Project title:	Integrated protection of horticultural crops through enhancing endogenous defence mechanisms
Project number:	CP 105
Project leader:	Professor Adrian C Newton, The James Hutton Institute
Report:	First annual report, September 2014
Previous report:	None
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, Invergowrie, Dundee
Industry Representative:	Neal Ward, Cantelo Nurseries Ltd, Isle Abbotts, Somerset.
Date project commenced:	1st September 2013
Date project completed (or expected completion date):	30th September 2016

DISCLAIMER

AHDB, operating through its HDC division seeks to ensure that the information contained within this document is accurate at the time of printing. No warranty is given in respect thereof and, to the maximum extent permitted by law the Agriculture and Horticulture Development Board accepts no liability for loss, damage or injury howsoever caused (including that caused by negligence) or suffered directly or indirectly in relation to information and opinions contained in or omitted from this document.

The results and conclusions in this report are based on an investigation conducted over a one-year period. 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.

Professor Adrian C Newton

Project leader

The James Hutton Institute, Dundee

Signature  Date 6th October 2014 (after editing)

Report authorised by:

[Name] [Position] [Organisation]

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

CONTENTS

GROWER SUMMARY

Headline

Resistance elicitors enable tomatoes to better defend themselves against Botrytis. Protection can be long-lasting but such products must be used as components of an IPM programme tailored to tomato cultivars.

Background

Many of our crop protectants either become ineffective as pathogens develop resistance, products are removed from the market for regulatory reasons, new pathogens emerge, or we need to grow varieties that are susceptible for market reasons. New active ingredients in crop protection products permitted for horticultural crops are all too rare and late, and for bacterial and viral pathogens there are few options available anyway. However, the plants themselves have highly effective resistance mechanisms that if primed and expressed in a focussed, specifically-targeted way, could not only lead to better crop protection, but also substantially reduce the need for toxic crop protectant interventions. This can be done with resistance ‘elicitors’. Resistance elicitors 'prime' plant defence mechanisms and enable the plants to respond to actual pathogen threats faster.

The underlying mechanism is mainly based upon a more effective induction and expression of defence mechanisms. However, being mediated through the plant's complex metabolic pathways where many feedback and trade-off mechanisms operate, the result of resistance priming and induction can potentially affect non-disease resistance mechanisms too. These may result in either positive or negative effects on yield quantity, quality and its components. 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 end-products 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. In particular we will use these approaches to determine both the phenotypic and molecular profiles of defence activator combinations that should prove synergistic. 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 work can be seen as under-pinning crop protection mechanisms.

In particular, the project aims to (i) establish a robust and reproducible beneficial effect with an elicitor regime on a 'model' plant pathogen system; (ii) to investigate the molecular basis to the plant defence response elicited by the treatment regime and (iii) to test whether this

same response is triggered in different plant species. The project will focus on a single plant-pathogen system: *Botrytis cinerea* on tomato plantlets, 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 similarities in the mode of elicitor action. We will also determine what the effects are on non-target organisms, and here we will focus on bacteria with the potential to a pose food safety risk.

Summary

Resistance elicitors are able to reduce disease in tomatoes caused by botrytis (*Botrytis cinerea*). Long-lasting defence is induced by B-amino-butyric acid (BABA), methyl jasmonate (MeJA), “Chitosan 23” and “ChitoPlant”. These substances can significantly reduce necrotic lesion expansion and tomato cultivars differ in their response. “ChitoPlant” defence induction is characterised by callose deposition in tomato cotyledons before pathogen challenge.

Elicitors that trigger one of the main defence pathways, the jasmonate-signalling pathway (i.e. MeJA and the chitosans) are able to provide long-lasting defence and limit *B. cinerea* infection progress in both cultivars. BABA is more effective in one tomato cultivar than another and is long-lasting but the level of protection is dependent on the pathogen inoculum concentration and aggressiveness. The cultivar difference is seen clearly in the amount of callose deposition induced. There is also a cost in plant fitness after elicitor- induced resistance, particularly with BABA.

The other main defence pathway, the salicylic acid (SA)-signalling pathway, is also involved in basal defence of tomato against *B. cinerea*, however its effect and efficacy in long-lasting resistance is still unclear. Further experiments with SA elicitors will be needed.

In summary, it has been demonstrated that both SA- and the JA-signalling pathways are involved in tomato resistance against *B. cinerea*. However, their efficacy may vary depending on the pathogen strain, tomato cultivar and infection time point.

A summary of some of the main findings are presented in the landscape figure below.

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 combinations and practices as identifying those that might be additive or synergistic.

Effectiveness of elicitor-IR in tomato Vs the necrotrophic fungus *Botrytis cinerea*

Long lasting defence induction (15 days prior infection)

Benefits of elicitor-Induced Resistance (IR)

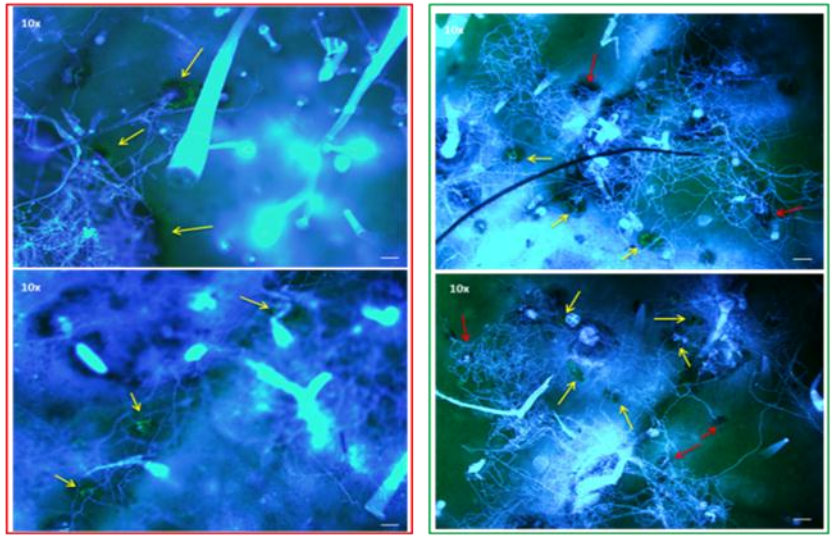
Jasmonic acid (MeJA and Chitosan) triggering elicitors

Salicylic acid (BABA) and Jasmonic acid (MeJA, Chitosan) elicitors

Costs of elicitor-Induced Resistance (IR) in plant fitness

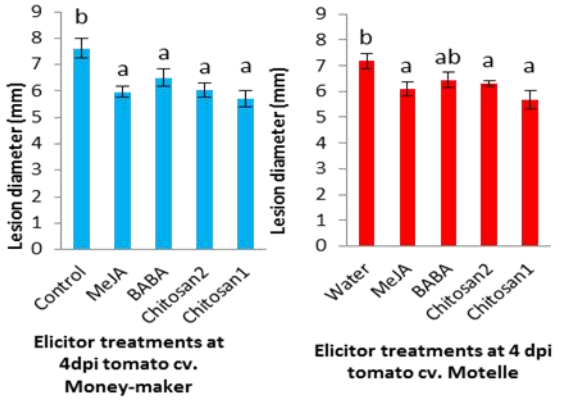
Elicitor-induced growth reduction

Cell-wall defences: Callose-rich papillae deposition

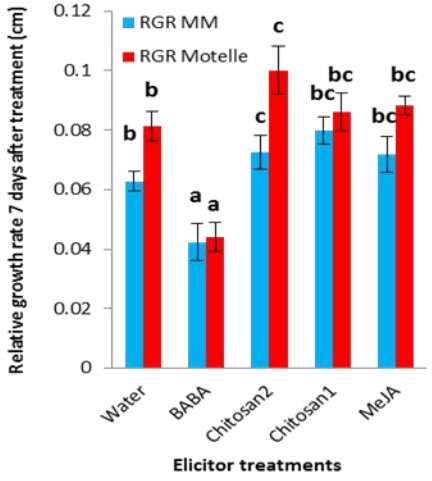


Mainly Jasmonic acid-dependent elicitors (**MeJA and Chitosan**) were able to **induce callose papillae deposition** before and **upon pathogen challenge** in both tomato cultivars (Money-maker and Motelle). Yellow arrows indicate callose papillae deposition surrounding pathogen mycelia penetration sites. Red arrows indicate fungal penetration sites without callose deposition.

Necrotic lesion reduction



BABA, MeJA and Chitosan can significantly reduce *B.cinerea* lesion growth



Quantification of relative growth rate (RGR) of 2 tomato cultivars after elicitor treatment. **BABA** caused the greatest growth inhibition of all elicitors.

Effectiveness of elicitor-IR in tomato Vs the necrotrophic fungus *Botrytis cinerea*

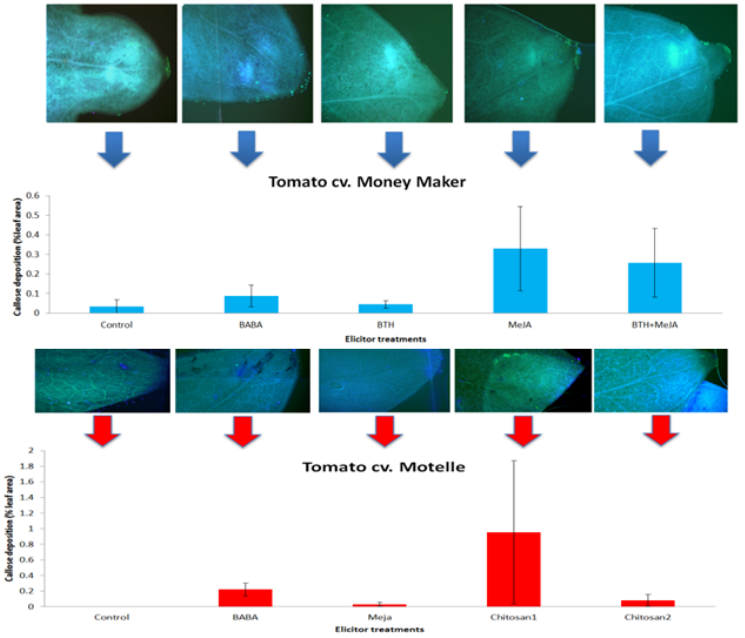
Long lasting defence induction (15 days prior infection)

Benefits of elicitor-Induced Resistance (IR)

Jasmonic acid (MeJA and Chitosan) triggering elicitors

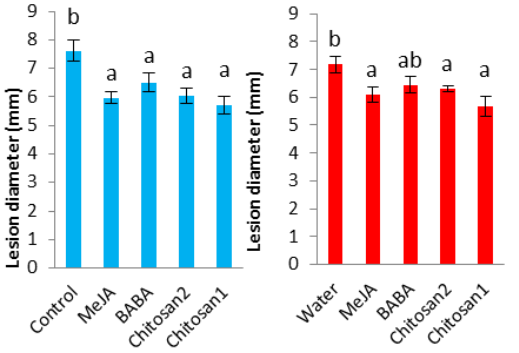
Salicylic acid (BABA) and Jasmonic acid (MeJA, Chitosan) elicitors

Cell-wall defences: Callose-rich papillae deposition



Jasmonic acid-dependent elicitors (**MeJA and Chitosan**) were able to **induce callose papillae deposition before** pathogen challenge in both tomato cultivars (Money-maker and Motelle).

Necrotic lesion reduction



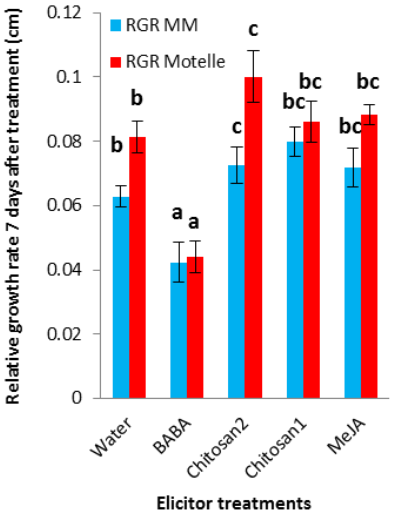
Elicitor treatments at 4dpi tomato cv. Money-maker

Elicitor treatments at 4 dpi tomato cv. Motelle

BABA, MeJA and Chitosan can significantly reduce *B.cinerea* lesion growth.

Costs of elicitor-Induced Resistance (IR) in plant fitness

Elicitor-induced growth reduction



Quantification of relative growth rate (RGR) of 2 tomato cultivars after elicitor treatment. **BABA** caused the greatest growth inhibition of all elicitors.

