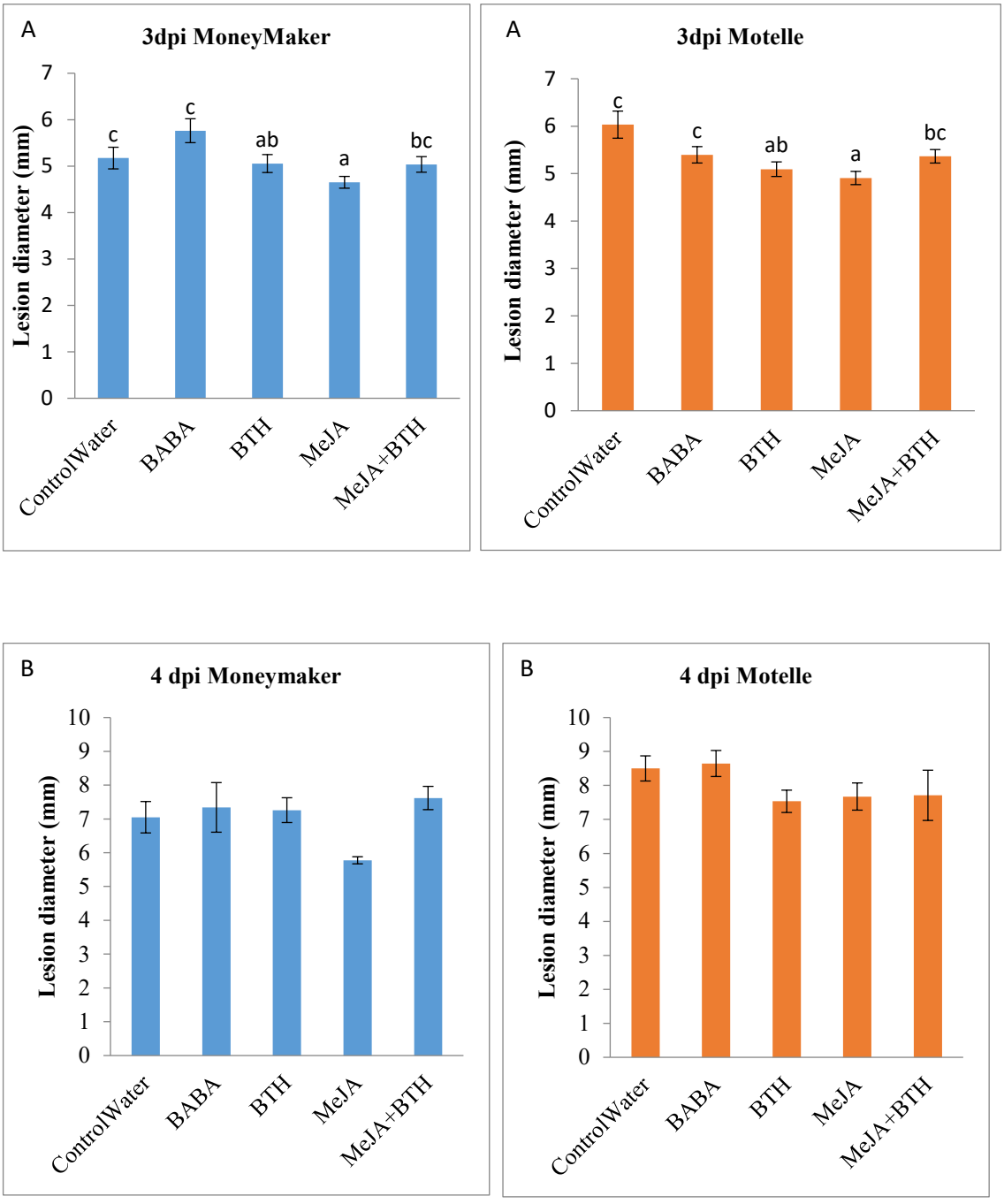


Figure 1. **A**. Quantification of BABA, BTH, MeJA and MeJA+BTH-induced resistance against *B. cinerea* (5*10^{4}spores/ml) in tomato cv. Moneymaker (blue bars) and Motelle (red bars) at 3 dpi. Values presented are means ± SEM. Different letters indicate statistically significant differences (Fisher's least

significant difference (LSD) test P<0.001, α=0.05). B. Quantification of BABA, BTH, MeJA and MeJA+BTH-induced resistance against *B. cinerea* in tomato cv. Moneymaker (blue bars) and Motelle (red bars) at 4 days post inoculation. Values presented are means ± SEM obtained from an ANOVA, significant differences were found between both tomato cultivars at 4 dpi (P=0.003 for cultivar interaction).

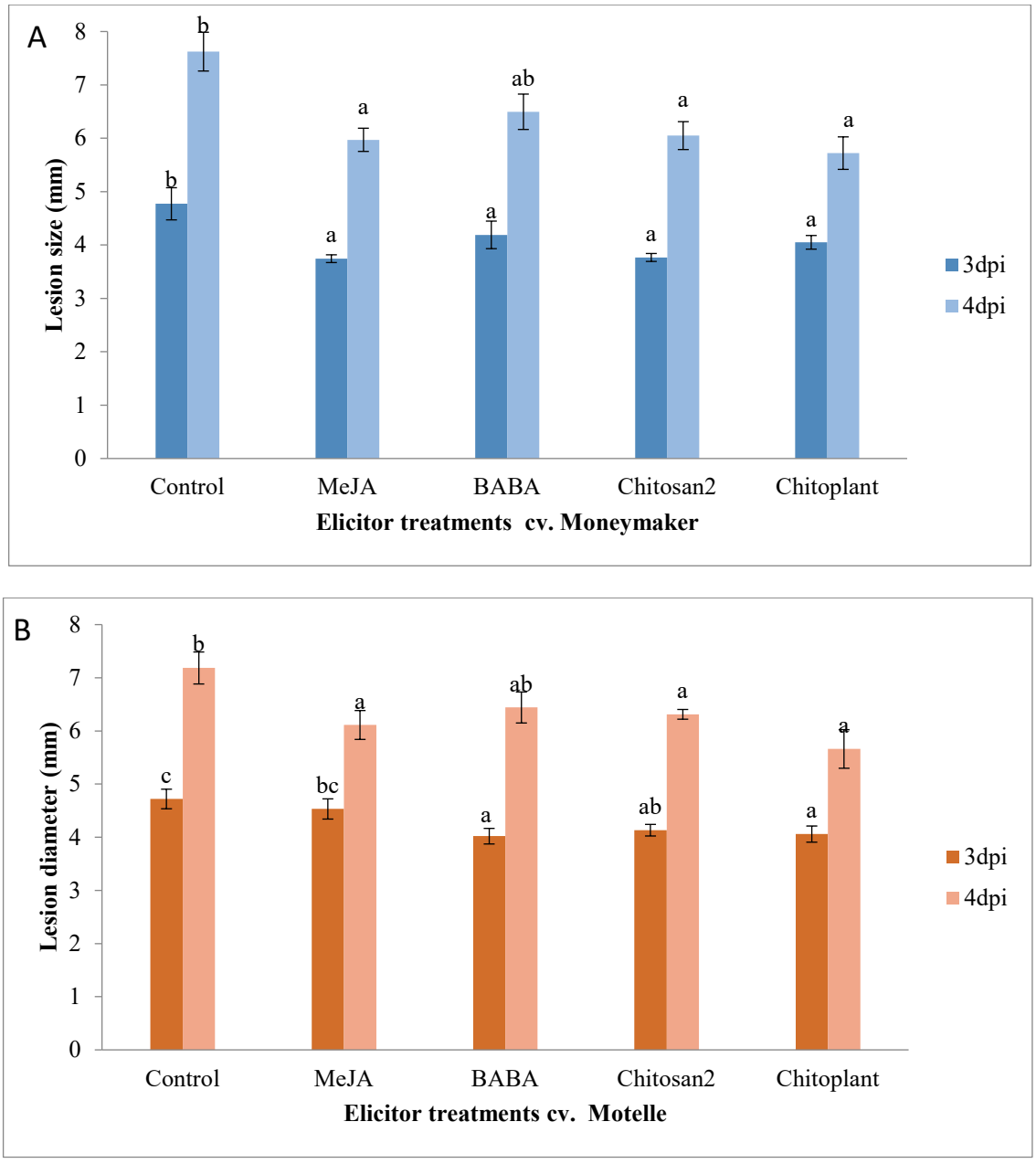


Figure 2. A. Quantification of MeJA, BABA, Chitosan 2 and commercial chitosan formulation ChitoPlant-induced resistance against *Botrytis cinerea* (2*10^4 spores/ml) at 3 and 4 days post inoculation in tomato cv. Moneymaker. Values presented are means ± SEM. Different letters indicate statistically significant differences (Fisher's least significant difference (LSD) test P=0.004 at 3 dpi and P<0.001 at 4 dpi, α=0.05). B. Quantification of MeJA, BABA, Chitosan 2 and commercial chitosan formulation ChitoPlant (ChiPro)-induced resistance against *Botrytis cinerea* at 3 and 4 dpi in tomato cv. Motelle. Values presented are means ± SEM. Different letters indicate statistically significant

differences (Fisher's least significant difference (LSD) test P=0.005 at 3 dpi and P=0.009 at 4dpi, α=0.05).

As seen before, normally tomato cv. Moneymaker expressed more induced resistance (IR) to *Botrytis cinerea* than Motelle in all treatments, except in BABA, where the level of IR was stronger in Motelle (Figure 1a and 1b). Similar results were seen in this experiment where Motelle was more susceptible to *B. cinerea* in general except in BABA-IR where Motelle defence was again stronger (Figure 3). MeJA-IR responses to *Botrytis cinerea* infection was significantly different between both varieties as well as Chitosan2-IR, which was responding better in Moneymaker variety (Figure 3). Interestingly, ChitoPlant-IR functioned similarly in both tomato varieties against *Botrytis cinerea* (Figure 3) which makes it a great candidate for further experiments.

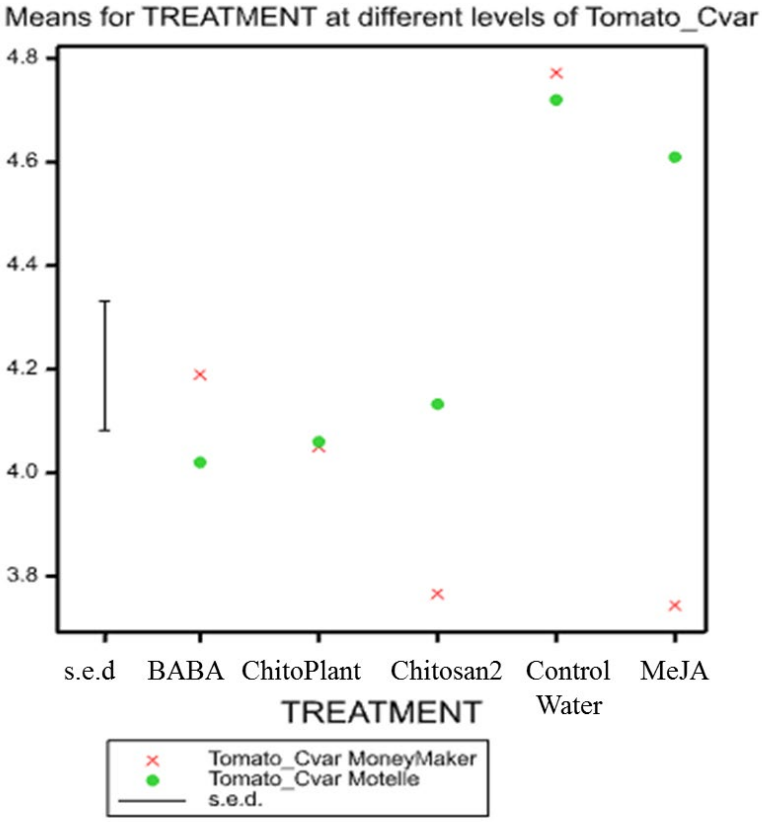


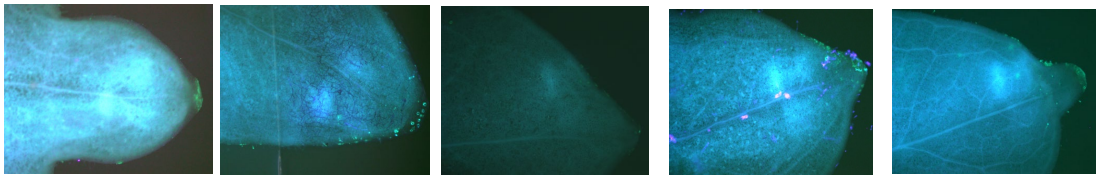
Figure 3. Quantification of BABA, MeJA and Chitosan 2 and ChitoPlant (ChiPro)-induced resistance against *B. cinerea* in tomato cv. Moneymaker and Motelle at 3 days post infection. Values represented are means (of the necrotic lesion diameter) ± SEM obtained from an ANOVA mean plot (P<0.05 for cultivar interaction; Treatment*Tomato_Cultivar).

Basal callose deposition induced by BABA, MeJA, BTH+MeJA and BTH, Chitosan1 (ChitoPlant) and Chitosan2-IR in tomato cv. Moneymaker and tomato cv. Motelle.

As stated previously, Callose is a plant polysaccharide that can reinforce the plant cell-wall and therefore delay pathogen cuticle penetration.

The aim of this experiment was to assess whether β-aminobutyric acid (BABA), methyl-jasmonate (MeJA), benzo (1,2,3)-thiadiazole-7-carbothioic acid S- methyl ester (BTH, BION; Syngenta), 2 different formulations of chitosan and the combination of BTH+MeJA can induce callose deposition in tomato varieties Motelle and Moneymaker.

For that, tomato cv. Money-maker and tomato cv. Motelle seedlings were treated with distilled water (as a control), MeJA, BTH, MeJA+BTH, Chitosan2, ChitoPlant (chitosan1) and BABA. Cotyledons of every treatment were excised one week after treatment and store in 100% ethanol previous Aniline Blue staining to dye callose deposition. Overall, there was the same trend in both tomato varieties apart from in treatments containing MeJA, where MeJA induced callose and this induction was higher in Money-maker (MM) than in Motelle (Figures 4 and 5). The combination of BTH and MeJA also induced callose deposition in tomato cotyledons in MM (Figure 4) and Motelle, although in less amount (Figure 5).



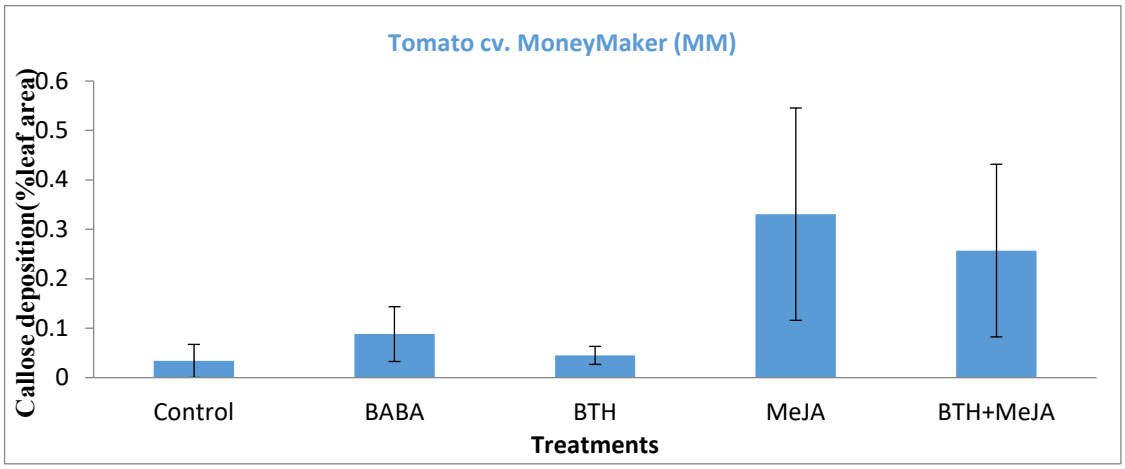


Figure 4. Basal callose deposition in tomato cv. Moneymaker leaves after Water-control, BABA, BTH, MeJA and MeJA+BTH treatments. 7 days after treatment leaves were harvested for Aniline Blue Staining. Pictures were taken under fluorescence microscopy at 4x magnifications (bright green dots represent callose deposits in epidermal cells). Callose was quantified as described previously (Material & Methods) (Luna et al., 2011). Values represent percentages of the mean ± SEM.

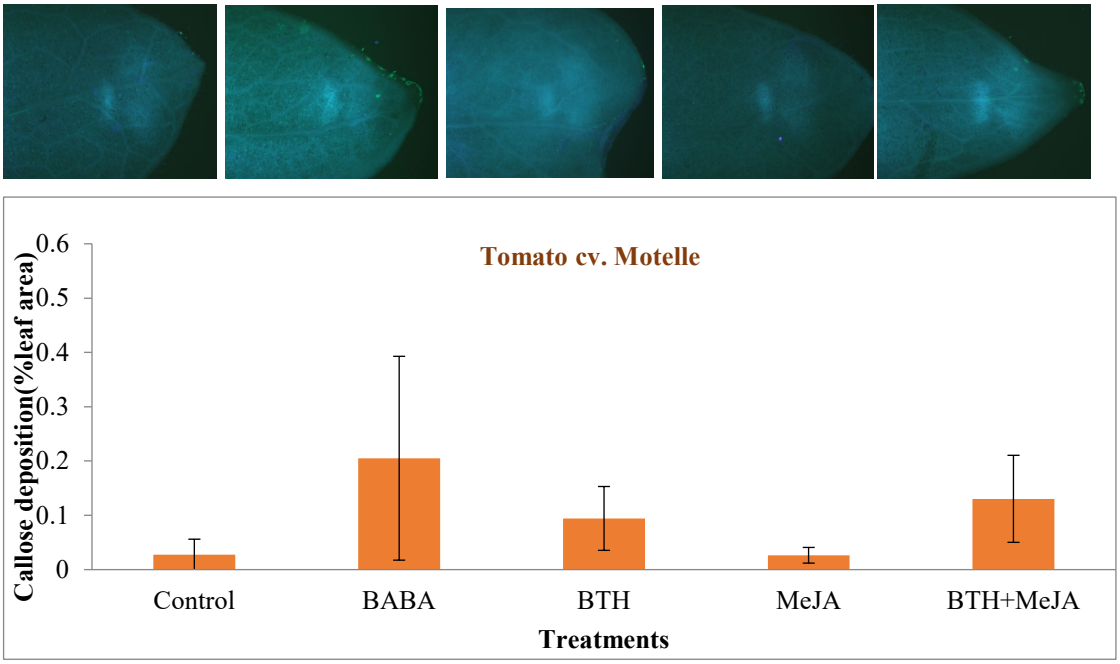


Figure 5. Basal callose deposition in tomato cv. Motelle leaves after Water-control, BABA, BTH, MeJA and MeJA+BTH treatments. 7 days after treatment leaves were harvested for Aniline Blue Staining. Pictures were taken under fluorescence microscopy at 4x magnifications (bright green dots represent callose deposits in epidermal cells). Callose was quantified as described previously (Material & Methods) (Luna et al., 2011). Values represent percentages of the mean ± SEM.

To define whether chitosan directly induces callose deposition the experiment was repeated and tomato cv.MM and Motelle were treated with BABA, MeJA and the 2 chitosan formulations. The trending in both varieties was similar, with almost no callose induction by any of the elicitors. BABA induced callose apposition was higher in tomato cv. Motelle than in Money-maker (MM) (Figures 6 and 7); nevertheless, callose induction was generally lower than expected in BABA treatments (as compared with *Arabidopsis thaliana*, (Ton & Mauch-Mani, 2004)). Interestingly, the commercial chitosan formulation ChitoPlant highly induced callose apposition in both cultivars in comparison with the rest of the elicitor treatments (Figures 6 and 7).

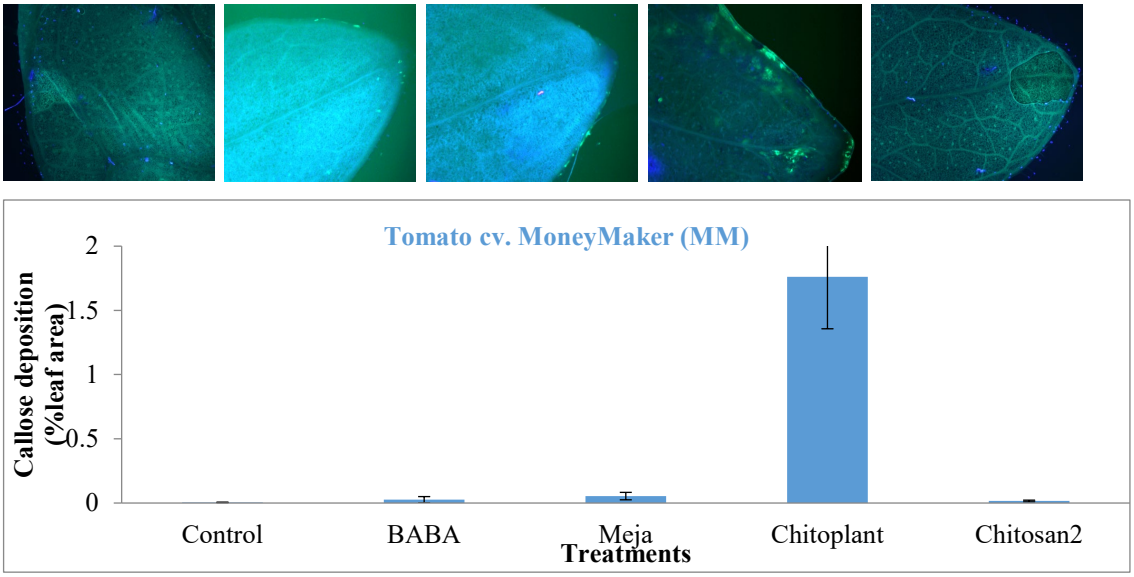


Figure 6. Callose deposition in tomato cv. Moneymaker leaves after Water-control, BABA, MeJA, ChitoPlant (ChiPro) and Chitosan 2 treatments. 7 days after treatment leaves were harvested for Aniline Blue Staining. Pictures were taken under fluorescence microscopy at 4x magnifications (bright green dots represent callose deposits in epidermal cells). Callose was quantified as described previously (Material & Methods) (Luna et al., 2011). Values represent percentages of the mean ± SEM.

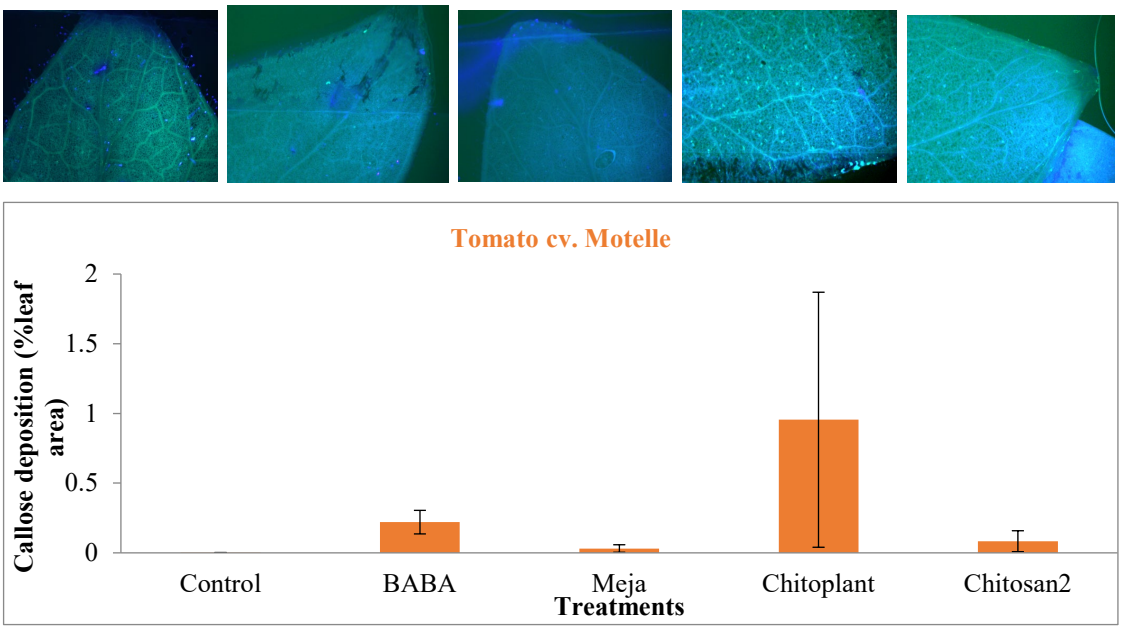


Figure 7. Callose deposition in tomato cv. Motelle leaves after Water-control, BABA, MeJA, ChitoPlant (ChiPro) and Chitosan 2 treatments. 7 days after treatment leaves were harvested for Aniline Blue Staining. Pictures were taken under fluorescence microscopy at 4x magnifications (bright green dots represent callose deposits in epidermal cells). Callose was quantified as described previously (Material & Methods) (Luna et al., 2011). Values represent percentages of the mean ± SEM.

Pathogen-induced callose deposition in BABA, MeJA, BTH+MeJA, two chitosan formulations and BTH-IR in tomato cv. Moneymaker and tomato cv. Motelle.

It's been demonstrated that MeJA, BABA, BTH, Chitosan2 and ChitoPlant can significantly induce resistance in tomato cv. Money-maker and Motelle against *Botrytis cinerea* (Figures 1 and 2). Furthermore, it was discovered that MeJA and mainly chitosan (ChitoPlant formulation) can induce callose deposition (Figures 6, 7) in both tomato varieties (cv. Moneymaker and Motelle) in the absence of pathogen.

MeJA primes callose deposition in tomato cv Moneymaker against aggressive *Botrytis cinerea* strain

To determine the effectiveness of BABA, MeJA, BTH+MeJA, two chitosan formulations and BTH treatments priming cell wall defences by inducing callose against *Botrytis cinerea*. Tomato cv. Moneymaker and tomato cv. Motelle seedlings were foliar sprayed with ddH_2O, BTH, MeJA, a combination of BTH+MeJA and BABA (soil drenched). 17 days after elicitor treatments (long-lasting defence induction) leaves of every plant were excised and prepared for infection/detached leaves assay. 3 days post inoculation double staining (Aniline Blue + calcofluor) was performed in order to see pathogen-induced callose deposition in all treatments.

Despite the high level of aggressiveness of the *Botrytis cinerea* R16 strain (strain used during all experiments for the PhD) and the high contrast of the calcofluor, which made difficult to measure callose deposition by microscopy, callose was found in tomato cv. Money-maker samples treated with MeJA (Figure 8), which correlates with the significant lesion size reduction of the same treatment (Figure 1 and 2). Callose was not seen in the rest of the elicitor-treated plants or in the water-treated control plants (Figure 8).

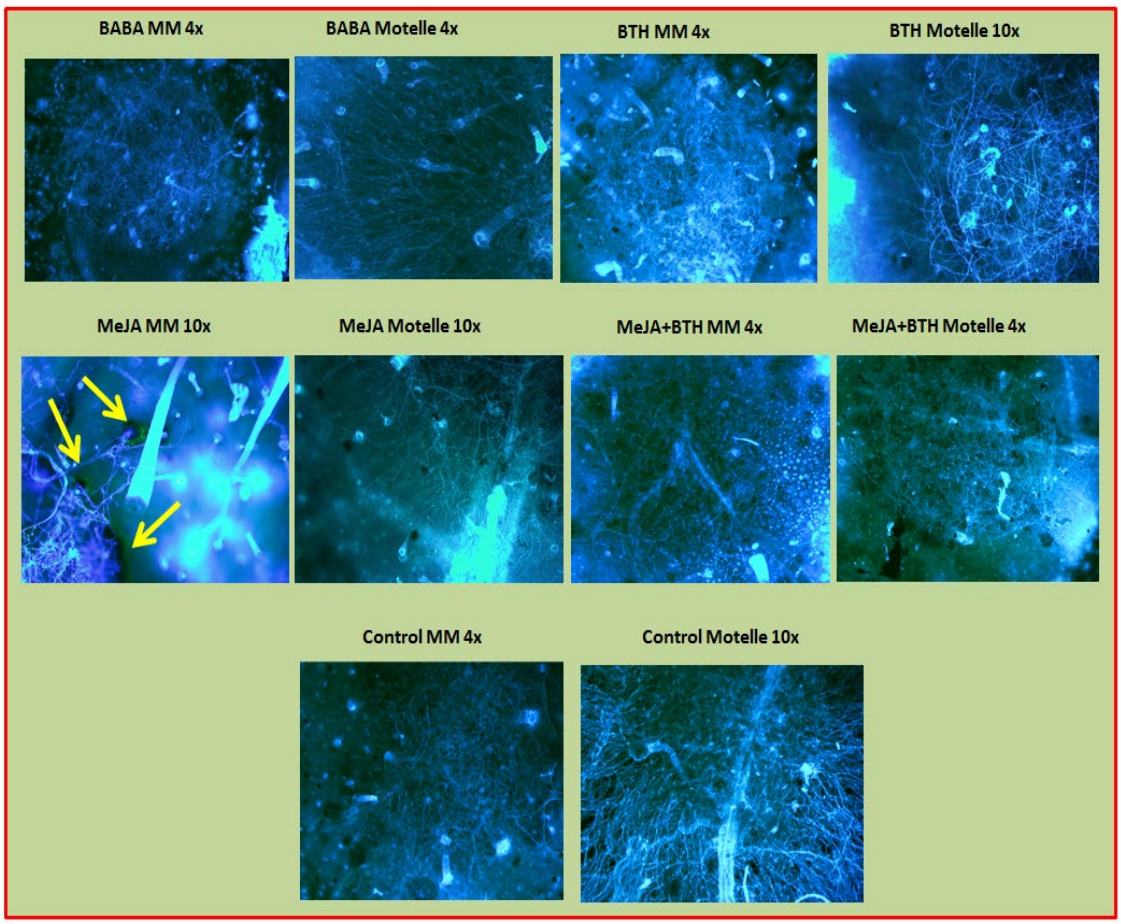


Figure 8. Callose deposition (bright green dots highlighted with yellow arrows represent callose deposits in tomato epidermal cells close to fungal penetration sites) in tomato cv. Moneymaker and tomato cv. Motelle leaves after *B. cinerea* infection in Water-control, BABA, BTH, MeJA and MeJA+BTH-treated plants. 3 days post inoculation, leaves were harvested for Double Staining (Aniline Blue + Calcofluor) and then pictures were taken under fluorescence microscopy at 4x and 10x magnifications.

