Project title:	Integrated protection of horticultural crops through enhancing endogenous defence mechanisms
Project number:	CP105
Project leader:	Professor Adrian C Newton, The James Hutton Institute
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Date project commenced:	1st September 2013
Date project completed	31st September 2016

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GROWER SUMMARY

Headline

Experiments have revealed that chitosan, the natural polysaccharide, and methyl-jasmonate (MeJA), the volatile organic compound and plant hormone derivative, can successfully induce resistance, having a positive effect at molecular level, in tomato, against the fungal pathogen *Botrytis cinerea*.

Background

Plants, especially crops, are threatened by numerous microbes; many of them can be actual pathogens causing devastating losses worldwide. Many of our crop protectants become ineffective as pathogens develop resistance due to extensive use or new pathogens emerge. Products are lost from the market also for regulatory reasons, and the market sometimes requires varieties to be grown that are susceptible. However, even susceptible plants have highly effective resistance mechanisms that, if triggered and expressed in a focussed, specifically-targeted way, could not only lead to better crop protection, but also substantially reduce the need for conventional pesticide use. This can be done with resistance 'elicitors' (RE). Resistance elicitors can mimic pathogen-induced defence mechanisms in the plant and thus trigger its defence mechanisms, enabling the plant to respond to actual pathogen threats faster, without damaging other species or the environment and with reduced operator hazard. Furthermore, pathogens don't develop resistance as elicitors target plant defences rather than killing pathogen directly, a common issue with other toxic crop protectants such as fungicides. 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 resistant *B. cinerea* strains (Pappas, 1997).

Pesticides are 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. Whilst elicitors can be regarded as pesticides as they result in pathogen control, their mode of action is mainly based upon a more effective plant defence induction and expression of defence mechanisms rather than direct toxicity against pathogens. However, elicitors can have costs in plant development due to the plant's complex metabolic pathways as defence induction can potentially have effects on yield quantity and quality. To develop resistance induction crop protection approaches, a detailed knowledge of the timing and amplitude of defence induction as well as the consequences on target and non-target pathways is required. The molecular tools for

such studies and our understanding of the mechanisms in model and crop systems have advanced considerably in recent years. We will use these approaches to determine both the phenotypic and molecular profiles of resistance elicitors. This will give crucial information about how key signalling pathways interact in various crops and the mechanisms of trade-offs associated with disease reduction.

The project will focus on a single plant-pathogen system: *Botrytis cinerea* on tomato plants, testing a range of treatment types and regimes. Once effective treatment components and combinations have been established and the response characterised, the treatments will then be tested on other plant species that are also infected by *B. cinerea* to determine whether there are commonalities in the mode of elicitor action.

In particular, the project aims to (i) Identify novel resistance elicitors that result in effective induced resistance in *Solanum lycopersicum* (tomato) against *B. cinerea* ; (ii) to test the cost of induced resistance in plant development; (iii) to investigate the molecular basis to the plant defence response elicited by the treatment regime and to characterize the molecular response of the induced plant to the pathogen infection; and (iv) to evaluate the role and expression of the defence hormonal pathways in the plant in response to the pathogen attack and to investigate the role of the elicitors in the phytohormone cross-talk.

The research has relevance to a number of different sectors because the nature of the research is to investigate common mechanisms of defence, rather than for example, focus on fungicides that are limited to a single group of crops. Therefore, the work can be seen as under-pinning crop protection mechanisms. Besides, this research will establish the principles and potential for using resistance elicitors in robust integrated crop protection strategies.

Summary

Below, summaries and main findings are discussed within the framework of the project's four main objectives:

1. Identify novel resistance elicitors that result in effective induced resistance in *Solanum lycopersicum* (tomato) against *B. cinerea*

Chitosan and Methyl-Jasmonate-induced resistance in tomato cv. Money-maker against Botrytis cinerea

As seen in last year's report, the plant hormone methyl jasmonate (MeJA) and chitosan, the novel natural elicitor, are able to reduce disease in tomato (cv. Money-maker and Motelle) caused by the aggressive pathogen Botrytis (*Botrytis cinerea*) (Figure 8A).

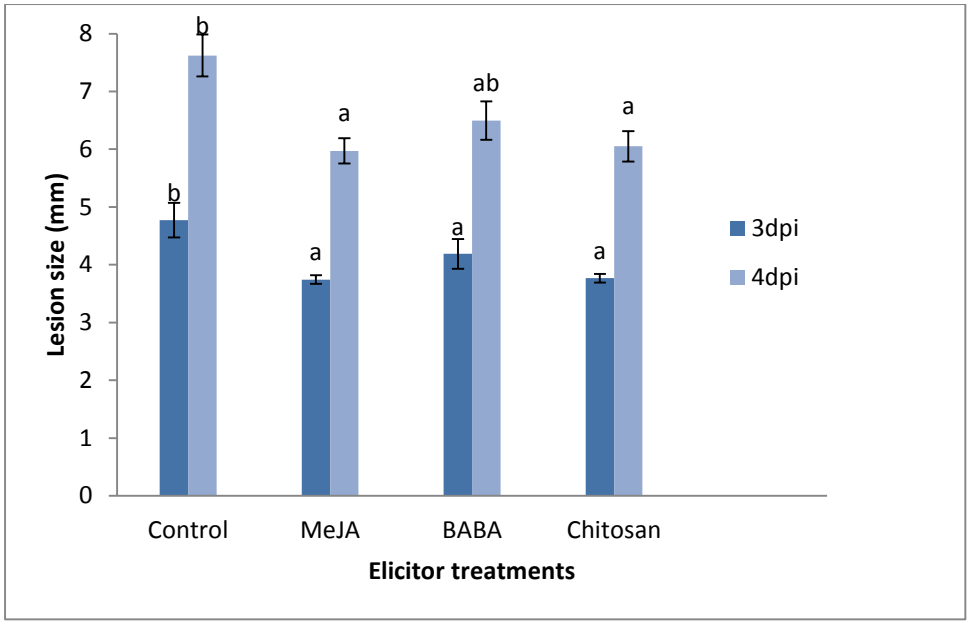


Figure 8A. Quantification of MeJA, BABA and Chitosan-induced resistance against *Botrytis cinerea* at 3 and 4 days post-infection in tomato cv. Money-Maker. Values presented are means ± SEM. Different letters indicate statistically significant differences.

Results also showed that beneficial bacteria *Bacillus subtilis* and *Bacillus amyloliquefaciens* GB03 strain can act as biocontrol agents and induce resistance against *B. cinerea* through significantly reducing necrotic lesion expansion at a late stage of the infection (72 hpi) (Figure 8).

2. Evaluate the cost of induced resistance in plant development

Elicitor-induced growth reduction in tomato cv. Money-Maker and Motelle

MeJA and the commercial chitosan formulation (ChitoPlant) were then chosen as the main significant elicitors to provide resistance in tomato against *B. cinerea* with no fitness costs in plant development. However, the synthetic elicitor BABA showed a strong growth repression in both tomato varieties (see 1st year annual report).

3. Investigate the molecular basis to the plant defence response elicited by the treatment regime and characterize the molecular response of the induced plant to the pathogen infection

***Early acting pathogen-inducible defence responses in tomato* - B.cinerea**

Plant's early defences, such as callose, the plant polysaccharide, and reactive oxygen species (i.e. H2O2), 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.

MeJA and Chitoplant (the chosen successful chitosan formulation) are able to significantly reduce botrytis necrotic lesion expansion in tomato and the model plant *Arabidopsis thaliana* (Figure 4). ChitoPlant defence induction is characterised by plant cell-wall fortification through callose deposition in tomato leaves before and after pathogen challenge (Figures 5 and 6).

Furthermore, both natural compounds can reduce and limit pathogen infection through localizing H_2O_2 production to the infection site (Figure 2), potentially reducing pathogen manipulation of its host defences (still to be further investigated).

4. Evaluate the role and expression of the defence hormonal pathways in the plant in response to the pathogen attack and to investigate the role of the elicitors in the phytohormone cross-talk

Jasmonic acid-dependent priming of gene expression in tomato -B.cinerea interaction

As expected, MeJA was able to trigger one of the main tomato defence pathways, the jasmonic acid-signalling pathway, through expression of key defence genes. Tomato leucine aminopeptidase (LapA) gene and tomato lipoxygenase D (LoxD) gene were triggered enabling the plants to respond to the pathogen infection faster (Figures 10 and 11). Both genes are involved in the jasmonic acid biosynthesis pathway, which is crucial for plant defence against necrotrophic pathogens as well as defence against herbivores.

In summary, it has been demonstrated that both chitosan and methyl-jasmonate play a crucial role in tomato defences against B. cinerea. Both elicitors act through "activating" the jasmonic acid-signalling pathway and through enhancing plant's early acting defences, such as cell-wall reinforcement (callose deposition) and H_2O_2 production surrounding the infection site. The chitosan findings may be extrapolated to the model plant Arabidopsis thaliana, which suggest a promising commonalty in chitosan mode of action between plant families. However, their efficacy may vary depending on the pathogen strain, crop cultivar and infection time point.

Financial Benefits

Outcomes of this project will be in the form of knowledge that enables product replacement with more benign alternatives, and principles for their use. We see this as maintaining profitability by providing the tools to continue to achieve effective crop protection that might

otherwise be compromised by loss of crop protection products or their reduced efficacy. Any specific knowledge that identifies either improved crop protection over conventional approaches or results in increased marketable or quality crop will be calculated in terms of financial benefit on a case-study basis as appropriate.