SCIENCE SECTION

Introduction

1. Integrated crop protection

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.

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. 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).

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. One of the main elements of the Regulation 1107/2009, unlike the Directive, is that it provides the possibility to reject active substances on the basis of their intrinsic properties (Williams, 2011). This is also critical for the control of bacterial pathogens, for which there are no other real alternatives.

Human Health	Environmental
Carcinogen C1A & C1B	PBT (Persistent, Bioaccumulative & Toxic)
Mutagen M1A & M1B	POP (Persistent Organic Pollutant)
Toxic for Reproduction R1A & R1B	vPvB (very Persistent, very Bioaccumulative)
Endocrine disruptor	Endocrine disruptor

Figure 1. Plant Protection Products Regulation 1107/2009. Criteria for the approval of active substances.

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 system. Plants have defence mechanisms that can prevent disease if

triggered at the right time in the right amount and in a targeted-specific way, reducing or removing the need for application of other toxic crop protection chemicals.

Thus, induced resistance can help achieve this through the use of non-specific inducers called elicitors. Resistance elicitors (RE), whether derived from natural or synthetic sources, can activate plant defence mechanisms, resulting in a broad-spectrum resistance against a wide range of pathogens and in a robust and more rapid way.

2. Plant Defence Systems

Plants are not unprotected against the pathogens and pests that attack them. They have a developed immune system that must be able to endure attacks from a wide variety of microorganisms, such as bacteria, oomycetes, fungi and viruses.

Despite the fact that every pathogen has a different host range depending of its nature and specialization level; it is well known that pathogens have co-evolved together with plants to develop a way to infect them, at the same time that plants have also developed more or less successful ways to resist pathogen infection and disease development. This evolutionary development of the plant immune system has recently been accepted and represented by a zig-zag model (Hein, Gilroy, Armstrong, & Birch, 2009; Jones & Dangl, 2006).

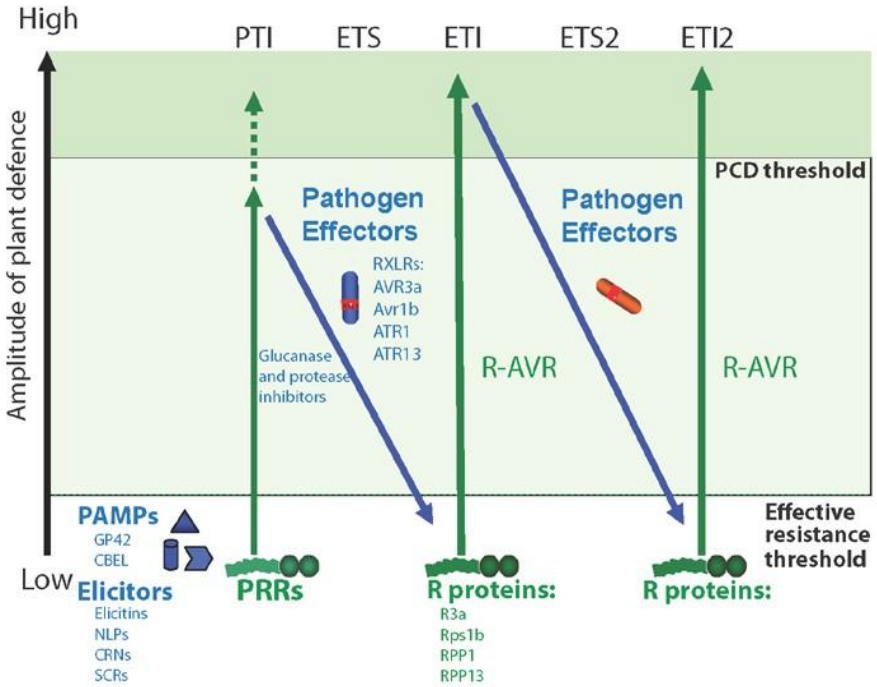


Figure 2. The zig-zag-zig in oomycete–plant interactions (Hein et al., 2009) (modified from Jones and Dangl, 2006).

Many pathogens, such as oomycetes, aphids and some fungi are able to penetrate directly their host cell wall, unlike plant viruses and bacteria, which depend on natural openings or vectors (Ahmad, Gordon-weeks, Pickett, & Ton, 2010). The plant pathogen *Botrytis cinerea* can infect undamaged plant tissue directly by penetration of the cuticle (J. a van Kan, van't Klooster, Wagemakers, Dees, & van der Vlugt-Bergmans, 1997). In order to fight pathogen infection, plants have created a series of resistance mechanisms. As a first physical defence, plants have a waxy layer on their leaf surfaces beneath which are a series of cell-wall defences, such as lignin and callose appositions, so-called papillae. If a pathogen attempts to infect and subsequently cause disease in the plant, firstly it needs to overcome these physical barriers. Compared with many plant defence responses, that can be specific to a phylum or even a species, the formation of callose-rich papillae can be regarded as a ubiquitous response because it appears to be induced in essentially all plants following pathogen challenge (Voigt, 2014).

However, if a pathogen does manage to penetrate through these layers, the plant needs to be able to combat them. As a primary defence response, plants have a range of specific proteins called pattern recognition receptors (PRRs) that respond to microbes through the sensitive recognition of conserved microbial features, such as flagellin, chitin, glycoproteins or lipopolysaccharides, called microbe-associated molecular patterns (MAMPs) and pathogen-associated molecular patterns (PAMPs) (Ahmad et al., 2010), as well as they can recognize endogenous plant elicitor molecules that are released on tissue damage called damage-associated molecular patters (DAMPs). This recognition triggers a set of defence mechanisms in the plant that results in the activation of PAMP-triggered immunity (PTI).

It has been discovered that successful pathogens have acquired specific (Avr) proteins and small RNAs, such as *Botrytis cinerea* which is able to use some small RNAs (Bc-sRNAs) to silence *Arabidopsis thaliana* and tomato genes involved in immunity (Weiberg et al., 2013). These avirulence proteins and small RNAs are called effectors, and they can overcome a plant's first layer of defence, either by preventing detection of their PAMPs by the host (Bardoel et al., 2011) or by suppressing PTI signalling. In a constant arms race, plants acquired a second line of defence in which resistance (R) proteins mediate recognition of these attacker-specific effectors, resulting in effector-triggered- immunity (ETI) (Pieterse, Van der Does, Zamioudis, Leon-Reyes, & Van Wees, 2012). This has been accepted as the classical avirulence (Avr) gene-for-gene model, where the pathogen gene evolves to escape host recognition while the host resistance (R) gene evolves to track the evolving pathogen elicitor (Stukenbrock & McDonald, 2009).

During this plant-pathogen interaction there is an onset of defence systems triggered by the

plant which leads to resistance or, in the worst case, disease development. This plant defence mechanisms can be a hypersensitive response (HR) produced by the plant, in order to avoid biotrophic pathogen expansion, or plant cell necrosis modulated by a necrotrophic pathogen for own benefit; however it is as yet unclear whether cell death caused by a necrotroph, such as *Botrytis cinerea*, is equivalent to cell death during a hypersensitive response (HR) to a biotrophic pathogen (J. A. L. Van Kan, 2005); it also includes production of reactive oxygen species (ROS), phytoalexin biosynthesis and accumulation of pathogenesis-related (PR) proteins (Walters & Heil, 2007).

2.1 Induced resistance

Till now, plant defence mechanisms were explained based on basal immune responses upon pathogen attack. This basal defence, in many cases, is not enough to survive and leads to an early death of the host. However, plants are capable of defending themselves and fight off pathogen attack through constitutive and inducible defence mechanisms. Upon specific stimulus, plants are able to induce both local and systemic responses, in a rapid and localized manner, such as quick synthesis of toxic metabolites and defensive proteins and on an extended scale of time and space (Zipfel, 2014). This way, plant resistance can be increased and confer greater protection against future pathogen attack, a phenomenon called induced resistance.

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).

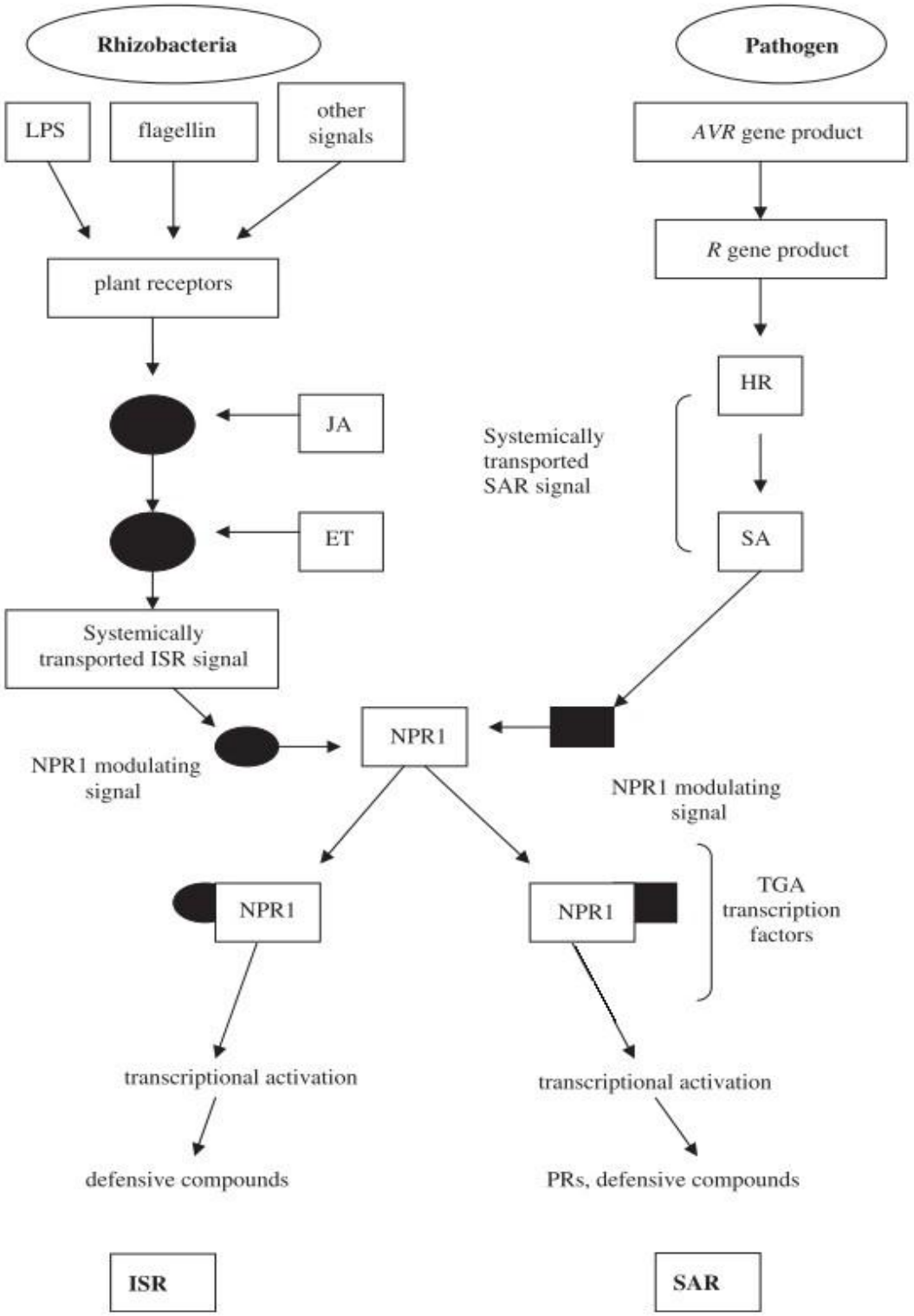


Figure 3. Signal transduction network controlling ISR mediated by PGPR and pathogen- induced-SAR. Adapted from (Walters & Heil, 2007).

In order to activate induced resistance in the plant there is a wide range of non-specific inducers known as resistance elicitors (RE). RE may be from biological, chemical or physical origin and may induce local acquired resistance (LAR), systemic acquired resistance (SAR) or induced systemic resistance (ISR) (Terry, 2004).