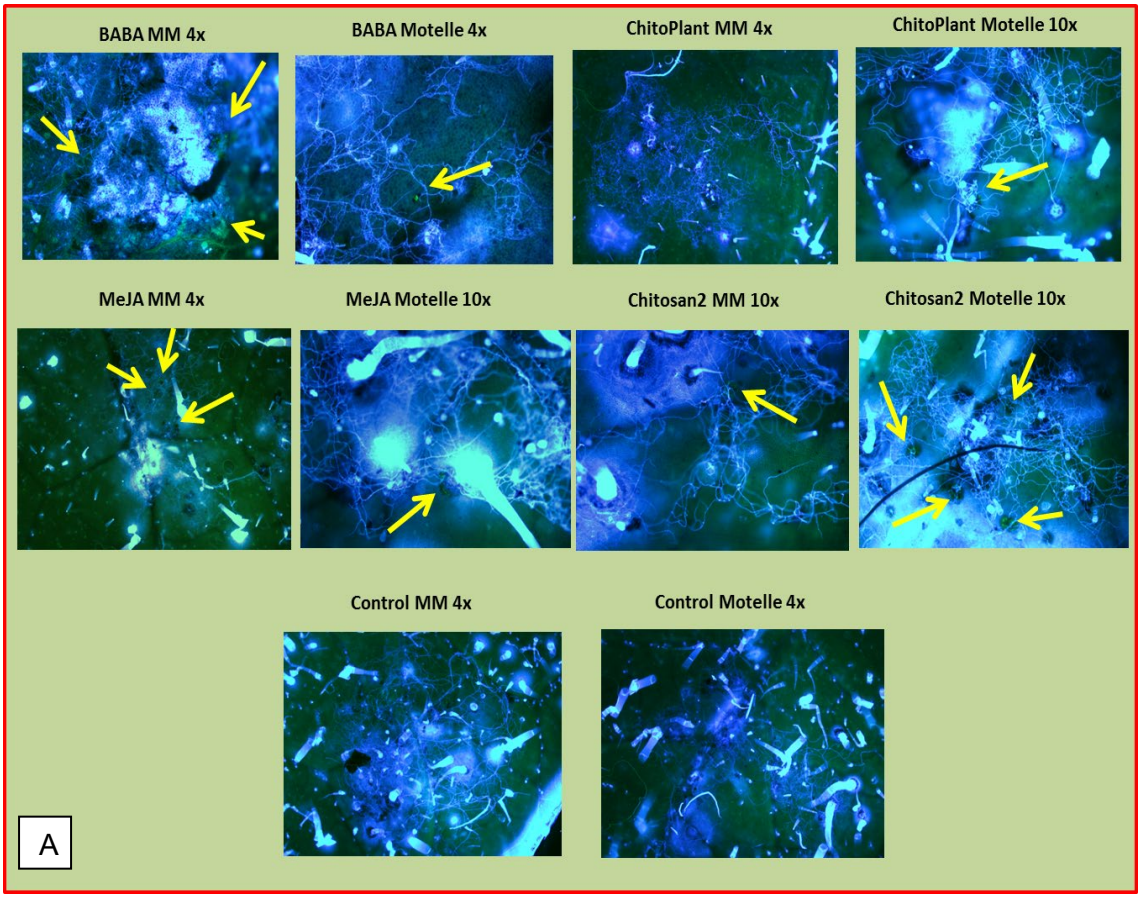
In tomato cv. Motelle leaves, callose was not seen in any of the elicitor-treated plants, results that correlate with the generally higher susceptibility of the variety Motelle to *Botrytis cinerea* (Figure 3).

<u>MeJA, BABA and chitosan can prime callose deposition in tomato cv Moneymaker and Motelle against less virulent *Botrytis cinerea*</u>

Another similar experiment was conducted in order to determine the effectiveness of chitosan treatment priming cell wall defences against fungal pathogen attack. Tomato cv. Moneymaker and tomato cv. Motelle seedlings were foliar sprayed with ddH_2O, BABA(soil drenched), Chitosan2, ChitoPlant and MeJA. 17 days after elicitor treatments (long-lasting defence induction) leaves of every plant were excised and prepared for infection/detached leaves

assay. 3 days post inoculation double staining (Aniline Blue + calcofluor) was performed in order to see pathogen-induced callose deposition in all treatments.

Due to the less concentrated pathogen spore inoculum (2*10^4 spores/ml), more differences were seen among treatments. In general, lesions in BABA-treated plants were smaller and visually with fewer amounts of mycelia. Water-treated control plants did not show callose deposition in any of the samples harvested (Figure 9A). Interestingly, as previously seen (Figure 8), tomato cv. MM callose papillae formation was greater than Motelle and callose was accumulated surrounding some parts of the inoculum droplet, which presumably slowed down *B. cinerea* expansion (Figure 9). MeJA-treated plants also produced callose around the penetration sites of the hypha with no differences between both varieties. ChitoPlant-treated plants deposited low amount of callose in comparison with the mock treatments in MM (Figure 6 and 7). In contrast, Chitosan2-treated motelle plants showed a greater callose apposition surrounding hypha penetration sites. Due to the unexpected results further experiments need to be done in order to see consistence or variance in BABA, MeJA and chitosan-induced callose deposition after pathogen challenge.



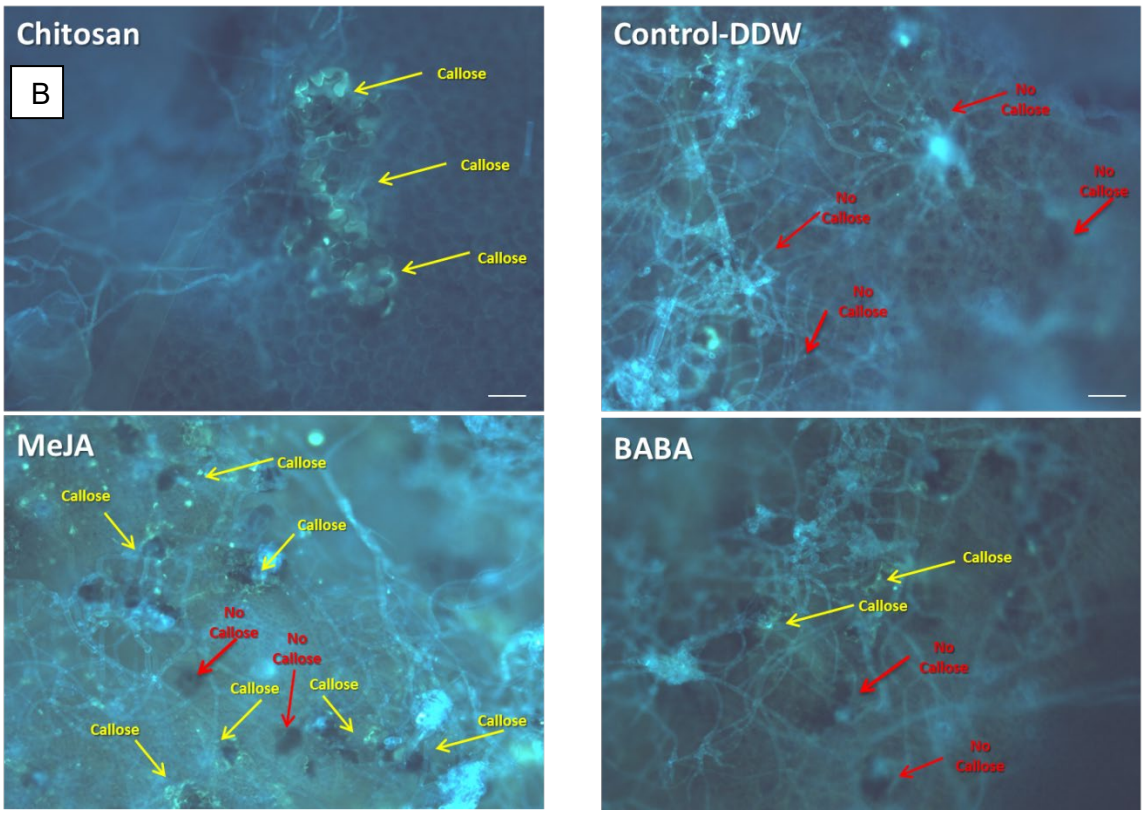


Figure 9. A. Callose deposition (brigh green dots highlighted with yellow arrows represent callose deposits in tomato epidermal cells close to fungal penetration sites) in tomato cv. Moneymaker and tomato cv. Motelle leaves after *Botrytis cinerea* infection in Water-control, BABA, MeJA, ChitoPlant (ChiPro Germany) and Chitosan2-treated plants. 2 dpi leaves were harvested for Double Staining (Aniline Blue + Calcofluor) and then pictures were taken under fluorescence microscopy at 4x and 10x magnifications. B. Callose deposition in tomato cv. Moneymaker leaves after *B. cinerea* infection in Water-control, BABA (as a positive control), MeJA and chitosan treated plants. 3 days post-inoculation leaves were harvested for double staining (Aniline Blue + calcofluor) and then pictures were taken under fluorescence microscopy at 4x and 10x magnifications.

Furthermore, results indicate that MeJA-treated plants highly induced callose around most of the penetration sites of the hypha as well as outside of Botrytis penetration sites (Figure 9B). ChitoPlant-treated plants also induced callose in a lesser extent; however it was more localized to Botrytis penetration sites (Figure 9B). BABA-treated plants rarely induced callose deposition towards Botrytis infection sites.

Bacillus spp. colonisation assay of tomato rhizosphere

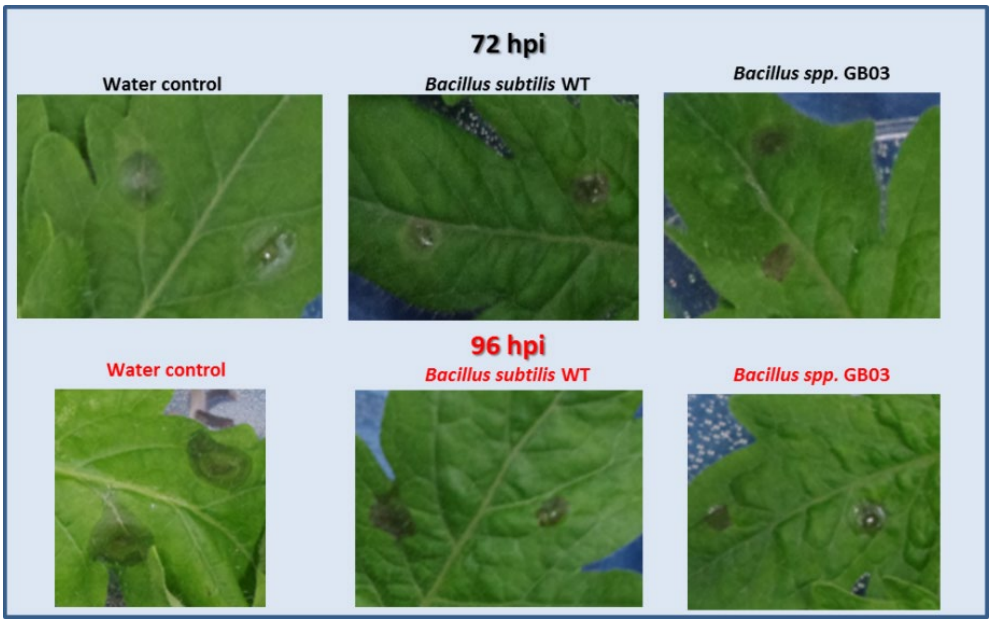
Bacillus subtilis is a gram-positive plant-associated rhizobacteria that is able to form microcolonies in the plant rhizosphere.

During the last decade, *Bacillus spp.* has been studied as a model biocontrol against various pathogens. Furthermore, it's been demonstrated that *Bacillus subtilis* can successfully

induced systemic resistance (ISR) in tomato against *Fusarium* wilt disease (Akram et al., 2014) and *Bacillus cereus* induced disease resistance against *Rhizopus* rot in peach fruit (Wang et al., 2015).

Thus, we hypothesized that our two *Bacillus* spp. strains may induce ISR in tomato cv. Money-maker against *B. cinerea*. In order to investigate the biocontrol properties of two *Bacillus spp.* strains and their ability to induce systemic resistance (ISR) in tomato against *B. cinerea*, 3 weeks-old tomato plants (20 plants per strain), growing in pesticide-free compost, were soaked with 2.5 L of bacterial culture at $1*10^7$ cells for one hour with a solution of a wild type strain (WT) of *Bacillus subtilis* and the *Bacillus* spp. GB03 strain. 4 days after inoculation, leaves of tomato plants were excised for infection assay and lesion diameters were measured to look for resistance phenotype at 3 and 4 dpi.

Results indicate that both strains can significantly delay Botrytis lesion expansion at both time points. However, *Bacillus* spp. GB03 strain induced a stronger resistance phenotype at 96 hpi (Figure 10) which correlates with the slower and more durable rhizosphere colonisation seen by this strain (data not shown).



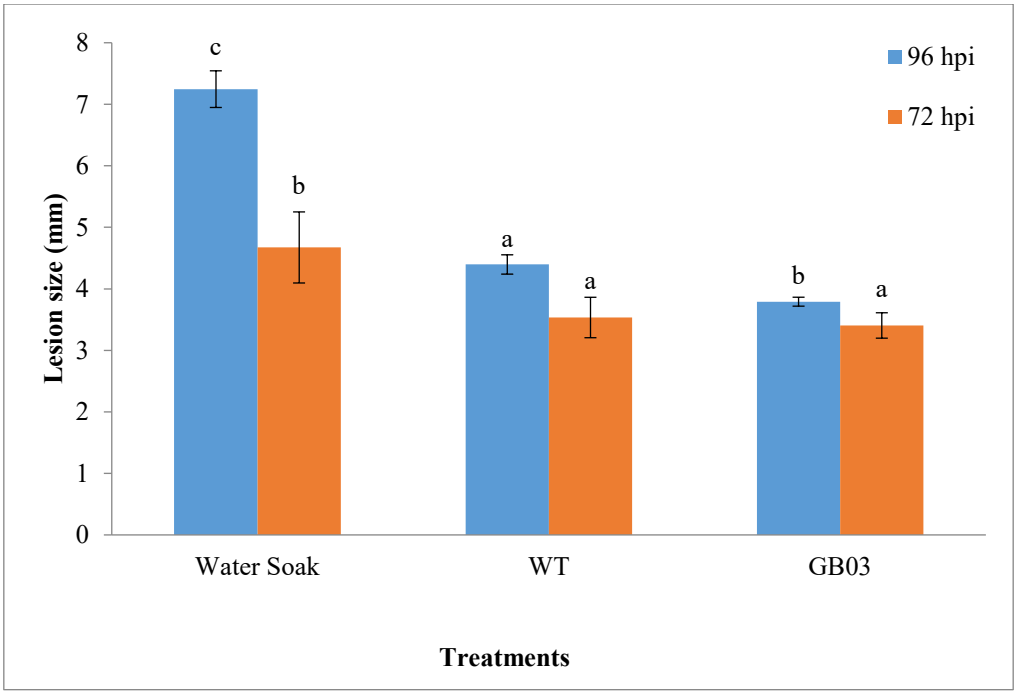


Figure 10. Quantification of *Bacillus subtilis* WT *and Bacillus spp.* GBO3 –induced systemic resistance (ISR) against *B. cinerea* in tomato cv. MoneyMaker at 3 and 4 dpi. Values presented are means ± SEM. Different letters indicate statistically significant differences (Values presented are means ± SEM obtained from an ANOVA and then pairwise Fisher's protected least significant difference (LSD) test P<0.001, α=0.05).

Elicitor-induced growth reduction in tomato cv. Moneymaker and Motelle

Resistance elicitors can induce resistance (IR) in tomato and other crops, and subsequently can enhance the plant basal resistance after perception of elicitor signals against pathogen attack (Luna, Beardon, Ravnskov, Scholes, & Ton, 2016). MeJA, BABA, BTH and chitosan significantly decreased disease lesion diameters by *Botrytis cinerea* in 4- week-old tomato cv. Moneymaker and Motelle plants.

However, it is widely known that there are costs and trade-offs associated with induced resistance (Luna et al., 2016; Redman, Jr., & Schultz, 2001; van Hulten, Pelser, van Loon, Pieterse, & Ton, 2006; Walters & Heil, 2007). Nevertheless, few studies have examined it in detail in crop systems, (Luna et al., 2016) showed that soil drench 1-week old tomato seedlings with high concentrations of BABA (10 mM) and JA (1 mM) abolished plant growth and had lethal effects. Elicitor-induced resistance (IR) on mainly tomato and other plants is the core of my PhD and therefore, to better understand how elicitor may affect the crop after treatment, and to achieve a more efficient induced resistance with minimal impact on plant fitness, it's crucial to evaluate the potential costs in plant development cause by elicitor interaction with the plant.

Finally, it's important to mention that all the elicitor concentrations were chosen from published articles where they used them at the mentioned concentrations (Material & Methods) to induce resistance in different plant-pathogen systems. BABA, BTH and Jasmonic acid (JA) were used by (Ton & Mauch-Mani, 2004) in Arabidopsis against *A.brassicicola* and *P.cucumerina* and (Barilli, Rubiales, Amalfitano, Evidente, & Prats, 2015) used 10 mM and 50 mM for BTH and BABA, respectively, previously proven to be effective in this Pea-*Uromyces pisi* system; (Terry, 2004) stated MeJA and BTH been used against *Fusarium semitectum* and *Botrytis cinerea* as postharvest and field treatments. (Meir, Droby, Kochanek, Salim, & Philosoph-Hadas, 2005; Walters, Walsh, Newton, & Lyon, 2005a) studied MeJA against *B. cinerea* and BTH-IR on wheat and barley against powdery mildew. Finally, (Romanazzi, Feliziani, Santini, & Landi, 2013) studied different chitosan formulations-IR and BTH-IR in strawberry against *Botrytis cinerea*.

Thus, to evaluate the costs of chitosan 1-IR (Chitoplant, ChiPro), Chitosan 2-IR (Naturcrop formulation), methyl-jasmonate-IR (MeJA), Benzo (1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester-IR (BTH, BION; Syngenta, Basel, Switzerland), the combination of MeJA + BTH-IR and β-amino-butyric acid (BABA)-IR in plant development, 1-2 weeks old tomato cv. Moneymaker and Motelle plants were foliar treated with Chitosan2, ChitoPlant MeJA, BTH, MeJA+BTH and BABA (soil drench). Plant height was measured every two days during seven days.

In 1- to 2-week-old plants, defence induction by BABA and MeJA lead to statistically significant reductions in RGR in both cultivars (figure 10). BABA reduced 39% RGR in Money-maker (MM) and 42% in Motelle, indicating that this cultivar is more susceptible to BABA-induced stress. Moreover, MeJA reduced RGR a 7% in MM and 15% in Motelle (Fig. 11, Table 1). RGR was significantly higher after the induction by BTH and the combination of MeJA+BTH in comparison with water-treated control plants, suggesting that BTH increases plant growth. However, plants developed thinner and smaller stems and less secondary leaves (data not shown), thus indicating that BTH does not affect plant height but it does alter normal plant development.

Thus, it can be observed that some of the elicitors, such as BABA, highly reduce plant growth in both varieties (Picture 1). However, it seems that the magnitude of the growth suppression is cultivar-dependent, as the tomato cv. Money-maker shows less BABA-induced growth reduction (Figure 11 and Table 1).

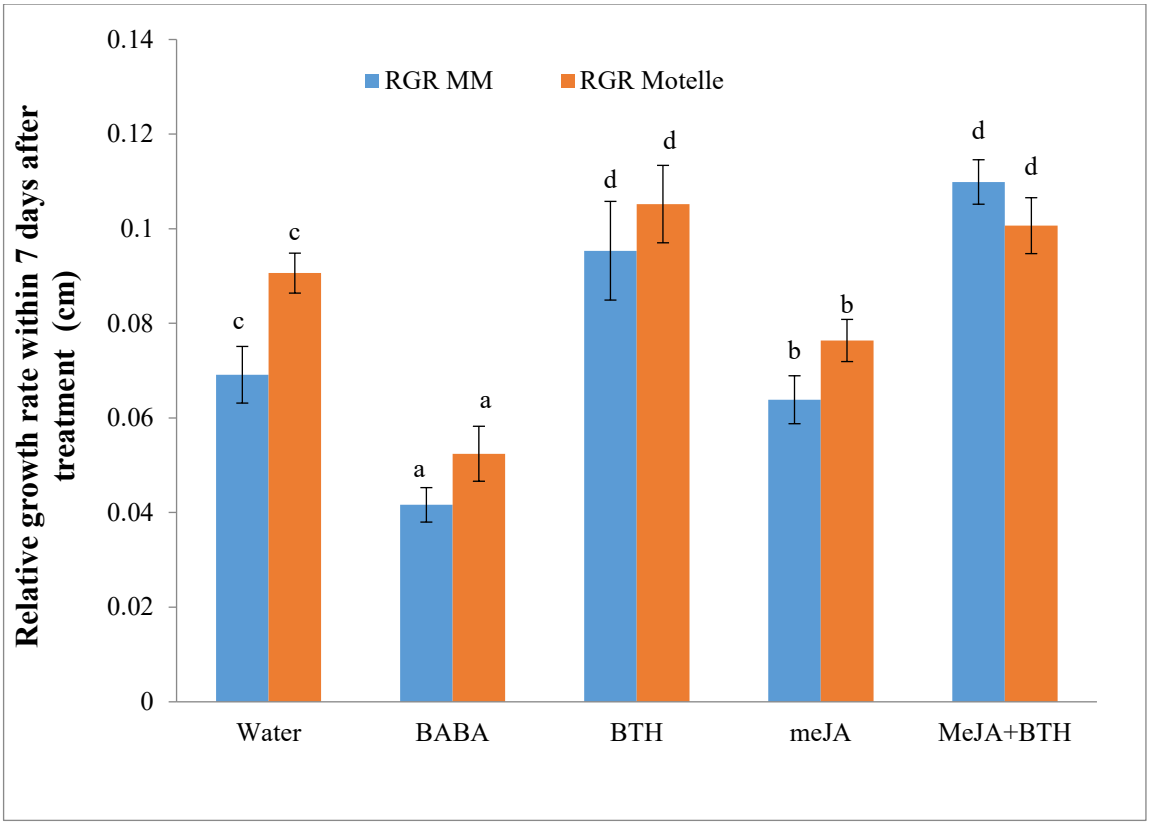


Figure 11. Relative growth rate of tomato cv. Moneymaker (MM) and cv. Motelle after elicitor treatment during seven days after treatment. Values presented are means ± SEM. Different letters indicate statistically significant differences (Fisher's least significant difference (LSD) test $P<0.001$, $\alpha=0.05$) between every elicitor treatment compared to water-control (Pairwise test independent per tomato variety).

% Repression in growth		
TREATMENT	**% Moneymaker**	**% Motelle**
BABA	39.7	42.1
MeJA	7.6	15.7
BTH	-37.9	-16.1
MeJA+BTH	-58.9	-11

Table 1. Percentage of repression of growth of tomato cv. Moneymaker (MM) and cv. Motelle after elicitor treatment seven days after treatment. Values are the percentage of the relative growth rate (RGR) of the elicitor divided by water-control RGR.

Picture 1. 2-week-old tomato cv. Motelle (red) and Money-maker (blue) seedlings 1 week after elicitor treatment. BABA-treated plants were smaller than the rest of the treatments and Motelle-BABA treated plants were even smaller than Money-maker BABA-treated seedlings.

Furthermore, the costs of Chitosan2-IR and Chitosan1-IR on plant fitness, such as seedling growth were also analysed by relative growth rate (RGR). Defence induction by BABA lead again to statistically significant reductions in RGR in both cultivars (Figure 12a). In contrast, Chitosan1 and MeJA RGR did not significantly differ from the control plants. Chitosan2 RGR was significantly higher than the water-control plants (Figure 12a) although plants under this treatment were creating thinner stems and less secondary leaves development (data not shown). BABA-growth repression was 32% in Money-maker (MM) and 45 % in Motelle, suggesting again that Motelle is more susceptible to BABA-IR (Table 2).

% Repression in growth (RGR)		
TREATMENT	**Moneymaker**	**Motelle**
BABA	32.6	45.8
MeJA	-14.1	-8.5
Chitosan2	-15.5	-22.9
ChitoPlant (Chitosan1)	-27	-5.7

Table 2. Percentage of repression of growth of tomato cv. Moneymaker (MM) and cv. Motelle after elicitor treatment seven days after treatment. Values are the percentage of the relative growth rate (RGR) of the elicitor divided by water-control RGR.

When relative-growth rates were compared between both tomato varieties, significant differences between the 2 tomato cultivars were also seen, verifying that every cultivar responds differently to elicitor-induced fitness costs for all elicitors except for Chitosan1 (ChitoPlant), which had a similar effect on the RGR of both cultivars (Figure 11B).

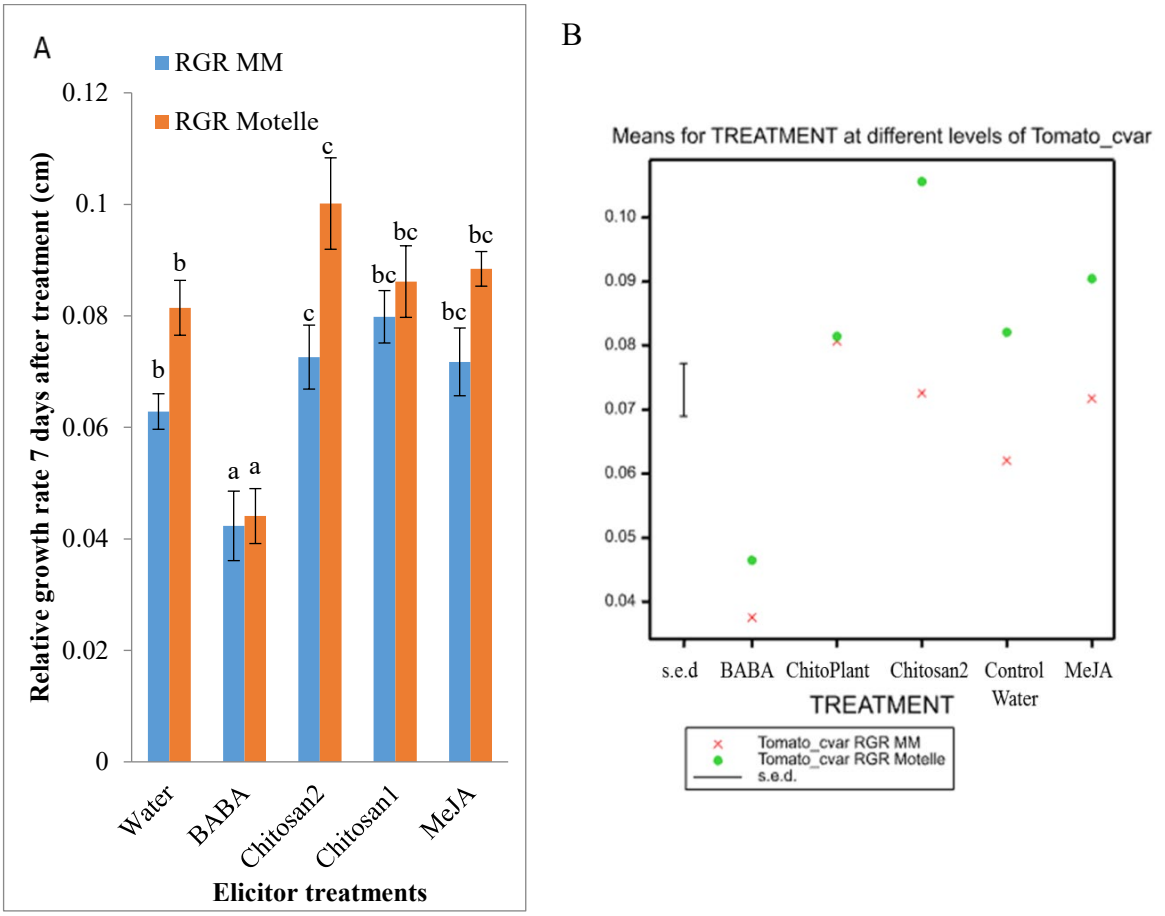


Figure 12. A. Quantification of relative growth rate of 2 tomato cultivars after elicitor treatment, during 7 consecutive days. Values presented are means ± SEM. Different letters indicate statistically significant differences (Fisher's least significant difference (LSD) test P<0.001, α=0.05) between every elicitor treatment compared to water-control (Pairwise test independent per tomato variety). B. Quantification of relative growth rate of 2 tomato cultivars after elicitor treatment, during 7 consecutive days. Values represented are means (of the RGR in cm) ± SEM obtained from an ANOVA mean plot (P<0.001 for Tomato_Cultivar).

Chitosan-induced cytotoxicity in tomato and aubergine

There are studies that recognize the ability of chitosan of inducing H_2O_2 followed by cell death (Iriti & Faoro, 2009). Furthermore, chitosan biological activity depends on the host, its degree of deacetylation, its concentration (Iriti & Faoro, 2009), and the chemical composition of the substrates (El Hadrami et al., 2010). Chitosan physicochemical complexity can ultimately affect plant recognition, which makes it a complex system for plant application and absorbance. Previous experiments needed to infiltrate chitosan into the plant to ensure

recognition and/or an effect (Ahmad et al., 2011; Scalschi et al., 2015) and others needed complex ways to dissolve chitosan oligosaccharides before plant application by dissolving practical grade chitosan in acetic, glutamic, formic and hydrochloric acids (Romanazzi, Feliziani, et al., 2013).

Here, the experiments were done following a novel approach of chitosan application by directly dissolving chitosan (ChitoPlant, commercial formulation) into distilled water and with the help of a surfactant (i.e. Silwet, Tween20) foliar spraying directly into the plant.

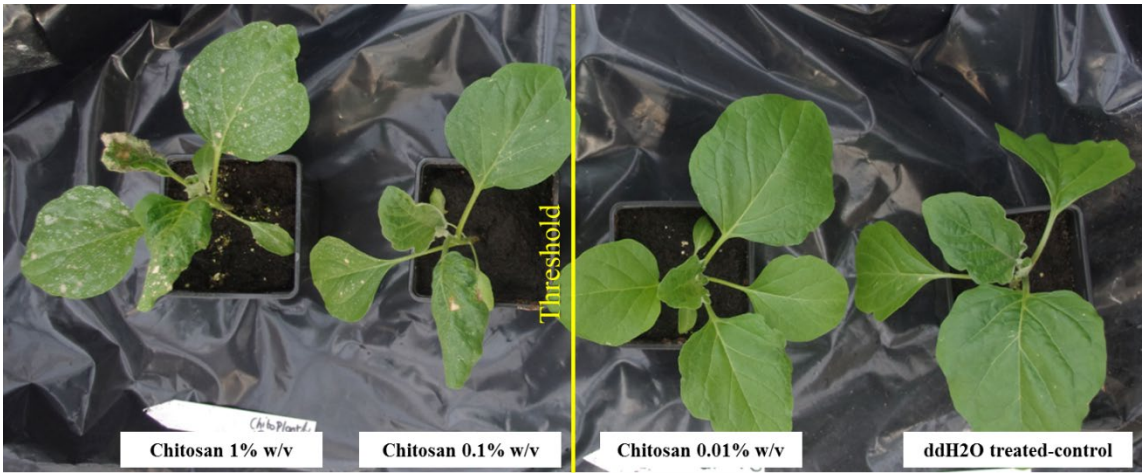
In order to achieve a more efficient defence strategy and less costly in terms of plant fitness, it is important, when using elicitors such as chitosan, to assess the effect of the concentration not only on the activation of plant endogenous defences, but also on the stress tolerance of the plant.

So far during the thesis experiments, chitosan treatment (ChitoPlant) has been used at high concentrations (1% w/v) which didn't have any detrimental effect on tomato growth although it caused cell death on 1-week old tomato cotyledons (Picture 2).