Action Points

There are few resistance elicitors currently licenced for use on horticultural crops and experimentation to determine which of these are effective for particular crops is being carried out in another project. This PhD project will help determine the principles whereby such products can be used, and particularly how they might be combined effectively. The latter will be as much about avoiding detrimental combination and practices as identifying those that might be additive or synergistic.

SCIENCE SECTION

Introduction

Plants are capable of defending themselves and fight off pathogen attack through constitutive and inducible defence mechanisms. Non-specific defence inducers, called elicitors, are able to mimic pathogen-induced defence mechanisms in the plant. Activation of plant endogenous defences by elicitors 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.

Conventional crop protectants (pesticides) can lose their efficacy due to pathogen resistance from their widespread use (Pappas, 1997). 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 (Figure 1).

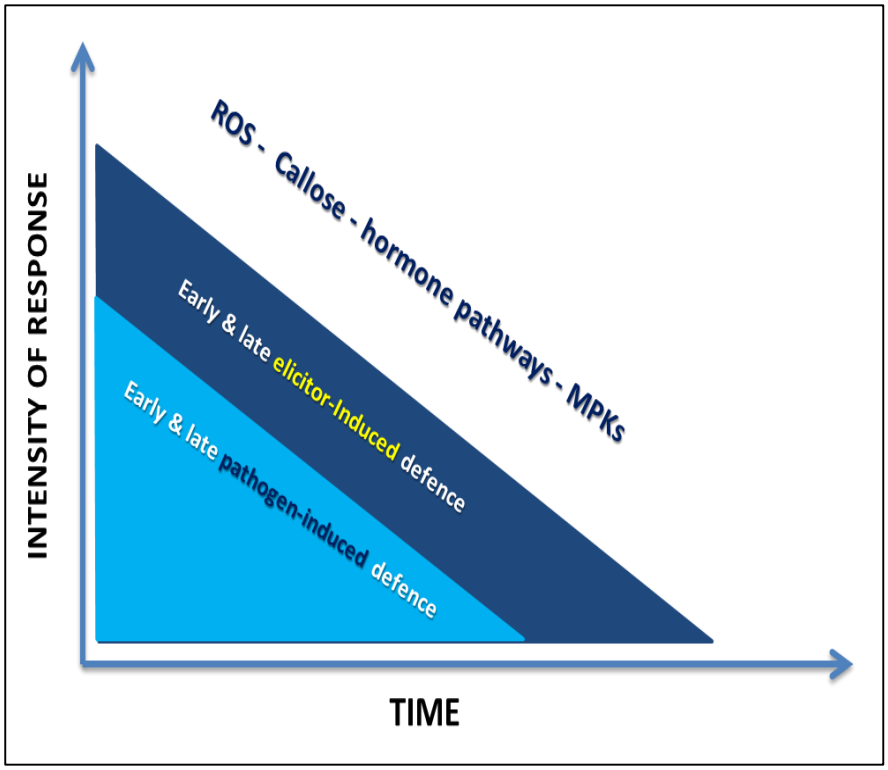


Figure 1. Model of series of inducible plant defences after pathogen challenge. Production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) is one of the most important plant defence responses to pathogens. Callose deposition is also an important factor for plant penetration resistance against invading pathogens. Plant hormones such as

jasmonic acid, salicylic acid and abscisic acid have emerged as important signals in the regulation of plant responses to pathogenic microorganisms.

Here we hypothesized that *Botrytis cinerea* is able to utilize tomato defences to promote disease and that novel elicitors such as chitosan, the natural compound, and the phytohormone methyl-jasmonate (MeJA) are able to reduce, in tomato, this pathogen manipulation alerting plant defences prior pathogen attack. However, their effective application requires understanding their expression and mode of action in the plant and the agronomy of the crop.

Moreover, Chitosan, the de-acetylated chitin derivative, can act as a Pathogen-associated molecular patterns (PAMP), inducing non-host resistance and priming for systemic acquired immunity in the plant. Nevertheless, chitosan-priming activity depends on the plant species and its physicochemical properties (Iriti & Faoro, 2009). Here, we demonstrate that chitosan-primed tomatoes are able to defend against *Botrytis cinerea* through induced callose deposition and a faster and stronger induction of its transcriptome. These data can lead to some exciting results on how tomato deploys its defences in response to specific priming agents, establishing the principles and potential for using resistance elicitors in robust integrated crop protection strategies.

Material and Methods

Basic methods were reported in the previous annual report. In addition the following methods were used:

Early acting pathogen-inducible defence responses in tomato-B.cinerea

Goal: To test whether marker elicitors can induce reactive oxygen species (ROS) and Callose apposition after pathogen challenge.

1. H_2O_2 temporal evolution after infection

4-5 weeks-old tomato seedlings were foliar sprayed with resistance elicitors to the following final concentrations:

- Control (non-treated): DDW + 0.02% Tween 20 (surfactant)
- Chitosan 1% w/v (Chitoplant, ChiPro, Germany) + 0.02% Tween20 (surfactant)
- MeJA (0.1 mM) + 0.02% Tween20 (surfactant)

Four days after elicitor treatments (after systemic defence induction), detached leaves were infected with *B.cinerea* spore inoculum (2x 10exp.4 spores/mL). Infected leaves of the different treatments were then harvested for 3, 3-diaminobenzidine (DAB) staining at 24 and

48 hours post-infection in order to evaluate temporal evolution of H_2O_2 accumulation (ROS). Polymerization-oxidation of the DAB molecule at the site of H_2O_2 accumulation results in a brown, reddish colour that is macroscopically visible (Asselbergh *et al*., 2007). For the positive control, leaves were infiltrated with 30% w/w H_2O_2 and with distilled water as a negative control (Figure 2).

Chitosan-induced Callose deposition in tomato and Arabidopsis thaliana

Goal: To test whether a commercial formulation of chitosan (Chitoplant, ChiPro, Germany), functions as PAMP inducing Callose deposition at different time points and concentrations in tomato and the model plant *Arabidopsis thaliana*.

Experimental procedures:

A.thaliana Col-0 plants were mass-seeded on soil, 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. 6 week-old plants were treated with:

- Control (non-treated): DDW + 0.02% Tween 20 (surfactant)
- Chitosan 1% w/v (Chitoplant, ChiPro, Germany) + 0.02% Tween20 (surfactant)
- Chitosan 0.1% w/v (Chitoplant, ChiPro, Germany) + 0.02% Tween20 (surfactant)
- Chitosan 0.01% w/v (Chitoplant, ChiPro, Germany) + 0.02% Tween20 (surfactant)

At one and two day's post-treatment (dpt), plant material was collected and fixed in ethanol and Callose deposition was analysed.

Chitoplant-induced resistance in Arabidopsis Col-0 against Botrytis cinerea

Goal: To test whether the commercial formulation of chitosan, called Chitoplant, induces resistance in the model plant *Arabidopsis thaliana* against *B. cinerea*.

Experimental procedures:

Col-0 plants were mass-seeded on soil, grown in cabinet and cultivated under Arabidopsis standard growth conditions: 8h-day (21ºC) and 16h-night (18ºC) cycle at ~60% relative humidity (RH). Two week-old plants were transplanted to individual 40 ml pots. 5 week-old plants were treated with water solution and 0.01%, 0.1% and 1% of Chitoplant (in 0.01% silwet) by spraying the solution onto the plants. 4 days after Chitoplant treatment, plants were infected with *B. cinerea* by drop inoculation of a solution containing $5*10^4$ spores/ml in

a potato dextrose broth solution (PDB). Disease was scored at 2 dpi by measuring the lesion diameter of the disease.

Basal callose deposition induced by Chitosan-IR in tomato cv. Money-Maker

To define whether ChitoPlant directly induces callose deposition and to confirm the phenotype in my model crop plant (tomato), 4-5 weeks-old tomato seedlings were foliar sprayed with Chitoplant at 3 different concentrations:

- Control (non-treated): DDW + 0.02% Tween 20 (surfactant)
- Chitosan 0.1% w/v (Chitoplant, ChiPro, Germany) + 0.02% Tween20 (surfactant)
- Chitosan 0.01% w/v (Chitoplant, ChiPro, Germany) + 0.02% Tween20 (surfactant)
- Chitosan 0.001% w/v (Chitoplant, ChiPro, Germany) + 0.02% Tween20 (surfactant)

Four days after treatment (after systemic defence induction), secondary leaves were excised and fixed in 100% ethanol previous Aniline Blue staining (see last year Annual Report for more detailed technical information).

Bacillus subtilis and Bacillus amyloliquefaciens GB03 as biocontrol agents (experiment done in collaboration with PhD student Emma Bissett of the UoD, supervisors Nicola Stanley-Wall and Nicola Holden)

Aim: To test the biocontrol properties of two Bacillus spp. strains and their ability to induced ISR in tomato against *B. cinerea.*

First, in order to test the ability of the two trains to colonize the tomato rhizosphere in compost/soil growing plants, 5 weeks-old tomato plants were soaked 4 days prior fungal infection with the 2 *Bacillus spp.* formulations (GB03 strain and WT). Subsequently, the colonization assay was performed at 0 hours, 24h and 72 hours post soaking.

After *Bacillus spp*. root colonization optimization, 4 days after, detached leaves of tomato seedlings (5 week-old) were challenged with a spore inoculum solution of *B.cinerea*. A total of 2 droplets per leaflet (5µl per droplet) were applied to the adaxial surface of leaves of the same chronological age. Necrotic lesion diameters were measured at 72 hpi and 96 hpi.