In the constant plant-pathogen fight, resistance elicitors can play a crucial role to help plants survive their attackers as elicitors can induce similar defence responses in plants as elicited by the pathogen infection. Different types of elicitors have been characterized, including carbohydrate polymers, lipids, glycopeptides, and glycoproteins (Thakur & Sohal, 2013). There are many examples of successful pathogen control, such as the fungal necrotroph *Botrytis cinerea,* through the application of these non-specific inducers (Achuo, Audenaert, Meziane, & Höfte, 2004; Häffner, Karlovsky, Splivallo, Traczewska, & Diederichsen, 2014; Romanazzi, Feliziani, Santini, & Landi, 2013; Ton & Mauch-Mani, 2004).

Table 1. Examples of chemical elicitors of natural disease resistance to postharvest pathogens in horticultural products (Terry, 2004).

Species	Elicitor	Target pathogen	Experiment type	Authors
Actinidia deliciosa (kiwifruit)	Salicylic acid	*B. cinerea*	Field/postharvest	Poole and McLeod (1994)
Apium graveolens (celery)	Gibberellic acid	*B. cinerea*	Postharvest	Afek et al. (1994)
Chamelaucium uncinatum (Geraldton waxflower)	Salicylic acid	*Alternaria sp. Epicoccum sp.*[a]	Field/postharvest	Beasley et al. (1999)
Citrus paradisi (grapefruit)	Jasmonic acid	*P. digitatum*	Postharvest	Droby et al. (1999)
	Methyl jasmonate	As above	As above	
Cucumis melo (rock and hami melon)	Acibenzolar	*Alternaria sp. Fusarium sp., Rhizopus sp.*	Field/postharvest	Huang et al. (2000)
Diospyros kaki (persimmon)	Gibberellic acid	*A. alternata*	Field/postharvest	Eshel et al. (2000)
Fragaria ananassa (strawberry)	Acibenzolar	*B. cinerea*	Glasshouse/postharvest	Terry and Joyce (2000)
Mangifera indica (mango)	Salicylic acid	*C. gloeosporioides*	Field/postharvest	Zainuri et al. (2001)
Passiflora edulis (passionfruit)	Acibenzolar[b]	*C. oxysporum*	Field/postharvest	Willingham et al. (2002)
Persea americana (avocado)	Carbon dioxide	*C. gloeosporioides*	Postharvest	Prusky et al. (1993)
	Cytokinins	As above	Field/postharvest	Beno-Moualem et al. (2001)
Rosa hybrida (rose)	Gibberellic acid	*B. cinerea*	Postharvest	Shaul et al. (1995)
	Methyl jasmonate	*B. cinerea*	Postharvest	Meir et al. (1998)
Solanum tuberosum (potato disks; tubers)	Acibenzolar	*Fusarium semitectum*	Field/postharvest	Bokshi et al. (2000)

[a] Reduced *Alternaria* sp., *Epicoccum* sp. but increased *B. cinerea*.
[b] Acibenzolar sprayed in combination with azoxystrobin and/or industry standard fungicides (copper oxychloride, mancozeb, iprodione).

2.2 Plant hormones as a pivotal defence system against pathogens

Throughout evolution, plants have developed a way to reduce fitness costs in order to fight against different types of pathogens. Hence, plants can trigger a specific defence signalling pathway depending on the pathogen nature. To date, it has been demonstrated that

primarily the three plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play key roles in different facets of plant defence.

As a generalisation, the immune pathway used by plants against biotrophic pathogens is the salicylic acid signalling pathway, whereas ethylene and jasmonic acid -dependent defences are generally triggered upon necrotrophic pathogen attack and insect wounding (Bari & Jones, 2009; Heil & Ton, 2008; Thakur & Sohal, 2013). However, there is evidence that these hormone-signalling pathways don't act independently and they can actually interact with each other. In the last few years it has been reported that there is a clear cross-talk between SA and JA/ET signalling pathways, which can act antagonistically, neutrally or synergistically (Kunkel & Brooks, 2002; Mur, Kenton, Atzorn, Miersch, & Wasternack, 2006) which may depend on the relative concentration of each hormone (Mur et al., 2006). Pathogens have also evolved to exploit these negative or positive pathway interactions to their benefit in order to promote disease development (El Oirdi et al., 2011a). Furthermore, the lifestyles of different pathogens are not often readily classifiable as purely biotrophic or necrotrophic (Newton, Fitt, Atkins, Walters, & Daniell, 2010).

Therefore, the positive or negative cross-talk between SA and JA/ET pathways may be regulated depending on the specific pathogen (Adie et al., 2007). With regard to this, although the role of SA in plant defence is commonly associated to resistance against would-be biotrophic pathogens, there is evidence that this not always the case and, thus, this specificity in the plant hormone pathway-pathogen interaction can also be seen in the tomato-*Botrytis cinerea* interaction. In this pathosystem it has been demonstrated that SA can contribute to basal defence of tomato against this necrotrophic pathogen, i.e., the activation of the SA-dependent defence pathway via its functional analogue benzothiadiazole (BTH) resulted in induced resistance against *Botrytis cinerea* in tomato but not in tobacco (Achuo et al., 2004).

To date, the hormones SA, JA and ET have been the target of plant-pathogen interactions research and they are known to play key roles in various aspects of plant defence (Kunkel & Brooks, 2002). However, recent studies indicate that other hormones that are usually associated to other aspects of plant growth and development, such as abscisic acid (ABA), auxin, gibberellic acid (GA), cytokinin (CK), brassinosteroids (BR) and peptide hormones are also implicated in plant defence signalling pathways but their role in plant defence is less well studied (Bari & Jones, 2009).

It is clear that induced resistance can be an effective tool to improve plant defence systems and help them to survive microbes challenge. However, it is well known that induced resistance can also carry some fitness costs in the plant development, and this has to be taken into account. To date, relatively few studies have quantified the costs of induced resistance, although there is evidence that chemical synthetic elicitors, such as DL-β-Aminobutyric Acid (BABA), 2,6- dichloroisoniciotinic acid (INA) and benzothiadiazole (BTH), can lead to negative effects on further plant development (Walters & Heil, 2007; Zhong et al., 2014).

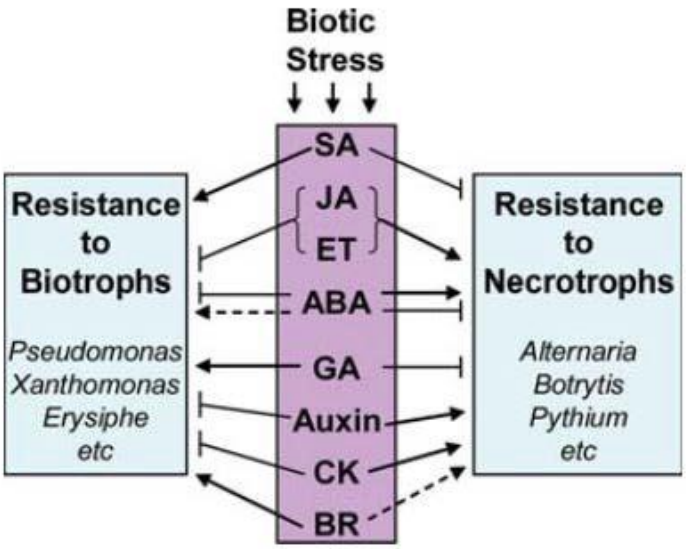


Figure 4. Simplified model showing the involvement of different hormones in the positive or negative regulation of plant resistance to various biotrophic and necrotrophic pathogens (Bari & Jones, 2009). The arrows indicate activation or positive interaction, blocked lines indicate repression or negative interaction and dashed arrows represent hypothesized connections.

There are studies also about the application of the plant hormone jasmonic acid (JA) or methyl jasmonate (MeJA) as resistance elicitors can have costs in terms of reduced seed production and leaf growth or of delayed flowering and fruiting (Darras, 2011; Redman, Jr., & Schultz, 2001). Nevertheless, in most cases, studies indicate that plants in the primed state (priming as the phenomenon that enables cells to respond to very low levels of a stimulus in a more rapid and robust manner than non-primed cells (Conrath, 2011)) are efficiently protected against a broad spectrum of stresses without major trade-off effects on commercially and ecologically important traits, such as growth and seed set (Ton, Ent, Hulten, & Pozo, 2009), therefore, benefits of priming-mediated resistance outweigh the costs in environments in which disease occurs (van Hulten, Pelser, van Loon, Pieterse, & Ton, 2006). In addition, other studies have already shown that low elicitor doses can enhance resistance to pests without

interfering with agricultural production (Redman et al.,2001).

3. The pathogen: Botrytis cinerea

3.1 Biology

Botrytis cinerea Pers. Fr. (teleomorph *Botryotinia fuckeliana*) also known as “grey mould fungus”, is a filamentous fungus that belongs to the phylum of *Ascomycota*. It is characterized by abundant asexual tree-branch like and grey structures called conidia (conidium in singular).

It also produces highly resistant structures called *sclerotia*, which are compact masses of tough fungal mycelium containing food reserves. One role of *sclerotia* is to overwintering environmentally extreme conditions such as cold and/or dry.

3.2 Life cycle

Botrytis cinerea is an airborne opportunistic pathogen with a broad host range, it is notoriously aggressive on fleshy fruit (Cantu et al., 2009) and it spreads its spores either through the air or rain water.

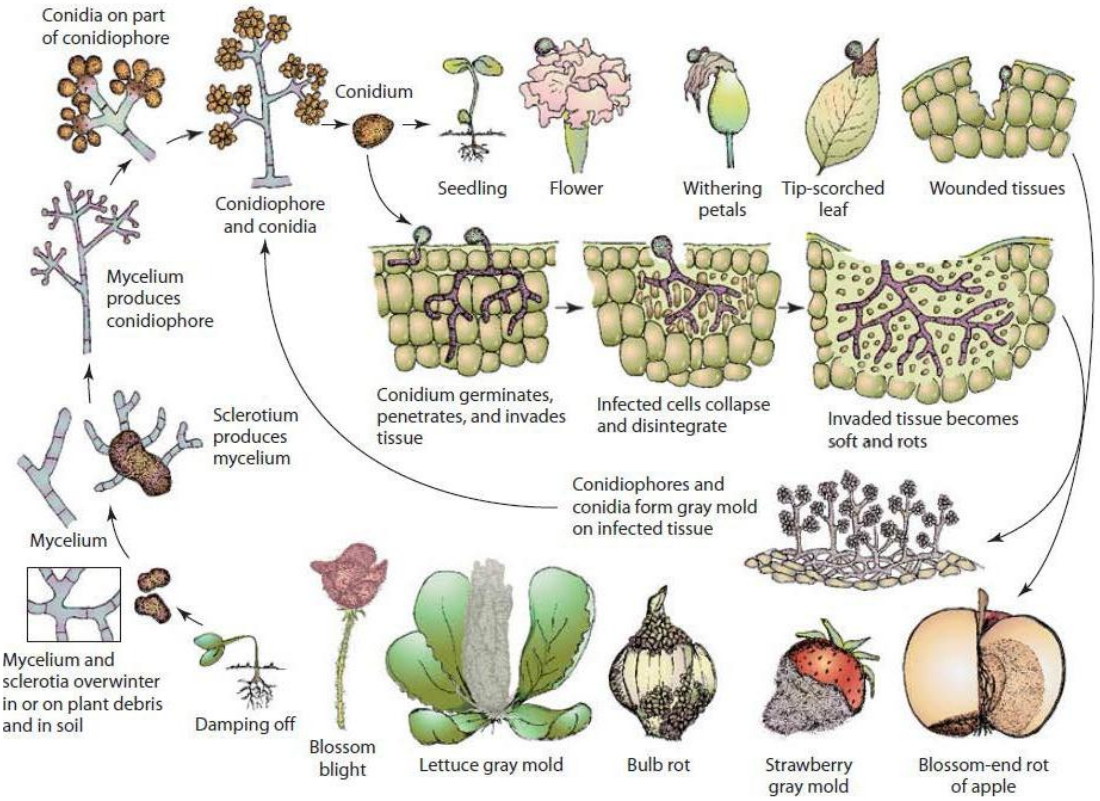


Figure 5. Disease cycle of *Botrytis cinerea* (Agrios, 2005).