Picture 2. Cell death/phytotoxic response in the cotyledons of 2 weeks-old tomato plantlets 1 week after chitosan 1% w/v treatment (commercial formulation, ChitoPlant)

Thus, due to the cytotoxic effect observed on tomato cotyledons (cell death brownish spots) after Chitosan1 (ChitoPlant) (Picture 2) when applied at high concentrations (1% w/v), 4-week-old *Solanum melongena* cv. Black beauty plants were foliar treated with water solution (Control); and ChitoPlant at 0.01%, 0.1% and 1% w/v. 4 days after treatment, plants were visually assessed for cytotoxic effects on the leaves surface in order to determine whether ChitoPlant has a phytotoxic effect in a concentration-dependent manner on aubergine plants, Highest concentrations of chitosan (1% and 0.1 % w/v) showed cytotoxic effect on treated and systemic leaves (Picture 4). However, this effect was decreasing with the concentration and had no visual effects on the lowest concentration (0.01% w/v). Thus, it seems to be a

threshold concentration able to switch cell-death (cytotoxicity) in aubergine plantlets (Picture 3) as well as tomato. Thus, lower concentrations of chitosan were used in subsequent experiments of chitosan-IR on tomato and other plants.



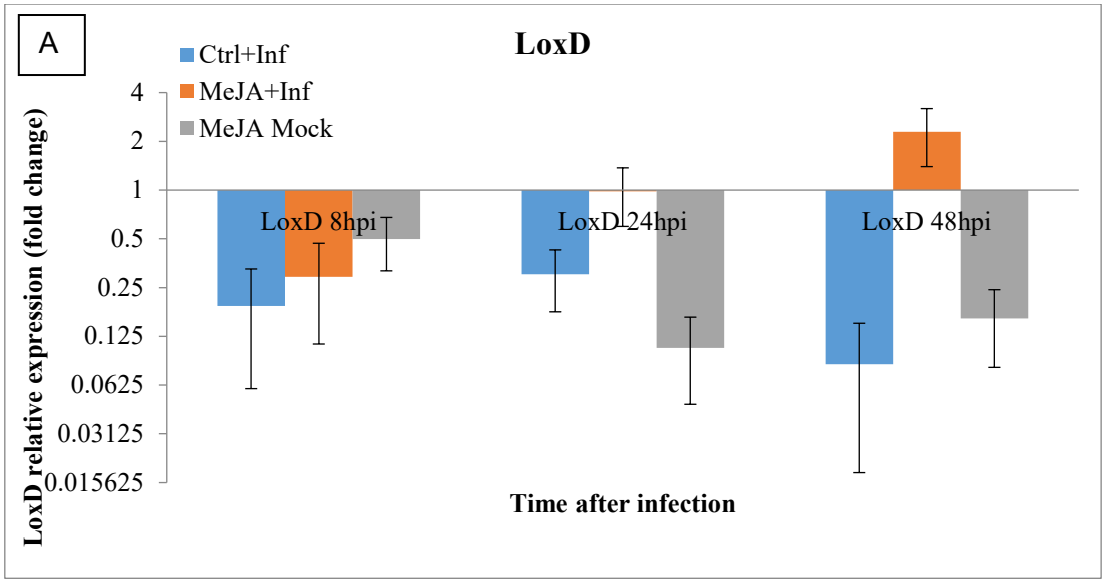
Picture 3. Cell death/phytotoxic response in the leaves of 4 weeks-old aubergine plantlets 4 days after (from left to right) chitosan 1%, 0.1%, 0.01% w/v and ddH_2O treatment (commercial formulation, ChitoPlant).

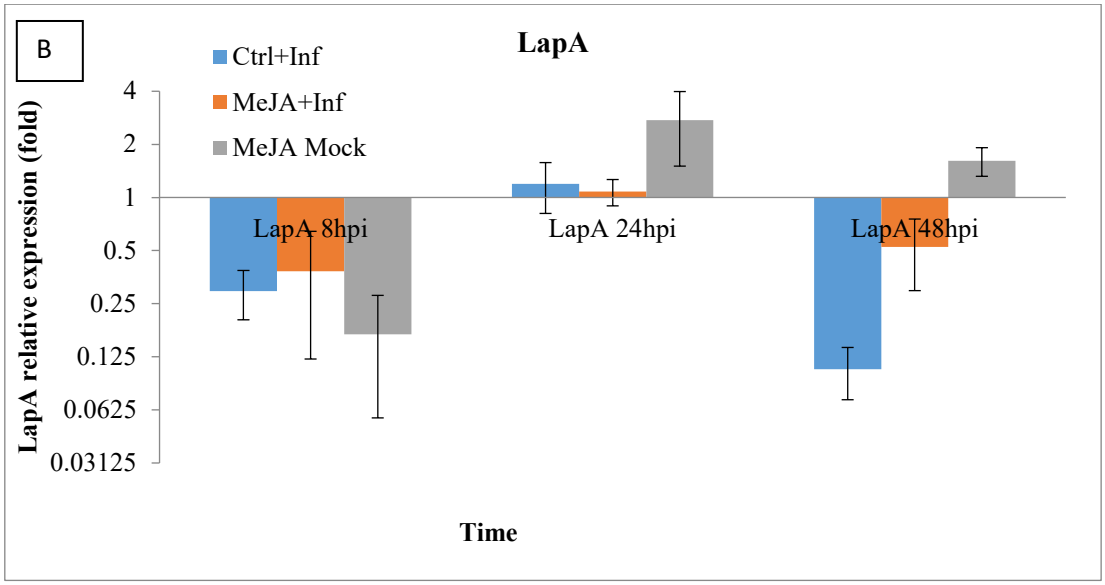
MeJA-priming of defence marker genes in tomato against *Botrytis cinerea*

Gene expression (qPCR) analysis of two tomato defence genes, Leucine aminopeptidase (Lap) A, a gene involved the plant-defence response against mechanical wounding, insect infestation, and in response to pathogen infection (Pautot, Holzer, Chaufaux, & Walling, 2001); and lipoxygenase (Lox)D, a gene involved in jasmonic acid defences and biosynthesis (Scranton, Fowler, Girke, & Walling, 2013), the main defence phytohormone against necrotrophic pathogens such as *Botrytis cinerea* was investigated.

LapA and LoxD transcript levels showed that both genes were repressed by both of the infected treatments at 8 hpi (Figure 13A). However, LoxD transcript was not down-regulated at 24 hpi and up-regulated at 48 hpi only by MeJA+ infected plants whilst MeJA mock plants and water-treated (control) + infected plants did not induce it. In contrast, LapA transcript levels were up-regulated by MeJA without pathogen and both infected treatments were not able to induce it at 24 hpi and 48 hpi (Figure 13B). MeJA mock plants were the only treatment that kept LapA expressed after 48 h while it was down-regulated by the infection treatments, being MeJA able to reduce LapA repression by the pathogen at both last time points (Figure 13B).

In order to test whether *Botrytis cinerea* is able to manipulate the antagonistic cross-talk between JA-SA pathways through NPR1 (El Oirdi *et al.*, 2011), NPR1 expression was tested. NPR1 expression did not differ among treatments although there was a down-regulation in MeJA-infected plants at 8 hpi whereas control-infected plants did not down-regulated it, and an up-regulation in MeJA-infected plants at 24 and 48 hpi (Figure 13C).





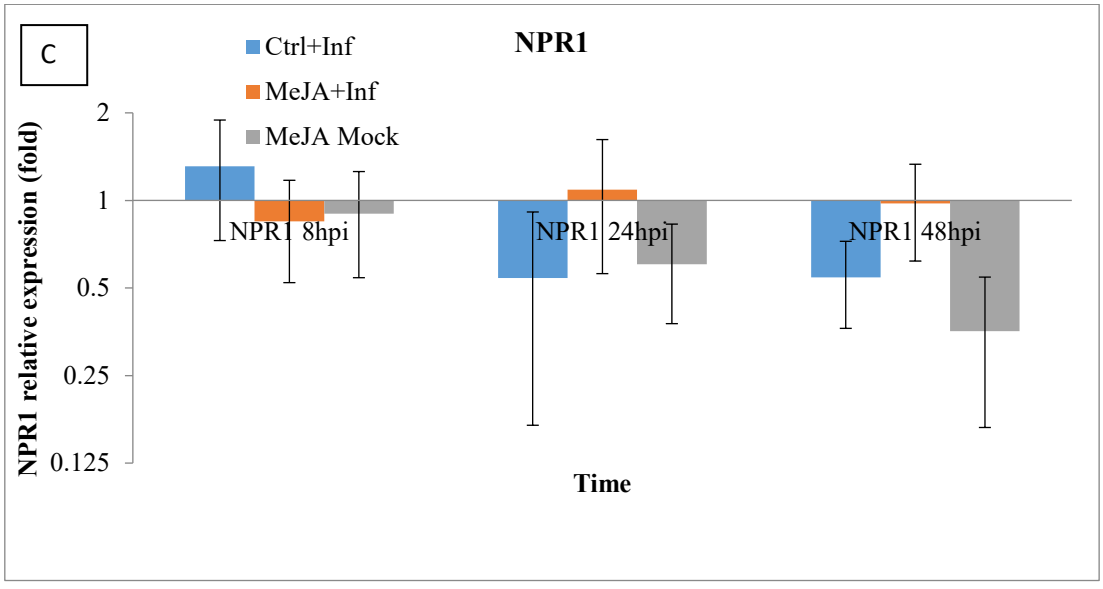


Figure 13. A. LoxD, B. LapA and C. NPR1 relative expression (fold change, Log2). Total RNA was extracted, and the levels of LoxD, LapA and NPR1 were determined by qRT-PCR. Ctrl+Inf, untreated and Botrytis-infected plants; MeJA, MeJA-treated and non-infected plants, MeJA+Inf, MeJA-treated and infected plants. EF1-α served as internal reference. The data shown belongs to the relative gene expression to the control-water mock treatment, means of three biological replicates for the qRT-PCR ± standard error of the mean (SEM).

Elicitor direct induction of SA & JA-defence genes in tomato against *Botrytis cinerea*

Pathogenesis-related protein 1 (PR-1) is one of the plant defence genes that has been extensively used as a marker for salicylic acid (SA)-mediated defence and systemic and local acquired resistance in various model plants (Laird, Armengaud, Giuntini, Laval, & Milner, 2004), such as *Solanum lycopersicum, Nicotiana tabacum* and *Arabidopsis thaliana* (Cohen, 2002). Among the defensive chemicals that are synthesized in response to either herbivore or pathogen attacks are proteinase inhibitor (PIs) proteins (Farmer & Ryan, 1990) that act as anti-nutritive defence compounds (Pluskota, Qu, Maitrejean, Boland, & Baldwin, 2007b). Also, wound-inducible proteinase inhibitors (PIs) in tomato plants provide a useful model system to elucidate the signal transduction pathways that regulate systemic defence response(Sun, Jiang, & Li, 2011).

As seen in the previous experiment, MeJA is able to prime Jasmonic acid dependent- LoxD (Figure 13) transcript against *Botrytis cinerea*. LoxD is a gene involved in the jasmonic acid biosynthetic pathway (reference). Thus, I decided to evaluate the contribution of BTH, BABA, MeJA, Sofguard (chitosan + chitin solution, Travena) in the expression profile of two tomato marker defence genes belonging to jasmonic and salycilic acid pathways respectively (PI I or Pin1 and PR-1).

Tomato cv. Moneymaker plants were foliar sprayed with ddH_2O (control), BTH, MeJA, BABA and Softguard and leaf tissue was harvested at 3, 9 and 24 hours after treatment for total RNA extraction and subsequent qRT-PCR was performed to test gene expression of PR1 and Pin1 (PI I).

Results showed that tomato SA-dependent PR1 was highly induced at 3 hours after treatment (hat) of BABA treatment in comparison with the other treatments (Figure 14). PR1 remained down-regulated at 9 hat in all treatments whilst expectedly, at 24 hat only BTH-Bion and BABA induced PR1 (Figure 14). Interestingly, the chitin + chitosan elicitor (Softguard) down-regulated or didn’t induce PR-1 at all time points.

JA-dependent tomato proteinase inhibitor I (PI I/Pin1) was induced at 3 hat after MeJA and BABA treatments in comparison with the other treatments (Figure 14). Interestingly, PI I was down-regulated by BABA at 9 hat and again up-regulated at 24 hat. The JA-positive control methyl jasmonate (MeJA) highly induced PI I at both time points and the chitin + chitosan commercial elicitor (Softguard, Travena) was able to induce PI I at both time points. PI I expression remained low in BTH-treated plants, however water-treated control plants induced PI I after 24 hours.

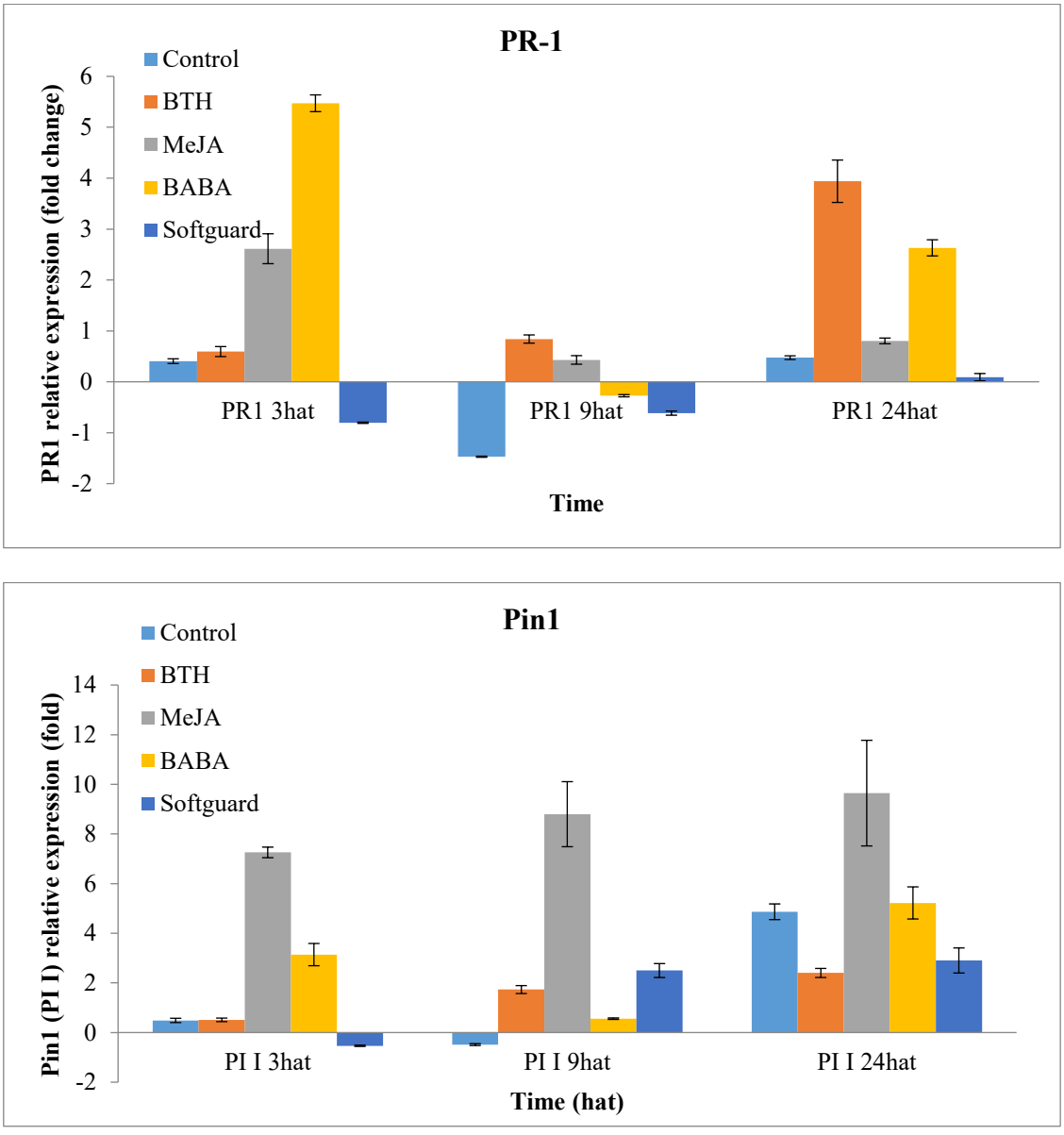


Figure 14. Expression levels of tomato SA-dependent PR-1 and JA- protein inhibitor Pin1 (also PI-I) relative to Actin (reference gene). 4 weeks-old plants were treated with BTH, MeJA, BABA, Softguard (chitin+chitosan) and water (control); Samples (leaves) were harvested at 3 time points (3h, 9h and 24 hours after treatment) for RNA extraction. qRT-PCR was performed with specific primers for tomato PR-1, Pin1 (PI I) and Actin (reference control gene) as described in Methods. Values represent means relative to Actin and 3 hours control treatment ±SD from three biological replicates.

PhD Years 2 & 3

Introduction

Chitosan is a polymeric and deacetylated derivative of chitin that is naturally present in arthropod shells and fungal cell walls. Chitosan has beneficial properties, including antimicrobial activity (Romanazzi, Murolo, & Feliziani, 2013), biocompatibility and biodegradability (Anusuya & Sathiyabama, n.d.), which makes it a perfect candidate for extensive applications in pharmacy, medicine, agriculture, food and textile industries, cosmetics, and wastewater treatment (Younes et al., 2014). Chitosan was approved by the European Commission as a "basic substance" in 2014 making it a great candidate for crop protection. Chitosan acts as a Pathogen-associated molecular pattern (PAMP) and is able to trigger a broad spectrum, long lasting and systemic immunity (systemic acquired resistance, SAR) by binding to a plant PRR (Iriti & Faoro, 2008). Chitosan can behave as a general elicitor, directly inducing systemic resistance or through priming the plant for a more efficient defence response upon pathogen attack (Iriti & Faoro, 2009).

Chitosan biological activity depends on the host, its degree of deacetylation, its concentration (Iriti & Faoro, 2009), and the chemical composition of the substrates (El Hadrami et al., 2010). The diverse mechanisms of action of chitosan have been studied, which include activation of antifungal proteins (Muñoz & Moret, 2010), oxygen-species scavenging and antioxidant activities, as well as the octadecanoid pathway activation (El Hadrami et al., 2010).

Despite these studies, experiments that specifically address the role of priming in the complex chitosan-plant interaction framework are still lacking. PhD Year 1 experiments showed that among all elicitors analysed, chitosan (ChitoPlant) induced resistance in tomato cv. Moneycv. Moneymaker and Motelle against *Botrytis cinerea* and induced callose deposition in tomato cv MM and Motelle. Furthermore, ChitoPlant-IR didn't have a negative effect on tomato cv. Moneymaker and Motelle growth and more interestingly, is seems that ChitoPlant-induced resistance does not depend on tomato cultivar as it was shown that ChitoPlant significantly decreases Botrytis lesion expansion similarly in both varieties.

Thus, due to the interesting results of Chitoplant (commercial chitosan formulation, ChiPro) previously observed, as well as the novelty of the complex interaction of chitosan-plant-*Botrytis cinerea* and the potential inclusion of chitosan in IPM strategies, the following research aims to investigate the role of chitosan in priming *Solanum lycopersicum*, *Arabidopsis thaliana*, *Solanum melongena* and *Nicotiana benthamiana* plants for a faster, stronger, fine-tuned resistance to the fungal aggressive necrotroph *Botrytis cinerea*.

Material and Methods

For PhD Year 1 long-lasting elicitor-defence induction experiments, tomato seeds were placed in petri-dishes containing wetted tissue paper, and maintained at 28°C in the dark for 3 days, to stimulate germination. Germinated seeds were planted in plant cell propagators containing Scott's M3 (pesticide-free) soil and cultivated under controlled standard growth conditions (16h- 8h/ day- night cycle; 23°C/ 20°C) until experimental procedures.

For the rest of the experiments, tomato and Aubergine seeds were placed into propagators containing Bulrush soil and a layer of vermiculite on the top and left into incubator at 20 °C for 1-2 weeks depending on the crop/variety. Germinated seeds were transplanted to individual pots containing pesticide-free compost (non-intercept) and grown in growth cabinets/glasshouse cubicles under controlled standard growth conditions (16h- 8h/ day-night cycle; 23°C/ 20°C) until experimental procedures.

Basal callose deposition induced by chitosan-IR in tomato cv. Moneymaker

In order to test whether chitosan (Chitoplant, ChiPro) induces the deposition of callose in *Solanum lycopersicum* 4 weeks-old plants were treated with ddH_2O-mock solution, 0.001%, 0.01% and 0.1% w/v of Chitoplant (in 0.01% Tween 20) by spraying the solution onto the plants. Plant material was collected at 3, 6 hours, 3 and 5 days after treatment (hat, dat), fixed in 96% ethanol before Aniline Blue staining as described previously (Luna et al., 2011). and callose deposition was analysed with an epifluorescence microscope with UV filter (BP 340 to 380 nm, LP 425 nm). Callose was quantified from digital photographs by the number of white pixels (callose intensity) or the number of depositions relative to the total number of pixels covering plant material, using ImageJ software.

To test whether chitosan (Chitoplant, ChiPro) induces the deposition of callose in *Arabidopsis thaliana* Col-0 were mass-seeded on soil (Sheffield compost), grown in cabinet 201 and cultivated under Arabidopsis standard growth conditions (8h-day (21ºC) and 16h-night (18ºC) cycle at ~60% relative humidity (RH). Ten day-old plants were transplanted to another pot with a total of 5 plants per pot. 6 week-old plants were treated with ddH_2O-mock solution, 0.01%, 0.1% and 1% of ChitoPlant (in 0.01% Silwet) by spraying the solution onto the plants. At one and two days after treatment, plant material was collected in 96% ethanol and callose deposition was analysed as above.

Chitosan and MeJA peroxidase (POD) activity and H_2O_2 production induction against *Botrytis cinerea*

4 weeks-old tomato cv. Moneymaker seedlings (3 plants per cultivar) were foliar sprayed with ddH_2O + 0.01% Tween 20 (surfactant); chitosan (ChitoPlant) 0.01% w/v + 0.01% Tween 20 (surfactant), MeJA (0.1 mM) (this time ethanol wasn't added to dissolve MeJA) + 0.01% Tween 20 and a combination of chitosan+MeJA (0.01% w/v + 0.1 mM) + 0.01% Tween 20. Last, the abscisic acid-deficient *sitiens* tomato mutant, known to highly induce POD activity, was used as a positive control. Extracellular peroxidase activity was examined with the tetramethylbenzoidine (TMB) assay described by Ros Barcelo (1998). Briefly, 4 days after treatment, tomato leaves were excised for infection assay (PhD Year 1 Material &

Methods) and detached leaves were inoculated with two 8-uL droplets of *B. cinerea* (BcR16 strain) spore inoculum (2*10^4 spores*ml^{-1}) (infected) or with the water/control (mock) solution. Finally, tomato leaf discs were harvested surrounding the infection site and fixed in ethanol at 6, 24 hpi (asymptomatic Botrytis infection stage) and 48 hpi.

After subsequent washing in distilled water, the discs were incubated in 1.5mL of 50mM Tris-acetate buffer (pH 5.0) containing 0.1mg/mL TMB and 0.03% H_2O_2 for 20 min. Peroxidation of the TMB molecule resulted in blue discoloration of both leaf tissue and incubation solution. Peroxidase activity of the discs was determined by measuring the absorbance of the incubation solution at 654 nm by mass spectrophotometry.

<u>Chitosan and MeJA may reduce *Botrytis cinerea*-triggered oxidation burst in tomato through localizing H_2O_2 production to infection sites</u>

In order to investigate chitosan and MeJA roles in H_2O_2 production after pathogen challenge, 4 weeks-old tomato cv. Moneymaker seedlings (3 plants per treatment) were foliar sprayed with ddH_2O + 0.01% Tween 20 (surfactant); chitosan (ChitoPlant) 1% w/v + 0.01% Tween 20 (surfactant) and MeJA (0.1 mM) (this time ethanol wasn't added to dissolve MeJA) + 0.01% Tween 20. 4 days after elicitor treatments, tomato leaves were detached (Chapter 2) and infected with two 6-uL droplets of *B. cinerea* spore inoculum (2*10 exp4 spores*ml^{-1}).

To evaluate temporal evolution of H_2O_2 accumulation, infected leaves of the different treatments were sampled and dyed at 24 and 48 hpi by using 3,3'-diaminobenzidine (DAB) staining. In this protocol, brown precipitates are formed at the sites of H_2O_2 accumulation (Thordal-Christensen et al., 1997; Bozo et al 2005).

Chlorophyll was removed from the leaf tissue samples with 96% ethanol to evaluate temporal evolution of H_2O_2 accumulation. Leaves were also infiltrated with H_2O_2 solution and ddH_2O as positive and negative controls respectively.

Chitosan-induced resistance in tomato, thale cress (*Arabidopsis thaliana*) and aubergine against *Botrytis cinerea* by significantly decreasing necrotic lesion expansion

To determine whether chitosan (Chitoplant, ChiPro) induces resistance in thale cress (*Arabidopsis thaliana*) against *Botrytis cinerea; Arabidopsis thaliana* Columbia-0 plants were mass-seeded on soil (Sheffield compost), grown in cabinet and cultivated under Arabidopsis standard growth conditions (8h-day (21ºC) and 16h-night (18ºC) cycle at ~60% relative humidity (RH). Ten day-old plants were transplanted to another pot with a total of 5 plants per pot. 5-week-old Columbia-0 *Arabidopsis* plants were treated, 4 days prior fungal infection, with ddH_2O solution, 0.01%, 0.1% and 1% of chitosan (in 0.01% Silwet L-77 as an adjuvant) by spraying the solution onto the plants. 4 days after treatment plants were infected with *Botrytis cinerea* as described in the Lancaster protocol with major modifications (Infection/Pathogenicity Assay). Infection was scored at 3 and 4 days after inoculation by measuring the diameter of the lesions with an electronic ruler.

To determine whether chitosan (Chitoplant, ChiPro) induces resistance in aubergine against *Botrytis cinerea*, 4-week-old aubergine (*Solanum melongena*) cv. Black beauty plants were treated, 4 days prior fungal infection, with ddH_2O solution, 0.01%, 0.1% and 1% of chitosan (in 0.01% Tween20) by spraying the solution onto the plants. 4 days after treatment, 2-3 leaves per plant were excised and subsequently infected with a spore solution of *Botrytis cinerea* ($2*10^4$ spores/ml) by drop inoculation as described in the Lancaster protocol with major modifications (Infection/Pathogenicity Assay). Infection was scored at 3 and 4 days after inoculation by measuring the diameter of the lesions with an electronic ruler.

To determine the benefits of ChitoPlant (chitosan) induces resistance in tomato against *Botrytis cinerea*, 4-5-week-old tomato cv. Moneymaker plants were treated, 4 days prior fungal infection, with ddH_2O solution, 0.01%, 0.1% and 1% of chitosan (in 0.01% Tween20) by spraying the solution onto the plants. 4 days after treatment, 2-3 leaves per plant were excised and subsequently infected with a spore solution of *Botrytis cinerea* ($2*10^4$ spores/ml) by drop inoculation as described in the Lancaster protocol with major modifications (Infection/Pathogenicity Assay). Infection was scored at 3 and 4 days after inoculation by measuring the diameter of the lesions with an electronic ruler.

Pathogenicity/Infection assays:

4-5 weeks-old active Botrytis cinerea hypha growing into potato dextrose agar (PDA) plates were kept in the dark at room temperature. Once *Botrytis cinerea* was sporulating, 20ml of ddwater (dd for distilled) with 0.01% Tween 20 (adjuvant) was added to the plate and it was subsequently scratched with a spatula to release and harvest spores. Spore concentration

was then counted with a cell counter Haemocytometer and adjusted to $2*10^4$ spores/ml. As a final inoculum solution, 3.3 ml of 1M glucose (freshly prepared/autoclaved) + 2.2 ml of 0.1M KH2PO4 (pH 5) (freshly prepared/autoclaved) were added and the incubation time was reduced to 10-15 min in order to decrease the virulence of the fungal strain.

Chitosan-induced resistance in Arabidopsis Col-0 against *Hyaloperonospora arabidopsidis*

To determine whether chitosan induces resistance in Arabidopsis (thale cress) against oomycete plant pathogens, 5 week-old *Arabidopsis thaliana* Col-0 plants were infected with the biotrophic pathogen H. arabidopsidis,(Hpa) treated with ddH2O solution or 0.01% of Chitoplant (in 0.01% silwet) by spraying the solution onto the plants. 4 days after Chitoplant treatment, plants were infected with Hpa by spraying an inoculum containing $1*10^5$ spores/ml. Disease was scored at 5 days by classifying trypan blue-stained leaves in different categories of disease colonization. Trypan blue stains dead cells and fungus/oomycete mycelium and spores. Leaf samples were submerged in trypan blue stain (50 ml Falcon tube), boiled for 1-2 minutes. Cooled trypan blue stain was poured away and washed once with SDW (sterile distilled water). Leaves were submerged in chloral hydrate and de-stained overnight. One day after, leaf samples were mounted on slides using 60% glycerol for microscopy visualization.

Chitosan direct fungicide effect on *Botrytis cinerea* spore germination and hypha growth: Concentration matters

In order to investigate the potential direct fungicide effect of chitosan against *B. cinerea*, the effect of chitosan (Chitoplant, Chipro) on fungal mycelial growth and spore germination of *Botrytis cinerea* was assessed in vitro using potato dextrose agar (PDA) as culture media amended with different concentrations of chitosan (1, 0.1, 0.05, 0.01 % w/v; g* $100mL^{-1}$). PDA was autoclaved and then ChitoPlant (water soluble chitosan) and fungicide Switch (as positive fungicide control) (1, 0.1, 0.05, 0.01 % w/v; g* $100mL^{-1}$) amounts were added directly to the PDA. Solutions were shacked till visually dissolved and then 15ml was added per petri dish (plates). Once media + chitosan/switch solutions were cooled down, 5 mm agar plug of actively growing *Botrytis cinerea* (BcR16 strain) mycelia was added per plate (5 plates per treatment) to test ChitoPlant fungicide effect on Botrytis mycelia growth. Finally, a 15ul droplet of *Botrytis cinerea* spores ($2*10^4$ spores*ml^{-1}) was added per plate (5 plates per treatment) as well to test effect chitosan on spore germination. Plates were covered with parafilm and then incubated under controlled conditions (darkness and 24°C). PDA itself was used as a regular fungal growth condition control. After incubation for 4 days, the mean radial

growth of the fungus was determined by measuring the fungal colonies in two perpendicular diameters and calculating the mean diameter.

Large scale transcriptome analysis (Microarray) on chitosan-treated and infected *Solanum lycopersicum* (tomato) with *Botrytis cinerea*

In order to evaluate whether that chitosan at low concentration (0.01% w/v) functions as a priming agent in tomato against *Botrytis cinerea* and therefore it can prime molecular pathways and specific genes that might be involved in the chitosan-IR phenotype a large-scale microarray gene expression study was performed in order to obtain information about the molecular mechanisms underlying chitosan-IR. This study reports the defence gene expression pattern of chitosan-primed and Botrytis-inoculated tomato plants.