Jasmonic acid-dependent priming of gene expression in tomato-B.cinerea interaction

Aim: to test whether MeJA act through JA-dependent priming of gene expression of marker genes.

Five days after elicitor treatment, plants were infected with *B.cinerea* spore inoculum. The time course analysis was done by testing the expression profile of defence-marker genes (PR1, LapA, LoxD, SlMkk6, NPR1/Nim1) at 0, 8, 24 and 48 hpi. Actin and EF1α serves as the internal reference genes for the analysis. Sample collection was done by harvesting leaf discs (with scalpel) surrounding infection droplet area.

Samples were then stored in 2mL tubes at -80ºC until RNA extraction. RNA extraction was performed with Tri Reagent and DNAse cleaning step was added (Turbo-DNA free, Ambion). Subsequently, cDNA was synthetized from 1ug RNA samples with SuperScript III Reverse transcriptase.

Genes of interest in the jasmonic acid (JA) pathway (JA precursor LoxD and the late acting gene LapA) and salicylic acid pathway (NPR1 and PR1.b) were chosen in order to evaluate the up/down regulation of these genes in 4 treatments:

Treatment	Elicitor	Infection
1	water	mock
2	water	Botrytis
3	MeJA	mock
4	MeJA	Botrytis

Results

H_2O_2 temporal evolution after infection in primed plants Vs. control

In order to evaluate the ability of Chitosan and MeJA to prime tomato plants for ROS defences against *B.cinerea*

At time points 24 hpi and 48 hpi, MeJA and chitosan treated plants displayed a more localized and reduced burst of H_2O_2 contained within the infection site, in comparison with non-treated (water control) plants which showed a stronger burst and expanded out of the local site of infection (Figure 2), which suggests that the pathogen might overcome ROS early defences. This results correlates with *(Finiti et al*., 2014) where they showed that hexanoic acid-primed tomatoes can reduce *B.cinerea* ROS scavenging abilities to stress and kill plant cells.

Furthermore, even though the role of ROS against necrotrophic pathogens remains unclear, it seems that timing, duration and intensity of reactive oxygen species burst are crucial factors in the plant-pathogen interaction outcome (Finiti et al., 2014).

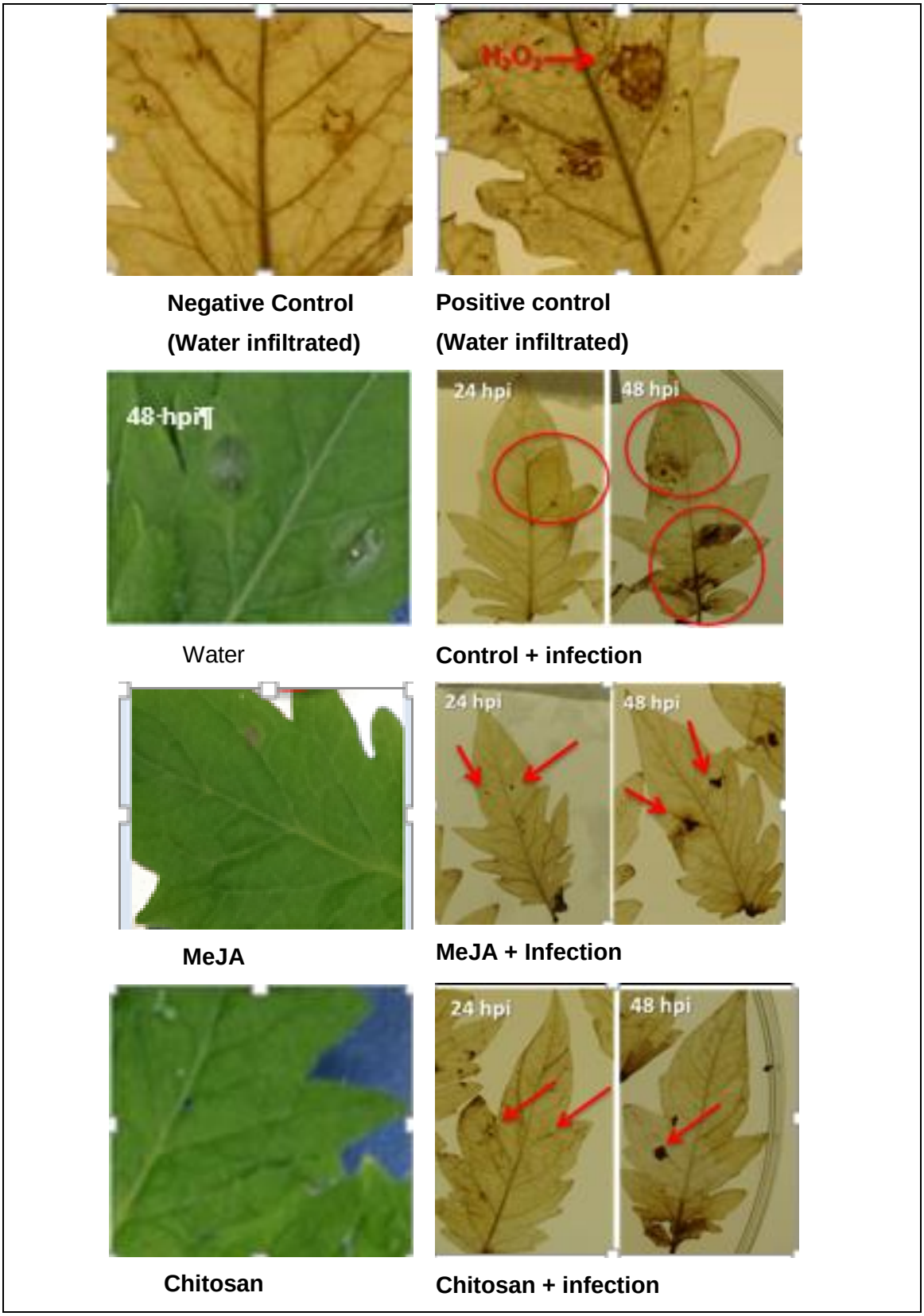


Figure 2. Temporal evolution of H2O2 accumulation (brownish dark spots) in tomato Money-maker, after infection with B. cinerea. DAB staining of detached leaves infected with two 6 µL drops of a spore suspension was performed at different time point's post-inoculation (24 and 48 hpi). One representative leaflet of three replicates is shown for each time point.

Chitosan-Induced Callose deposition before and after pathogen attack

Chitosan has also 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 *et al*., 2010). Nevertheless, its concentration and physicochemical properties are decisive in being recognized by the plant receptors and thus, inducing plant resistance and determining the induction of priming or activation of plant direct defences (Iriti & Faoro, 2009). In this study I aim to show whether the commercial formulation ChitoPlant is enhancing plant cell-wall defences through inducing callose deposition in both *Arabidopsis thaliana* and tomato cv. Money-maker. At 1 and 2 days post-treatment (dpt), all chitosan-treated plants deposited higher percentage of callose than non-treated/water plants, being chitosan at 0.01% sprayed plants statistically significant in comparison with water-control plants (Figure 3).