Conidia are released from hyphae and blown onto leaf surface. Once conidia have landed on the surface of a susceptible host, there are several factors that can affect the asexual spore germination. Free surface water or high relative humidity (>93% RH) is essential to germinate and penetrate the host epidermis (B. Williamson, B. Tudzynski, P.Tudzynski, 2007). It also requires cool (18–23°C) weather for best growth, sporulation, spore release, germination, and establishment of infection. During active growth, it produces a range of hydrolytic enzymes and metabolites to facilitate penetration and colonization of host tissues (J. Kars, G. Krooshof, L. Wagemakers, R. Joosten, 2005). Conidia form a grey mould on infected tissue and subsequently, infected cells collapse and disintegrate. *B.cinerea* can survive through extreme conditions creating highly resistant dormant structures called "sclerotia". Sclerotia can germinate in spring or warmer conditions to create new hypha and conidiophores.

3.3 Pathogenicity

Botrytis cinerea is the causative agent of grey mould disease in several horticultural crops, ornamental crops and softfruits, such as tomato, grape, lettuce and raspberry. The pathogen can cause spots, rot and blight in the field (post-harvest) as well as in greenhouses (Finkers, van den Berg, et al., 2007). Furthermore, it can infect and cause disease on at least 235 host species (Jarvis, 1977), most of which are dicotyledonous, including important plant species that are used for oil, protein and fibre extraction. Some monocotyledonous plants are also susceptible to attack by *B. cinerea*, in turn there is a group of related *Botrytis* species specialized to infect about a dozen monocots hosts (B. Williamson, B. Tudzynski, P.Tudzynski, 2007). Product quality of horticultural crops has been the main area of research the past decades. Growers and sellers have been seeking for best possible product quality and highest possible profits (Darras, 2011). *Botrytis cinerea* is able to reduce the yield of the crop before it is harvested (pre-harvest yield loss) causing massive losses of crops grown in greenhouses.

Its aggressive nature makes it very destructive in mature or senescent tissues of crops and fruits, which strongly reduces the quality of the product, making it one of the major causes of post-harvest waste and spoilage worldwide. However, there is evidence that *Botrytis* can be an opportunistic pathogen and infect at a much earlier phase of the plant development. It is able to colonize unripe plant tissue and remain in a quiescent and/or asymptomatic state, waiting for favourable environmental conditions to start spore germination and rot host tissues. Furthermore, it has been reported that *B.cinerea* can infect some plants, such as *Primula x polyantha*, and grow systemically without any symptoms, as an endophytic infection (Barnes

& Shaw, 2002). In this study it was discovered that the infections caused by this fungus, although they usually are from airborne spores, can also be seed-borne, commonly from external infection but sometimes also within the seed. Experiments also show that *Botrytis cinerea* is often present in symptomless lettuce plants as a systemic, endophytic, infection which may arise from seed (Sowley, Dewey, & Shaw, 2009).

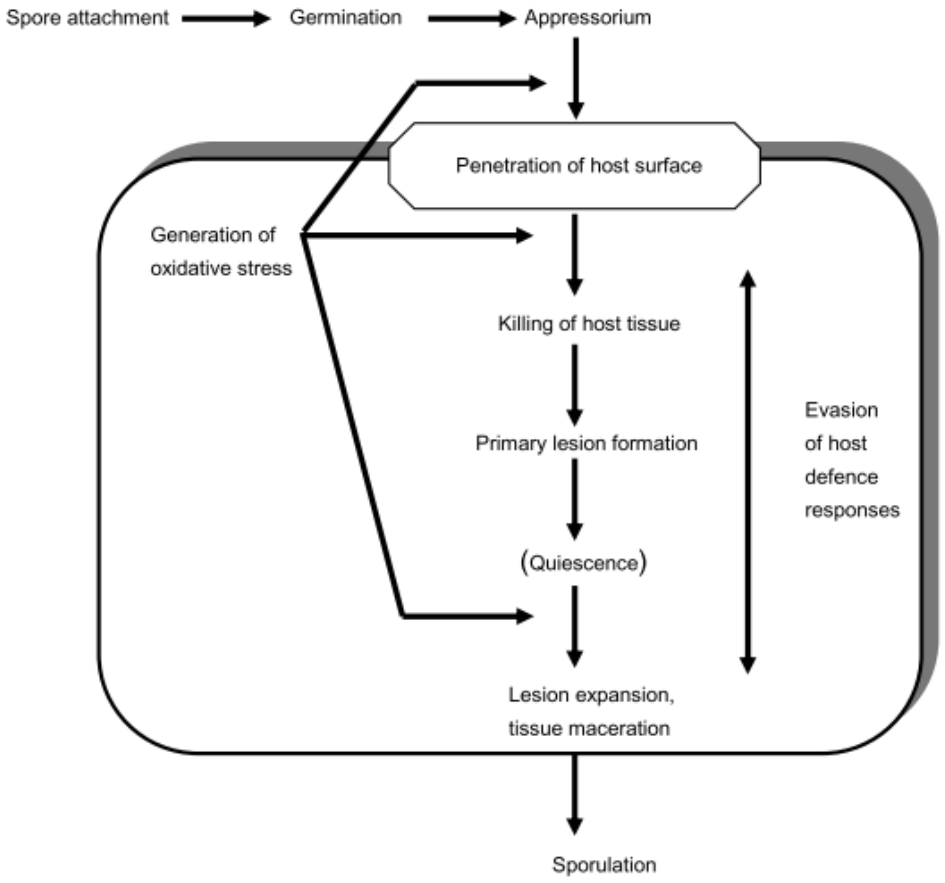


Figure 6. Different stages in the infection process of *Botrytis cinerea*. The shaded box represents the host tissue (J. A. L. Van Kan, 2005)

Botrytis cinerea is a difficult fungal pathogen to control, as it has a variety of attack modes, diverse hosts as inoculum sources, and it can survives as mycelia and/or conidia or for longer periods as sclerotia in crop debris (B. Williamson, B. Tudzynski, P.Tudzynski, 2007). Efforts to investigate and fight against this pathogen are numerous , and include biocontrol agents, non-specific plant defence inducers and fungicides (J. A. L. Van Kan, 2002; Sellal, Dahmani, Benkirane, Touhami, & Douira, 2013; Terry, 2004). Several groups have also studied the molecular mechanisms that *Botrytis cinerea* uses to infect plants and to develop disease (El Oirdi et al., 2011a; J. A. L. Van Kan, 2005; Weiberg et al., 2013).

There is evidence that degradation of the plant cell wall by secreted enzymes is an important aspect in the infection by *B. cinerea*. The action of plant-cell-wall degrading enzymes is believed to facilitate intercellular fungal growth and destabilize host cell integrity (ten Have, Dekkers, Kay, Phylip, & van Kan, 2004). *B. cinerea* is able to secrete many enzymes and metabolites that are presumed to enable the fungus to degrade plant cuticle and cell wall components, killing the cells and subsequently feed on them (J. A. L. Van Kan, 2005; ten Have et al., 2010). In addition, it is well known that fungal and bacterial pathogens are able to generate specific "effector" molecules, such as high molecular weight carbohydrates or exopolysaccharide (EPS). In turn, *Botrytis cinerea* is able to produce an EPS known as β-(1,3)(1,6)-D-glucan (Stahmann et al., 1995) that acts as a type of virulent factor or effector.

It has recently been discovered that this pathogen can manipulate the antagonistic effect between SA and JA signalling pathways through this effector β-(1,3)(1,6)-D-glucan, circumventing the JA-related defence pathway (El Oirdi et al., 2011a) in order to be able of spread and infect all plant tissue whilst the host immune system is deteriorated.

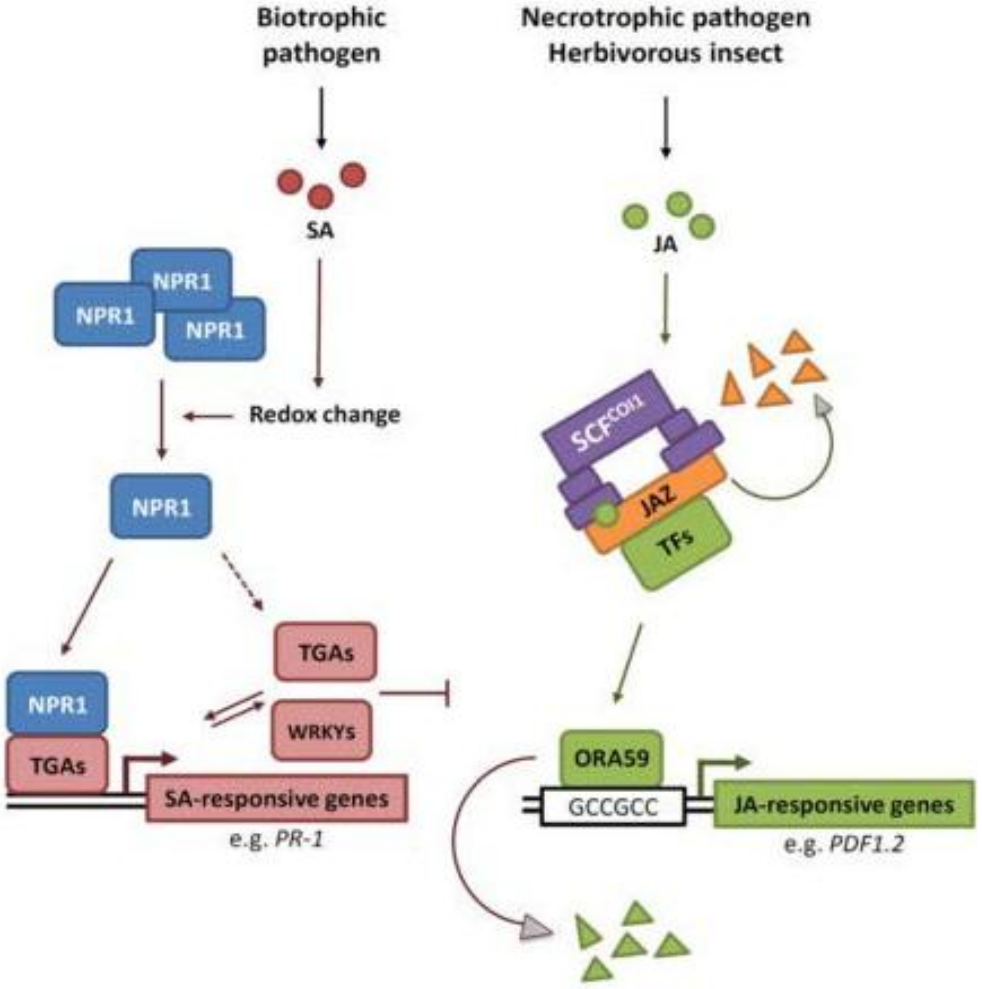


Figure 7. Model for SA/JA Signal Interaction on GCC-Box-Containing Promoters of JA-Responsive Genes (Van der Does et al., 2013). Infection by a biotrophic pathogen results in the accumulation of SA and monomerization of NPR1 through SA-mediated redox changes in the cell. Monomeric NPR1 is then translocated into the nucleus where it interacts with TGA transcription factors, ultimately leading to the activation of SA-responsive genes. Insect wounding or necrotrophic pathogen attack, results in the accumulation of JA. Binding of JA to the SCFCOI1-E3 ubiquitin-ligase complex leads to degradation of JAZ transcriptional repressor proteins via the proteasome, which results in the release of transcriptional activators. Transcription factors, such as ORA59 and ERF1, are induced that activate the ERF branch of the JA pathway. Binding of ERFs to the GCC-box induces JA-responsive gene expression, which can be suppressed by SA. SA can negatively affect ORA59 protein accumulation, which provides an explanation for the antagonistic effect of SA- on JA-responsive gene expression in wild-type plants.

4. The host: Solanum lycopersicum

Tomato (*Solanum lycopersicum*) belongs to the third most economically important plant taxa, after legumes and grasses. Tomato belongs to the *Solanaceae* family, which comprise more than 3000 species, some of which are economically very important for the agriculture industry, including, potato, tobacco, aubergine, and pepper.

During the last decade tomato has become one of the most important model research plant together with *Arabidopsis thaliana* and *Solanum tuberosum* (potato). *Solanum lycopersicum* and *Nicotiana attenuate,* have served as model organisms to understand wound responses in the Solanaceae (Scranton, Fowler, Girke, & Walling, 2013).