Tomato cv. Moneymaker seeds were placed into propagators containing Bulrush soil and a layer of vermiculite on the top and left into incubator at 20 °C for 1-2 weeks till germination. Germinated seeds were transplanted to individual pots containing pesticide-free compost (non-intercept) and grown in growth cabinets under controlled tomato standard growth conditions (16h- 8h/ day- night cycle; 23°C/ 20°C) until experimental procedures.

4-week-old tomato cv. Moneymaker plants were treated, 4 days prior fungal infection, with ddH_2O solution and 0.01% of chitosan (in 0.01% Tween20) by spraying the solution onto the plants. 4 days after treatment, 1-2 whole leaves per plant were excised and subsequently infected with a spore solution of *B. cinerea* ($2*10^4$ spores/ml) or ddH_2O mock by drop inoculation (Figure 1). Furthermore, four conditions were analysed: non-treated (water) non-infected control plants (watermock), non-treated (ddH_2O) and Botrytis-infected plants (WaterInf or Inf), chitosan-treated and non-infected/mock plants (ChitoMock), and chitosan-treated and infected plants (ChitoInf) (Figure 1). Leaf discs from four plants per treatment (4 biological replicates) were sampled for total RNA extraction at three early (asymptomatic) Botrytis infection stages (6, 9 and 12 hpi). RNA was extracted with RNeasy Plant MiniKit (Qiagen) and RNA quality was confirmed by Bioanalyser and RNA electrophoresis. Once RNA area, concentration, and RNA integrity number (RIN) was confirmed, samples were processed by the Microarray and Sequencing department of The James Hutton Institute. The transcriptomic analysis was designed with a joint 60k Agilent array design made with 16.365 *Botrytis cinerea* probes and 34.510 *Solanum lycopersicum* probes (Figure 1).

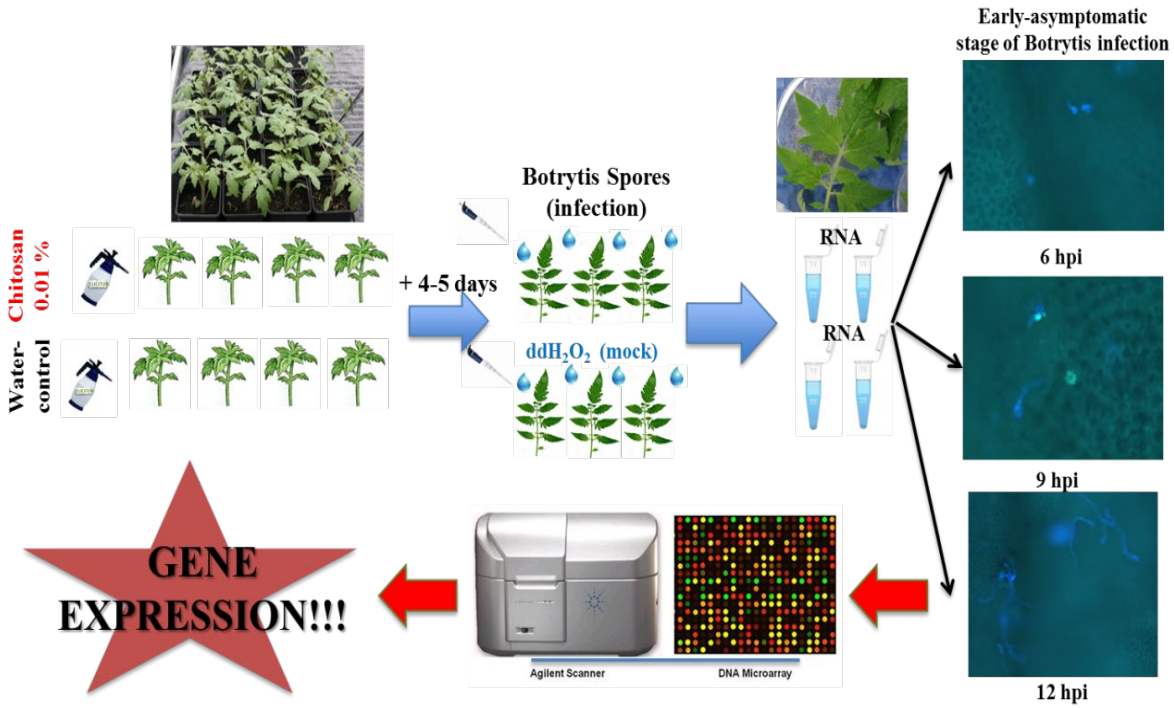


Figure 1. Experimental design of the Transcriptomic analysis (Microarray) of chitosan-primed and Botrytis-inoculated tomato plants. Four conditions: non-treated (water) non-infected plants (Ctrl), non-treated (water) and Botrytis-infected plants (Inf), chitosan-treated and non-infected/mock plants (ChitoMock), and chitosan-treated and infected plants (ChitoInf). 4 days after treatment (dat) leaf discs from four plants per treatment were sampled at three early (asymptomatic) time points; 6, 9 and 12 hours post-inoculation (hpi) of Botrytis spores or water-control mock

Microarray Data Analysis and Bioinformatics

Data was pre-processed (Quality Control) as follows. Quality control was performed in order that all 4 biological replicates behaved in a similar way. Due to the 2-colour probed array, of 34.616 *Solanum lycopersicum* probes used, gene expression data was filtered to eliminate probe sets for which the mean signal from the four replicate arrays did not exceed the minimum signal (Raw>50), which narrowed down to 20.000 transcripts from the original 34.616 tomato probes. Raw data from the 20.000 probes were normalised using GeneSpring GX (Agilent Genomics). Within the normalization process, normalized values of each of the 20.000 probes were divided to the control sample (water-treated and non-infected/mock plants) for which value was close to 1. To identify differentially-expressed genes 2-way ANOVA was performed with a cut-off at P-value ≤ 0.05 and a Benjamini Hochberg false discovery rate correction, which resulted in 3.713 differentially-expressed genes (DEGs).

Phythormone analysis on chitosan, MeJA and chitosan+MeJA-treated and infected *Solanum lycopersicum* (tomato cv. Moneymaker) with *Botrytis cinerea* by LC/MS/MS

Tomato cv. Moneymaker seeds were placed into propagators containing Bulrush soil and a layer of vermiculite on the top and left into incubator at 20 °C for 1-2 weeks till germination. Germinated seeds were transplanted to individual pots containing pesticide-free compost (non-intercept) and grown in growth cabinets under controlled tomato standard growth conditions (16h- 8h/ day- night cycle; 23°C/ 20°C) until experimental procedures.

4-week-old tomato cv. Moneymaker plants were treated, 4 days prior fungal infection, with ddH_2O solution, 0.01% of chitosan (in 0.01% Tween20), MeJA (0.1mM) (in 0.01% Tween20) and a combination of chitosan 0.01% + MeJA (0.1mM) (in 0.01% Tween20) by spraying the solution onto the plants. 4 days after treatment, 1-2 whole leaves per plant were excised and subsequently infected with a spore solution of *Botrytis cinerea* (BcR16 strain) by droplet infection ($2*10^4$ spores/ml) and/or water inoculation as a control (mock). Tomato leaf tissue was harvested at 6, 9 and 24 hours after infection/inoculation and freeze in liquid nitrogen. Tissue was then stored at -80°C until freeze dry. Freeze dried samples were grinded (adding a tungsten ball) into a beat beater. 10 mg of each sample was used for hormone extraction. Hormones (JA,SA and ABA) were extracted following (Forcat, Bennett, Mansfield, & Grant, 2008) protocol. Briefly 10 (+/- 0.1) mg freeze dried leaf powder was accurately weighed and extracted in 2 x 0.4 ml 10% methanol containing 1% acetic acid. Two extraction stages were performed to ensure effective extraction of hormones. For accurate quantification, solvent A also contained the following amounts of stable isotope labelled hormone standards: 20ng 2H6 salicylic acid (SA) (C/D/N Isotopes, Quebec, Canada), 10ng 2H5 jasmonic acid (JA) (C/D/N Isotopes, Quebec, Canada) and 10ng 2H6 abscisic acid (ABA) (OlChemlm, Czech Republic). Samples were vortexed every 10 minutes, for 30 minutes. Each step involved incubation on ice followed by 15 minutes sonication in an ice water bath. Supernatants from both extractions were pooled after centrifugation (10 min at 16,100 x g, 4 °C) followed by filtering through a 0.4 µm (RC) syringe filter (Phenomenex, UK). Finally, quantitative analysis of plant hormones was performed using an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Technologies, Palo Alto, USA) hyphenated to a 1200 series Rapid Resolution HPLC system. 5 µl of sample extract were loaded onto an Eclipse Plus C18 3.5 µm, 2.1 x 150 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA).

Mass-spectrophotometer data analysis

Data analysis was undertaken using Agilent Mass Hunter Quantitative analysis software for QQQ (Version B.07.01). Accurate quantification of ABA, SA and JA used the deuterated internal standards added during sample extraction (Plant Methods 2008, 4:16 and Plant, Cell

and Environment 2011, 35:2, pp 388–404). For the other compounds normalised peak areas were compared and concentrations were calculated using standard concentrations curves.

Functional analysis: Transient overexpression of ACRE75 and ACRE180 on *Nicotiana benthamiana*

Transient overexpression approach was performed under Gateway compatible plasmids (destination vector pB7WGF2-GFP). Tomato SlACRE75, SlCARE180 and *Nicotiana benthamiana* orthologues NbACRE75 and NbACRE180 proteins were transformed into *Agrobacterium tumefaciens* GV3101 strain and they were agroinfiltrated into leaves of *N.benthamiana* plants. In order to decrease variation within plants, every construct was agroinfiltrated on the right side of the leaf whilst *Agrobacterium tumefaciens* containing pB7WGF2-empty vector was infiltrated on the left side of every leave as a non-protein negative control.

2 days after ACRE proteins agroinfiltration, leaves were excised for pathogenicity test and subsequently challenged with *Botrytis cinerea* spore inoculum ($2x10^4$ spores*ml^{-1}) by drop inoculation; lesion size measurements were annotated to look for a resistance phenotype. The experiment was repeated twice with similar results.

RESULTS

Below, summaries and main findings are discussed within the framework of the PhD project main objectives:

Early acting pathogen-inducible defence responses in tomato-*Botrytis cinerea*

As stated before (PhD Year 1), plant's early defences, such as callose, the plant polysaccharide, and reactive oxygen species (i.e. H_2O_2), can play a crucial role in reducing pathogen penetration and giving the plant "more time" to display its late acting and fine-tuned defences, such as hormone pathways and chromatin/DNA modifications (reference).

MeJA and ChitoPlant (water-soluble chitosan formulation) were able to significantly reduce botrytis necrotic lesion expansion in tomato, aubergine and the model plant *Arabidopsis thaliana* with minimal or null costs on plant yield. Furthermore, chitosan (ChitoPlant) defence induction is characterised by plant cell-wall fortification through callose deposition in tomato leaves before and after pathogen challenge (PhD Year 1).

Chitosan has been extensively utilized as a foliar treatment to control the growth, spread and development of many diseases involving viruses, bacteria, fungi and pests (El Hadrami, Adam, El Hadrami, & Daayf, 2010). Nevertheless, its concentration and physicochemical properties are critical for recognition by plant receptors and thus inducing plant resistance and determining the induction of priming or activation of plant direct defences (Iriti & Faoro, 2009). There are studies that recognize the ability of chitosan of directly inducing H_2O_2 followed by programmed cell death (PCD) in a concentration-dependent manner, for which it exists a threshold for each chitosan switching PCD to necrotic cell death (cytotoxicity) (Iriti & Faoro, 2009). Furthermore, chitosan biological activity can also depend on the host, its degree of deacetylation (Iriti & Faoro, 2009), and the chemical composition of the substrates (El Hadrami et al., 2010). Chitosan physicochemical complexity can ultimately affect plant recognition, which makes it a complex system for plant application and recognition. To ensure plant absorbance and recognition, previous experiments have shown harmful ways such as chitosan infiltration into the plant (Ahmad et al., 2011; Scalschi et al., 2015) whilst others needed complex ways to dissolve chitosan before plant application by dissolving it chitosan in acetic, glutamic, formic or hydrochloric acids (Luna et al., 2011; Romanazzi, Feliziani, et al., 2013; Yu, Li, & Zheng, 2007)

In order to achieve a more efficient defence strategy and less costly in terms of plant fitness, it is important, when using elicitors such as chitosan, to assess the effect of the concentration not only on the activation of plant endogenous defences, but also on the stress tolerance of the plant. Here, the experiments were done following a novel approach of chitosan

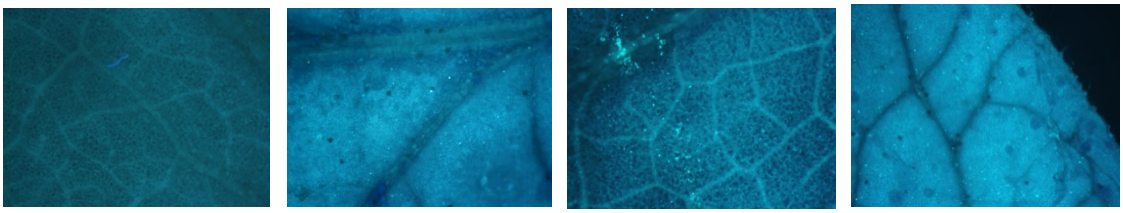
application by directly dissolving chitosan (ChitoPlant, commercial formulation) into distilled water and with the help of a surfactant (i.e. Silwet, Tween20) foliar spraying directly into the plant.
So far during the thesis experiments, chitosan treatment (ChitoPlant) has been used at high concentrations (1% w/v) which didn't have any detrimental effect on tomato growth although, as previously seen, it caused cell death on 1-2-weeks old tomato cotyledons (Picture 2).

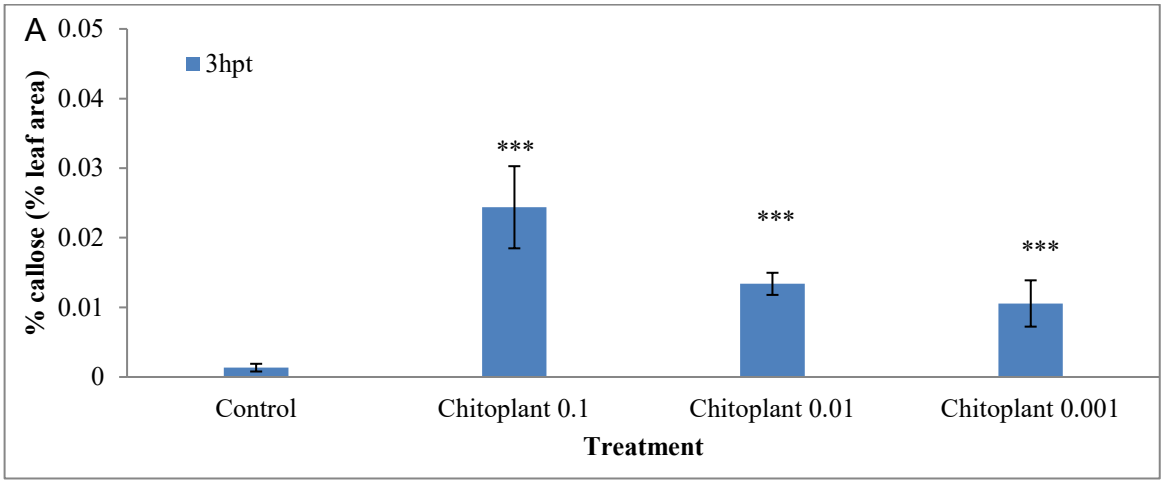
To elucidate whether ChitoPlant induces callose deposition in a concentration-dependent manner, in this study we aim to show whether different concentrations of the commercial formulation ChitoPlant can induce callose deposition in tomato cultivar Moneymaker and *Arabidopsis thaliana* (the model plant) wild type accession Col-0 in order that ChitoPlant can be used for fundamental studies.

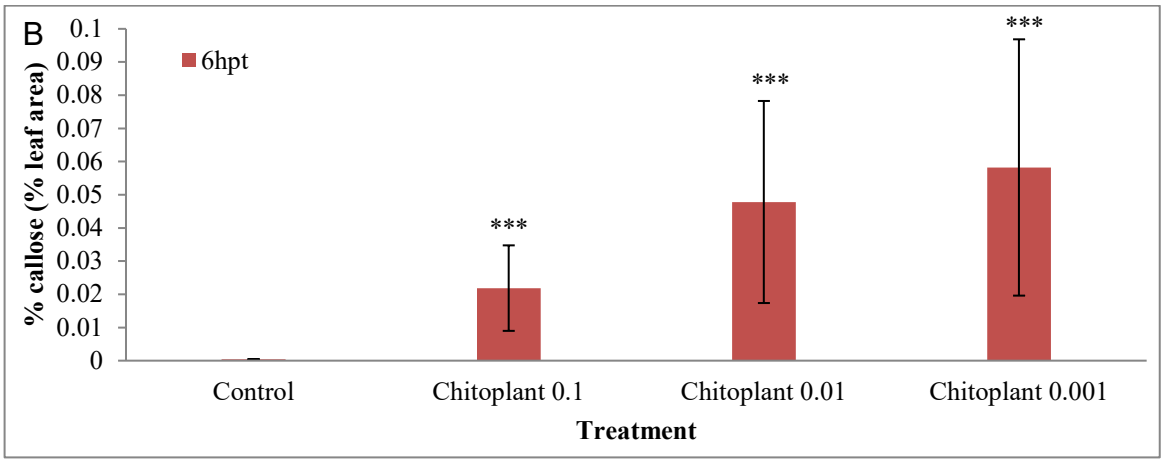
Basal callose deposition induced by chitosan in tomato cv. Moneymaker and *Arabidopsis thaliana* Col-0

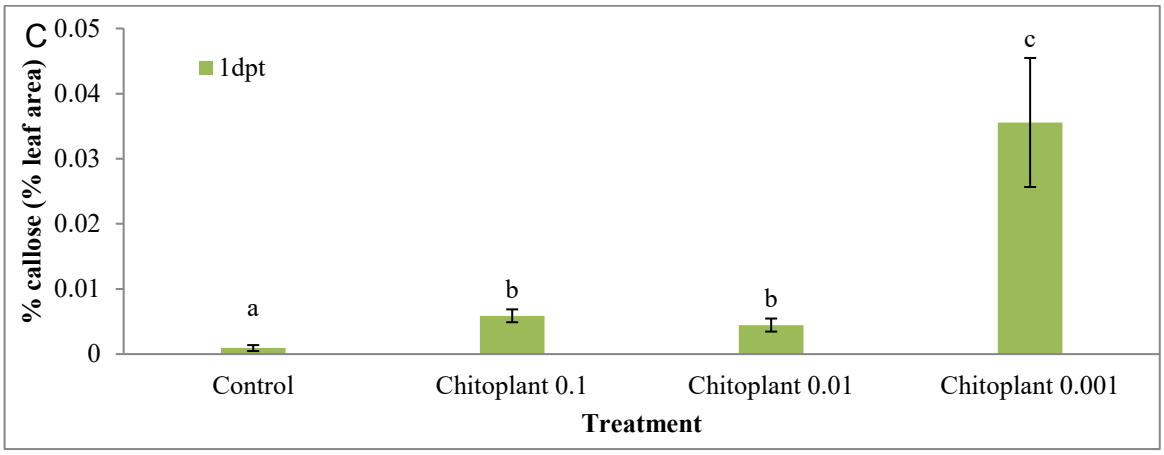
In order to test whether chitosan (Chitoplant, ChiPro) induces the deposition of callose in *Solanum lycopersicum,* plants were treated with ddH_2O-mock solution, 0.001%, 0.01% and 0.1% w/v of ChitoPlant by spraying the solution onto the plants. Plant material was collected at 3, 6 hours, 3 and 5 days after treatment (hat, dat), fixed in ethanol and callose deposition was analysed (M&M). Due to the ChitoPlant-induced necrotic cell death seen before (PhD Year 1) when applied at 1% w/v, this concentration was not included in the experiment.

Overall, during the first hours (3 and 6 hpt) and 1 day after chitosan treatment all concentrations resulted in statistically significant callose deposition whilst at later time (5 dpt) none of the concentrations displayed significant callose accumulation (P=0.06) in comparison to water/control treated plants (Figure 2D), which suggest that chitosan-induced callose decreases gradually after 1 dpt. The lowest concentration (0.001% w/v) significantly induced the highest amount of callose deposits in tomato epidermal cells at 1 dpt (Figure 2C), followed by the other 2 concentrations which were significantly higher than water-treated control plants but lower than the low concentrated 0.001% (Figure 2C).









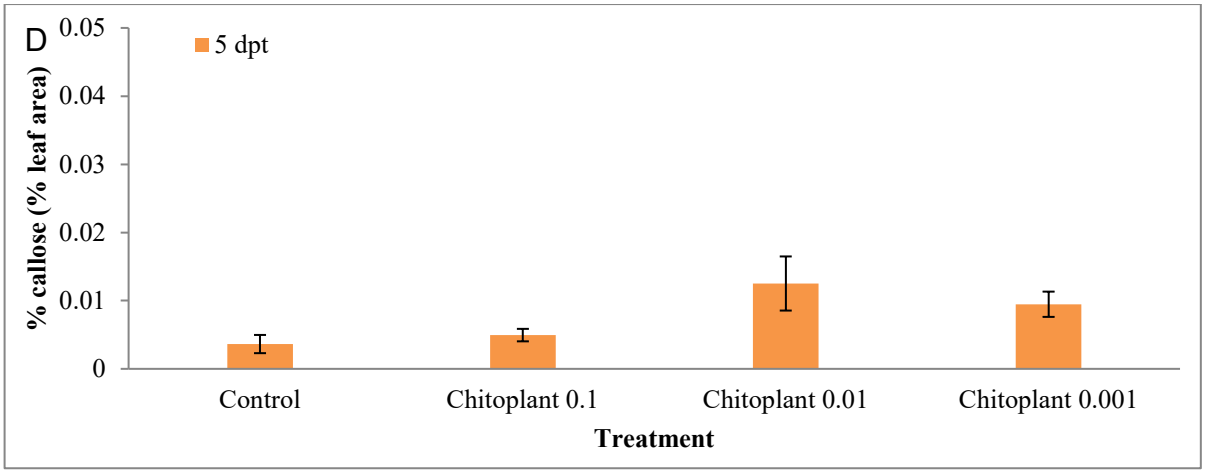


Figure 2. Basal callose deposition in tomato *Moneymaker* leaves after water-control and chitosan (ChitoPlant) treatments at 3 different concentrations. 3 and 6 hours, and 1 and 5 days after treatment leaves were harvested for Aniline Blue staining (M&M). Photographs were taken under fluorescence microscopy (bright green dots represent callose deposits in epidermal cells). Callose was quantified as described by (Luna et al., 2011) (M&M). Values represent percentages of the mean ± SEM. Asterisk indicates statistically significance between the treatment and the water control (t-test $p<0.05$) Kruskal-Wallis test (3 hpt $p< 0.001$; 6 hpt $p< 0.001$; 1 dpt $p< 0.001$; 5 dpt $p=0.06$).

Callose induction by ChitoPlant in tomato was concentration-dependent at 3 hat (Figure 2A), where the highest concentration (0.1%) induced more callose sooner and decreased faster than the lower concentrations. However it did not follow the same pattern at 6 hat and 1 dpt and 5 dpt (Figure 2B, 2C, 2D), where lowest concentrations (0.01% and 0.001%) significantly induced callose deposition for a longer period (1 dpt) and higher than the highest concentration (0.1%), indicating that elicitor-priming does not follow a classical dose-response curve and the duration of the response is affected by concentration. However, this duration may be inversely related to chitosan concentration.

Basal callose deposition induced by chitosan in Arabidopsis thaliana Col-0

To test whether chitosan (Chitoplant, ChiPro) induces the deposition of callose in *Aarabidopsis thaliana* Col-0 (wild type) plants were treated with distilled water (control), 0.01%, 0.1% and 1% w/v of Chitoplant by spraying the solution onto the plants. Plant leaves were harvested at 1 dat and callose was quantified (M&M).

Chitosan induced callose deposition in Arabidopsis but the amount varied depending on the concentration, being significantly different from water-control treatment only at 0.01% w/v one day post-treatment (Figure 3). As seen before, at high concentrations (1%w/v) chitosan (ChitoPlant) induces chunks of callose which can ultimately damage the plant (PhD Year 1). This effect lead to a big variability and affected statistical significance. Thus, although not significantly different from the other concentrations, chitosan 1% w/v induced the highest

concentration of callose followed by chitosan 0,01% w/v (lowest concentrated) (Figure 3), which suggests that there is no clear dose-response curve for such a priming agent.

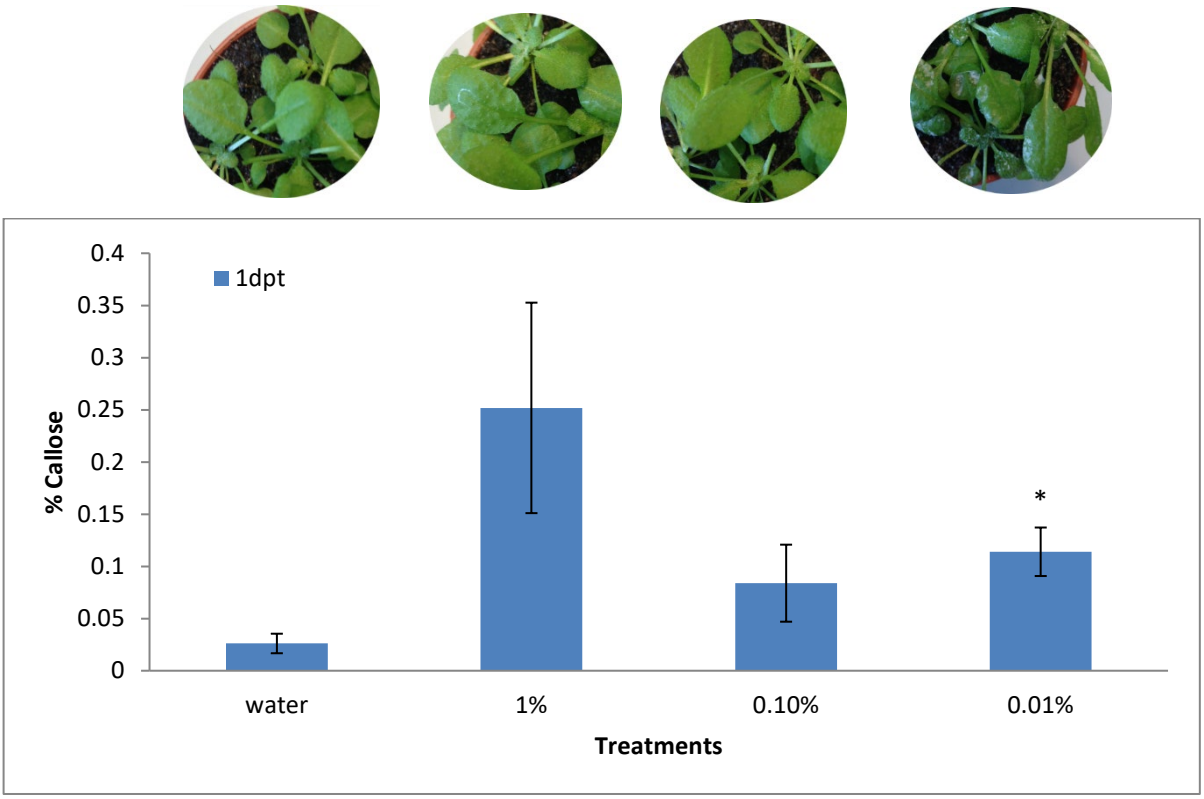


Figure 3. Basal callose deposition in *Arabidopsis thaliana* Col-0 leaves after water-control and chitosan treatments at 3 different concentrations. 1 day after treatment leaves were harvested for Aniline Blue staining. Pictures were taken under fluorescence microscopy. Callose was quantified as described by (Luna et al., 2011) (M&M). Values represent percentages of the mean ± SEM. Asterisk indicates statistically significance between the treatment and the water control (t-test $p<0.05$ Kruskal-Wallis test (1 dpt $p=0.02$))

Chitosan and MeJA peroxidase (POD) activity and H_2O_2 production induction against *Botrytis cinerea*

In the fight against pathogens, plants have developed an onset of defence mechanisms that can delay or even avoid disease in the early and crucial stages of the infection. One of the main early defences, together with callose deposition, is the production of reactive oxygen species (ROS) by the plant. However, production of ROS can be manipulated by necrotrophic fungi such as *Botrytis cinerea* in order to promote hypersensitive response (HR) and local cell-death to facilitate fungal infection (Govrin & Levine, 2000; Smith et al., 2014a). Furthermore, it's been previously shown that chitosan can induce peroxidase (POD) activity (El Hadrami, Adam, El Hadrami, & Daayf, 2010) and it's efficacy against various pathogens

and in several plants has been also proven which made it, together with MeJA, great candidates to study POD activity against *Botrytis cinerea*. Chitosan synergistic effects have also been shown, such as chitosan synergy with *Cryptococcus laurentii* on inhibition of *Penicillium expansum* infections in apple fruit (Yu et al., 2007). Thus, to elucidate whether chitosan and MeJA may prime tomato plants for peroxidase and/or H_2O_2 production against *Botrytis cinerea*:

Chitosan and MeJA may prime tomato peroxidase (POD) activity against *Botrytis cinerea* at different infection stages

The effect of chitosan (ChitoPlant formulation), MeJA and the combination of chitosan + MeJA on increasing peroxidase activity in tomato cv. Moneymaker and tomato *sitiens* (resistant mutant tomato line) after *Botrytis cinerea* infection was investigated. For that, tomato plants were foliar sprayed with the elicitors and 4 days after treatment leaf tissue discs were harvested for extracellular peroxidase activity evaluation with the tetramethylbenzoidine (TMB) assay, as described in Material & methods.