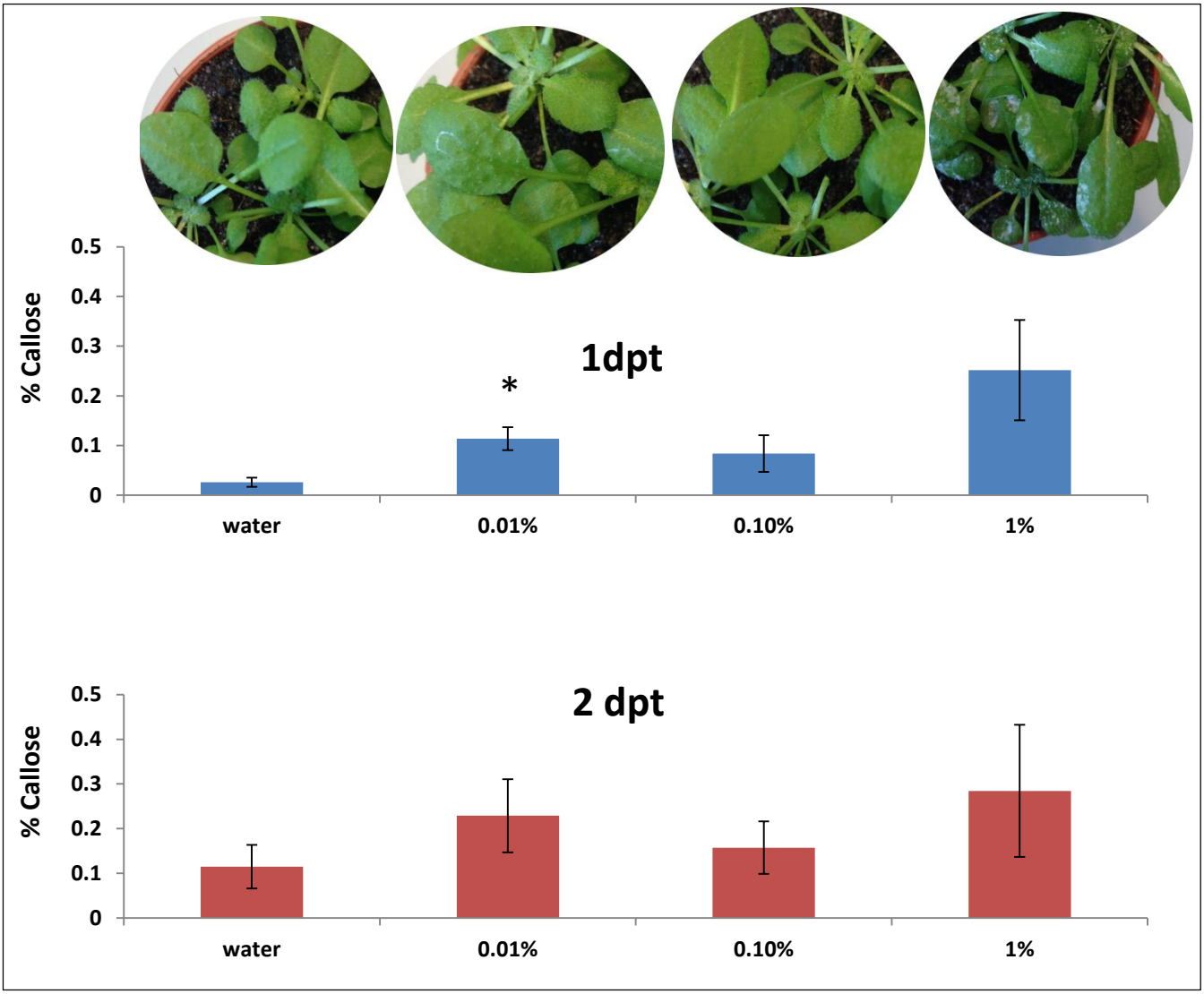


Figure 3. Basal callose deposition in A.thaliana leaves after Water-control and ChitoPlant treatments. 1 and 2 days after treatment leaves were harvested for Aniline Blue staining.

Pictures were taken under fluorescence microscopy. Callose was quantified as described by (Luna *et al*., 2011). 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).

Chitoplant-induced resistance in Arabidopsis Col-0 against Botrytis cinerea

In order to investigate the priming properties of Chitosan, I performed an infection/pathogenicity assay similar than with tomato, in the model plant *Arabidopsis thaliana*. 4 days after applying Chitosan at 1%, 0.1% and 0.01% w/v, all Chitoplant-treated plants at all concentrations were able to successfully induce resistance, in comparison with control (non-treated) plants at 2 days post-infection (Figure 4). This gives evidence of the ability of ChitoPlant to induce resistance in various species plants against *B.cinerea*. Further experiments in tomato are needed in order to reveal the defence mechanisms involved in this plant-chitosan interaction.

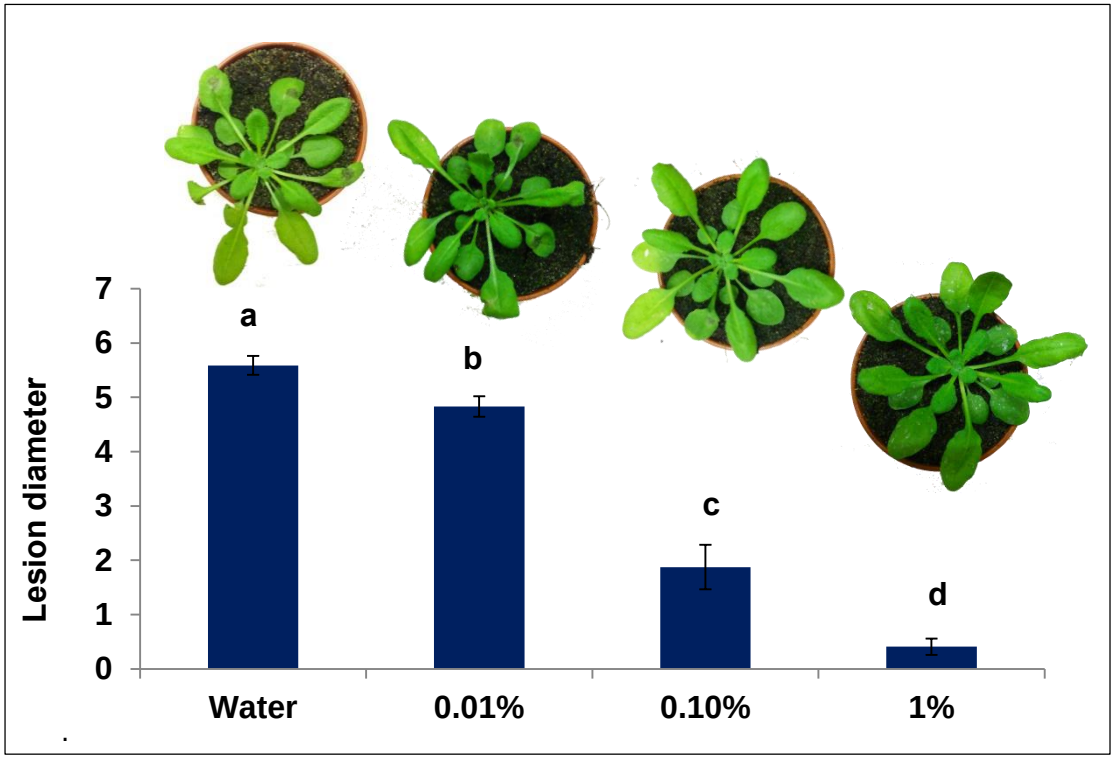
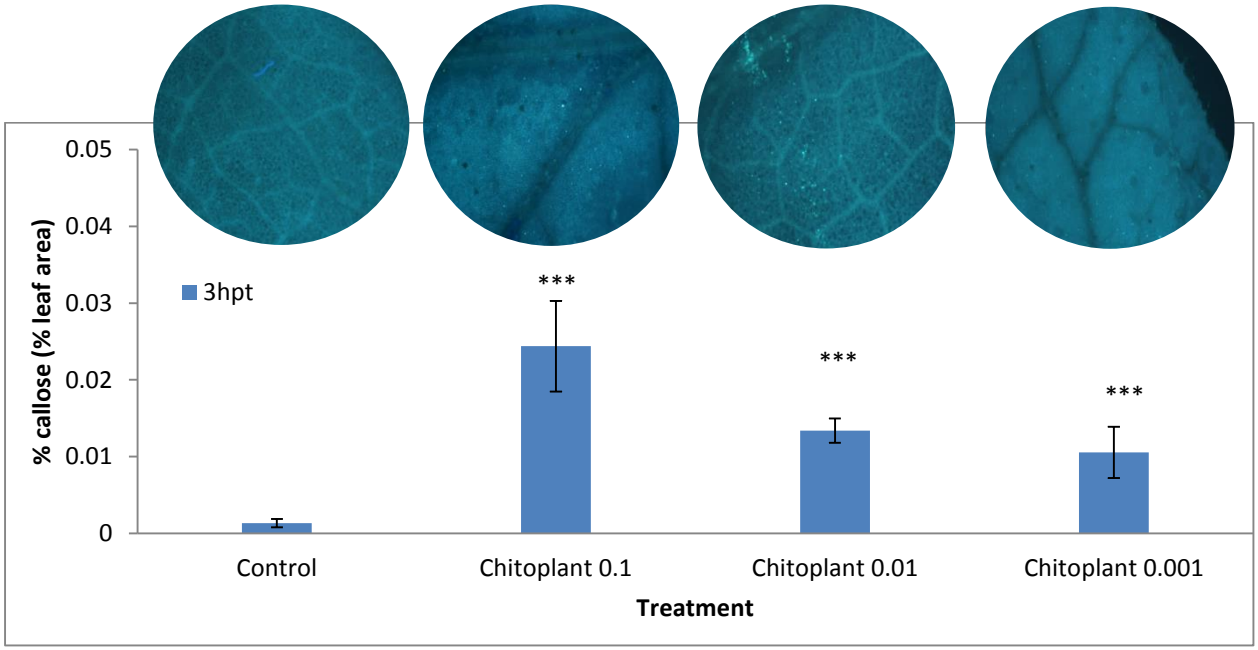
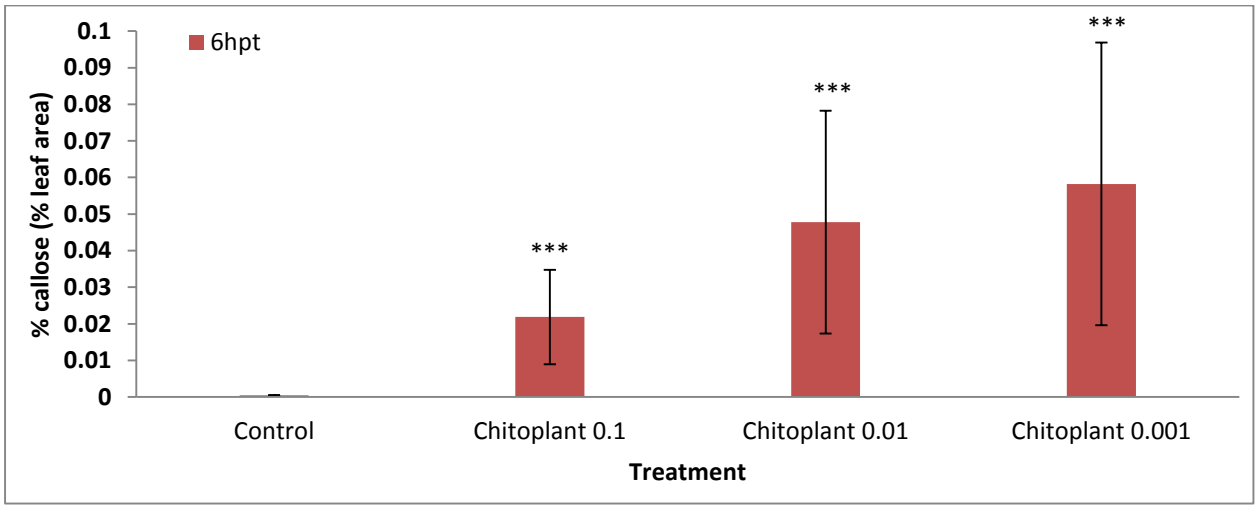


Figure 4. Quantification of the commercial chitosan formulation ChitoPlant-induced resistance *Arabidopsis thaliana* at 3 different concentrations (1% w/v, 0.1 % and 1%) against *Botrytis cinerea* at 2 days post-inoculation. Values presented are means ± SEM. Different letters indicate statistically significant differences (Fisher's least significant difference (LSD) test P<0.001 at 2 dpi, α=0.05).