In addition, tomato (*Solanum lycopersicum*) has served as a model organism to study fruit ripening (Giovannoni, 2004) and has emerged as an informative experimental system to characterize the molecular regulation of the ripening-related susceptibility to pathogens, in particular to necrotrophic fungi, such as *Botrytis cinerea* (Flors, Ton, & Jakab, 2007; Powell et al., 2000). Unlike other model plants with relatively small number of pathogens, such as *Arabidopsis thaliana*, tomato is a susceptible species to many pathogens of different types, including fungi, nematodes, viruses, bacteria and insects. This diversity of pathogens emphasizes the importance of the tomato pathosystem as a favourable model for studying plant-pathogen interactions (Arie, Takahashi, Kodama, & Teraoka, 2007). Besides, the tomato genome was published in Nature on May 31st, 2012, culminating years of work by the Tomato Genome Consortium, a multi-national team of scientists from 14 countries. Due to this joint work, it is currently known that common tomato has 12 chromosomes with a modest diploid genome size of 950Mb as well as it has short generation time, has abundant genetic resources, such as mutants collections, microarrays, etc., making it a perfect crop model to study induced resistance, pest and diseases of dicotyledons among others disciplines.

Materials and methods

Experiment 1: Relative gene expression of 2 genes of interest after elicitor-induced resistance in *Solanum lycopersicum*.

Aim: To investigate the contribution of resistance elicitors in the cross-talk of 2 main signalling pathways (JA and SA) involved in tomato resistance to *Botrytis cinerea*.

Experimental Procedures:

This experiment aims to evaluate the effect of different elicitors in the gene expression profile of 2 tomato genes of interest that belong to the SA and JA-defence pathways. To check which gene is up or down regulated by the elicitors, I chose 2 marker genes, one for each signalling pathway (SA-PR1 and JA-Proteinase inhibitor I) and Actin as a reference (housekeeping) gene. To address this question gene expression was measured in leaves of tomato plants after the following treatments thought to induce either SA or JA-dependent defence genes (BTH, BABA, MeJA, Softguard=chitin+chitosan, and Control-DDW). Resistance elicitors were sprayed to 3-4-week-old plants (3 biological reps), and at 3 time points (3h, 9h and 24 hours after treatment). Leaves were harvested and total RNA was extracted with TRIzol Reagent (Invitrogen, UK) and then an extra step was added to further clean the RNA with Phenol-chloroform. Equal amounts of RNA (4 µg of 250 ng/µl) were used to synthesize cDNA with random hexamer primers and SuperScript™ III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR (qRT-PCR) reactions were performed using SYBR Green Mastermix (Qiagen, UK) according to the manufacturer's protocol. qRT-PCR was performed with specific tomato primers (Sigma-Aldrich), *PI I* and *Actin* (El Oirdi et al., 2011b) and PR-1 (Song, Ma, Tan, & Zhou, 2011). Amplification and detection of specific products were performed with following the cycle profile: denaturation step at 95°C for 15 min, 95°C for 15 sec, annealing and extension at 60°C for 1 min. Each qPCR reaction contained three non-template controls. All reactions were run in technical triplicate for each biological replicate, and the average values were used for quantification. The relative quantification of target genes was determined using the ΔΔCt method.

Experiment 2 (Part I): SA/JA-induced resistance in tomato against *Botrytis cinerea*

Aim: To assess whether treatments with the chemical inducers BABA, MeJA, benzo (1,2,3)-thiadiazole-7-carbothioic acid S- methyl ester (BTH, BION; Syngenta, Basel, Switzerland) and the combination of BTH+MeJA induce resistance in tomato varieties Motelle and Money-Maker.

Experimental Procedures:

Seeds of the two tomato cultivars 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 1 spray DDW + 0.02% Silwet (surfactant)+ 0.05% ethanol + Control 2 soil-drench of DDW (150ml water per tray)
- DL-β-aminobutyric acid (BABA) (Positive callose control) 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) foliar sprayed (1mM) + 0.02% Silwet (surfactant) + 0.05% ethanol
- MeJA (Positive JA-IR control) foliar sprayed (0.1 mM) + 0.02% Silwet (surfactant)
- BTH+MeJA (0.25mM+0.1 mM)+ + 0.02% Silwet (surfactant)

Plant height was measured every two days during the treatment week to determine elicitor-induced growth reduction (fitness costs in tomato seedlings development).

One week later, 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), plants were infected with *B.cinerea* as described in the Lancaster protocol with major modifications:

4-5 weeks-old *Botrytis cinerea* PDA plates were kept in the dark at room temperature. Once *B.cinerea* was sporulating, 20ml of DDW with 0.01% Tween 20 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 Haemocytometer and adjusted to $5*10^4$ 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 strain.

Infection was scored at 3 and 4 days after inoculation by measuring the diameter of the lesions and the % of spreading lesions.

Material required:

- 12 plants per treatment and 2 cultivars → **120 plants**
- 24 plants per propagator/tray → **5 trays**
- 8 plants per treatment after elicitor treatment (1 plant per pot – **80 pots**) – **2 shelves**

Experiment 2 (Part II): Chitosan-induced resistance in tomato against *Botrytis cinerea*

Aim: To test whether complex formulated elicitors, known to induce multiple defence responses, result in effectiveness induced resistance against *B. cinerea* in tomato cv. Money-Maker and cv. Motelle.

Experimental Procedures:

This experiment aims to assess whether treatments with BABA, MeJA and two chitosan formulations (commercial chitosan formulation-Chitoplant and Chitosan 23) induce resistance in tomato varieties Motelle and Money-Maker.

Seeds of 2 tomato cultivars (Money-Maker 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 1 spray DDW + 0.02% Silwet (surfactant)+ 0.05 % ethanol
- Chitosan NC 23 (1:100) + 0.02% Silwet (surfactant) + 0.05% ethanol

- ChitoPlant (ChiPro) 1% w/v + 0.02% Silwet (surfactant) + 0.05% ethanol
- MeJA (Positive control) foliar sprayed (0.1 mM) + 0.02% Silwet (surfactant)
- BABA soil drenched (5mM stock solution= 0.5mM final concentration)

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

Plant height was measured every two days during the treatment week to determine elicitor-induced growth reduction (fitness costs in tomato seedlings development). 17 days after elicitor treatments, plants were infected with *B. cinerea* as described in the Lancaster protocol with major modifications (see above). Spore inoculum concentration was adjusted to $2*10^4$ spores/ml and incubation time after adding the nutrients was significantly reduced to 15min before the pathogenicity test. Infection was scored at 3 and 4 days after inoculation (due to the *B. cinerea* strain high level of aggressiveness) by measuring the diameter of the lesions and the % of spreading lesions.

Material required:

- 12 plants per treatment and 2 cultivars → 120 plants
- 24 plants per propagator/tray → 5 trays
- 8 plants per treatment after elicitor treatment (1 plant per pot – 80 pots) – 2 shelves

Results

Experiment 1: Relative gene expression of 2 genes of interest after elicitor-induced resistance in *Solanum lycopersicum*.

In the present study I evaluated the expression profile of two tomato marker *R* genes (PI I and PR-1) involved in plant induced resistance. Pathogenesis-related gene/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, 2007). 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).

In these results, due to the lack of 0 hours-time point, data was compared to the first time point (3 hours). Results show that tomato SA-dependent PR1 was highly induced at 3 hours after treatment (hat) of BABA treatment in comparison with the other treatments. PR1 remained down-regulated at 9 hat in all treatments whilst expectedly, at 24 hat only BTH- Bion and BABA induced PR1.

JA-dependent tomato proteinase inhibitor I (PI I), was induced at 3 hat after MeJA and BABA treatments in comparison with the other treatments. 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 and chitosan commercial elicitor (Softguard, Travena) also primed 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.

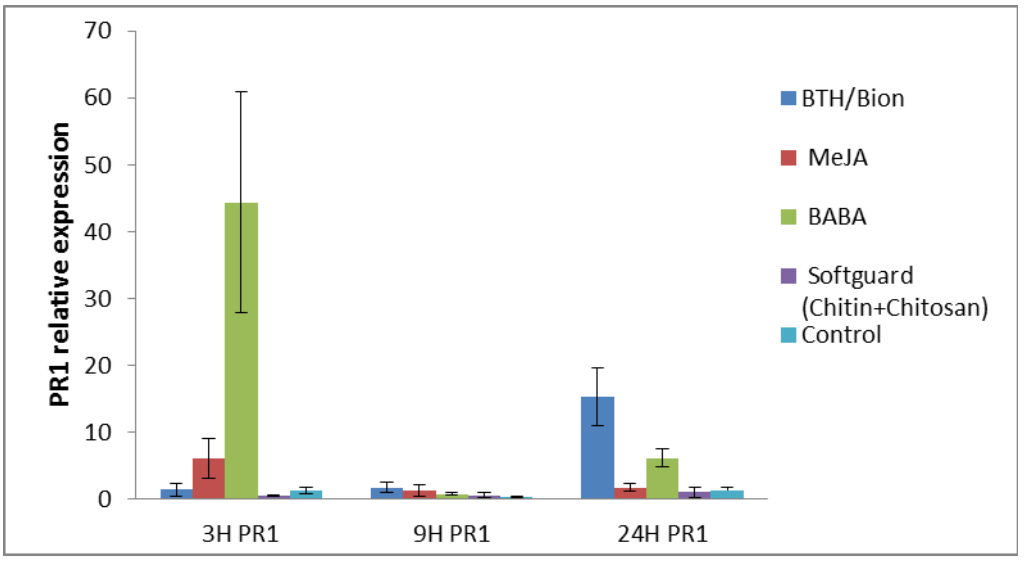


Figure 1. Expression levels of tomato SA-dependent PR-1 relative to Actin. Four-week-old plants were treated with BTH, MeJA, BABA, Softguard (chitin+chitosan) and water (control); Samples (leaves) were harvested at 3 time points (3h, 9h and 24 hours after treatment) for RNA extraction. qRT-PCR was performed with specific primers for tomato PR-1 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.

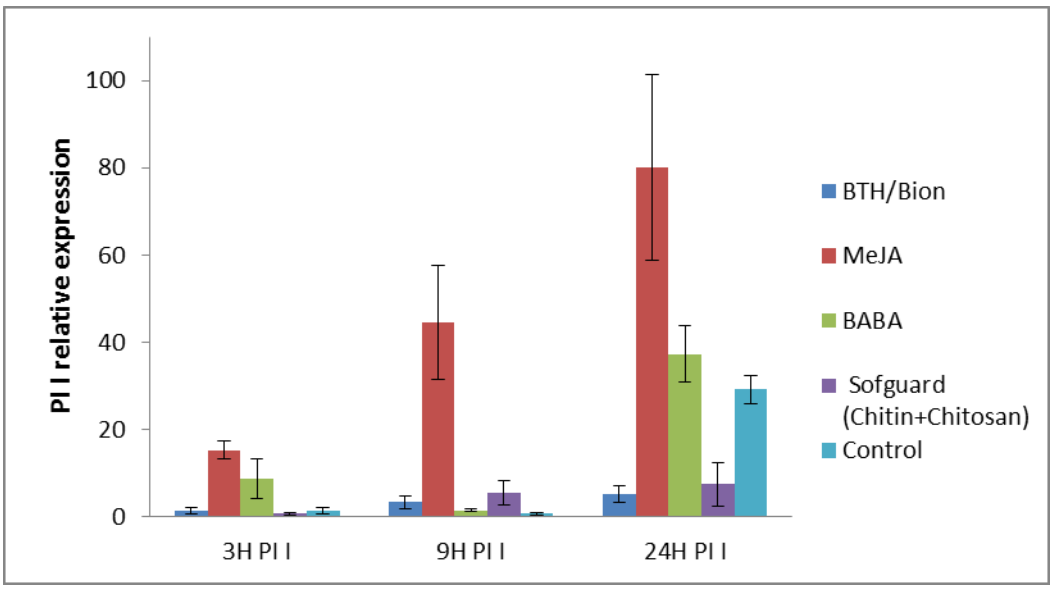


Figure 2. Expression levels of tomato JA-dependent protein inhibitor PI I (also called PinI) relative to Actin. Four-week-old plants were treated with BTH, MeJA, BABA, Softguard (chitin+chitosan) and water (control); Samples (leaves) were harvested at 3 time points (3h,9h and 24 hours after treatment) for RNA extraction. qRT-PCR was performed with specific primers for tomato PI I and Actin (reference control gene) as described in Methods. Values represent means ±SD from three biological replicates.

Experiment 2 (Part I): SA/JA-induced resistance in tomato against *Botrytis cinerea*

Basal callose deposition induced by BABA, MeJA, BTH+MeJA and BTH-IR in tomato cv. Money-Maker and tomato cv. Motelle.