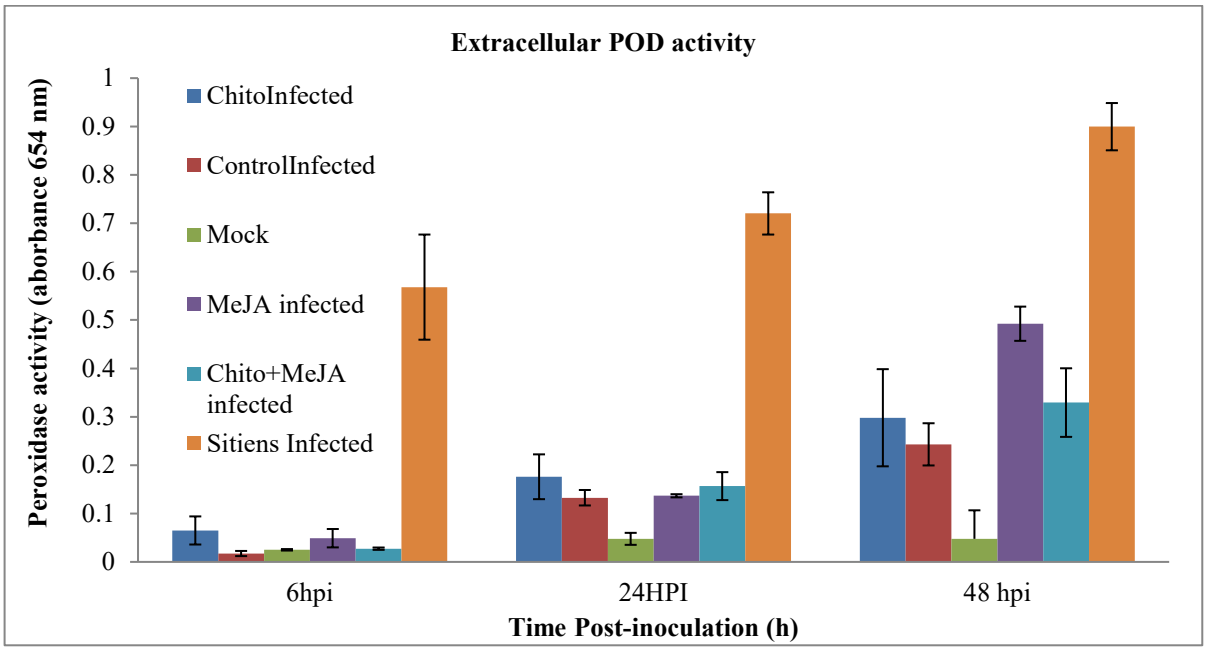


Figure 4. Extracellular peroxidase activity in tomato susceptible Money-maker-wild-type (treated with MeJA, chitosan, MeJA+chitosan and water/control) and untreated tomato ABA deficient-Sitiens (resistant mutant line to *Botrytis cinerea*) leaf discs infected with *B. cinerea*. Tissue sampling was done at 24 and 48 hours post infection (hpi). Values presented are means of 3 biological replicates ± SEM.

Results indicate that, as Botrytis infection progresses, there is a higher production of peroxidases. The resistant tomato line *sitiens* highly increased POD activity during all time points after infection (Figure 4). In tomato cv. Money-maker (susceptible wild type line) chitosan and MeJA elicitors were able to increase POD activity at all time points although not

significantly, only chitosan-treated WT plants were able to induce POD activity at early stages of the infection (6 hpi) (Figure 4). MeJA-treated WT plants were able to increase POD production at 48 hpi (when the necrotic lesions are visible) in comparison with control infected plants which suggests the potential ability of chitosan to prime tomato peroxidases at early stages of the infection, while MeJA may prime tomato peroxidases at later stages against *Botrytis cinerea*. However, the combination treatment chitosan + MeJA did not have a synergistic effect on extracellular peroxidase expression against *Botrytis cinerea* at any time point.

Chitosan and MeJA may reduce *Botrytis cinerea*-triggered oxidation burst in tomato through localizing H_2O_2 production to infection sites

Production of H_2O_2 can result from increased peroxidase activity and peroxidases mediate many H_2O_2-related defence responses (Asselbergh et al., 2007).

Thus, in order to investigate chitosan and MeJA roles in temporal evolution of H_2O_2 accumulation after pathogen challenge, 4 weeks-old tomato cv. Moneymaker seedlings were foliar sprayed with ddH_2O, chitosan (ChitoPlant) 1% w/v and MeJA (0.1 mM). 4 days after elicitor treatments, tomato leaves were detached and infected with *Botrytis cinerea*.

Infected leaves of the different treatments were sampled and then dyed at 24 and 48 hpi by using 3,3'-diaminobenzidine (DAB) staining. Results indicate that mostly in all MeJA and in some chitosan-treated leaves, H_2O_2 was contained around the local site of infection at 24 hpi and 48 hpi; however in non-treated control leaves H_2O_2 was not restricted at the infection sites and spread throughout a bigger area of the leave (Figure 5), suggesting a possible manipulation of *Botrytis cinerea* host defences as stated before (El Oirdi et al., 2011). However, in order to confirm this theory, further experiments (more time points, different chitosan concentrations and quantitative methodology) need to be done.

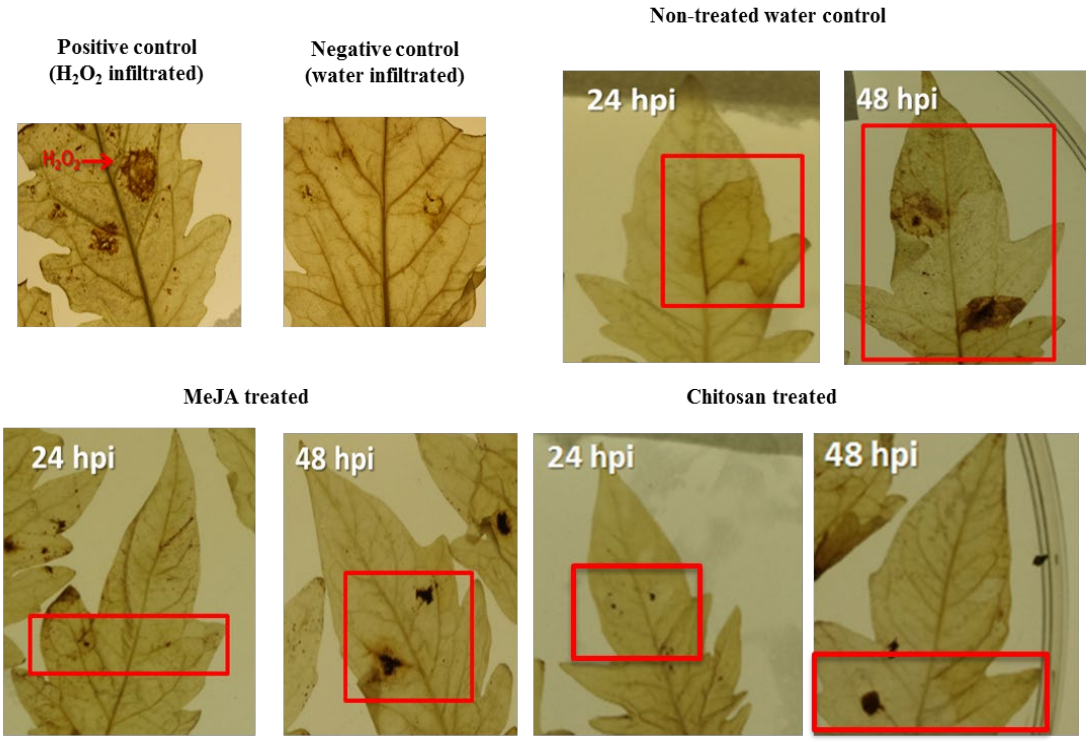


Figure 5. Temporal evolution of H_2O_2 accumulation (brown dark dots) in tomato cv. Moneymaker sprayed with either 100 uM MeJA, 1% w/v chitosan and ddH_2O as control after infection with *B. cinerea*. DAB staining of detached leaves infected with two 6 µL drops of a spore suspension was performed at different time points after Botrytis infection (24 and 48 hpi). One representative leaflet of three replicates is shown for each time point

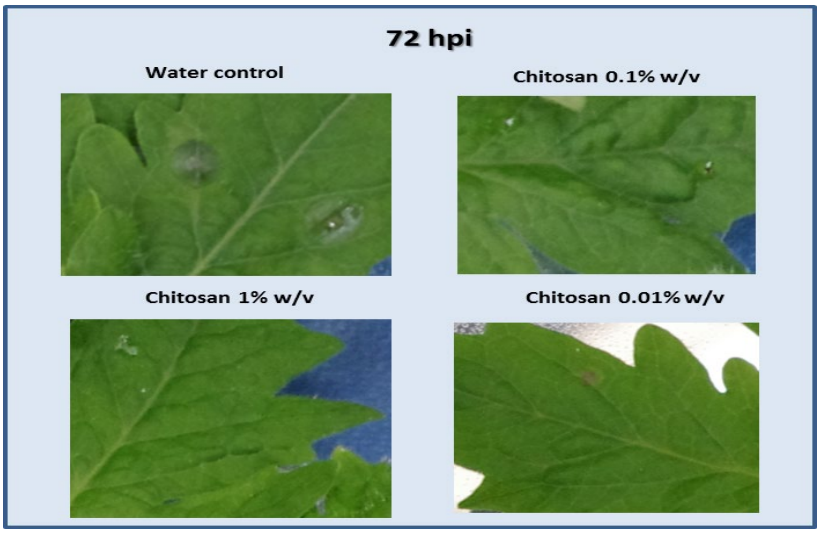
Thus, MeJA and ChitoPlant can reduce and limit pathogen infection through localizing H_2O_2 production to the infection site (Figure 5), potentially reducing pathogen manipulation of its host defences (still to be further investigated).

Chitosan protect tomato, *Arabidopsis* and aubergine against *Botrytis cinerea* by significantly decreasing necrotic lesion expansion

To determine whether chitosan (Chitoplant, ChiPro) induces resistance in a concentration-dependent manner in thale cress (*Arabidopsis thaliana*), aubergine and tomato against *Botrytis cinerea*, tomato cv. Money-maker, thale cress and Aubergine cv. Black beauty plants were treated, 4 days before fungal infection, with ddH_2O solution (as a control), 0.01%, 0.1% and 1% w/v of chitosan by spraying the solution onto the plants.

In tomato, chitosan significantly decreased necrotic lesion size against Botrytis in all concentrations compared to water-treated control plants at 3 dpi (Figure 6). Interestingly, the resistance phenotype increased with the concentration at 48 hpi; being chitosan 1% w/v the strongest resistance phenotype of the three concentrations and chitosan 0.01% the lowest level of protection although it was still significant. However this concentration-dependent

resistance differed at 72 hpi, where the lowest concentration (0.01%) was significantly smaller than the medium 0.1% (Figure 6).



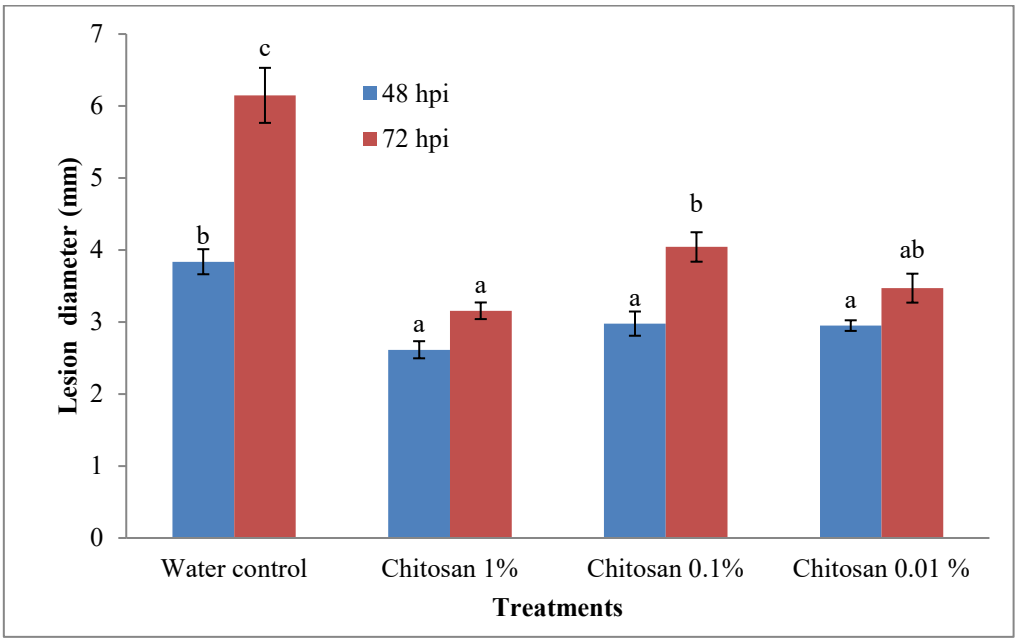


Figure 6. Quantification of chitosan-induced resistance against *B. cinerea* in tomato cv. MoneyMaker at 2 and 3 days post-inoculation. Values presented are means ± SEM. Different letters indicate statistically significant differences (Values presented are means ± SEM obtained from an ANOVA and then pairwise Fisher's protected least significant difference (LSD) test $P<0.001$, $\alpha=0.05$). One representative leaflet of eight-twelve replicates is shown for each treatment at 72 hpi.

In thale cress, chitosan induced resistance in a concentration-dependent manner; it significantly decreased Botrytis necrotic lesion size in all concentrations compared to water-treated control plants (Figure 7). The resistance phenotype decreased with the concentration,

being chitosan 1% w/v the strongest resistance phenotype of the three concentrations and chitosan 0.01% the lowest level of protection but still significant (Figure 7).

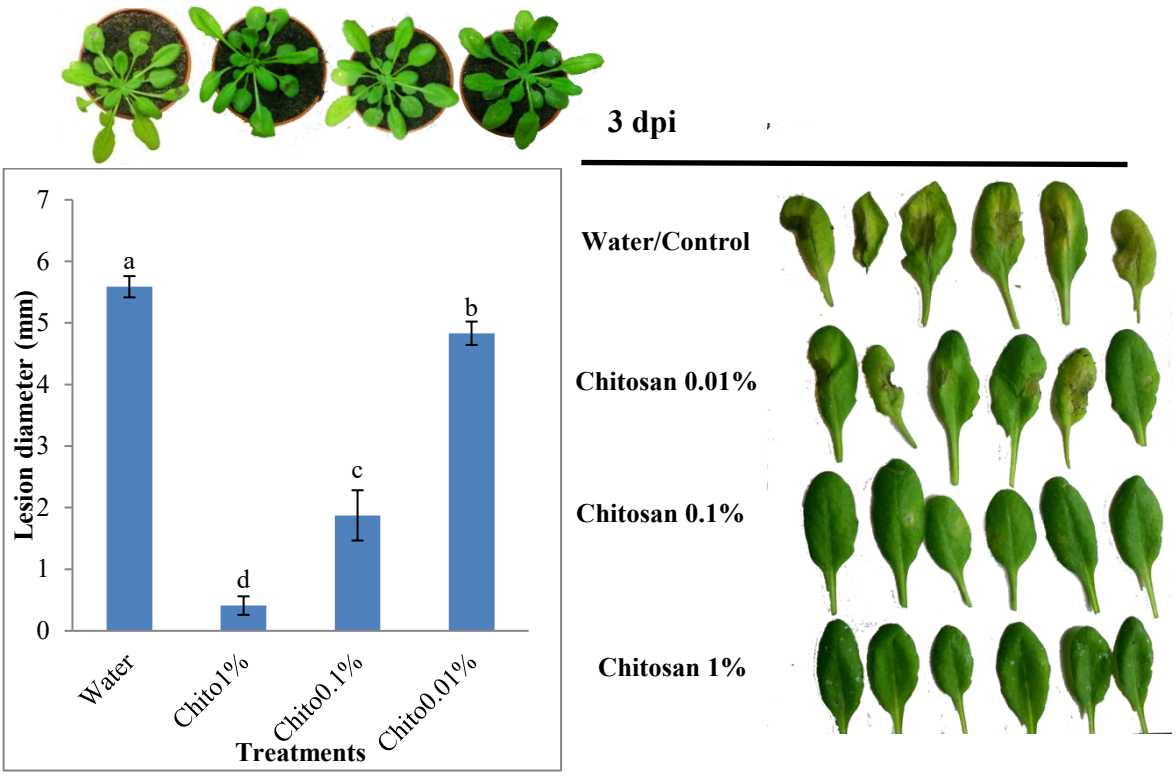


Figure 7. Quantification of chitosan-induced resistance in *Arabidopsis thaliana* at 3 different concentrations (0.01%, 0.1 % and 1% w/v) against *Botrytis cinerea* at 48 hours post-inoculation. Values presented are means ± SEM. Different letters indicate statistically significant differences (Games-Howell's Post-Hoc test P<0.05 at 2 dpi). One representative leaflet of eight-twelve replicates is shown for each treatment at 3 dpi.

In aubergine, chitosan significantly induced resistance in tomato against *Botrytis cinerea* in all concentrations compared to water-treated control plants at 3 dpi (Figure 8). Chitosan induced resistance didn't have a concentration-dependent effect as seen before. Chitosan 1% w/v-treated plants had significantly bigger lesion size at 4 dpi than any of the treatments including water-control (Figure 8) as well as it had a disease phenotype as high as control at 5 dpi. Interestingly, this concentration (Chito 1%) induced cell-death prior to infection (Additional Data) and that presumably facilitated Botrytis infection producing the biggest necrotic lesions surpassing water-treated control plants (Figure 8) at 4 and 5 dpi. However, the two lowest concentrations were able to induce resistance in a concentration-dependent manner at 3 and 5 dpi, where chitosan 0.1% and 0.01% w/v significantly reduced Botrytis necrotic lesion size in aubergine during all time points after Botrytis infection.

These results suggest a possible threshold in chitosan-priming for resistance that depends on its concentration and if trespassed, it may overstress plant defences in benefit of necrotrophic pathogens (that feed upon death tissue) such as *Botrytis cinerea*.

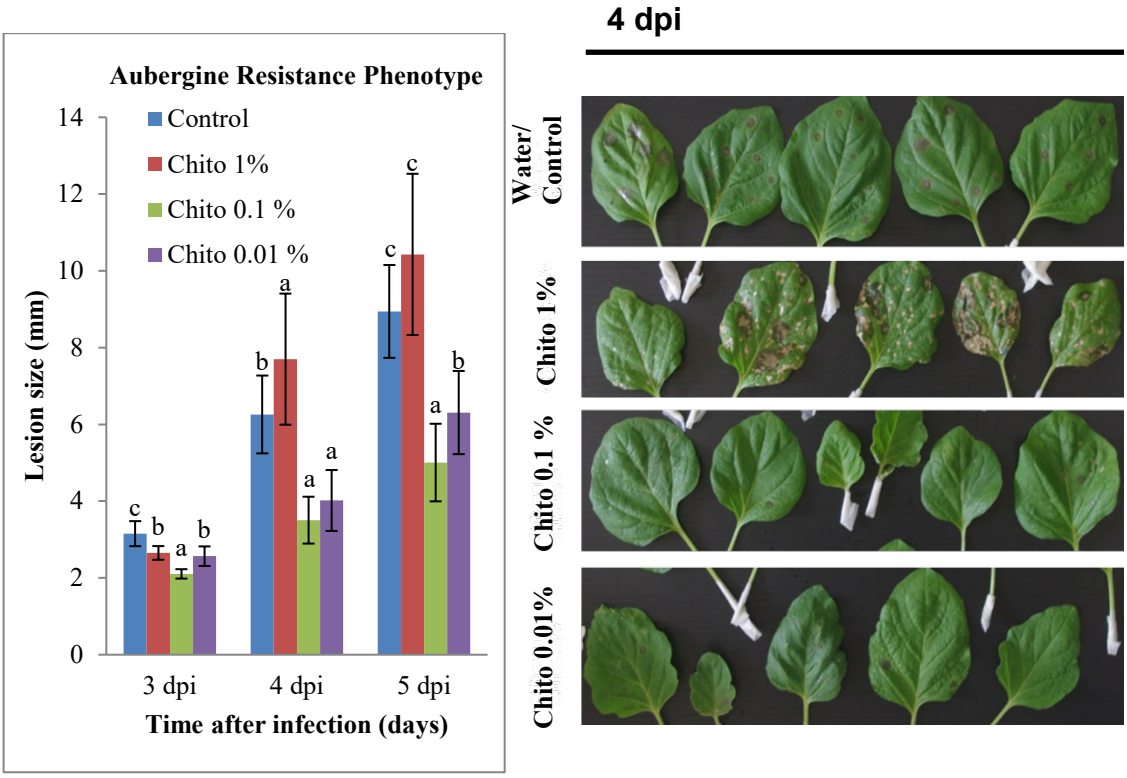


Figure 8. Quantification of chitosan-induced resistance in aubergine at 3 different concentrations (0.01%, 0.1 % and 1% w/v) against *B. cinerea* at 3, 4 and 5 days post-inoculation. Values presented are means ± SEM. Different letters indicate statistically significant differences (ANOVA followed by Bonferroni's Post-Hoc test P<0.001 at 3, 4 and 5 dpi).

Chitosan-induced resistance in Arabidopsis Col-0 against *Hyaloperonospora arabidopsidis*

To determine whether chitosan induces resistance in Arabidopsis against unrelated plant pathogens, thale cress (Columbia 0) plants were infected with the biotrophic pathogen *H. arabidopsidi* (*Hpa*) previously treated with ddH_2O solution or 0.01% of chitosan (ChitoPlant). 4 days after ChitoPlant treatment, plants were infected with *Hpa* and disease was scored at 5 days after infection.

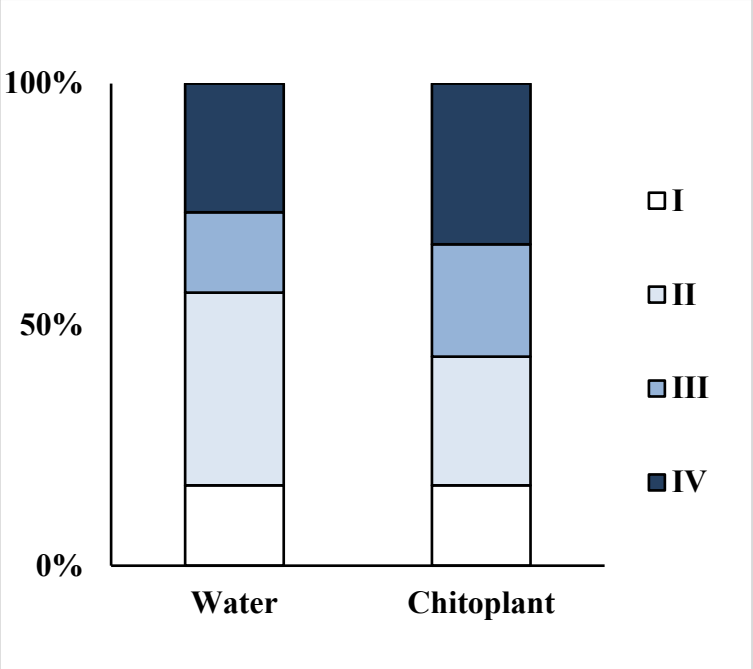


Figure 9. Quantification of chitosan-induced resistance at 0.01% w/v in *A. thaliana* against *H. arabidopsidis* at 5 days post-inoculation. Values presented belong to 4 disease classes. Class I, Healthy leaf, no Hpa growth, Class II, Hpa growth less than 25% of the leaf, Class III, Hpa growth more than 25% of the leaf with no sponrangiophores and Class IV, Hpa growth with sponrangiophores. Percentage indicate statistically significant differences (Chi-square p value P<0.716).

Results indicated that there are no significant differences between both treatments (P<0.716) having both treatments a similar Hpa infection profile (Figure 9). Thus, we can conclude that chitosan did not induce resistance in thale cress against the oomycete *H. arabidopsidis.* This result indicates that chitosan-induced resistance works differently depending on the plant and pathogen species.

Chitosan direct fungicide effect on *Botrytis cinerea* spore germination and hypha growth: Concentration matters

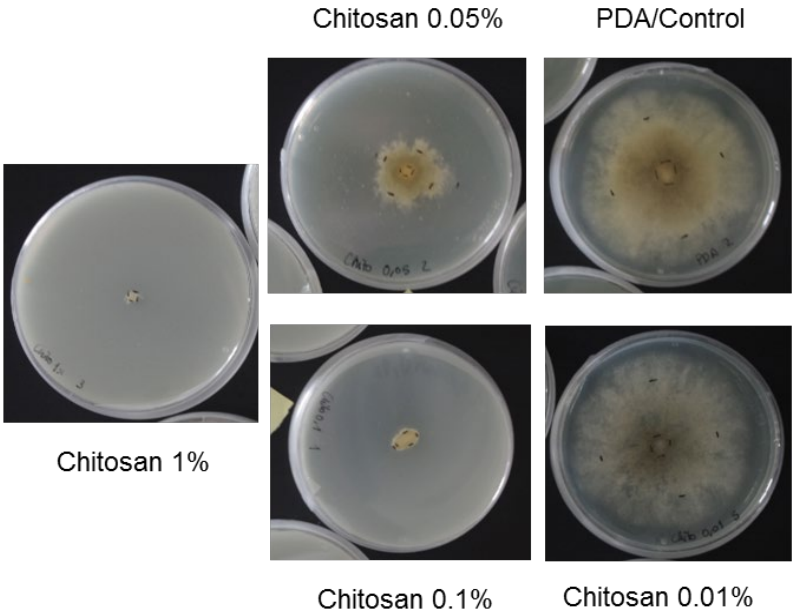
It's well-known that chitosan has antifungal properties (Badawy & Rabea, 2014; Romanazzi, Feliziani, et al., 2013); however this activity may depend on the physicochemical properties as well as the concentration.

The main focus of my PhD project is to investigate resistance elicitors (e.g. chitosan) role in priming plant own defences rather than having a direct effect on the pathogen such as conventional fungicide mode of action. Thus, in order to investigate the potential direct fungicide effect of chitosan against *Botrytis cinerea*, the effect of chitosan (ChitoPlant, Chipro) on fungal mycelial growth and spore germination was assessed *in vitro.*

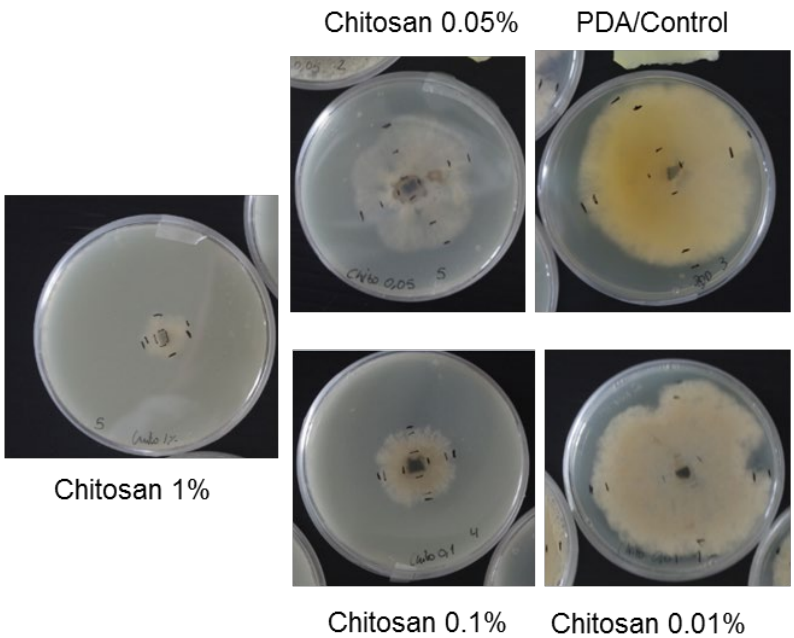
Chitosan had a fungicide effect both on fungal mycelia growth and spore germination in a concentration-dependent manner (Figure 10). Both, the highest concentrations (1 and 0.1% w/v) and, to a lesser extent, the medium concentrated (0.05% w/v) had a direct effect on mycelia growth repression as well as suppression of spore germination (Figure 10). However, the lowest concentrated chitosan (0.01% w/v) didn't have any direct fungicide effect on Botrytis mycelia and spores in comparison with control non-treated PDA (Figure 10), which suggest a concentration threshold for chitosan-direct fungicide effect against *Botrytis cinerea*. As expected, the positive control fungicide (Switch, Syngenta) supressed both Botrytis mycelia and spores in all concentrations (Figure 10).

These results clearly support the hypothesis of the concentration-dependence of priming of elicitors (chitosan in particular), suggesting a concentration threshold in which chitosan might not directly affect fungal growth instead of priming the plant own defence mechanisms to fight back pathogen challenge.

Botrytis Spore Germination at 3 dpi



Botrytis hypha growth at 4 dpi



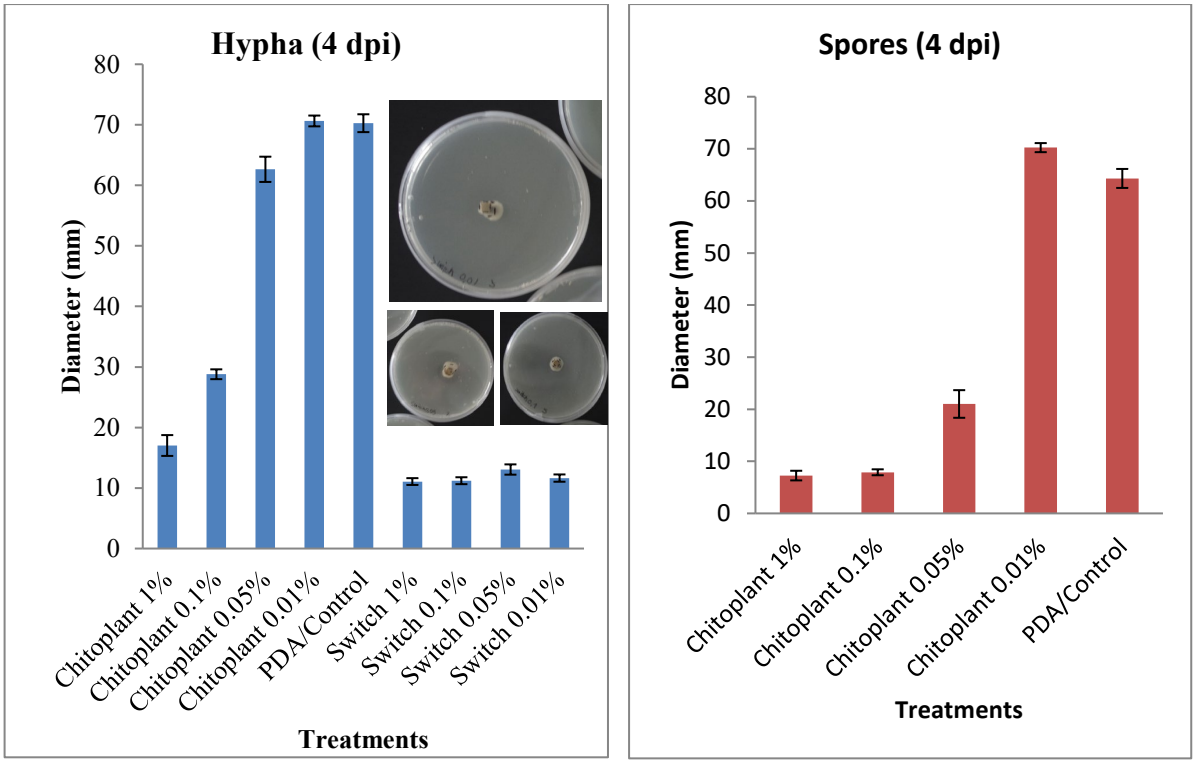


Figure 10. Quantification of chitosan-fungicide effect on *Botrytis cinerea* in PDA media plates. 5 mm agar plug of actively growing *B. cinerea* (BcR16 strain) mycelia was added per plate (5 plates per treatment) to test chitosan (Chitoplant, ChiPro) fungicide effect on Botrytis mycelia growth and a 15ul droplet (2*10 exp4 spores*ml^{-1}) with *B. cinerea* spores was added per plate as well to test effect chitosan effect on spore germination. Plates were covered with parafilm and were placed in 24C incubator in the dark. After incubation for 4 days, the mean radial growth of the fungus was determined by measuring the fungal colonies in two perpendicular diameters and calculating the mean diameter.