Basal callose deposition induced by Chitosan-IR in tomato cv. Money-Maker

To define the ability of chitosan in being recognized by the plant membrane receptors and thus, enhancing cell-wall though Callose deposition an experiment was performed testing this elicitor under various concentrations. Overall, during first hours (3 and 6 hours post treatment, dpt) 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 in comparison to water/control treated plants (Figure 5); which suggest that





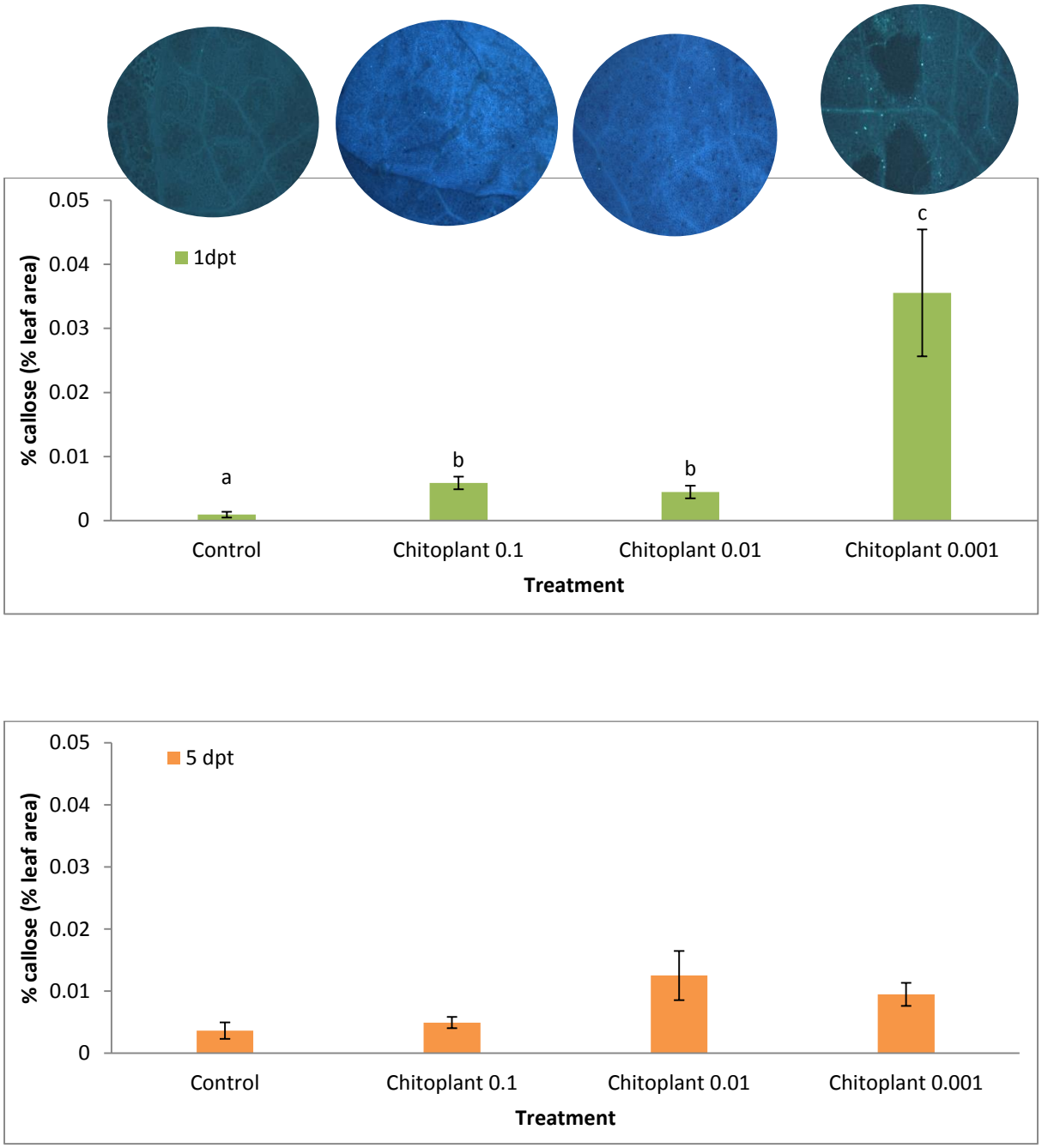


Figure 5. Basal callose deposition in tomato *Money-maker* leaves after water-control and ChitoPlant treatments at 3 different concentrations. 3 and 6 hours, and 1 and 5 days after treatment leaves were harvested for Aniline Blue staining. Pictures were taken under fluorescence microscopy (greenish points represent callose deposits in epidermal cells). Callose was quantified as described by (Luna *et al*., 2011). 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 $p< 0.001$). Different letters (a, b and c) indicate statistically pairwise significance between treatments (Fisher's protected least significant difference test $p<0.001$).

Pathogen-induced callose deposition in chitosan, BABA and MeJA-primed plants

Induced resistance is based on two general mechanisms: direct activation of defence responses in systemic tissue after local stimuli and priming, which implies activation of systemic responses, but only when the pathogen reaches these sites. Previously it was demonstrated that ChitoPlant is able to induce direct callose deposits after treatment and can confer resistance in tomato against *B.cinerea*. To determine whether ChitoPlant can also prime for callose deposition after pathogen challenge in tomato, double staining (Aniline Blue + calcofluor, see last year Annual Report for detailed methodology) was performed to see pathogen-induced callose deposition in all treatments.

Despite the high level of aggressiveness of the *Botrytis cinerea* R16 strain, which made measurement of callose difficult due to the high contrast of the calcofluor, callose was found in samples of Chitosan and MeJA-treated plants (Figure 6) which correlates with the lesion size significant reduction of the same treatment (see 1st year Annual report). In contrast, callose was barely seen in the BABA-treated (not expected, although BABA can be problematic and it may depend on the pathosystem and concentration) plants and in the water-treated control plants (Figure 6).

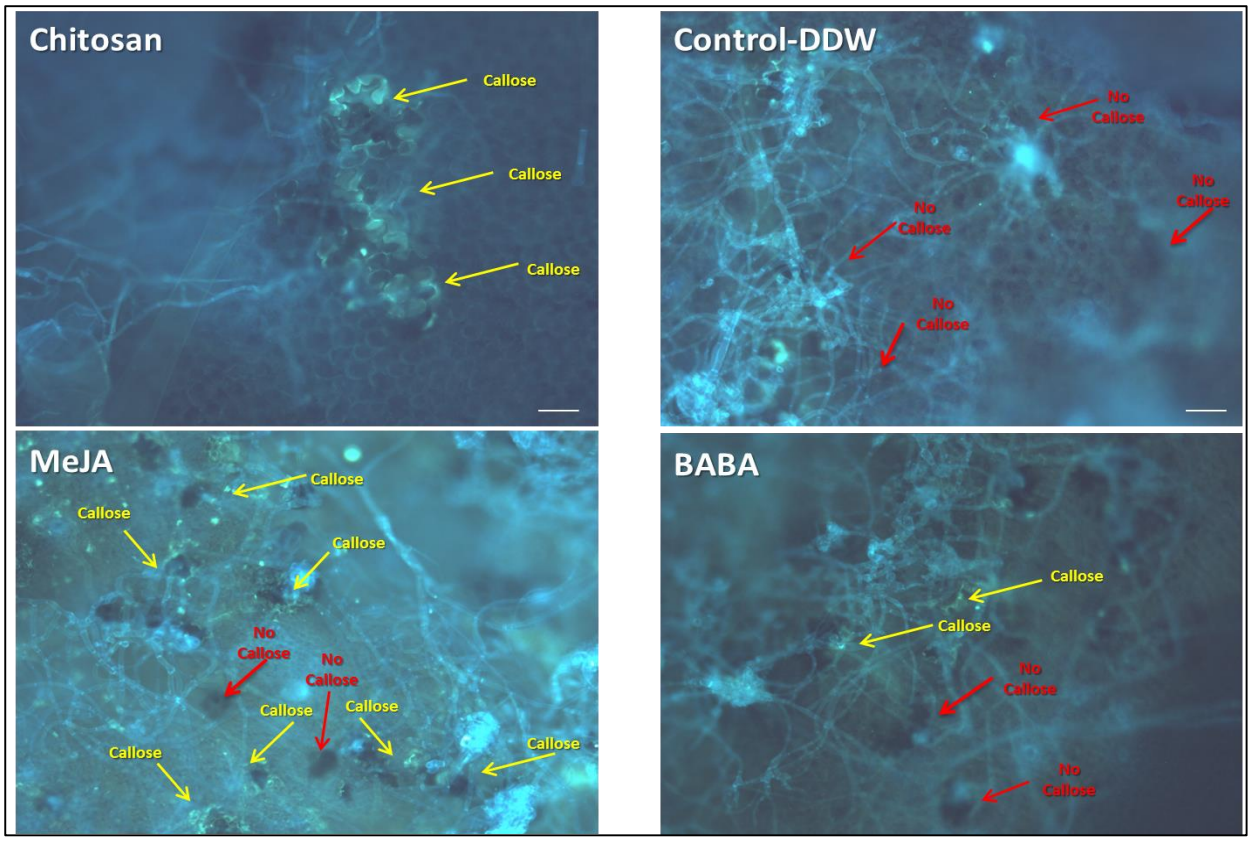


Figure 6. Callose deposition in tomato cv. Money-Maker leaves after B.cinerea infection

in Water-control, BABA (as a positive control), MeJA and Chitosan treated plants. Three 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.