To define whether elicitors directly induce callose deposition, cotyledons of every treatment were excised one week after treatment and store in 100% ethanol previous Aniline Blue staining. Overall, there was the same trend in both varieties apart from in treatments containing MeJA, where MeJA induced callose and this induction was higher in Money- maker (MM) than in Motelle. The combination of BTH and MeJA also induced callose deposition in tomato cotyledons (Figure 1) in MM but not in Motelle plants.

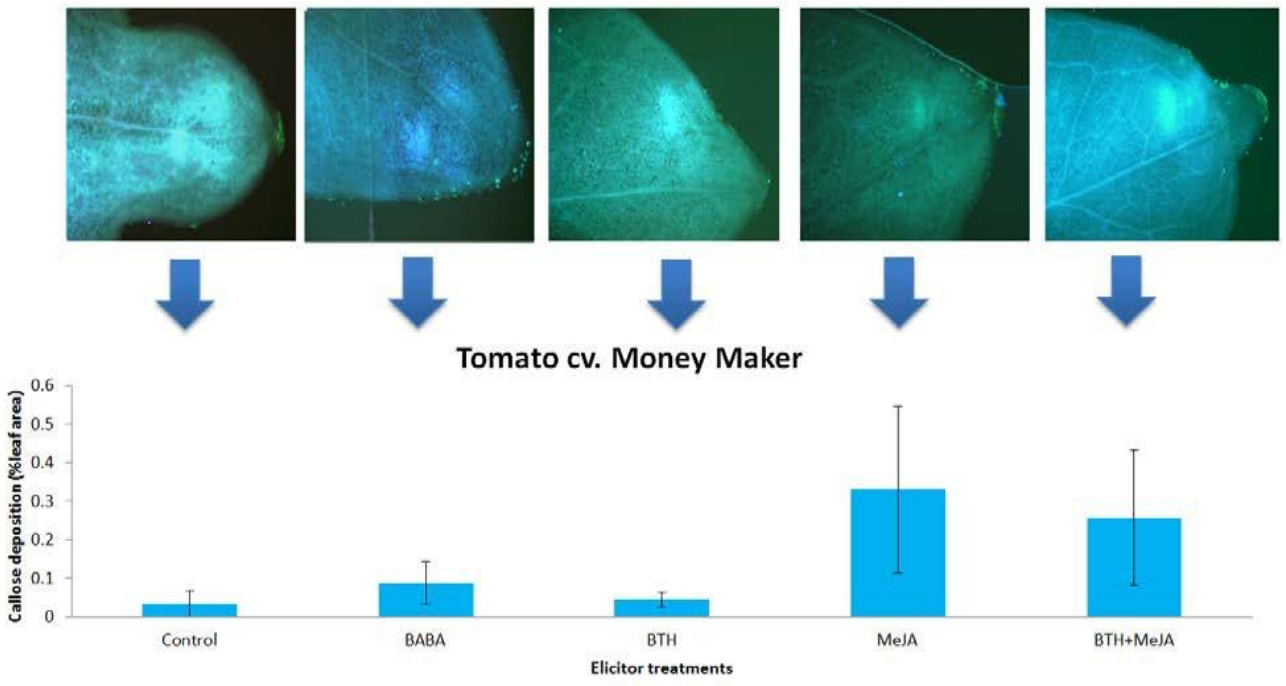


Figure 1. Basal callose deposition in tomato cv. Money-Maker leaves after Water-control, BABA, BTH, MeJA and MeJA+BTH treatments. 7 days after treatment leaves were harvested for Aniline Blue staining. Pictures were taken under fluorescence microscopy at 4x magnifications. Callose was quantified as described previously (Luna et al., 2011). Values represent percentages of the mean ± SEM.

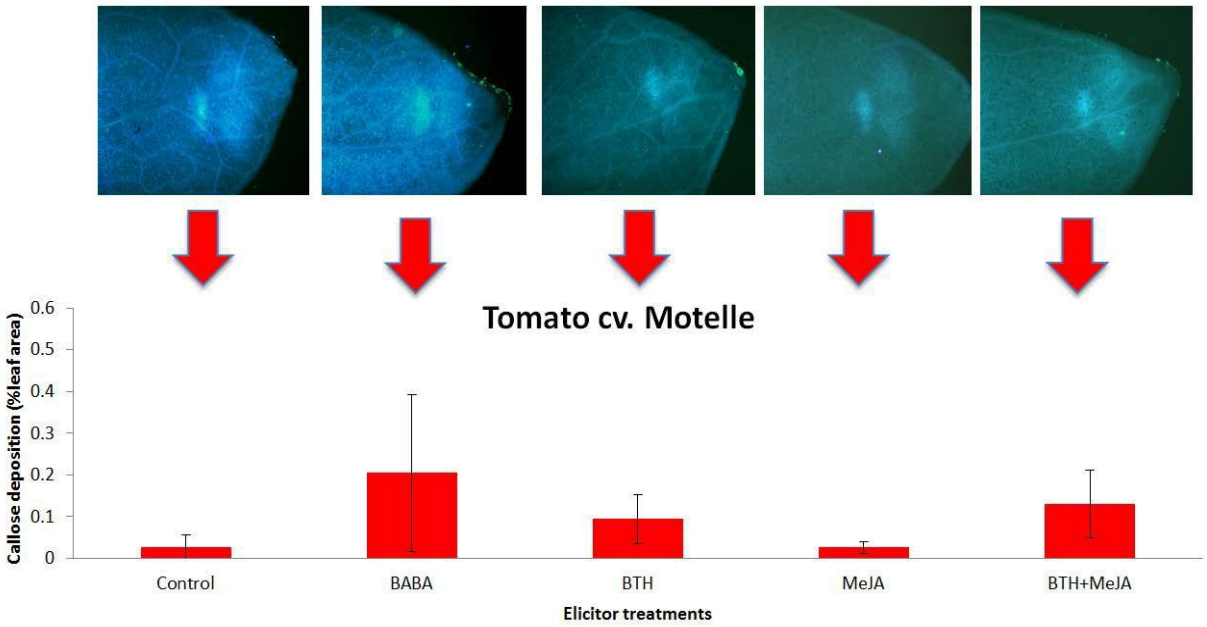


Figure 2. Basal callose deposition in tomato cv. Motelle leaves after Water-control, BABA, BTH, MeJA and MeJA+BTH treatments. 7 days after treatment leaves were harvested for Aniline Blue staining. Pictures were taken under fluorescence microscopy at 4x magnifications. Callose was quantified as described previously (Luna et al., 2011). Values represent percentages of the mean ± SEM.

Elicitor-induced resistance in tomato cultivars (Money-Maker and Motelle) against Botrytis cinerea

Both tomato cultivars are susceptible to the aggressive necrotrophic fungus *B.cinerea*. To determine the benefits of BABA-IR, BTH-IR, MeJA-IR and the combination of MeJA+BTH- IR against *B. cinerea*, 1-week-old tomato plantlets were treated with different elicitors and water as control. 7 days post-treatment, plants were transplanted to larger pots and 17 days after elicitor treatments, plants were challenged with *B. cinerea* ($5*10^4$spores/ml) by drop inoculation.

Interestingly, the level of resistance induced by the 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 3a). In contrast, BABA-induced resistance provided no protection against *B. cinerea*. No 'basal' differences between the two tomato cultivars (P=0.086) were observed 3 days post-inoculation, while these differences were significant at 4 d.p.i. (P=0.003) (Figure 3b). In contrast, basal differences were found in the response of the two varieties to the elicitors (P=0.05) at 3 d.p.i.

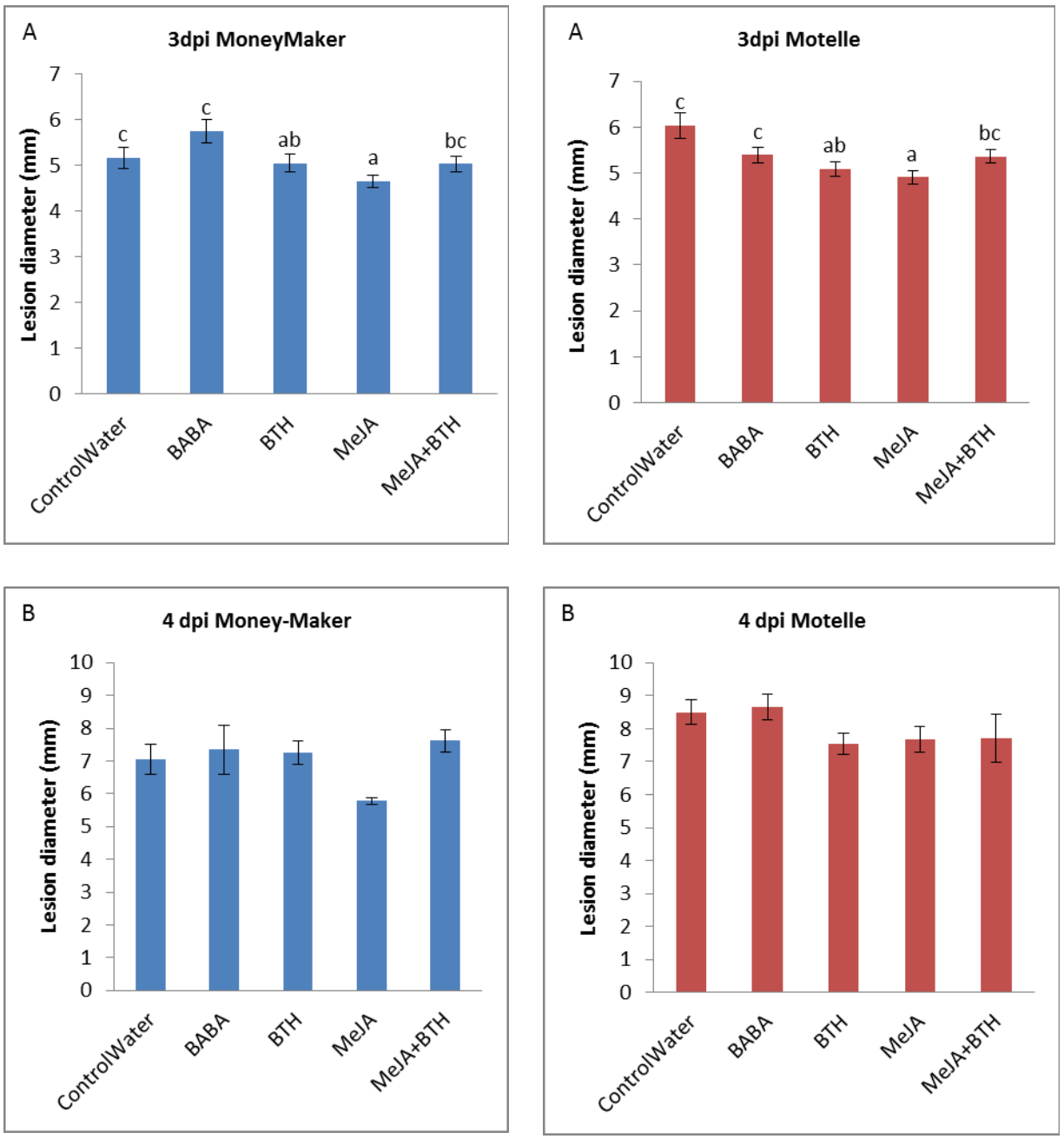


Figure 3.

A. Quantification of BABA, BTH, MeJA and MeJA+BTH-induced resistance against *B. cinerea* in tomato cv. Money-Maker (blue bars) and Motelle (red bars) at 3 days post-inoculation. Values presented are means ± SEM. Different letters indicate statistically significant differences (Fisher's least significant difference (LSD) test $P<0.001$, $\alpha=0.05$).

B. Quantification of BABA, BTH, MeJA and MeJA+BTH-induced resistance against *B. cinerea* in tomato cv. Money-Maker (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 varieties ($P=0.003$ for Tomato-Cultivar).

Pathogen-induced callose deposition in BABA, MeJA, BTH+MeJA and BTH-IR in tomato cv. Money-Maker and tomato cv. Motelle.

To determine the effectiveness of the elicitor treatments inducing cell wall defences, double staining (Aniline Blue + calcofluor) was performed to see pathogen-induced callose deposition in all treatments. The high level of aggressiveness of the *Botrytis cinerea* R16 strain made measurement of callose difficult due to the high contrast of the calcofluor. Nevertheless, callose was found in samples of MeJA-treated plants (Figure 4) which correlates with the lesion size significant reduction of the same treatment. In contrast, callose was not seen in the rest of the elicitor-treated plants or in the water-treated control plants (Figure 4). On the other hand, the rest of the elicitor treatments did not have a strong effect on infection reduction since the pathogen was too virulent and host defences were overwhelmed.