Transcriptome analysis on chitosan-primed and Botrytis infected *Solanum lycopersicum*

This PhD project has shown that chitosan induces resistance in tomato, aubergine and thale cress against *Botrytis cinerea*. Furthermore, it was demonstrated that chitosan can induce callose deposition on tomato epidermal cells, it has an effect on ROS and might prime extracellular peroxidase activity at early stages of Botrytis infection, as well as it has a priming effect when used at low concentrations (0.01% w/v), being capable to induced resistance in the plant whilst not having a direct fungicide effect against Botrytis (Figures 3, 4, 5, 6, 7 and 10). The diverse mechanisms of action of chitosan have been studied before, which include activation of antifungal proteins (Muñoz & Moret, 2010), oxygen-species scavenging and antioxidant activities, as well as the octadecanoid pathway activation (El Hadrami et al., 2010). Despite these studies, experiments that specifically address the role of priming in the complex chitosan-plant interaction framework are still lacking. This research aims to investigate the role of chitosan (ChitoPlant commercial formulation) in priming tomato plants for a faster, stronger, fine-tuned resistance to the fungal aggressive necrotroph *Botrytis cinerea.* For that, tomato cv. Money-maker plants were treated (primed), 4 days before fungal infection, with ddH_2O solution (as a control) and low-concentrated chitosan (0.01% w/v) by spraying the solution onto the plants. 4 days after treatment, detached leaves were challenged with *Botrytis cinerea* spores and tissue was harvested at 3 asymptomatic and crucial early time points after infection (6, 9 and 12 hours post infection). Total RNA was extracted and samples were sent for processing.

Statistical analysis on the Microarray (GeneSpring GX, Agilent Genomics) revealed chitosan mode of action on chitosan-treated and Botrytis-infected tomato cv. Moneymaker plants. Chitosan-treated plants were able to display stronger and faster defence mechanisms by differentially expressing more than 2000 defence-related genes after early stages of Botrytis infection (no symptoms visible), while water-treated tomatoes only differentially expressed 363 genes (Figures 11 and 12).

Chitosan-treated tomatoes were also able to repress Botrytis virulence genes expression (used by the pathogen to facilitate infection) while water-treated plants could not avoid Botrytis gene expression.

Figure 11. Venn Diagram showing the total differentially expressed genes (DEGs) activated specifically in every condition. Chitosan + infection (red), Chitosan non-infected (mock) (green) and control-water-treated + infection (blue).

Furthermore, Gene ontology analysis on the 2133 genes induced by chitosan revealed key pathways involved in tomato defences against Botrytis, such as cell-wall modification genes (lignin and cellulose synthesis), regulatory and signalling genes (protein kinases, transcriptional factors, involved in the transmission of the defence signal throughout the plant cells), jasmonate and ethylene-dependent genes, redox state (glutaredoxins, that reduce cell oxidative stress) and secondary metabolites (phenylpropanoids).

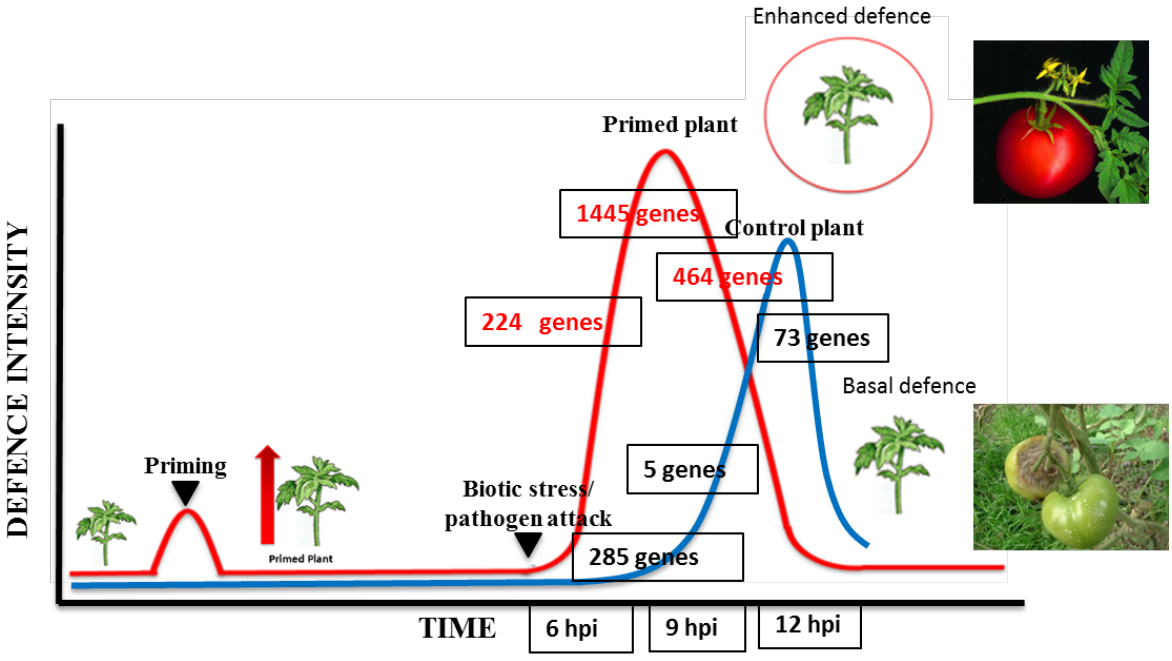


Figure 12. Model of basal (blue line) and induced resistance (red line) by chitosan as a priming agent in tomato-*Botrytis cinerea*. Text boxes shows the total differentially expressed genes (DEGs) activated specifically in every condition. Chitosan + infection (red) and control-water-treated + infection (blue) at 3 asymptomatic stages/times after Botrytis infection (6, 9 and 12 hour post infection, hpi).

These results unveiled potential molecular pathways involved in chitosan-induced priming for resistance in tomato (and potentially applicable to other crops, i.e. Aubergine) against the aggressive fungal pathogen *Botrytis cinerea*. This information might help breeders to target commercial crop varieties with a stronger expression of the mentioned pathways in order to cultivate more resistant domestic varieties against fungal pathogens.

Investigating gene molecular functions with MapMan software:

This large-scale transcriptomic analysis gives us the opportunity to identify tomato defence pathways that might be crucial for chitosan-induced resistance in tomato against *Botrytis cinerea* in the poorly studied early stages of the infection. So far it has been revealed that chitosan does prime tomato for a faster response against Botrytis in comparison to control plants, inducing the expression of more than 2100 genes whilst control plants are only able to induce 363 genes (Figures 11 and 12).

Looking in more detail at the biological functions of chitosan-primed genes, it was found that chitosan can significantly down-regulate redox state genes (Glutaredoxins), which can be susceptible factors that benefit pathogen infection. Interestingly, transcriptional factors (ERF, WRKYs and MYB) were also uniquely activated by chitosan against *Botrytis cinerea*. Chitosan activated Auxin, Ethylene pathways and cell-wall genes and triggered peroxidases, PR-proteins, proteolysis and signalling processes earlier at 6hpi and 9 hpi whilst non-treated (control) plants didn't triggered these processes until later during the infection.

Molecular Pathway Activated	
Chitosan + Infection	**Non-treated (Control) + Infection**
Cell-wall modification genes	Ethylene and Auxins
Ethylene and Auxins	Signalling
Proteolysis	Proteolysis
Signalling and MAPKinases	Salicylic acid
Jasmonic acid	Stress abiotic (heat, drought)
Redox state genes	Transcriptional regulators
Transcriptional regulators	
PR-proteins	
Stress abiotic (heat, drought)	

Table 1. Total molecular pathways significantly triggered by chitosan+Infected and Control+Infected with MapMan software.

Functional study of Gene Ontology (GO) terms and Gene set enrichment

So far we've seen which pathways were uniquely triggered by chitosan-treated plants after Botrytis infection in comparison with non-treated plants. However, to further investigate which classes of genes are over-represented ("enriched") within all the significantly expressed transcripts from the microarray a gene set enrichment analysis was performed. This analysis may help us to to identify molecular pathways that may have an association with chitosan-induced resistance phenotype. Here we compared chitosan+ infected with non-treated + infected DEGS per time point.

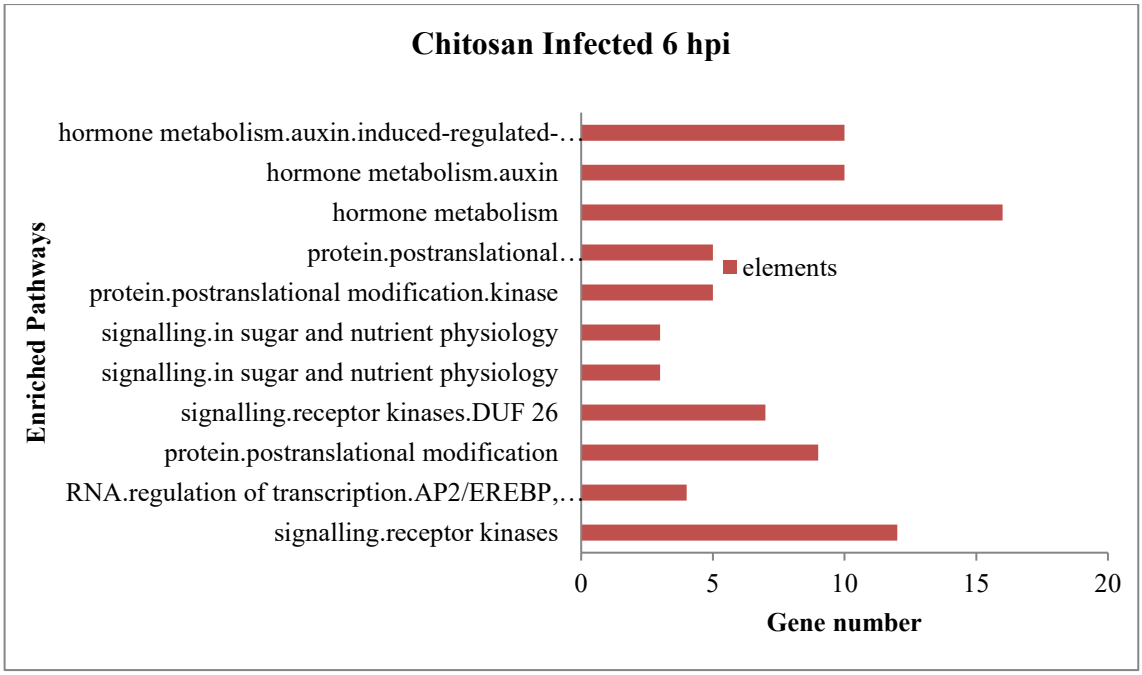


Figure 13. GO terms enriched with MapMan for chitosan + infected at 6 hpi. Data was loaded into MapMan tool and statistical analysis was performed (Wilcoxon Rank Sum Test (P<0.05)).

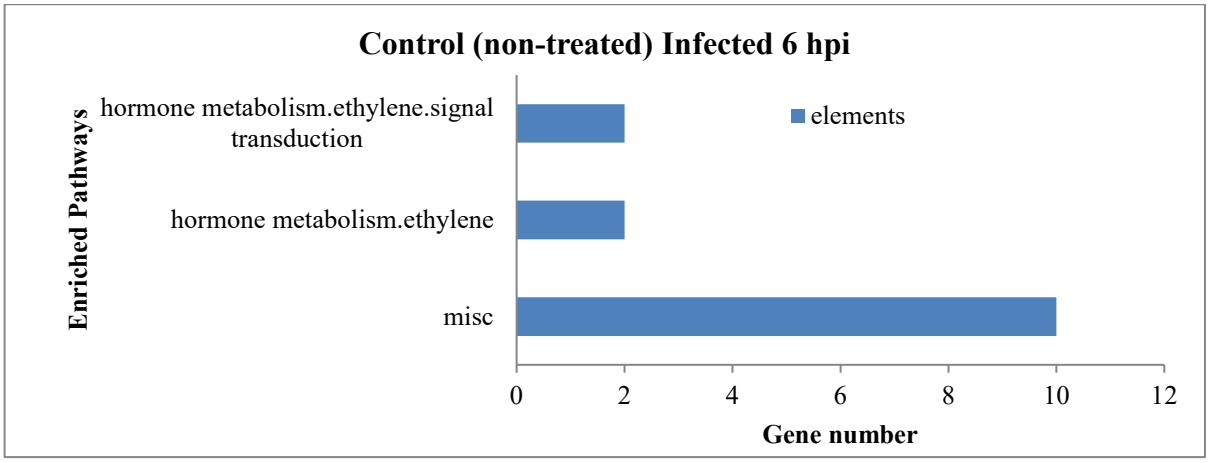


Figure 14. GO terms enriched with MapMan for non-treated + infected at 6 hpi. Data was loaded into MapMan tool and statistical analysis was performed (Wilcoxon Rank Sum Test (P<0.05)).

Functional enrichment study of DEGs belonging to the two infected conditions (Inf and ChitoInf) revealed 11 molecular pathways that were significantly enriched for ChitoInf at 6 hpi (Figure 13). Response to AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family, signalling in receptor kinases, signalling in sugar and nutrient physiology, hormone metabolism/Auxins, and protein translation were significantly enriched (Figure 13). For non-treated + infected plants 3 pathways were significantly enriched at 6 hpi, including ethylene signal transduction (Figure 14).

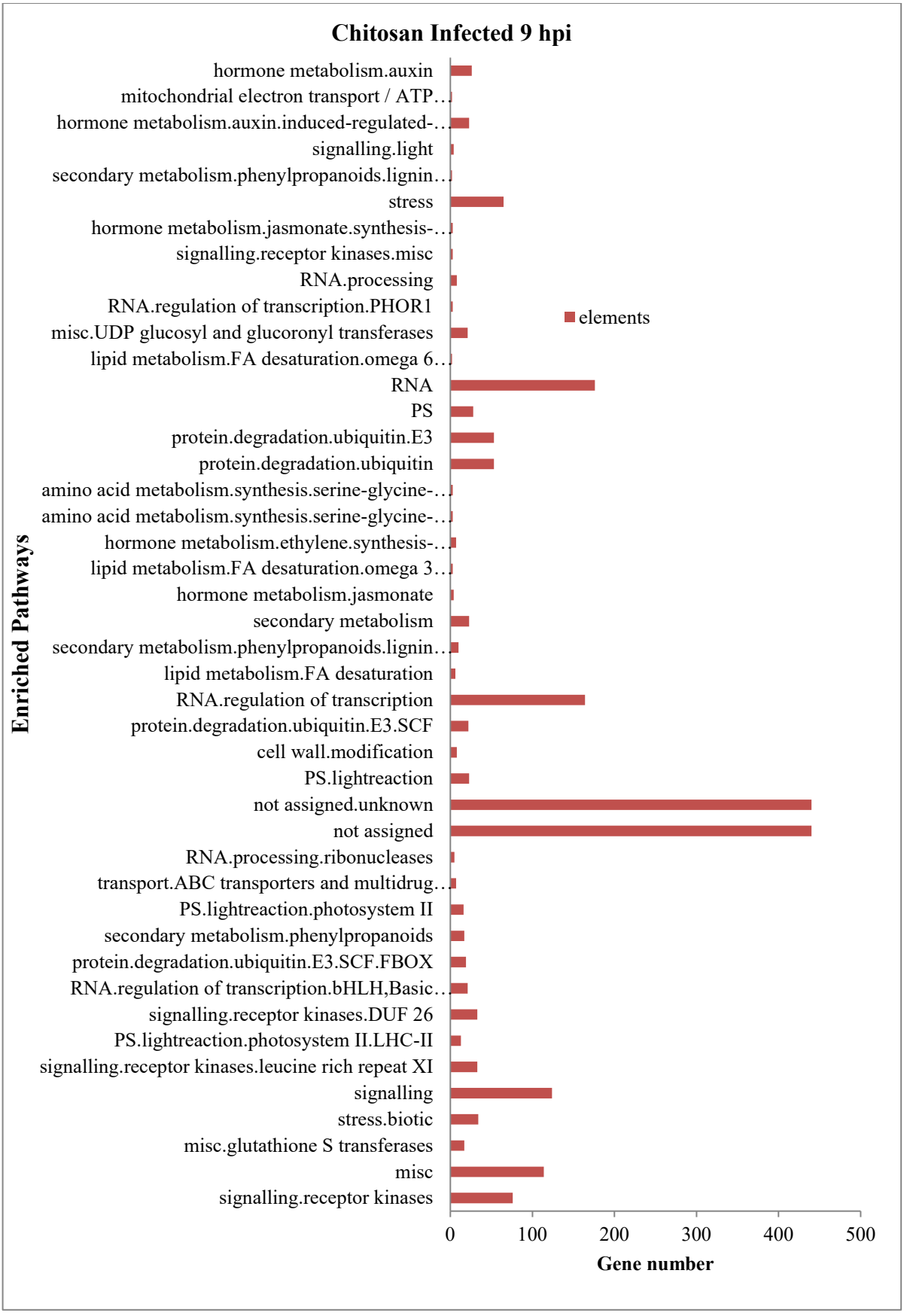


Figure 15. GO terms enriched with MapMan for chitosan + infected at 9 hpi. Data was loaded into MapMan tool and statistical analysis was performed (Wilcoxon Rank Sum Test (P<0.05)).

A functional enrichment study of DEGs belonging to the two infected conditions (Inf and ChitoInf) at 9 hpi revealed 44 enriched pathways for chitosan + infected plants, including cell-wall, secondary metabolism such as wax, phenylpropanoids and lignin; peroxidases, receptor kinases, lipid metabolism, FA desaturation, aminoacid and ethylene and jasmonate metabolism (Figure 15); whilst non-treated + infected plants hadn't any enriched pathways. At 12 hpi chitosan + infected showed 7 enriched pathways, such as pectin methylesterase inhibitor family protein and glutaredoxins; whilst non-treated + infected plants had 5 enriched pathways including secondary metabolism and abiotic stress (Figures 16 and 17).

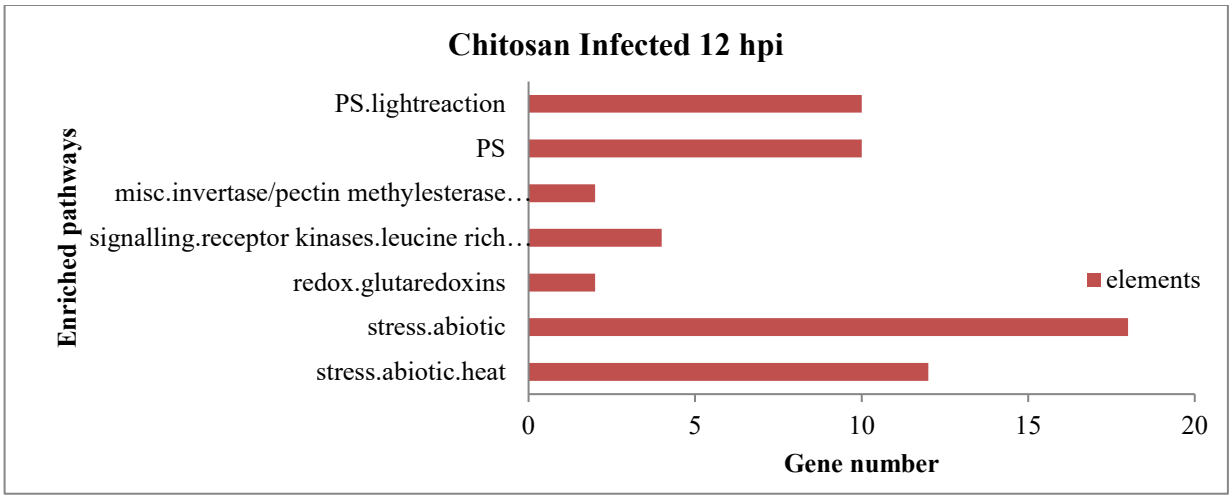


Figure 16. GO terms enriched with MapMan for chitosan + infected at 12 hpi. Data was loaded into MapMan tool and statistical analysis was performed (Wilcoxon Rank Sum Test (P<0.05)).

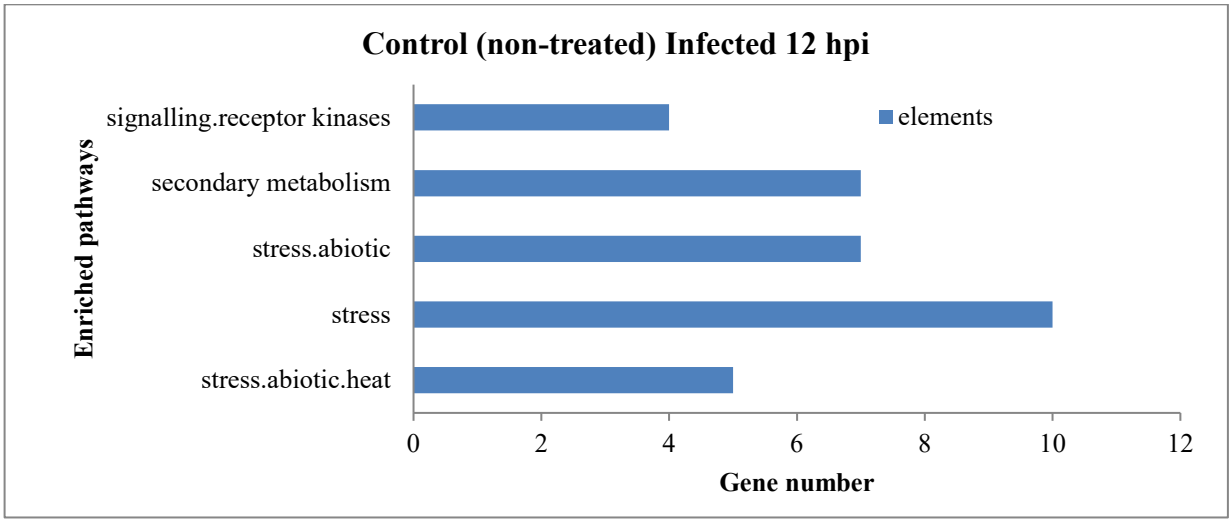


Figure 17. GO terms enriched with MapMan for non-treated + infected at 12 hpi. Data was loaded into MapMan tool and statistical analysis was performed (Wilcoxon Rank Sum Test (P<0.05)).

Microarray selected gene candidates involved in chitosan-induced resistance against *Botrytis cinerea*

Functional enrichment analysis helped us to further understand chitosan priming mechanisms on tomato plants against fungal necrotroph *Botrytis cinerea*, by inducing a stronger and faster set of genes (Figures 11 and 12) and by significantly inducing (enriching) key defence pathways to fight Botrytis infection (Table 1, Figures 13,14, 15, 16 and 17). However, analysis of large data sets, such as Microarrays, with software tools that identify metabolic pathways or other molecular processes (like MapMan) can filter important genes which functions are not yet assigned or well-characterised. For this reason, genes that were significantly expressed (from the ANOVA) ealier and/or stronger during the infection on chitosan-primed plants (Venn Diagrams, Figure 11) were selected to further validate the transcriptome analysis with real time PCR (qRT-PCR).

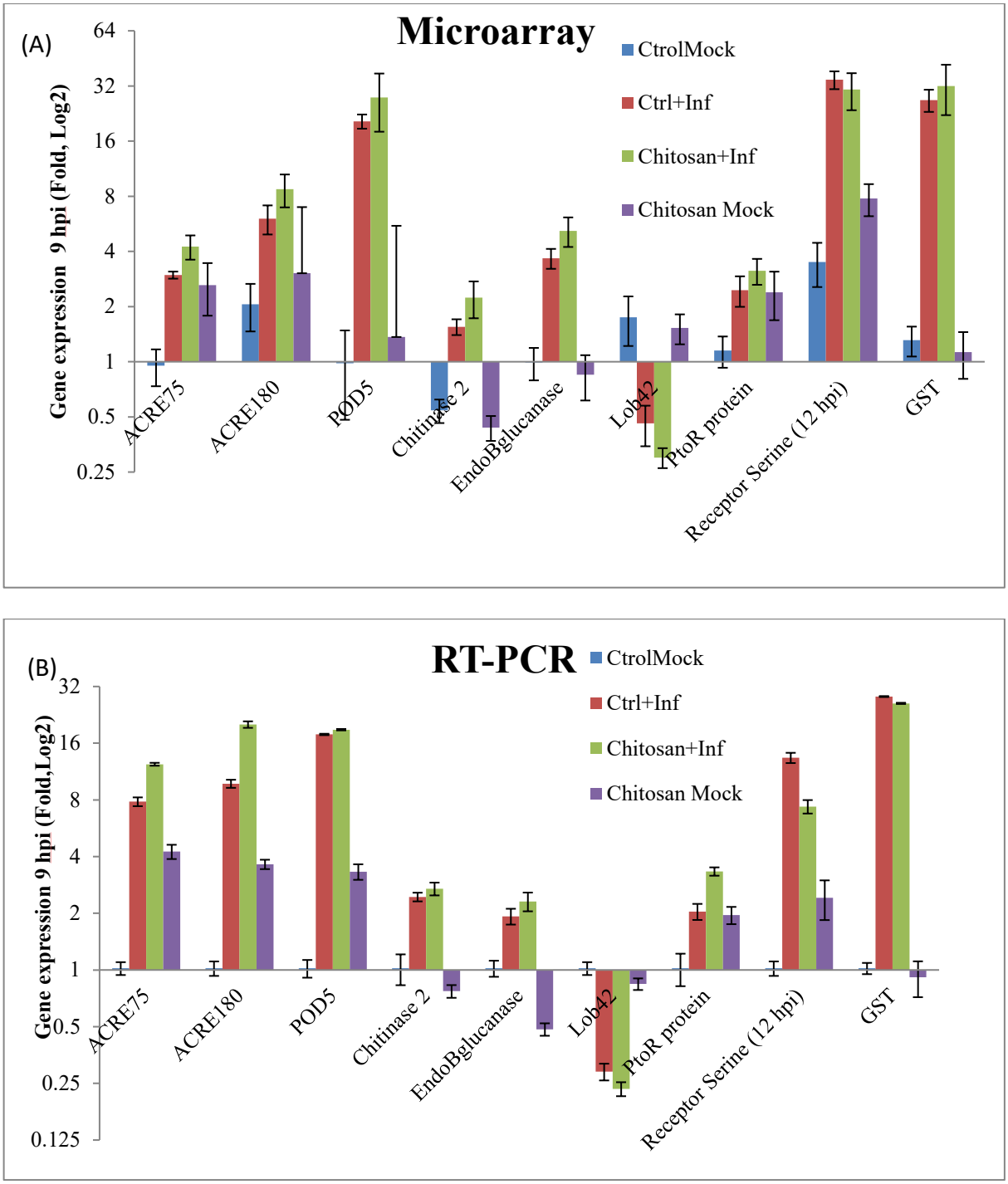


Figure 18. Microarray data validation by reverse transcription-quantitative polymerase chain reaction (qRT-PCR) at 9 hpi. (A) Fold change (Log2) normalized values obtained by microarray hybridization. (B) Fold change (Log2) values obtained by RT-PCR. The data shown are the means of three biological replicates for the qRT-PCR and 4 biological replicates for the Microarray ± standard error of the mean (SEM). Ctrl+Inf, untreated and Botrytis-infected plants; chitosanMock, chitosan-treated and non-infected plants; chitosan+Inf, chitosan-treated and infected plants at 9 hpi. ACRE75, ACRE180, Avr9/Cf-9 rapidly elicited protein 75/180; POD5, Peroxidase 5, LOB42, lateral organ boundaries domain protein 42, PtoR protein, Pto-like, Serine/threonine kinase protein, resistance protein; receptor serine, Receptor serine/threonine kinase-like protein, GST, Glutathione S-transferase.

Jasmonic acid is a key hormone in chitosan-priming for resistance against *Botrytis cinerea*

Microarray has revealed key molecular pathways in chitosan-priming tomato to fight back Botrytis early stages of infection, such as cell-wall modification genes, pathogenesis-related (PR) proteins, secondary metabolites, signalling and redox processes. However, it's well-accepted by the scientific community that phytohormones play crucial roles in the regulation of the defence signalling network upon perception of biotic or abiotic stress (Forcat et al., 2008; Pieterse, Van der Does, Zamioudis, Leon-Reyes, & Van Wees, 2012). It's traditionaly accepted that plants use Salicylic acid (SA) to fight biotrophic and hemibiotrophic-type of pathogens whereas jasmonic acid (JA) and ethylene (ET) primarily regulate plant resistance to infections by necrotrophic pathogens (Glazebrook, 2005; Zhu, 2014). In recent years it has become clear that there are more hormone pathways that might play important roles in plant defences, including abscisic acid, auxins and gibberellins (Figure 19). These hormone pathways can act positively or negatively depending on the nature of the pathogen. Furthermore, there is clear evidence that there is a cross-talk among hormone pathways and there is a fine line to which these can act synergistically or antagonistically. Transcriptomic analysis showed stress-related hormonal pathways primed by chitosan after *Botrytis cinerea* infection, including auxins, ethylene and jasmonate related genes (Figures 13 and 15).

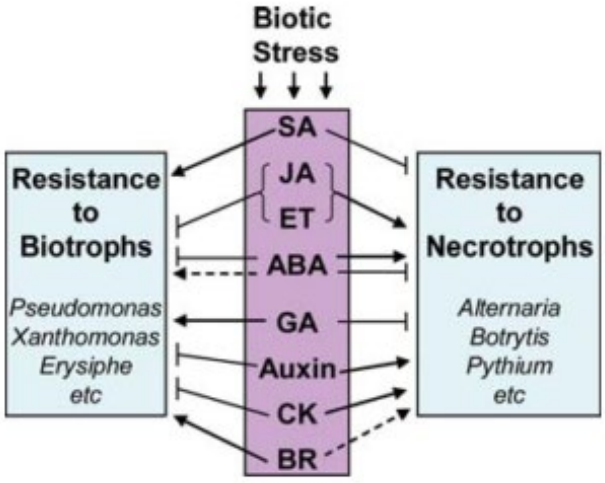


Figure 19. Simplified model showing the involvement of different hormones in the positive or negative regulation of plant resistance to various biotrophic and necrotrophic pathogens (Bari & Jones, 2009). Arrows indicate activation or positive interaction and blocked lines indicate repression or negative interaction.

In order to elucidate role of key hormonal pathways in chitosan-tomato-*Botrytis cinerea* interaction, significant expression of jasmonic and salycilic acid-related genes was investigated. MYC2, a basic helix-loop-helix (bHLH) domain–containing transcriptional factor

(TF) that can act both as an activator and repressor of distinct JA-responsive gene expression in Arabidopsis (Lorenzo et al., 2004) was down-regulated at 6 hpi and 9 hpi for both non-treated and infected (inf) and chitosan-primed and infected (ChitoInf) treatments. However, SlMYC2 was only down-regulated at 12 hpi by ChitoInf. Jasmonate Zim Domain proteins (JAZ) are negative regulators of JA-induced gene expression (Wasternack & Hause, 2013); SlJAZ1 was antagonistically expressed to SlMYC2, were it was up-regulated by both inf and in a higher extent by chitoinf at 6 and 9 hpi, however at 12 hpi is was noticed that SlJAZ1 induction was lower that inf treatment. This suggests a possible shift of JA-induced defences at 12 hpi where chitosan no longer represses JA-induced defence expression in favour of other pathways in the early stages of the infection and after the pathogen manages to overcome these “relative early defences” chitosan instead primes JA-defence expression.