Bacillus subtilis and Bacillus amyloliquefaciens GB03 as biocontrol agents

In the present study I evaluated the ability of two bacterial strains (*Bacillus amyloliquefaciens* and *Bacillus subtilis*) to work as biocontrol agents against the pathogen *B.cinerea*.

Colony forming units per root tissue (CFU/g) were counted in selective media plates (LB + kanamycin) following a dilution series:

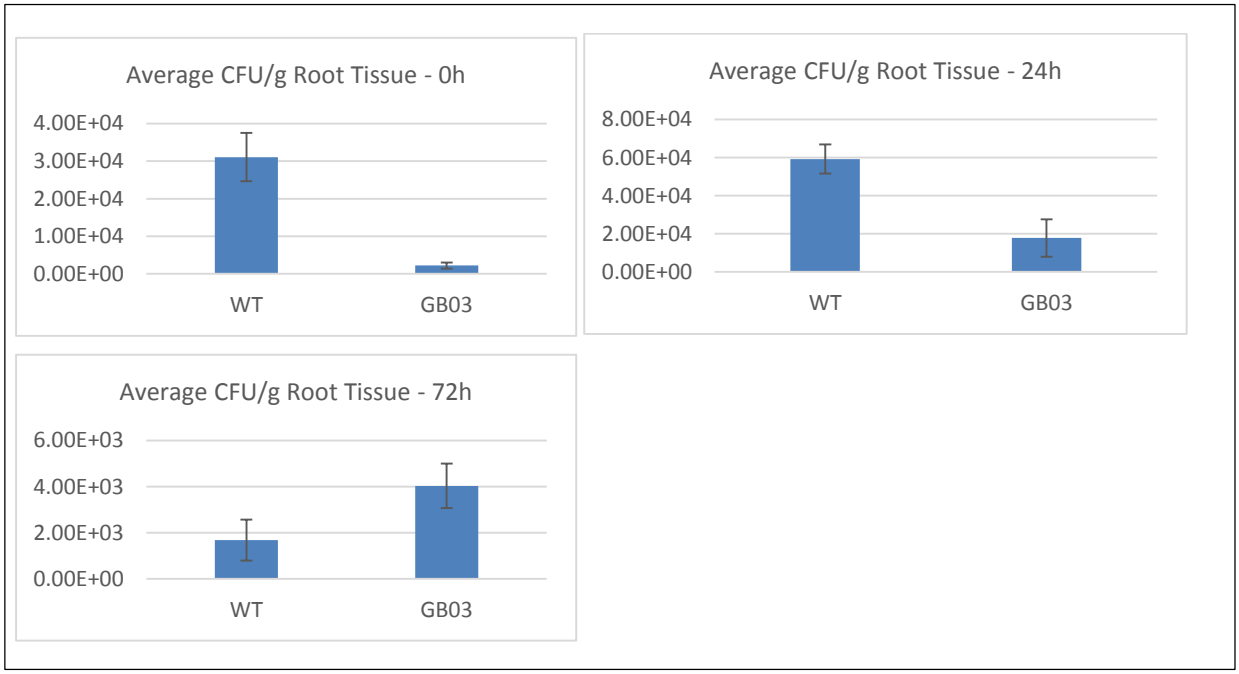


Figure 7. Graph plotting the average of colony forming units (CFU/g) per root tissue for the *Bacillus subtilis* WT and *Bacillus amyloliquefaciens* GB03 strains at 0 hours, 24h and 72 hours after soaking plants with bacterial solution. Error bars are standard error of the mean.

Results showed that both Bacillus WT and GBO3 strains were able to induce resistance against *B.cinerea* through significantly reducing necrotic lesion expansion at 72 hpi (Figure 8). Furthermore, at 96 hours after infection both strains still significantly reduced lesion size in comparison to control, WT strain lesion reduction was similar to Chitoplant 0.1% w/v whilst the biocontrol GBO3 strain was able to reduce lesions to a level comparable to ChitoPlant 1% w/v (used as my strongest positive control) and ChitoPlant 0.01% (used as the potentially right concentration for priming properties in tomato-Botrytis interaction).

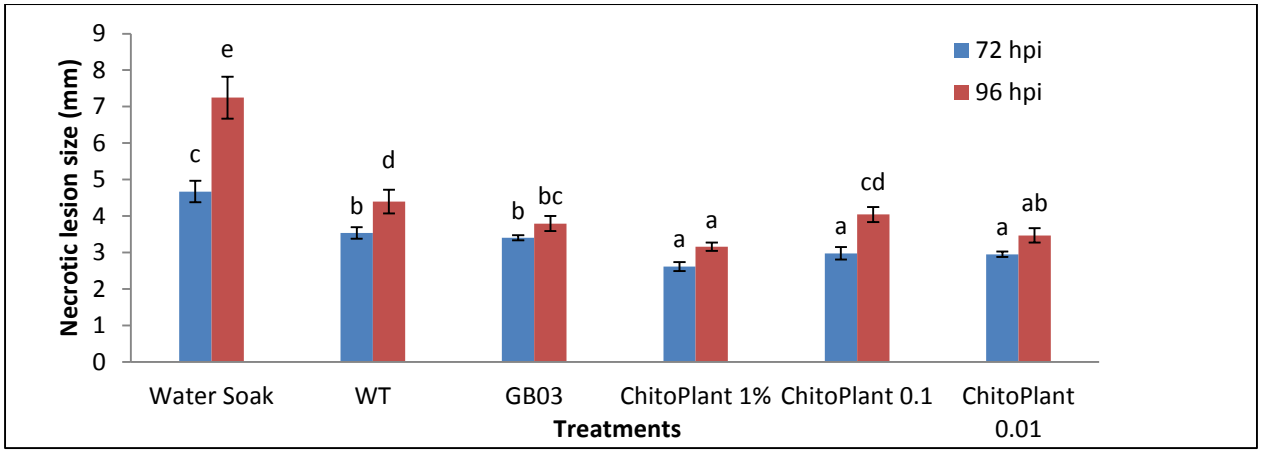


Figure 8. Quantification of *Bacillus subtilis* and *Bacillus amyloliquefaciens* GB03 and ChitoPlant-induced resistance against *B. cinerea* in tomato cv. Money-Maker at 3 days post-inoculation (dpi) (blue bars) and 4 dpi (red bars). Values presented are means ± SEM. Data shows ANOVA for significant differences among all treatments, p value <0.01, letter means significant differences according to Fisher's LSD test). Data includes ChitoPlant (as a positive control in resistance phenotype) at 3 different concentrations 1% w/v, 0.1% w/v and 0.01% w/v, to compare lesion growth repression.

Jasmonic acid-dependent priming of gene expression in tomato-B.cinerea interaction

The experiment was conducted in order to test the following hypothesis:

MeJA can induce resistance through priming tomato against *Botrytis cinerea*, thus it can interfere in the hormone pathways JA/SA/ABA cross-talk.

The first marker gene to test was Leucine Aminopeptidase (LapA). LAP proteins appear to be part of the plant-defence response because the LapA RNAs and proteins accumulate to high levels in tomato leaves after mechanical wounding, insect infestation, and in response to *Pseudomonas syringae* pv. *tomato* infection. LapA genes are activated by the jasmonate synthesis pathway (octadecanoid pathway) (Figure 9) because methyl jasmonate (MeJA) cause increases in the levels of LapA RNA, protein, and activity (Pautot, Holzer, Chaufaux, & Walling, 2001). I also tested the relative expression of Lipoxygenase D (LoxD), a gene induced early by the fungus, which encodes a chloroplast-targeted lipoxygenase that initiates JA synthesis and constitutes a marker gene of the JA pathway (Heitz et al., 1997; Zhao et al., 2003).

In addition, salicylic acid (SA) suppresses wound induction of LapA genes (Chao et al. 1999), consistent with SA's role in blocking JA biosynthesis and JA action (Doares et al. 1995; Peña-Cortés et al. 1993).

The second marker gene to test was tomato lipoxygenase D (LoxD), Lipoxygenase is involved in the oxylipin pathway (Figure 9), leading to OPDA and JA synthesis (the main plant defence pathways against necrotrophic pathogens such as *B.cinerea*) (Flors et al., 2007)

In order to test the hypothesis that *Botrytis cinerea* is able to manipulate the antagonistic cross-talk between JA-SA pathways (Figure 9) through NPR1 (El Oirdi et al., 2011), I tested NPR1 expression (Figure 12), a key regulator gene of systemic acquired resistance (SAR) (Pieterse & Van Loon, 2004). Nevertheless, NPR1 wasn't up-regulated in infected plants as expected; however, this could be due to various reasons, JA-pathway repression by *Botrytis cinerea* could be strain-dependent or that NPR1 is simply expressed at very low levels and so it is difficult to detect.