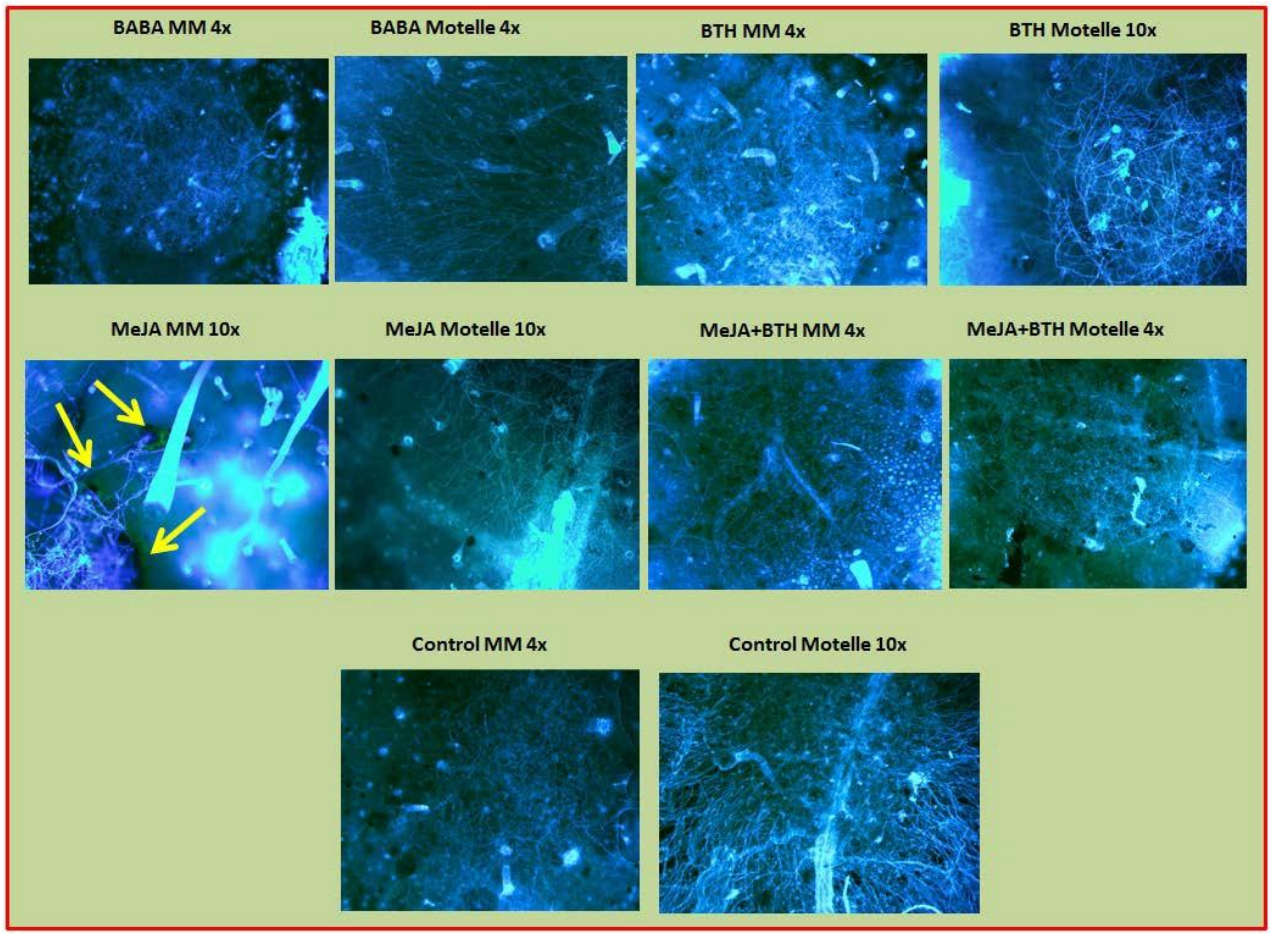


Figure 4. Callose deposition in tomato cv. Money-Maker and tomato cv. Motelle leaves after *B.cinerea* infection in Water-control, BABA, BTH, MeJA and MeJA+BTH-treated plants. 3 days post-inoculation leaves were harvested for double staining (Aniline Blue + calcofluor) and then pictures were taken under 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 *B.cinerea* (Figures 3a and 3b). Yellow arrows indicate callose papillae (greenish points) deposition surrounding pathogen mycelia (light blue colour) penetration sites into tomato epidermal surface.

Costs of elicitor-induced resistance

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

The costs of BABA-IR, MeJA-IR, MeJA+BTH-IR and BTH-IR on plant fitness were analysed by relative growth rate (RGR). Every other day after elicitor treatment, height measurements were obtained during the treatment week in both tomato cultivars.

Defence induction by BABA and MeJA lead to statistically significant reductions in RGR in both cultivars. 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 27% in Motelle (Fig. 5).

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, as appreciable in the pictures, plants developed thinner and smaller stems and less secondary leaves (see appendix), thus indicating that BTH does not affect height but it does alter normal plant development.

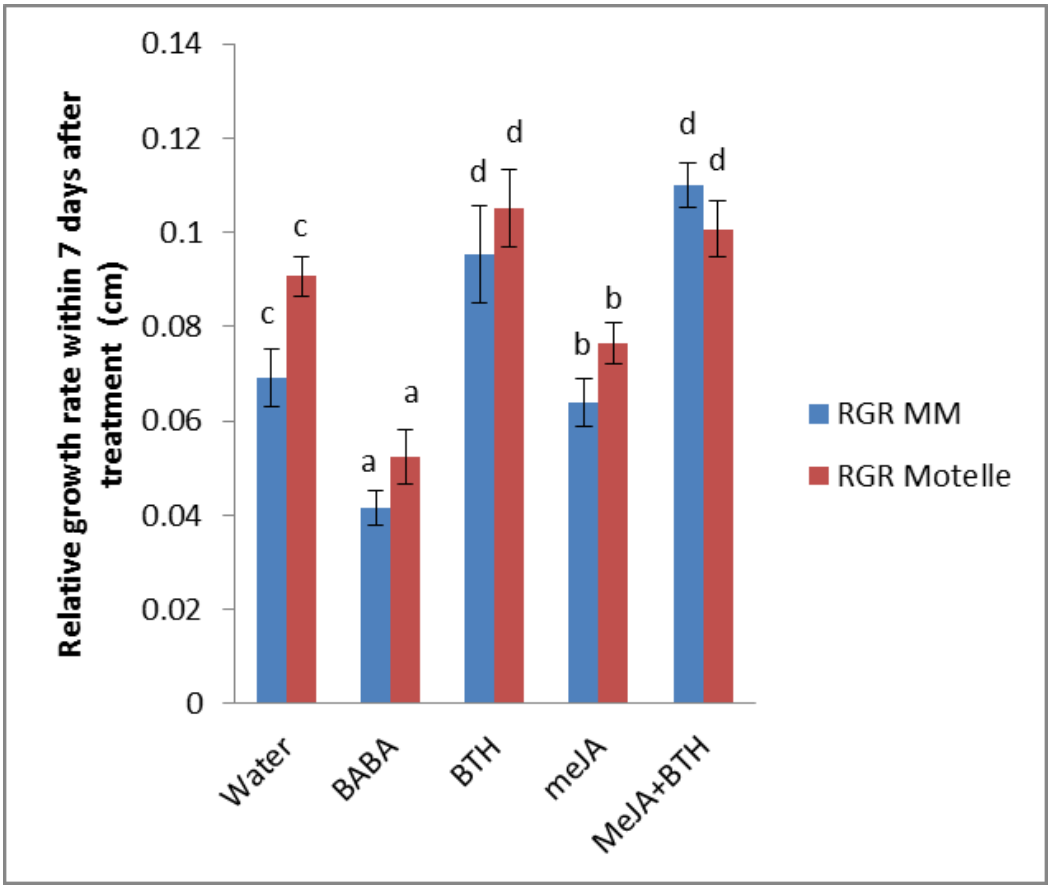


Figure 5. Relative growth rate after elicitor treatment during the week after treatment. Values presented are means ± SEM. Different letters indicate statistically significant differences (Fisher's least significant difference (LSD) test P<0.001, α=0.05).

Experiment 2 (Part II): Chitosan-induced resistance in tomato against *Botrytis cinerea*

Basal callose deposition induced by BABA, MeJA, ChitoPlant, Chitosan23-IR in tomato cv. Money-Maker and tomato cv. Motelle.

To define whether elicitors directly induce callose deposition, 2-week-old cotyledons of every treatment were excised one week after treatment and store in 100% ethanol previous Aniline Blue staining. Although 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); nevertheless, callose induction was much lower than expected in BABA treatments (Figures 6 and 7). Interestingly, the commercial chitosan formulation ChitoPlant induced callose apposition in comparison with the rest of the elicitor treatments (Figure 6).

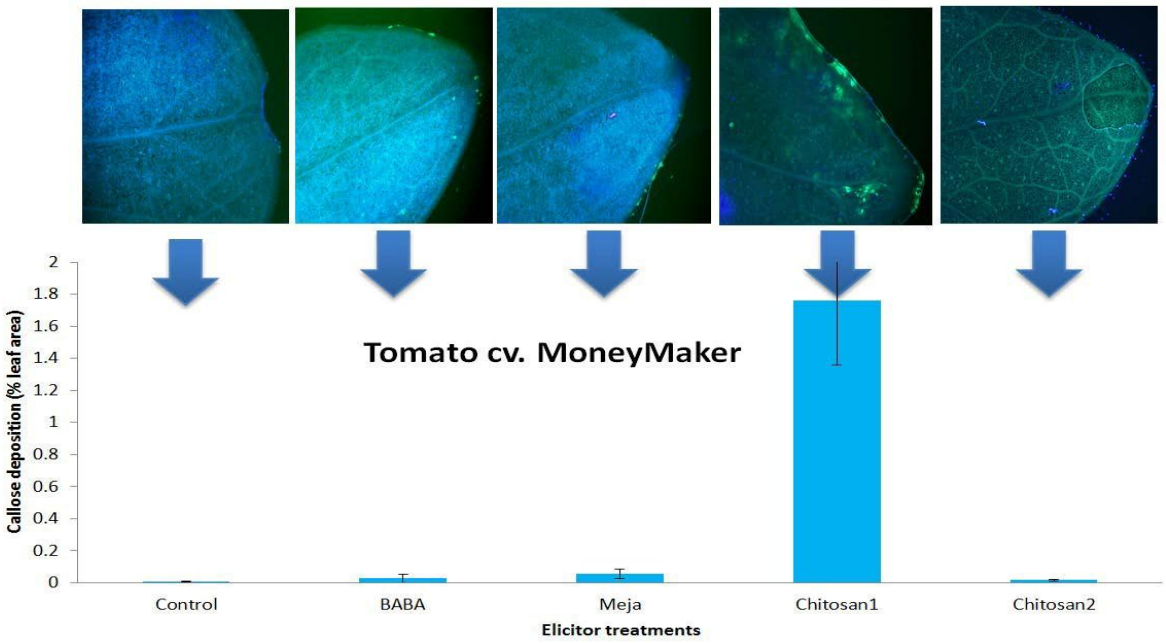


Figure 6. Callose deposition in tomato cv. Money-Maker leaves after Water-control, BABA, MeJA, Chitosan 1/ChitoPlant (ChiPro) and Chitosan 2/Chitosan 23 (NC) treatments. 7 days after treatment leaves were harvested for Aniline Blue staining. Pictures were taken under fluorescence microscopy at 4x magnifications. Callose was quantified as described previously (Luna et al., 2011). Values represent percentages of the mean ± SEM.