Furthermore, there is a complex network in the JA-SA cross-talk in response to pathogen attack (Van der Does et al., 2013). JA and SA are generally considered antagonistic pathways (Takahashi et al., 2004), in Arabidopsis, the defence regulatory protein NONEXPRESSOR OF PR GENES1 (NPR1) was identified as a key signalling protein in the regulation of SA/JA crosstalk (Van der Does et al., 2013); (Spoel et al., 2003) showed that in mutant npr1-1 plants, the antagonistic effect of SA on JA-defence gene PDF1.2 was completely abolished. Thus, antagonism between JA and SA signalling pathways requires the activation of SA-dependent proteins such as NPR1 and WRKY70 that repress JA-responsive genes (Adie et al., 2007; Van der Does et al., 2013). It is well-known that biotrophic, hemi-biotrophic and in the last decade it has also been revealed that necrotophic pathogens are able to manipulate the cross-talk between immune pathways to promote disease (Abd, Rahman, Oirdi, Gonzalez-lamothe, & Bouarab, 2012; Birch et al., 2008; Boevink et al., 2016; Xin & He, 2013), such *as Botrytis cinerea* that manipulates the antagonistic effects between SA and JA through NPR1 to spread disease in tomato (El Oirdi et al., 2011). To further support this hypothesis and therefore to elucidate whether chitosan is able to reduce Botrytis SA-JA manipulation, NPR1 transcripts expression was measured. SlNPR1-1 was significantly expressed in the ANOVA-BH and it was down-regulated by both inf and chitoinf at 6 hpi. However, at 9 and 12 hpi, SlNPR1-1 expression changed and it was only down-regulated by chitoinf whereas non-treated control and infected plants were not able to down-regulate it at the 2 last time points.

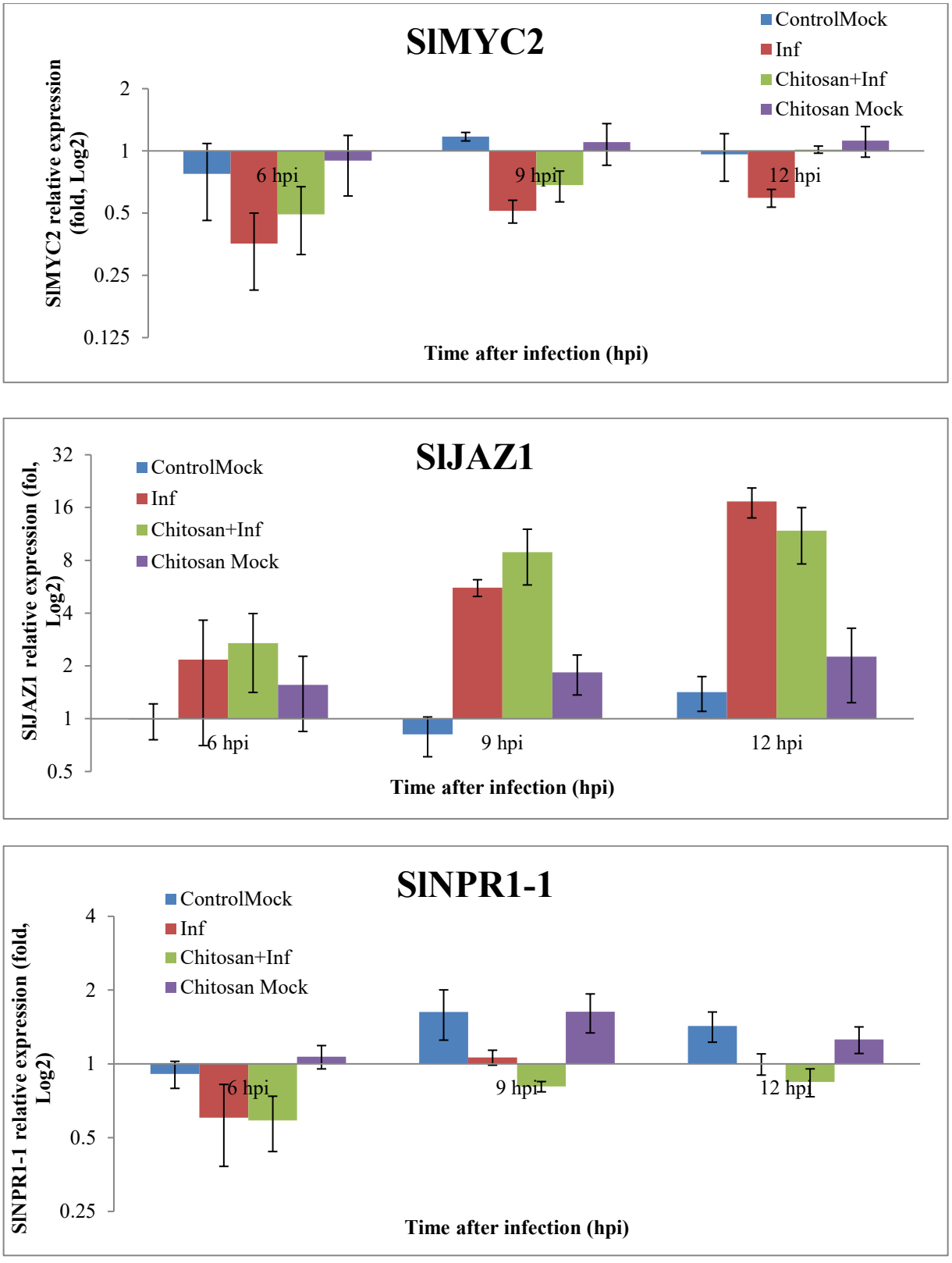


Figure 20.Expression of JA-dependent SlMYC2, SlJAZ1 and SA-dependent SlNPR1-1. Data shown are the means of 4 biological replicates from the normalized values obtained by ANOVA-Benjamini Hochberg ($p<0.05$) from the microarray. Relative expression folds (Log2) were calculated with control mock samples.

Phytohormone role on chitosan and MeJA-priming for resistance tomato against Botrytis cinerea: HPLC/MS analysis

Microarray analysis revealed jasmonic acid as a key hormone in chitosan-priming tomato against *Botrytis cinerea*, together with a possible cross-talk among other plant hormones such as abscisic acid, salicylic acid and ethylene (Figures 13,14 and 15). Furthermore, transcriptomic data revealed that JA positive and negative regulator genes (SlMYC2 and SlJAZ1 respectively) together with ABA and ET-dependent genes, such as ABAPYL4 and SlERF1 (data not shown), play a key role in chitosan-priming for tomato resistance against *Botrytis cinerea* during the early infection stages.

Thus, to determine the role of main phytohormone pathways SA, JA and ABA in chitosan-IR in tomato-*Botrytis cinerea* interaction we performed a quantitative determination of the abundance of three acidic plant hormones from a single crude extract directly by LC/MS/MS following (Forcat et al., 2008) novel methodology. The method exploits the sensitivity of MS and uses multiple reaction monitoring and isotopically labelled samples to quantify the phytohormones abscisic acid, jasmonic acid and salicylic acid in tomato leaf tissue (Forcat et al., 2008).

Phytohormones SA/JA/ABA expression was measured in tomato cv. Money-maker plants infected with *Botrytis cinerea* under four different conditions. 4-5 weeks-old tomato plants were treated with two different resistance elicitors, Methyl-jasmonate (MeJA) (0.1mM), chitosan (0.01% w/v) and a combination of both elicitors (chitosan + MeJA) in order to look for potential synergies, and ddH_2O (in 0.01% Tween 20). 5 days later, tomato whole leaves were excised and challenged with *Botrytis cinerea*. Leaf discs were harvested and freeze dried at three different time points during symptomless stage of the infection (6,9 and 24 hpi) and evaluated for hormone analysis through HPLC/MS.

Resistance phenotype (infection assay) analysis revealed that chitosan and MeJA alone and in combination were able to induced resistance and protect tomato against Botrytis, being able to significantly reduce necrotic lesion expansion at 48, and 72 hours post infection (hpi) (data not shown). Interestingly, we saw that the combination treatment highly protected tomato plants and they looked healthier and stronger and the levels of hormones were not higher in the combination treated tomatoes in comparison with the single-treated plants. These results suggests that low doses of chitosan may be used in combination with other crop protectants which can potentially lead into synergistic effects and a more efficient and less stressful plant defence.

HPLC/MS hormone analysis revealed jasmonic acid and its bioactive compound jasmonic acid-isoleucine as significantly induced by chitosan and/or MeJA in pathogen presence and absence. However, abscisic acid and salicylic acid were not significantly expressed in any of the treatments. Methyl-jasmonate (MeJA) significantly induced jasmonic acid (JA) without infection (MM) as well as it was able to prime JA earlier and stronger during *Botrytis cinerea* infection (MI) in comparison with non-treated control plants (WI) by significantly increase JA levels at 9 and 24 hpi (Figure 21). Interestingly, chitosan was also able to induce JA and the bioactive compound jasmonic acid-isoleucine (JA-Ile) in a time-dependent manner, later than MeJA, during the infection, whilst the combination treatment primed JA faster and quicker than chitosan itself (Figure 21). Besides, chitosan itself (non-infection) and in combination with MeJA (ChiM and CoM) supressed MeJA-induced JA levels (MM) at 9 and 24 hpi (Figure 21). This suggests a different strategy of chitosan in protecting tomato plants against Botrytis cinerea during early stages of the infection by repressing JA-responsive defences in favour of PTI-ETI type of responses, including, resistance-type genes activation, MAP-kinase signalling cascades, cell-wall reinforcement, peroxidase and PR-proteins activity, as showed by microarray analysis, and which also correlates with repression and activation of JA-dependent SlMYC2 and SlJAZ1 transcripts seen during microarray analysis, were SlMYC2 was down-regulated and JAZ1 was significantly up-regulated by chitosan at 6hpi and 9 hpi and this trending changed at the latest time point 12 hpi were SlJAZ1 and SlNPR1-1 expression was lower than control + infected plants (Figure 20).

Hormone analysis showed that MeJA was able to, although not significantly, trigger synergistically the 3 hormones ABA, SA and JA at 9 hours after the infection (data not shown). However, MeJA and chitosan weren’t able to significantly induce Salicylic and abscisic acid. Apparently, there was not any salicylic acid manipulation from the pathogen during the infection and ABA was much more concentrated than the other hormones (data not shown); however ABA doesn't seem to play a big role during the early stages of the infection, apart from its putative pathogen-caused reduction at 6hpi (data not shown).

Hence, HPLC/MS hormone analysis discovered jasmonic acid and its bioactive derivative jasmonic acid-isoleucine (JA-Ile) as the key hormones involved in chitosan-IR and MeJA-induced resistance in tomato against *Botrytis cinerea* during early stages (symptomless) of the infection (at 9 and 24 hours after infection (hpi)). These results correlate with the well-known role of jasmonic acid in plant resistance against necrotrophic pathogens such as *Botrytis cinerea*, *Sclerotinia sclerotium* and *Fusarium oxysporum*.

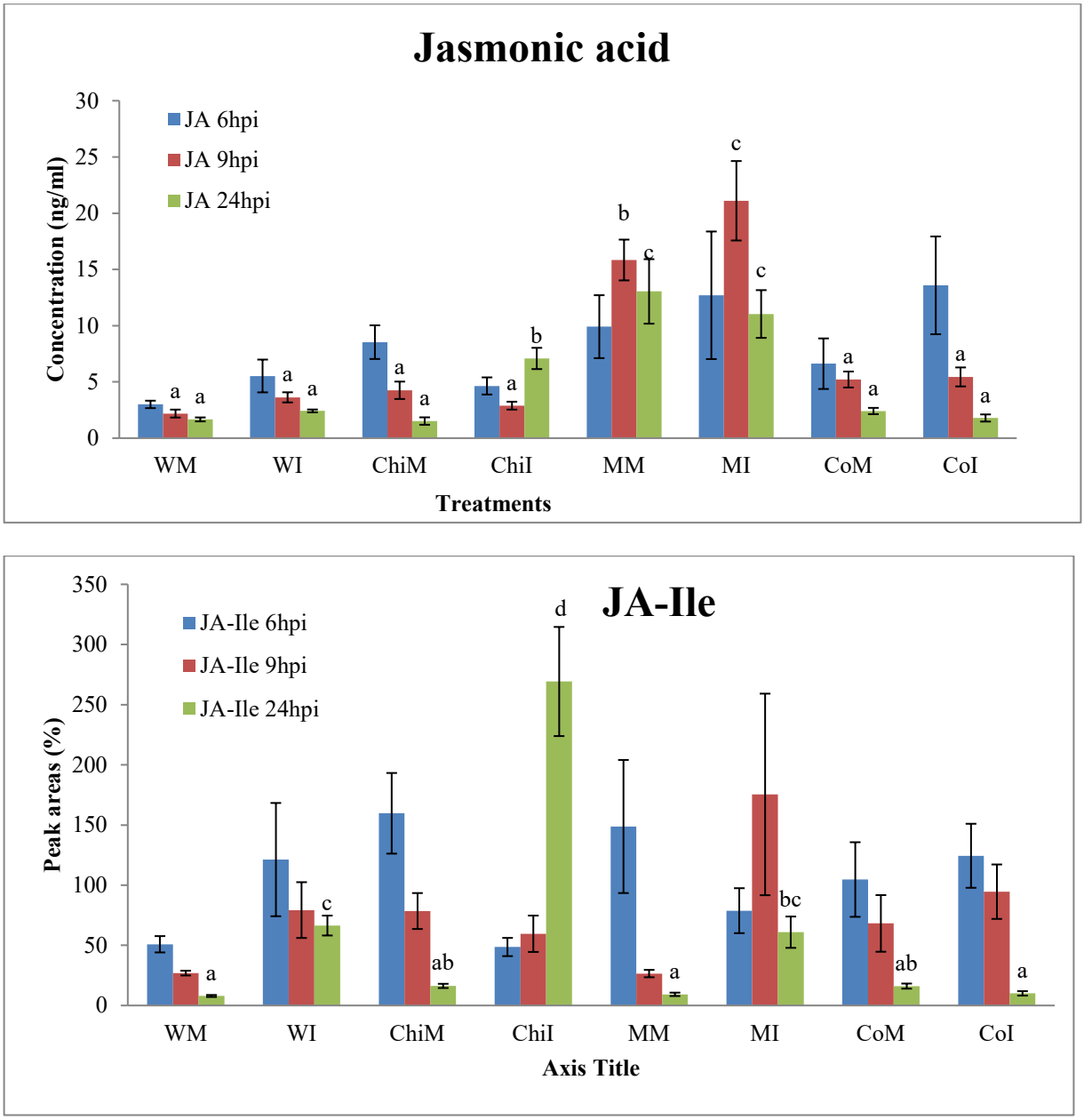


Figure 21. Quantification of endogenous JA, SA and ABA (ng/g dry weight) and bioactive hormone JA-Ile (% peak area) levels in tomato cv. Moneymaker plants following infection with *Botrytis cinerea* infection for 6, 9 and 24 hpi. WM, Water-treated and non-infected; WI, water-treated + infected, ChiM; chitosan-treated + non-infected; ChiI, chitosan-treated + infected; MM, MeJA-treated + non-infected; MI, MeJA-treated + infected; CoM, chitosan+MeJA-treated + non-infected; CoI, chitosan+MeJA-treated + infected. Values presented are means ± SEM and different letters mean significant differences among all treatments per time point and they were obtained from an ANOVA and then pairwise Fisher's protected least significant difference (LSD) test ($P<0.001$ at 9hpi and 24 hpi, $\alpha=0.05$; $P=0.194$ at 6hpi for JA; $P=0.221$ for 6hpi and 9hpi and $P<0.001$ at 24 hpi for JA-Ile).

Avr9/Cf-9 rapidly elicited (ACRE) genes role in chitosan-priming tomato for resistance

ACRE genes are usually expressed as part of the defence response of tobacco and tomato to the biotrophic fungus *Cladosporium fulvum* and other stress responses (Durrant, Rowland, Piedras, Hammond-Kosack, & Jones, 2000), such as elicitors, effector proteins and pathogen/microbe-associated molecular patterns (PAMPS, MAMPs). Many ACRE genes role have been deciphered in previous studies (Durrant et al., 2000), and they usually encode components of signalling pathways, including transcription factors, protein kinases, and ubiquitination pathway-related proteins, such as F-box and U-box proteins (González-Lamothe et al., 2006). Some ACRE genes have been associated to pamp-triggered immunity (PTI) responses, such as ACRE31 (Boevink et al., 2016; Sonnewald et al., 2012) while others, such as ACRE74, ACRE276 and ACRE189 are related to hypersensitive response (HR) (González-Lamothe et al., 2006; Sadanandom, Bailey, Ewan, Lee, & Nelis, 2012) as part of the effector-triggered immunity (ETI) response. Low doses of the avirulence gene Avr9, of the biotrophic tomato pathogen *Cladosporium fulvum*, independently to HR, can delay *Botrytis cinerea* development in tomato and and *Scletotinia sclerotium* in Cf9 oilseed rape plants (Hennin, Diederichsen, & Höfte, 2002). Many of the ACRE genes encode putative signalling components and thus may play pivotal roles in the initial development of the defence response (Rowland et al., 2005). However, there are still ACRE genes, such as ACRE65, ACRE180, ACRE75, ACRE194 and ACRE169 which molecular functions remains unknown (Durrant et al., 2000). Thus, many ACRE gene roles in plant defences are still poorly understood and their part in the defence against necrotrophic pathogens is still unclear. Among all differentially expressed genes (DEGs) from the ANOVA, Avr9/Cf-9 rapidly elicited (ACRE) genes were identified within the DEGs lists for ChitoInf and they were differentially expressed only by chitosan + infected at 6 and 9 hpi (Figure 22).

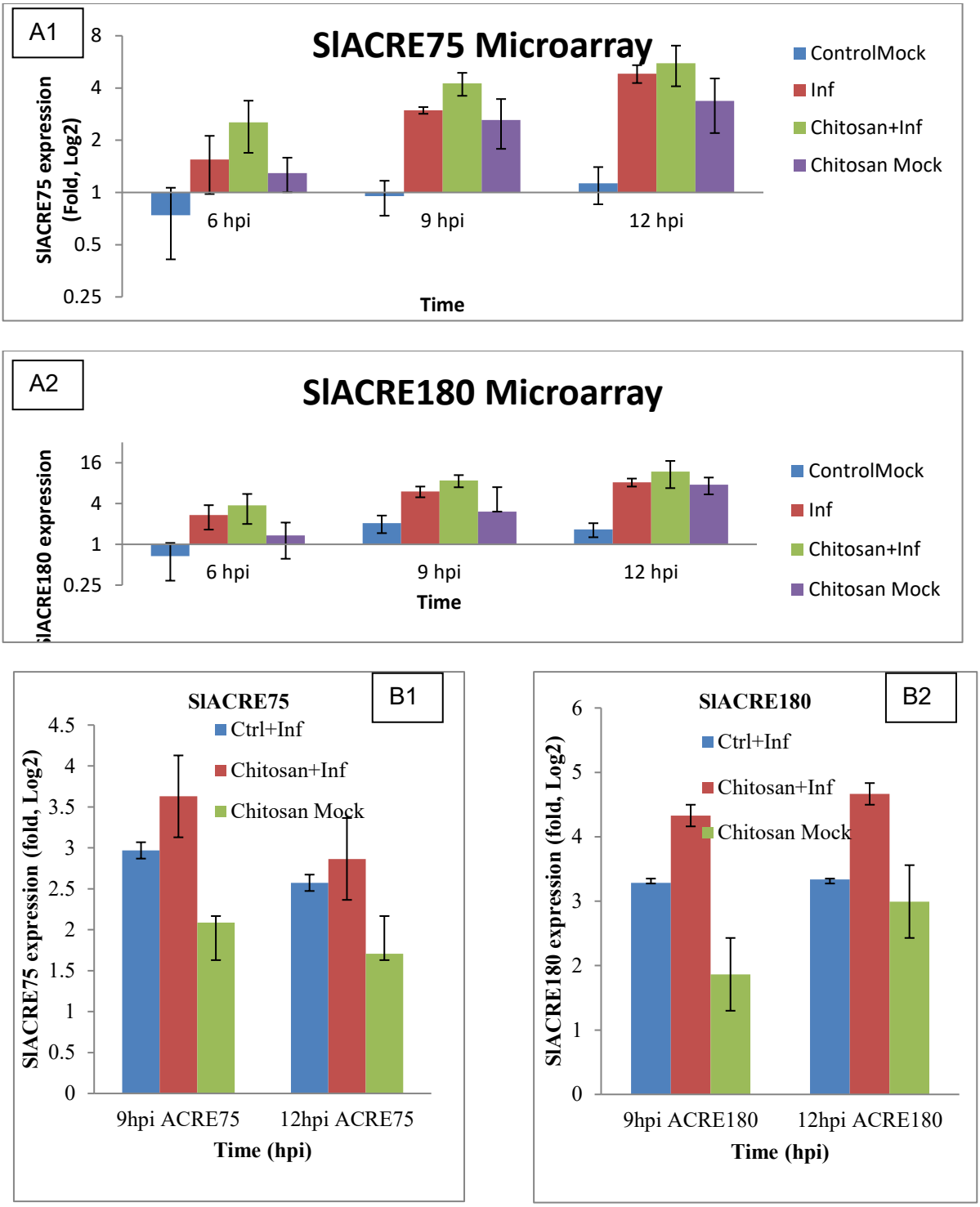
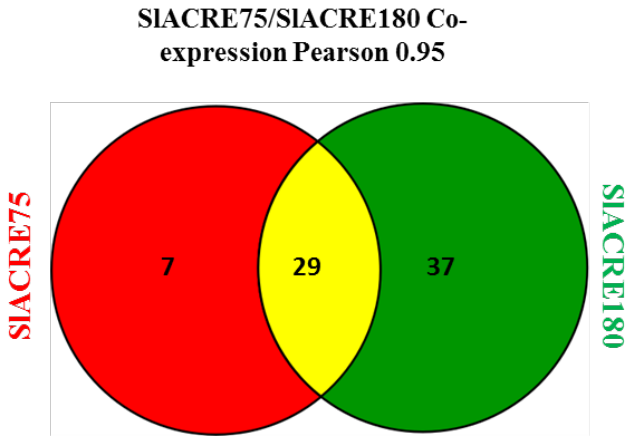


Figure 22. A1,A2. SlACRE75 and SlCRE180 relative expression values obtained by normalized gene expression relative to watermock (control) in the Microarray within the ANOVA Benjamini-Hochberg corrected ($p<0.05$). B1,B2. SlACRE75 and SlCRE180 relative expression values obtained by normalized gene expression relative to watermock (control) by qRT-PCR. The data shown are the means of three biological replicates for the qRT-PCR and 4 biological replicates for the Microarray ± standard error of the mean (SEM). ControlMock, untreated and non-infected; Ctrl+Inf, untreated and Botrytis-infected plants; chitosan-Mock, chitosan-treated and non-infected plants; chitosan+Inf, chitosan-treated and infected plants.

SlACRE180 and SlACRE75 were primed (triggered earlier and/or stronger by Chitoinf in comparison to WaterInf) by chitosan, being differentially expressed only by chitosan + infected, also triggered by chitosan mock (non-infected) (Figure 22). Furthermore, Pearson co-expression analysis revealed that both genes are co-regulated, SlACRE75 had 36 genes co-regulated and SlACRE180 had 66, all of them were DEGs and they shared 29 DEGs including themselves (Figure 23). Some of 29 co-expressed genes with SlACRE75 and SlACRE180 were calmodulin, Calcium-transporting ATPase2, Exocyst protein, harpin-induced protein, gibberellin 2-oxidase, auxins, BCS1, WRKY53/30/11, Matrix metalloproteinase (Sl2-MMP) and receptor-like kinase. Some of these genes have been reported to be involved in cell-wall papillae formation and plant defence against various pathogens including *Botrytis cinerea* (H. Dong, Delaney, Bauer, & Beer, 1999; D. Li et al., 2015; X. Li, Huang, et al., 2014) (X. Li, Zhang, et al., 2014) (Peenková et al., 2011).



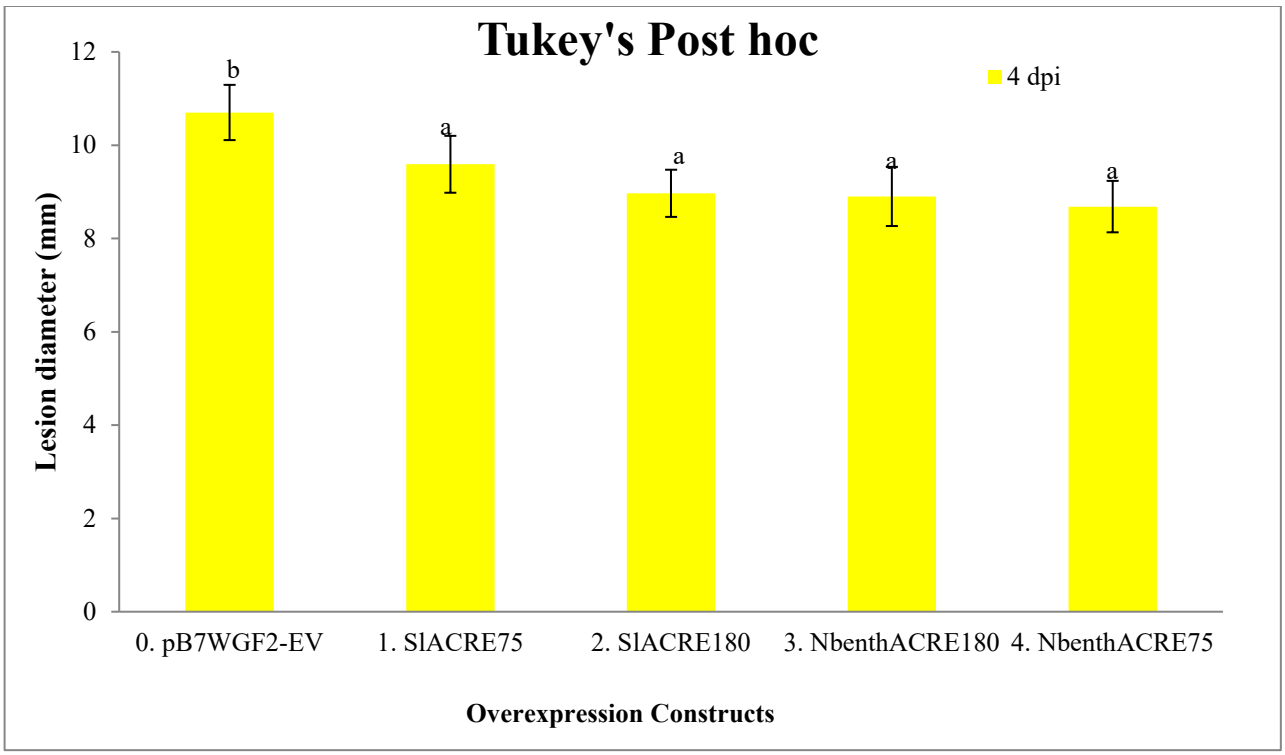
Co-expressed genes SlACRE75	Co-expressed genes SlACRE180
Calmodulin	BCS1
Calcium-transporting ATPase2	WRKY53,11,30
Exocyst complex protein	Receptor-like kinase
Harpin-induced protein	CHP-rich zinc finger protein
Gibberellin 2-oxidase	Matrix metalloproteinase (Sl2-MMP)
Auxin-related protein	SlACRE75
SlACRE180	

Figure 23. (a) Venn diagram illustrating the number of common and specific genes co-regulated with SlACRE75 (red circle) and SlACRE180 (green circle). All 29 genes were included in the ANOVA-Benjamini Hochberg corrected (3713 DEGs).

Functional analysis: Transient overexpression of ACRE75 and ACRE180 on *Nicotiana benthamiana*

Microarray analysis has shown that ACRE genes have an important role on tomato resistance to fungal aggressive pathogen *Botrytis cinerea*. Furthermore, tomato SlACRE75, SLACRE180 and their *Nicotiana benthamiana* orthologues, NbACRE75 and NbACRE180 are induced by the pathogen and also primed by chitosan at 24 hpi (data not shown). Thus, it seems logical to study them further by functional analysis. Hence, to investigate whether these 4 proteins can delay *Botrytis cinerea* lesion expansion, using transient overexpression approach, the 4 ACRE candidate proteins and an empty vector (as a control) were agroinfiltrated into *Nicotiana benthamiana* (model plant for cloning experiments and well-stablished methodology in The James Hutton Institute) plants. 2 days after ACRE proteins agroinfiltration, leaves were excised for pathogenicity test and subsequently challenged with *Botrytis cinerea* spore inoculum ($2x10^4$ spores ml^{-1}) by drop inoculation; lesion size measurements were annotated to look for a resistance phenotype.

Lesion size measurements indicated that all 4 proteins significantly decreased Botrytis infection in comparison with empty vector (non-protein plasmid) (Figure 24), which suggests a possible role of ACRE75 and ACRE180 in tomato and benthamiana resistance against *Botrytis cinerea*.



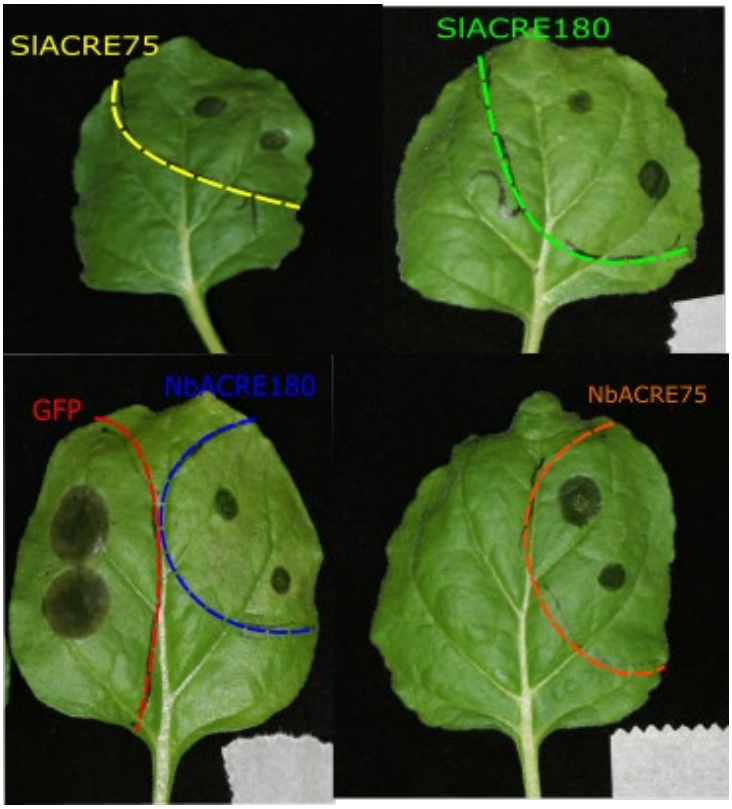


Figure 24. Transient expression of constitutively active SlACRE75, SlACRE180, NbACRE75 and NbACRE180 in *Nicotiana benthamiana* resulted in increased disease resistance against *Botrytis cinerea*. Pictures show disease symptoms in SlACRE75, SlACRE180, NbACRE75, NbACRE180-and empty vector (GFP)/non-protein-infiltrated plants at 3 days post *Botrytis cinerea* infection (dpi). Opposite part of the leaves infiltrated with constructs was inoculated by dropping spore suspension (2 × 10exp4 spores/mL) of *Botrytis cinerea*. Lesion sizes were measured at 3 and 4 days after inoculation on 3 independent plants and 3 leaves contruct^{-1}. Values presented are means ± SEM. Different letters indicate statistically significant differences (ANOVA P<0.01 followed by Tukey‘s Post-hoc at 4 dpi).