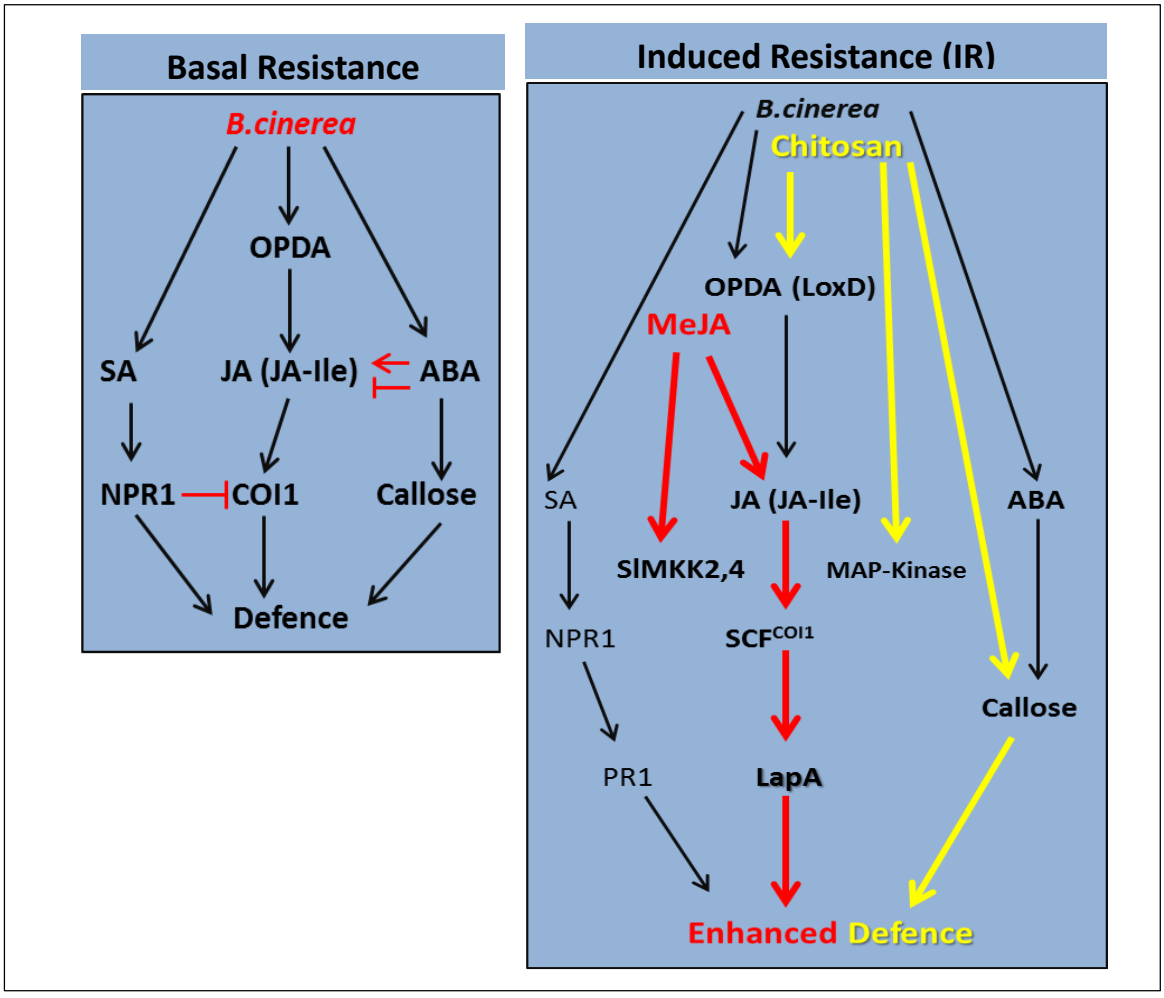


Figure 9. Model for basal/induced resistance in tomato against Botrytis cinerea

(adapted from (Vicedo et al., 2009). Infection by B. cinerea leads to activation of jasmonic acid (JA)-dependent defence pathway while abscisic acid (ABA) and salicylic acid (SA) could act as susceptibility and antagonistic factors respectively, depending on the experimental conditions. On the other hand, priming agents (MeJA) could specifically up-regulate JA-dependent genes by priming JA-dependent LapA and 12-oxophytodienoic acid (OPDA)-dependent LoxD.

At 8 hpi in all cases LoxD and LapA genes weren't induced in comparison to control plants. At 24 hpi and 48 hpi only MeJA+ infected plants were able to induced LoxD whilst MeJA mock plants and water+infected plants didn't induced it (Figure 10). LapA in the downstream pathway was induced at 24 hpi in all treatments compared with the control. MeJA mock plants were the only treatment that kept the induction after 48 h whilst the others were down-regulated (Figure 11).

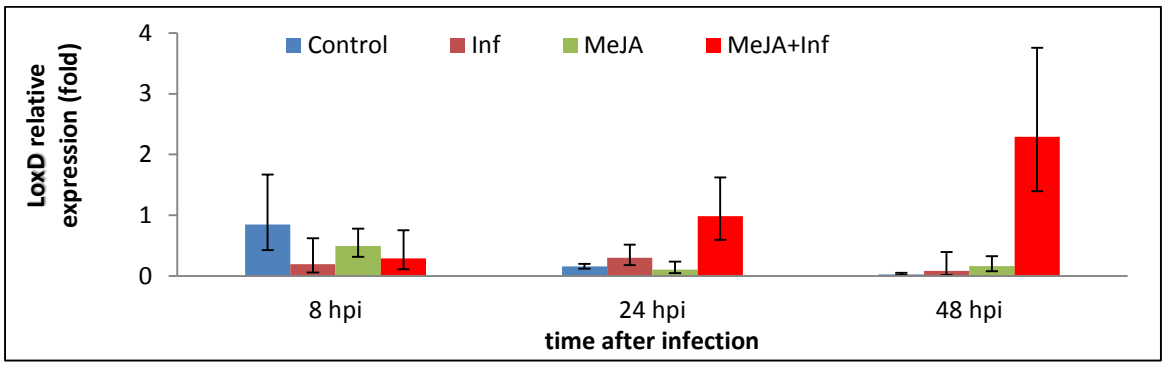


Figure 10. Expression levels of tomato JA-dependent LoxD relative to housekeeping gene EF1alpha. Four-week-old plants were treated with MeJA and water (control); Samples (leaves) were harvested at 3 time points (8h, 24h and 48 hours after infection/inoculation) for RNA extraction. QRT-PCR was performed with specific primers for tomato LoxD and EF1alpha (reference control gene) as described in Methods. Values represent means relative to EF1a and control treatment ±SD from three biological replicates.

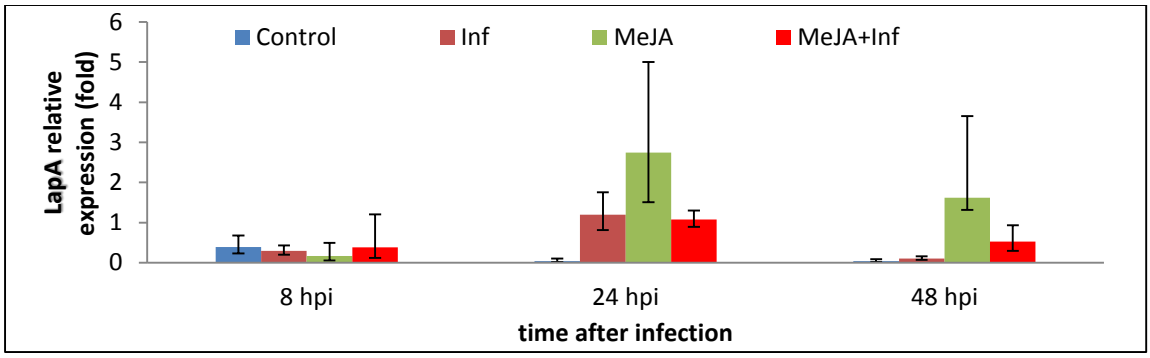


Figure 11. Expression levels of tomato JA-dependent LapA relative to housekeeping gene EF1alpha. Four-week-old plants were treated with MeJA and water (control); Samples (leaves) were harvested at 3 time points (8h, 24h and 48 hours after infection/inoculation) for RNA extraction. QRT-PCR was performed with specific primers for tomato LapA and EF1alpha (reference control gene) as described in Methods. Values represent means relative to EF1a and control treatment ±SD from three biological replicates.