Botrytis cinerea **Microarray analysis**

As previously stated this transcriptomic analysis was a joint 60k Agilent array design made with 16,365 *Botrytis cinerea* probes & 34,510 *Solanum lycopersicum* probes. For the transcriptomic analysis on the pathogen, statistical test (T-test pairwise) on non-treated (control) + infected and chitosan-primed + infected samples revealed that chitosan was able to stop the expression of Botrytis virulence genes (Figure 25), including hexokinase, hexokinase is required for pathogenicity (Rui & Hahn, 2007), novel virulence genes involved in Botrytis germination such as BcPG2, a pectinase gene to degrade plant cell-wall pectin, superoxide Dismutase, required for lesion expansion on *Phaseolus vulgaris* (Smith, Mengesha, Tang, Mengiste, & Bluhm, 2014b) which was expressed at 9 and 12 hpi, which correlates with spore germination observed under microscope (data not shown); Uracil phosphoribosyltransferase, recently found to be new potential virulence factor involved in Botrytis spore germination (Gonzalez-Rodriguez et al., 2014), thus key for the establishment of the infection, which was expressed in Inf plants at 9 hpi, and the well-known polygalacturonase genes that play a role in tissue colonization (Frías, González, González, & Brito, 2016; Gonzalez-Rodriguez et al., 2014; Rui & Hahn, 2007) that were expressed later at 12 hpi.

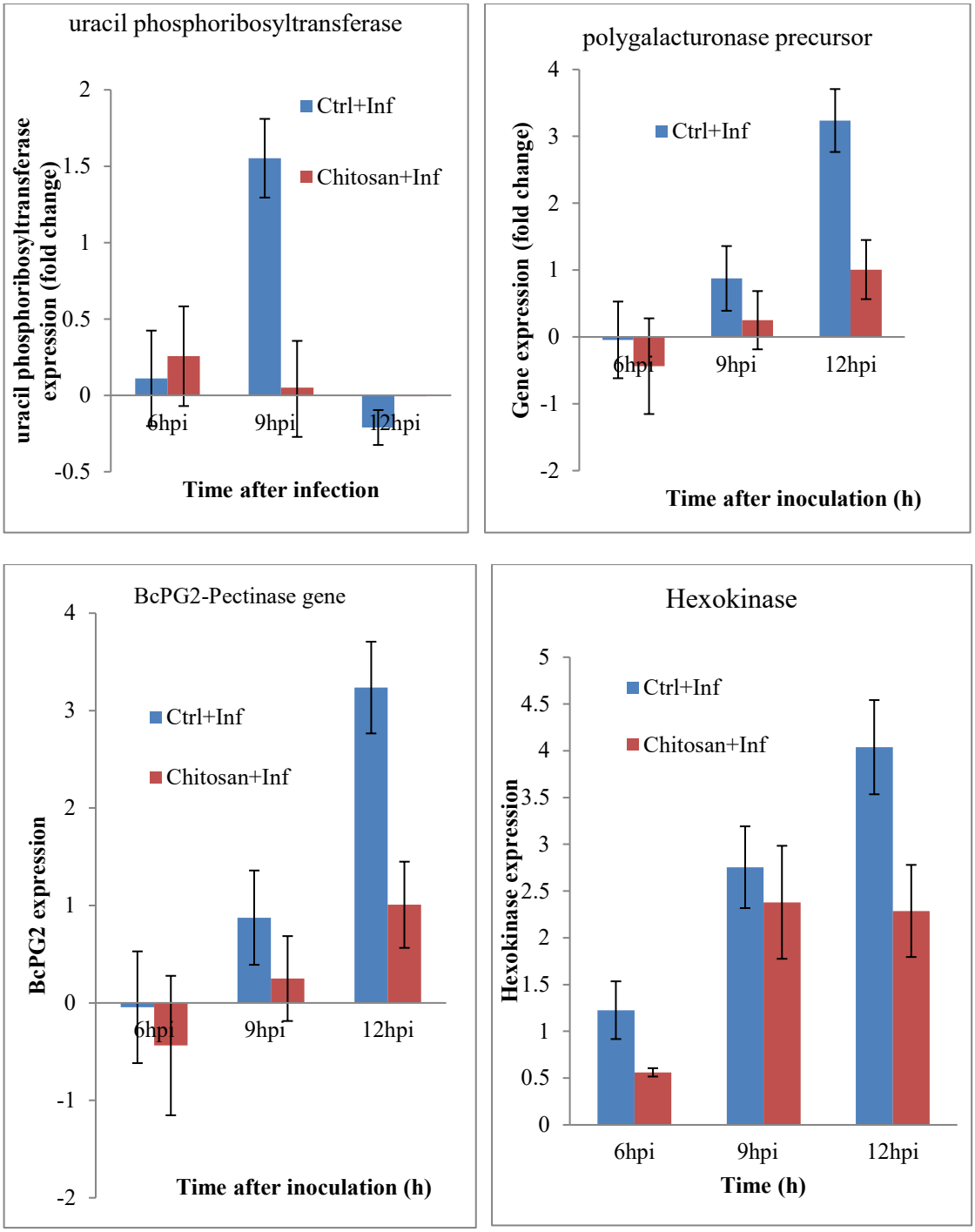


Figure 25. Normalized gene expression. The data shown are the means of four biological replicates ± standard error of the mean (SEM). Ctrl+Inf, untreated and Botrytis-infected plants; chitosan+Inf, chitosan-treated and infected plants. Test Type: Parametric test, differentially expressed genes were defined by Fold Difference: 2 and a P-value < 0.05.

Discussion

In this PhD project, the effectiveness and mode of action of resistance elicitors BABA, BTH, MeJA and two formulations of chitosan-induced resistance in two tomato varieties against the necrotrophic pathogen *Botrytis cinerea* has been investigated It has been shown that treatment with BABA, BTH, MeJA and chitosan induces “long-lasting resistance” in tomato cv. Moneymaker and tomato cv. Motelle against *Botrytis cinerea*. However, elicitor-induced resistance seemed to be cultivar-dependent, as BTH and MeJA-induced resistance was more effective in Moneymaker whilst BABA-IR was more effective in Motelle. This suggests a stronger ability of Motelle to respond to BABA-IR against *Botrytis cinerea*. Interestingly, chitosan (water soluble formulation) functioned similarly in both tomato varieties against *Botrytis cinerea* (Figure 3) which suggests a potential extrapolation of chitosan-induced resistance to other varieties and/or crops. It’s well known that plants can display “relatively early acting defences” like reactive oxygen species (ROS) production, including superoxide (O_2^-) and H_2O_2, which are generated following the recognition of a variety of pathogens, and they function as a threshold trigger for the hypersensitive response (HR) (Mouekouba et al., 2014); or callose deposition, a plant beta-1,3-glucan polymer, which is rapidly synthesized and deposited just beneath the sites of attempted pathogen penetration, and has long been considered as an important factor for plant penetration resistance against invading pathogen (Oide et al., 2013). These plant defences may be crucial for stopping or slowing pathogen expansion, thus leaving the plant time to trigger its fine tuned, late and endurable defences. To elucidate whether BTH, MeJA, chitosan and BABA act through inducing “relatively early acting defences”, we examined callose deposition after elicitor treatment. Interestingly, chitosan (ChitoPlant) was able to induce callose in both tomato varieties while MeJA-induced callose was higher in Moneymaker and BABA-induced callose was higher in Motelle, which suggests a Motelle higher affinity towards BABA. These results seem to confirm earlier theory of plant cultivar-dependence of elicitor-IR (Walters, Walsh, Newton, & Lyon, 2005b).

As stated before, reactive oxygen species (ROS) are quickly accumulated after pathogen attack (Temme & Tudzynski, 2009) which make them crucial for the disease development. However, even though there is a controversy in the role of ROS against necrotrophic pathogens, together with callose accumulation seems to be crucial in the early stages of the infection (Finiti et al., 2014).

Results on extracellular peroxidase (POD) activity showed that sitiens, the ABA deficient tomato mutant line, had a strong POD induction against *B. cinerea*, which confirms earlier discoveries on sitiens resistance to *B. cinerea* based on a timely fine-tuned H_2O_2 production (Asselbergh et al., 2007). In tomato wild type (Moneymaker), although not significantly, chitosan and MeJA showed to prime POD in comparison with control infected plants, however

chitosan induced POD at early stages of the infection, while MeJA induced tomato peroxidases at later stages against *Botrytis cinerea*, which suggest a difference in MeJA and chitosan mode of action against *B. cinerea*. Furthermore, MeJA and chitosan, in a less extent, were able to contained H_2O_2 accumulation on tomato infected leaves at later stages of the infection whereas non-treated control leaves H_2O_2 was not restricted at the infection sites and spread throughout a bigger area of the leave, suggesting a possible manipulation of *Botrytis cinerea* host defences as stated before (El Oirdi et al., 2011). However, in order to confirm this theory, further experiments need to be done.

Another PhD objective was to assess the, previously seen, priming properties of MeJA in tomato and other plant systems after pathogen attack as well of the ability of this elicitor interfering in the plant hormone cross-talk (Biswas, Dey, Karmakar, & Satpathy, 2014; Koornneef et al., 2008; Pluskota, Qu, Maitrejean, Boland, & Baldwin, 2007a; Wang et al., 2009). It is well-known that cross talk between salicylic acid (SA) and jasmonic acid (JA) signalling pathways plays an important role in the regulation and fine tuning of plant induced defences that are activated after pathogen attack (Dong, 2004; Pieterse & Van Loon, 2004; Koornneef et al., 2008). Moreover, there is evidence that *Botrytis cinerea* can manipulate plant antagonistic effects between immune signalling pathways in order to promote disease development (El Oirdi et al., 2011); however this manipulation may depend on the plant species and pathogen strain.

However, it is not simply the manipulation of SA/JA pathways by a necrotrophic pathogen since exogenous application of SA to tomato plants can be sufficient to inhibit the JA-induced expression of genes encoding proteinase inhibitors (PI I and PI II), suggesting that SA targets the JA pathway downstream of JA biosynthesis (Doares, Narvaez-Vasquez, Conconi, & Ryan, 1995). In the present study MeJA, known to trigger JA-dependent defence genes, was used in order to investigate whether it can interfere in SA/JA cross-talk and prime tomato to potentially stop pathogen hormone manipulation.

In order to investigate whether that *Botrytis cinerea* BcR16 strain is able to manipulate the antagonistic cross-talk between JA-SA pathways through NPR1 (El Oirdi et al., 2011), NPR1 expression was tested (Figure 13), a key regulator gene of systemic acquired resistance (SAR) (Pieterse & Van Loon, 2004).

Interestingly, SlNPR1 was only up-regulated by control plants at 8hpi, whereas MeJA was able to repress NPR1 expression. However, control-infected plants repressed NPR1 at 24 and 48 hpi whereas MeJA-infected plants were not able to neither repress nor induce it; this suggest an early manipulation of the JA-pathway in favour of SA expression and MeJA can supress this manipulation during early stages of the infection, however in order to test this

theory further experiments need to be done. Gene expression analysis of two tomato JA-defence genes, Leucine aminopeptidase (Lap) A, a gene involved the plant-defence response against mechanical wounding, insect infestation, and in response to pathogen infection (Pautot et al., 2001); and lipoxygenase (Lox)D, a gene involved in jasmonic acid defences and biosynthesis (Scranton et al., 2013), and the main defence phytohormone against necrotrophic pathogens such as *Botrytis cinerea* were investigated. LapA and LoxD transcript levels showed that both genes were repressed by both of the infected treatments. However, LoxD transcript was repressed by the pathogen during early stages of the infection and MeJA was able to prime it at 48 hpi, which suggest that MeJA is able to reduce JA-defence gene repression by the pathogen.

So far, plant hormone jasmonic acid was revealed as a key hormone in elicitor-tomato-*Botrytis cinerea* interaction, which correlates with previous studies of JA key importance against necrotrophs (Aubert, Widemann, Miesch, Pinot, & Heitz, 2015; Glazebrook, 2005; Kravchuk et al., 2011). Furthermore, induced systemic resistance (ISR) has been associated with the expression of jasmonic acid-dependent genes (van Wees et al., 1999). In order to test the role of induced systemic resistance (ISR) in tomato protection against *Botrytis cinerea*, two *Bacillus amyloliquefaciens* and *Bacillus subtilis* strains, which are well-characterised PGPR capable to induce ISR in multiple crops and other plants (Akram et al., 2014), were used to test their biocontrol properties. Results showed that both Bacillus WT and GBO3 strains were able to induce resistance against *B. cinerea* through significantly reducing necrotic lesion expansion. Besides, both strains induced resistance abilities were compared with chitosan-induced resistance which is ultimately triggering JA pathway (Iriti & Faoro, 2009). This result suggest a possible similar role of ISR in tomato than Arabidopsis, where the ISR pathway functions independently of salicylic acid (SA) but requires responsiveness to jasmonate and ethylene (van Wees et al., 1999).

Also, as previously stated, elicitor-induced resistance can lead to potential costs and trade-offs in plant (Luna et al., 2016; Redman et al., 2001; van Hulten et al., 2006; Walters & Heil, 2007). Nevertheless, few studies have examined it in detail in crop systems, (Luna et al., 2016) showed that soil drench 1-week old tomato seedlings with high concentrations of BABA (10 mM) and JA (1 mM) abolished plant growth and had lethal effects. Elicitor treatment on tomato cv. Moneymaker and Motelle seedlings showed that BABA had a strong growth repression on both cultivars, reducing 39% the relative growth rate (RGR) in Money-maker (MM) and 42% in Motelle, indicating that this last cultivar is more susceptible to BABA-induced stress. Furthermore, it was shown that two of all resistance elicitors used during the PhD project (MeJA and chitosan) were able to significantly reduced *Botrytis cinerea* expansion in two tomato cultivars with no fitness costs in plant growth. These results verify a cultivar-

dependence to elicitor-induced fitness costs. Interestingly, water-soluble chitosan (ChitoPlant) had a similar effect on the RGR of both cultivars, which again confirms a potential extrapolation of chitosan-induced resistance to other varieties and/or crops. However, chitosan had a cytotoxicity effect on tomato and aubergine leaves when applied at high concentrations (1% and 0.1% w/v) but not at lower concentrations (0.01%), which made us decide to investigate whether chitosan-induced resistance and callose deposition is concentration dependent.

Resistance phenotype assays revealed that chitosan was able to induced resistance in tomato, aubergine and Arabidopsis in a concentration-dependent manner, however at high concentration had a detrimental phytotoxicity effect on Botrytis infected aubergine plants, which suggest a possible threshold in chitosan-priming for resistance that depends on its concentration and when trespassed, it may overstress plant defences in benefit of necrotrophic pathogens such as *Botrytis cinerea*, which correlates with Botrytis host defence manipulation ability (Angulo et al., 2014; El Oirdi et al., 2011; Smith et al., 2014a).

On the other hand, chitosan-induced callose deposition did not depend on the concentration where lowest concentrations (0.01% and 0.001%) significantly induced callose deposition for a longer period (1 dpt) and higher than the highest concentration (0.1%), indicating that elicitor-priming does not follow a classical dose-response curve and the duration of the response is affected by concentration. However, this duration may be inversely related to chitosan concentration.

So far, PhD results have shown that chitosan might prime crops for resistance against *Botrytis cinerea*; however this priming effect might be concentration-dependent. Thus, we decided to further investigate chitosan direct fungicide effects against *Botrytis cinerea*. In vitro assay showed that chitosan abolished fungal hypha growth and spore germination under high concentrations whilst at low concentrations chitosan showed no direct fungicide effect. These results clearly support the hypothesis of the concentration-dependence of priming of elicitors (chitosan in particular), suggesting a concentration threshold in which chitosan might not directly affect fungal growth instead of priming the plant own defence mechanisms to fight back pathogen challenge.

The diverse mechanisms of action of chitosan have been well-studied, which include activation of antifungal proteins (Muñoz & Moret, 2010), oxygen-species scavenging and antioxidant activities, as well as the octadecanoid pathway activation (El Hadrami et al., 2010), however the majority of previous studies seem to focus on chitosan direct defence induction rather than upon its priming properties. Despite these studies, experiments that specifically address the role of priming in the complex chitosan-plant interaction framework are still lacking.

During my PhD we have seen that chitosan under low concentrations can still protect crops against aggressive necrotrophic fungal pathogens without having a direct interaction with the pathogen. For this reason, we decided to perform a microarray to unveil chitosan priming activity in tomato against *Botrytis cinerea*.

Transcriptomic analysis (microarray) clearly showed that chitosan primed tomatoes for a faster and stronger defence response with the significant expression of more than 2100 genes whereas non-treated control plants only significantly expressed 363 genes. In order to further understand chitosan-priming activity on tomato-*B. cinerea* interaction, functional enrichment study of DEGs belonging to the two infected conditions (Inf and ChitoInf) revealed important molecular pathways that were significantly enriched for chitosan after Botrytis infection, including APETALA2/Ethylene-responsive element binding protein family, signalling in receptor kinases, signalling in sugar and nutrient physiology, hormone metabolism/Auxins, cell-wall, secondary metabolism such as wax, phenylpropanoids and lignin early during Botrytis infection; and peroxidases, receptor kinases, lipid metabolism, FA desaturation, amino acid, pectin methylesterase inhibitor family protein, glutaredoxins and ethylene and jasmonate metabolism later during the infection. These results start to unveil chitosan strategies to prime tomatoes against *Botrytis cinerea*, by firstly inducing a stronger and faster signalling network, together with cell-wall reinforcement and modification, all to reduce pathogen penetration and reduce and/or avoid pathogen induced-stress, and later during the infection by inducing transcriptional regulation, protein ubiquitination, reduce pathogen-induced redox stress and induction defence hormone metabolism such as jasmonic acid.

It is well-known that plant hormones play important roles in the regulation of the defence signalling network upon perception of biotic or abiotic stress (Forcat et al., 2008; Pieterse et al., 2012). In order to elucidate role of key hormonal pathways in chitosan-tomato-*Botrytis cinerea* interaction, significant expression of jasmonic, salycilic, abscicic (ABA) and ethylene-related genes in the microarray was investigated. MYC2, a basic helix-loop-helix (bHLH) domain–containing transcriptional factor (TF) that can act both as an activator and repressor of distinct JA-responsive gene expression in Arabidopsis (Lorenzo et al., 2004) was down-regulated at 6 hpi and 9 hpi for both non-treated and infected (inf) and chitosan-primed and infected (ChitoInf) treatments. However, SlMYC2 was only down-regulated at 12 hpi by ChitoInf. Jasmonate Zim Domain proteins (JAZ) are negative regulators of JA-induced gene expression (Wasternack & Hause, 2013); SlJAZ1 was antagonistically expressed to SlMYC2, were it was up-regulated by both inf and in a higher extent by chitoinf at 6 and 9 hpi, however at 12 hpi is was noticed that SlJAZ1 induction was lower that inf treatment. This suggests a possible shift of JA-induced defences at 12 hpi where chitosan no longer represses JA-induced defence expression in favour of other pathways in the early stages of the infection

and after the pathogen manages to overcome these “relative early defences” chitosan instead primes JA-defence expression. Furthermore, there is a complex network in the JA-SA cross-talk in response to pathogen attack (Van der Does et al., 2013). JA and SA are generally considered antagonistic pathways (Takahashi et al., 2004), in Arabidopsis, the defence regulatory protein NONEXPRESSOR OF PR GENES1 (NPR1) was identified as a key signalling protein in the regulation of SA/JA crosstalk (Van der Does et al., 2013).Thus, NPR1 expression was studied, SlNPR1-1 was significantly expressed in the ANOVA-BH and it was down-regulated by both inf and chitoinf at 6 hpi. However, at 9 and 12 hpi, SlNPR1-1 expression changed and it was only down-regulated by chitoinf whereas non-treated control and infected plants were not able to down-regulate it at the 2 last time points. This suggests that chitosan might reduce Botrytis JA-dependent defence manipulation through NPR1 in tomato at later stages of the infection.

Transcriptomic results on hormone expression in chitosan-primed tomatoes vs *B. cinerea* were further verified by phytohormone analysis. HPLC/MS SA/JA/ABA analysis revealed jasmonic acid and its bioactive compound jasmonic acid-isoleucine as significantly induced by chitosan and/or MeJA in pathogen presence and absence. However, abscisic acid and salicylic acid were not significantly expressed in any of the treatments. Methyl-jasmonate (MeJA) significantly induced jasmonic acid (JA) without infection as well as it was able to prime JA earlier and stronger during *Botrytis cinerea* infection in comparison with non-treated control plants by significantly increase JA levels at 9 and 24 hpi. Interestingly, chitosan was also able to induce JA and the bioactive compound jasmonic acid-isoleucine (JA-Ile) in a time-dependent manner, later than MeJA, during the infection, whilst the combination treatment primed JA faster and quicker than chitosan itself. Besides, chitosan itself and in combination with MeJA were able to supress MeJA-induced JA levels. This suggests a different strategy of chitosan in protecting tomato plants against *Botrytis cinerea* during early stages of the infection by repressing JA-responsive defences in favour of PTI-ETI type of responses, revealed in the microarray, including, resistance-type genes activation, MAP-kinase signalling cascades, cell-wall reinforcement, peroxidase and PR-proteins activity, as showed by microarray analysis, and which also correlates with repression and activation of JA-dependent SlMYC2 and SlJAZ1 transcripts seen during microarray analysis, were SlMYC2 was down-regulated and JAZ1 was significantly up-regulated by chitosan at 6hpi and 9 hpi and this trending changed at the latest time point 12 hpi were SlJAZ1 and SlNPR1-1 expression was lower than control + infected plants.

Furthermore, hormone analysis showed that MeJA was able to, although not significantly, trigger synergistically the 3 hormones ABA, SA and JA at 9 hours after the infection. However, MeJA and chitosan weren’t able to significantly induce Salicylic and abscisic acid. Apparently,

there was not any salicylic acid manipulation from the pathogen during the infection and ABA was much more concentrated than the other hormones; however ABA doesn't seem to play a big role during the early stages of the infection, apart from its putative pathogen-caused reduction at 6hpi.

Hence, HPLC/MS hormone analysis discovered jasmonic acid and its bioactive derivative jasmonic acid-isoleucine (JA-Ile) as the key hormones involved in chitosan-IR and MeJA-induced resistance in tomato against *Botrytis cinerea* during early stages (symptomless) of the infection (at 9 and 24 hours after infection (hpi)). These results correlate with the well-known role of jasmonic acid in plant resistance against necrotrophic pathogens such as *Botrytis cinerea*, *Sclerotinia sclerotium* and *Fusarium oxysporum*.

Conclusions

1. Resistance elicitors methyl-jasmonate (MeJA, plant phytohormone) (Sigma-Aldrich), BTH (BION, Syngenta), the amino-acid compound (beta-aminobutyric acid-BABA, Sigma) and chitosan (ChitoPlant, low molecular weight and water soluble formulation, ChiPro, Germany) induced resistance by significantly reducing gray mould disease (*Botrytis cinerea*) on two 4-weeks old tomato varieties (tomato cv. Moneymaker and cv. Motelle). Also, the biocontrol strains *Bacillus subtilis* and Bacillus spp. GB03 induced systemic resistance in tomato cv. Moneymaker by significantly decreasing Botrytis necrotic lesion diameter.

2. Besides, MeJA and ChitoPlant also induced resistance in 4-week-old *Arabidopsis thaliana* (thale cress) cv. Columbia0 and Aubergine cv. Black beauty plants and significantly decreased *Botrytis cinerea* infection in comparison to water-treated control plants, just by foliar spraying the solutions once into the shoot 4 to 17 days (called long-lasting induced resistance) before Botrytis infection.

3. MeJA, BTH, BABA and Chitosan2-induced resistance (resistance phenotype) behave differently depending on tomato cultivar. Interestingly, ChitoPlant-induced resistance does not depend on tomato cultivar.

4. To help understand more about the balance between the value of priming crop defences and elicitor interaction with the plant development, the costs of the elicitor-induced resistance in crop yield were measured. Elicitors can reduce plant growth when applied at medium-high concentrations. Treatment of 1-2 weeks old tomato cv. Moneymaker and cv. Motelle with MeJA (0.1mM), the amino-acid compound BABA (Sigma), 2 formulations of chitosan (chitosan from NaturCrop and ChitoPlant, water soluble chitosan from ChiPro), BTH (Bion, Syngenta) revealed that BABA, when soaked (5mM stock solution= 0.5mM final concentration) into trays containing 12 tomato cv. Moneymaker and 12 tomato cv. Motelle seedlings had a strong growth repression effect in both varieties. BABA reduced 39% the relative growth rate (RGR) in Money-maker (MM) and 42% in Motelle, indicating that this last cultivar is more susceptible to BABA-induced stress. Chitosan (ChitoPlant formulation) may also induce cell-death in tomato and aubergine plants at high concentrations.

5. MeJA and chitosan (chitoplant) can induce callose deposition in tomato cv. Moneymaker and Motelle. However, BABA induces callose deposition mainly in tomato cv. Motelle.

6. Large transcriptomic analysis revealed chitosan mode of action on chitosan-treated and Botrytis-infected 4-weeks old tomato plants. Chitosan-treated 4-weeks old tomato cv. Moneymaker seedlings were able to display stronger and faster defence mechanisms by differentially expressing more than 2100 defence-related genes after Botrytis infection (no symptoms visible), while water-treated tomatoes only differentially expressed 363 genes. Chitosan-treated tomatoes were also able to practically abolish Botrytis *virulence gene*

expression (used by the pathogen to facilitate infection) while water-treated plants were not able to repress Botrytis gene expression. Furthermore, analysis on the 2100 genes induced by chitosan revealed key pathways involved in tomato defences against Botrytis, such as cell-wall modification genes (lignin and cellulose synthesis), regulatory and signalling genes (protein kinases, transcriptional factors, involved in the transmission of the defence signal throughout the plant cells), jasmonate and ethylene-dependent genes, redox state (glutaredoxins, involved in the cell oxidative stress) and secondary metabolites (phenylpropanoids). These results unveiled potential molecular pathways involved in chitosan-induced priming for resistance in tomato (and potentially applicable to other crops, i.e. Aubergine) against the aggressive fungal pathogen *Botrytis cinerea*. This information might help breeders to target commercial crop varieties with a stronger expression of the mentioned pathways in order to cultivate more resistant domestic varieties against fungal pathogens.

7. HPLC/MS phytohormone analysis on chitosan and methyl-jasmonate treated and Botrytis infected tomatoes (4 weeks-old) of the 3 main hormones (salicylic acid, jasmonic acid and abscisic acid) involved in plant defences against pathogens. Jasmonic acid was revealed as the key hormone in chitosan-induced resistance and methyl-jasmonate-induced resistance in tomato against Botrytis. This result is of high interest as it shows that jasmonic-acid dependent elicitors (such as chitosan, methyl-jasmonate) are effective against necrotrophic (they feed on death plant tissue/cells) pathogens such as *Botrytis cinerea*.

8. As part of the PhD project, the effects of an elicitor combination treatment were evaluated. Low-dose combination of chitosan (0.01% w/v) + methyl-jasmonate (0.1mM) (in Tween 20 0.01%, adjuvant) foliar treated-tomato plants showed a stronger protection against Botrytis (healthier plants after infection), which suggest a potential synergistic effect of chitosan in combination with other elicitors (i.e. Methyl-jasmonate). Thus, chitosan might be a good candidate to include in IPM antifungal strategies and therefore reduce fungicide use.

Knowledge and Technology Transfer

- Vegetable Consultants Association (VCA) annual meeting: 1st November 2016, James Hutton Limited, Dundee. Talk: Elicitor-Induced resistance in crops for a more efficient fungal disease management
- AHDB Crops PhD annual Conference 2016. Talk: CP105-Priming tomato with chitosan for a more efficient fungal disease management
- Tomato Growers Association (TGA) annual conference 2016. Talk: Resistance elicitors protect tomato against *Botrytis cinerea* (gray mould) through inducing defences
- AHDB-Horticulture Grower.No225. Jul/August 2016. Report of my PhD: Crop Protection: The Science of self-defence. Priming against pathogens.
- 5th International Symposium on Tomato Diseases that was held in Málaga, Spain, June 13-16, 2016**.** Poster presentation: Chitosan primes resistance in tomato plants against *Botrytis cinerea* through inducing expression of 1,745 genes including Avr9/Cf-9 rapidly elicited genes and cell-wall processes
- IOBC-Induced resistance PR-proteins. Joint meeting of the 'PR Proteins Workshop' and the 'Working Group Induced Resistance in Plants against Insects and Diseases' of the International Organization of Biological Control (IOBC-WPRS), Aachen, Germany. September 2015. Poster and pitch presentation: chitosan protects tomato plants against *Botrytis cinerea* by priming defence responses
- The 2015 Molecular Biology of Plant Pathogens (MBPP) conference, University of the West of England (UWE), Bristol, UK, April 2015**.** Talk
- AHDB-horticulture 2014 and 2015. Annual reports and summary report for AHDB March 2016
- AHDB-HDC annual conferences 2015 and 2014. Poster and talk: Integrated protection of horticultural crops through enhancing endogenous defence mechanisms
- Tomato Growers Association (TGA) annual Conference 2015. Poster and Pitch presentation: Resistance elicitors protect tomato against *Botrytis cinerea* (grey mould) through inducing defences
- Proceeding: Induced resistance in crop protection: an overview. Adrian C Newton, Nicola Holden, Daniel De Vega Perez, Clement Gravouil, Dale R Walters 2014 IOBC-WPRS Working Group "Integrated Control in Protected Crops, Temperate Climate", At Ghent, Belgium, Volume: IOBC-WPRS Bulletin Vol. 102
- “Integrated crop protection through enhancing endogenous defence mechanisms”. Poster for the James Hutton Institute annual 2014 Postgraduate competition.

- Attendance to Crop Protection Northern Britain 2014 that was held in Dundee

Glossary

Induced resistance (IR); Pathogen-associated molecular pattern (PAMP); Microbe-associated molecular pattern (MAMP); Avr9/Cf-9 rapidly elicited (ACRE) genes; hypersensitive response (HR); β-aminobutyric acid (BABA); reactive oxygen species (ROS); methyl-jasmonate (MeJA); programmed-cell death (PCD); High Performance Liquid Chromatography (HPLC) mass-spectrophotometry (MS); PAMP-triggered immunity (PTI); effector-triggered immunity (ETI); plant growth-promoting rhizobacteria (PGPR), induced-systemic resistance (ISR), systemic-acquired resistance (SAR); salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA)

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