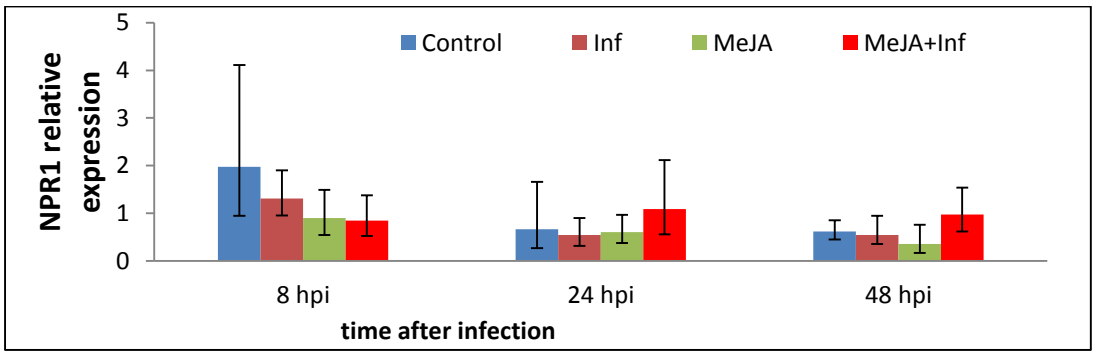


Figure 12. Expression levels of tomato SA-dependent NPR1 relative to Actin. Four-week-old plants were treated with MeJA and water (control); Samples (leaves) were harvested at 3 time points (8h, 24h and 48 hours after infection/inoculation) for RNA extraction. QRT-PCR was performed with specific primers for tomato NPR1 and EF1alpha (reference control gene) as described in Methods. Values represent means relative to EF1a and control treatment ±SD from three biological replicates.

Discussion

Plants are able to express defence responses against pathogen attack. However, these defences may be overcome by some aggressive pathogens. Thus, plant response timing and robustness can be crucial for a successful pathogen challenge outcome (Finiti et al., 2014). Plants can display “relatively early acting defences” like 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 (Figure 1), 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.

As shown in last year annual report, two marker elicitors (MeJA and chitosan) were able to significantly reduced *Botrytis cinerea* expansion in two tomato cultivars with no fitness costs

in plant growth. Hence, one of the main objectives of this year study was to unveil the role MeJA and chitosan in tomato defences against this necrotrophic pathogen.

Here we show that, in mock (non-infected) plants, treatment with chitosan triggered the deposition of callose in tomato and *A.thaliana* leaves in a concentration and time-dependent manner, which suggests that the biological activity of chitosan, besides the plant model, may depend on its physicochemical properties (deacetylation degree, molecular weight and viscosity) (Iriti & Faoro, 2009). Due to the efficacy of Chitoplant inducing resistance in both plants, further experiments were conducted in order to test the role of chitosan and MeJA in plant ROS and callose production after pathogen infection. As seen in Figures 2 and 6, both elicitors were able to deposit callose in tomato epidermal cells surrounding most of the pathogen penetration sites and both were able to trigger and contain H_2O_2 at the local site of the infection, in comparison with control (non-primed) plants that weren't able to induce callose in the pathogen penetration sites. Besides, control plants weren't able to contain H_2O_2 production, which was expanded beyond the infection sites, results that suggest Botrytis may manipulate plant defences (ROS production) in its own benefit (Temme & Tudzynski, 2009).

Other objective was to assess the priming properties of MeJA in tomato after pathogen attack as well of the ability of this elicitor interfering in the plant hormone cross-talk. 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 *B.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 test the hypothesis that *Botrytis cinerea* is able to manipulate the antagonistic cross-talk between JA-SA pathways (Figure 9) through NPR1 (El Oirdi et al., 2011), I tested NPR1 expression (Figure 12), a key regulator gene of systemic acquired resistance (SAR) (Pieterse & Van Loon, 2004). Nevertheless, NPR1 wasn't up-regulated in infected plants as

expected; however, this could be due to various reasons, JA-pathway repression by *Botrytis cinerea* could be strain-dependent or that NPR1 is simply expressed at very low levels and so it is difficult to detect.

At 24 hpi (not visible symptoms yet) and 48 hpi only MeJA-primed plants were able to induced LoxD whereas control/non-primed plants weren't able to up-regulate this marker gene. LapA gene in the downstream pathway was induced at 24 hpi in primed and non-primed infected plants whilst at 48 hpi only MeJA-primed and infected plants were able to keep this gene up-regulated in comparison with non-primed/infected plants were LapA was down-regulated, which correlates with the previous studies about pathogen defence manipulation. This suggests a role of MeJA in priming tomatoes against *B.cinerea* through faster gene expression.

In order to test the role of induced systemic resistance (ISR) in tomato protection against *B.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 at both time points after infection (72 and 96 hpi) (Figure 8). Besides, both strains induced resistance abilities were compared with chitosan-induced resistance which is ultimately triggering JA-dependent defences (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).

Due to the positive results with ChitoPlant (chitosan), MeJA and biocontrol agents in inducing tomato defences against this aggressive pathogen, further elicitor co-treatment experiments will be conducted in order to investigate their potential synergistic effects on the tomato-*B.cinerea* model system.

Conclusions

- The two main elicitors chosen, MeJA and Chitosan, can significantly reduce necrotic lesion expansion without reducing plant growth, thus they have low costs in plant development, suggesting priming properties.
- MeJA and Chitosan may reduce oxidative burst stress after *B.cinerea* infection, thus they may potentially avoid pathogen hosts defence manipulation.
- MeJA can prime JA-dependent defences through marker tomato gene expression (LoxD and LapA), which ultimately lead to "long lasting inducible defences"

- Chitosan can induce callose deposition in tomato before and after pathogen challenge in a timing and concentration-dependent manner, thus chitosan can reinforce tomato cell-wall stopping or reducing pathogen expansion.
- *Bacillus subtilis* and *Bacillus amyloliquefaciens* can induce resistance in tomato against *B.cinerea*, which suggest a promising role of ISR producers in conferring protection in tomato and acting as biocontrol agents.

Future Plans

Microrray: Transcriptome analysis on Chitosan-primed tomato-B.cinerea

Chitosan is a polymeric and deacetylated derivative of chitin that is naturally present in arthropod shells and fungal cell walls. 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, depending on the dose, the chitosan derivative and the plant (Iriti & Faoro, 2009). The diverse mechanisms of action of chitosan have been studied, which include oxygen-species scavenging and antioxidant activities, as well as octadecanoid pathway activation (El Hadrami et al., 2010). During my PhD research I have shown that chitosan is able to induced resistance in tomato cv. Money-maker, tomato cv. Motelle and the model plant *Arabidopsis thaliana* against *Botrytis cinerea*. Chitosan was also able to induced callose deposits before and after pathogen attack as well as contain the H_2O_2 accumulation to the infection site and thus, reduce the pathogen manipulated oxidative stress. Despite these studies, experiments that specifically address the role of priming in the complex chitosan-plant interaction framework are still lacking.

Aim: to test whether Chitosan (commercial formulation ChitoPlant, ChiPro) functions as a priming agent in tomato versus *B. cinerea* (BcR16 *strain*).

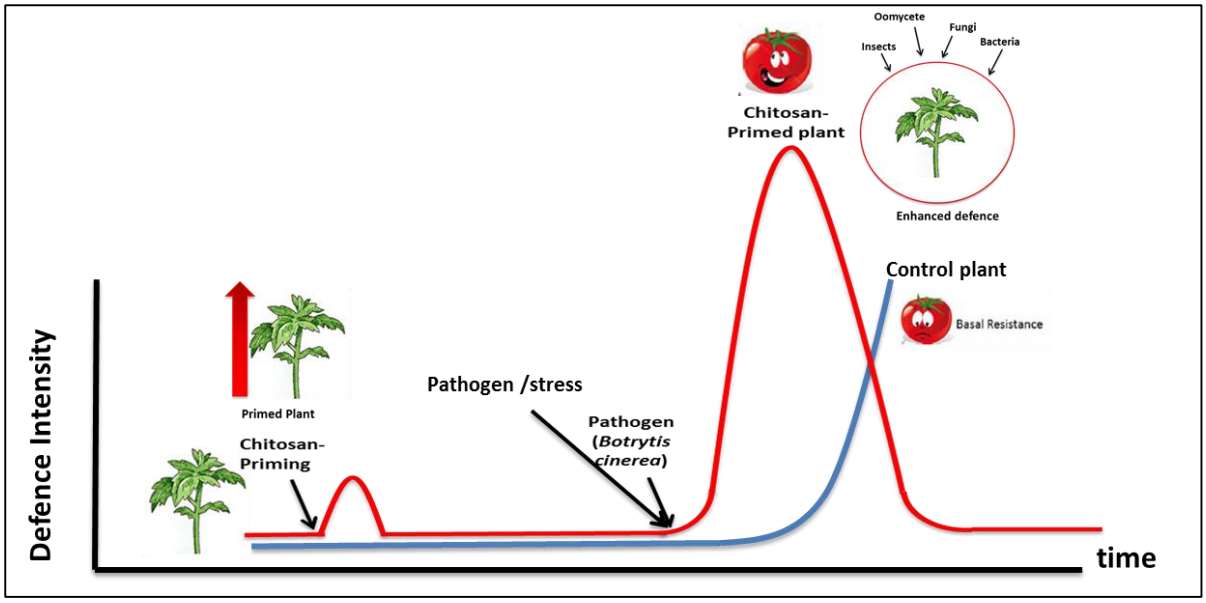


Figure 13. Model of basal/induced resistance by chitosan as a priming agent in tomato-*B.cinerea*

- After microarray analysis, selected candidate genes by qRT-PCR will be validated in order to test their role in tomato defences.
- Elucidate the “potential synergistic effect” of Chitosan combination with MeJA and other elicitors against *Botrytis cinerea* and the human pathogen *Salmonella enterica.*
- Investigate the effect of Chitosan and MeJA in genotypes compromised in the main hormone signalling pathways.

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