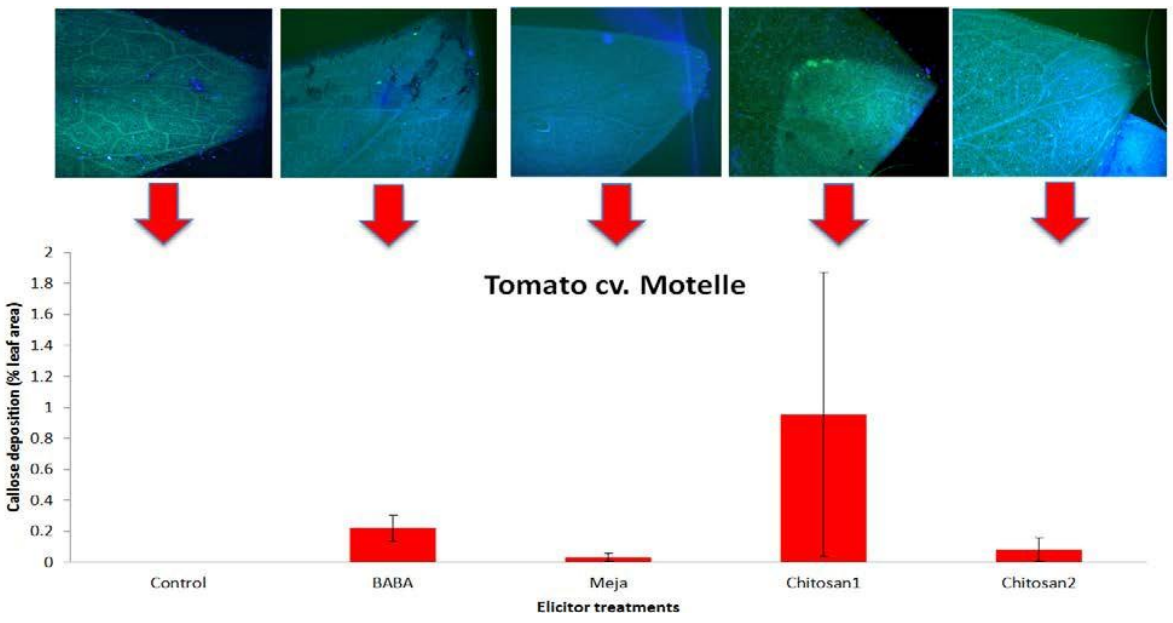


Figure 7. Callose deposition in tomato cv. Motelle leaves after Water-control, BABA, MeJA, Chitosan 1/ChitoPlant (ChiPro) and Chitosan 2/Chitosan 23 (NC) treatments. 7 days after treatment leaves were harvested for Aniline Blue Staining. Pictures were taken under fluorescence microscopy at 4x magnifications. Callose was quantified as described previously (Luna et al., 2011). Values represent percentages of the mean ± SEM.

Comparison between various elicitor-induced resistance in 2 tomato cultivars (Money-Maker and Motelle) against Botrytis cinerea

Both tomato cultivars are susceptible to the aggressive necrotrophic fungus *B.cinerea*. To determine the benefits of BABA-IR, MeJA-IR and the Chitosan-IR against *Botrytis cinerea*, 1-week-old tomato plantlets were treated with different elicitors and water as a negative control. Subsequently, 17 days weeks after elicitor treatments, tomato plants were challenged with *B.cinerea* according to Lancaster Protocol with major modifications ($2*10^4$ spores/ml). Interestingly, 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 8). In tomato Money-Maker, all elicitor treatments behaved similarly in significantly reducing disease expansion, in comparison with the water- treated control plants at both time points (Figure 8a). In contrast, there were more differences among treatments in Motelle at both time points (figure 8b). In tomato cv. Motelle, BABA-IR and ChitoPlant-IR were more effective against *B. cinerea* than the other treatments, at 3 d.p.i . In adittion, at 4 d.p.i. Motelle MeJA-IR and both Chitosans-IR were slightly more effective than BABA-IR in the fight against grey mould disease. After 4 days post-inoculation, all elicitors still significantly reduced *B. cinerea* lesion expansion, in comparison with the control in both cultivars; besides some differences among treatments were still seen in Motelle (Figure 8b). Mainly, BABA and the two chitosan formulations significantly reduced necrotic lesion size at 3 d.p.i. in comparison with water-treated control plants. At 4 d.p.i. MeJA and again both chitosans behaved similarly in significantly reducing disease expansion compared to control treatment (Figure 8b). There were also significant differences among treatments and the 2 tomato cultivars (Motelle and Money-Maker) according to statistical analyses (ANOVA) conducted with GenStat after 3 d.p.i. (Figure 8), although these significant differences disappeared after 4 days post-inoculation.

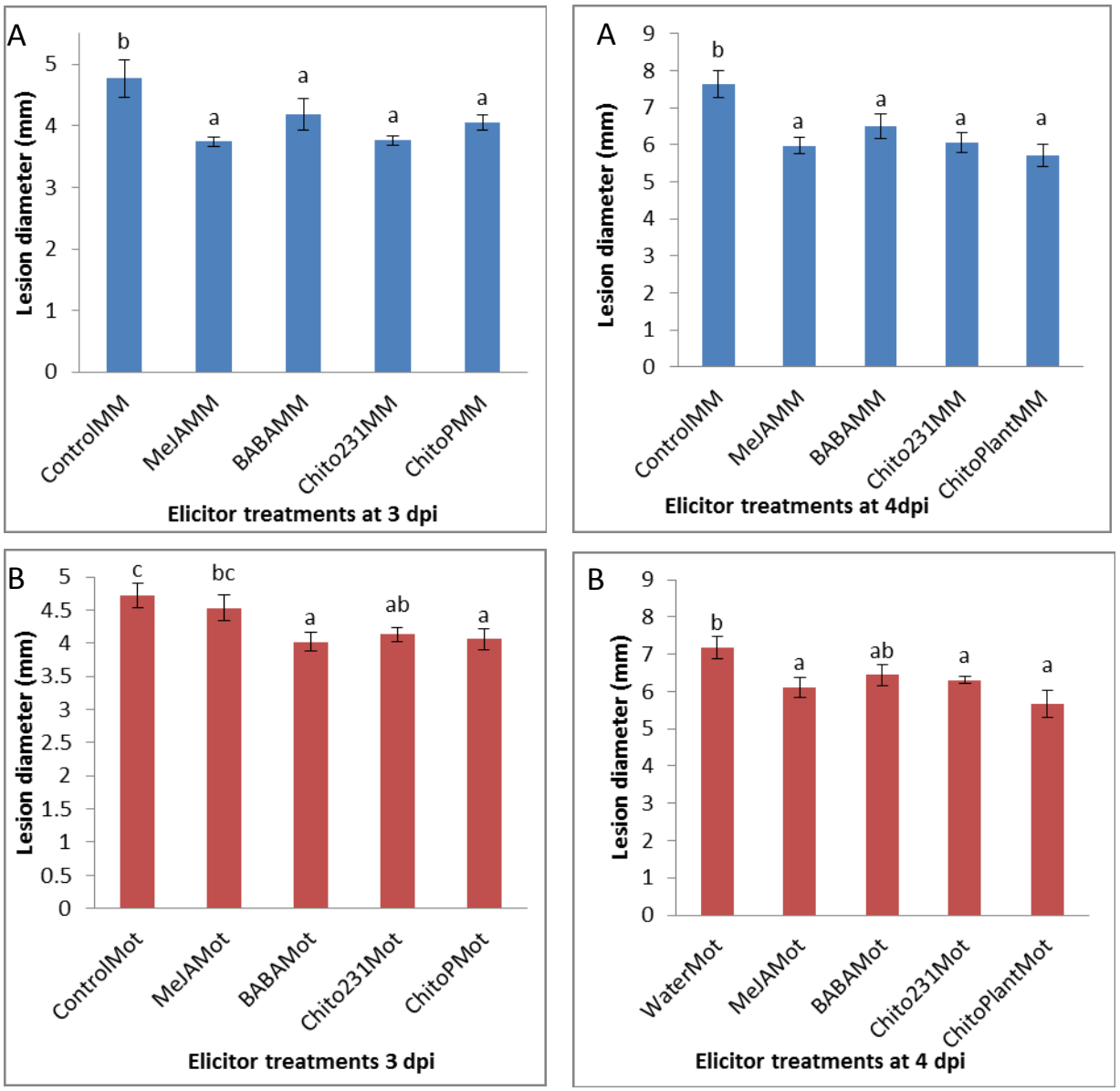


Figure 8.

A. Quantification of MeJA, BABA, Chitosan 23 (NC) and commercial chitosan formulation ChitoPlant (ChiPro)-induced resistance against *Botrytis cinerea* at 3 and 4 days post-inoculation in tomato cv. Money-Maker. 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 23 (NC) and commercial chitosan formulation ChitoPlant (ChiPro)-induced resistance against *Botrytis cinerea* at 3 and 4 days post-inoculation 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).

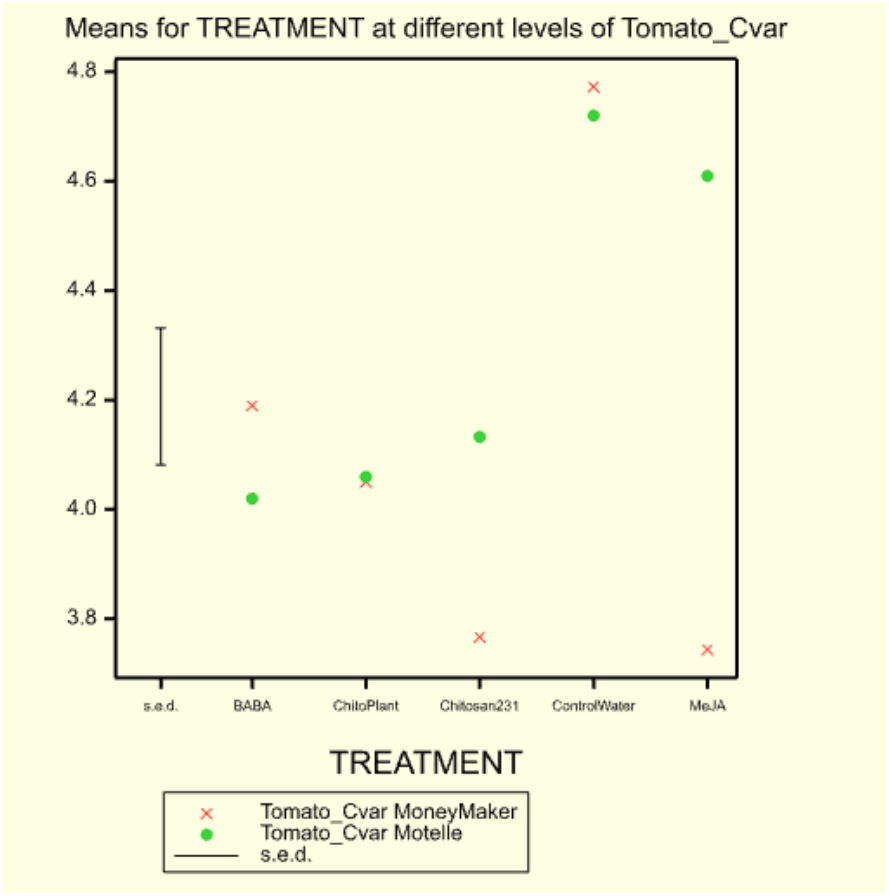


Figure 9. Quantification of BABA, MeJA and Chitosan 23(NC) and ChitoPlant (ChiPro)-induced resistance against *B. cinerea* in tomato cv. Money-Maker and Motelle at 3 days post-inoculation. Values represented are means (of the necrotic lesion diameter) ± SEM obtained from an ANOVA mean plot ($P<0.05$ for TREATMENT*Tomato_Cultivar).

As seen before, normally tomato cv. Money-Maker induced resistance to *B. cinerea* was higher than Motelle in all treatments, except in BABA, where the level of IR was stronger in Motelle (Figure 3a and 3b). 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 9). MeJA-IR responses to *B. cinerea* infection was significantly different, according to GenStat statistical analyses, between both varieties as well as Chitosan23-IR, which was responding better in MM. Interestingly, ChitoPlant-IR functioned similarly in both tomato varieties against grey mould disease (Figure 9).

Pathogen-induced callose deposition in BABA, MeJA, Chitosan23 (NC) and ChitoPlant (ChiPro)-IR in tomato cv. Money-Maker and tomato cv. Motelle.

To determine the effectiveness of the elicitor treatments inducing cell wall defences, double staining (Aniline Blue + calcofluor) was performed to see pathogen-induced callose deposition in all treatments. In this experiment measures were done at 2 days post-*Bc* inoculation and the spore inoculum concentration was previously reduced to get a less aggressive and slower infection. Due to this modification more differences were seen among treatments. In general, lesions in plants treated with BABA 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 10). Interestingly, as previously seen (Figure 4), 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 10). 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). In contrast, Chitosan23-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.

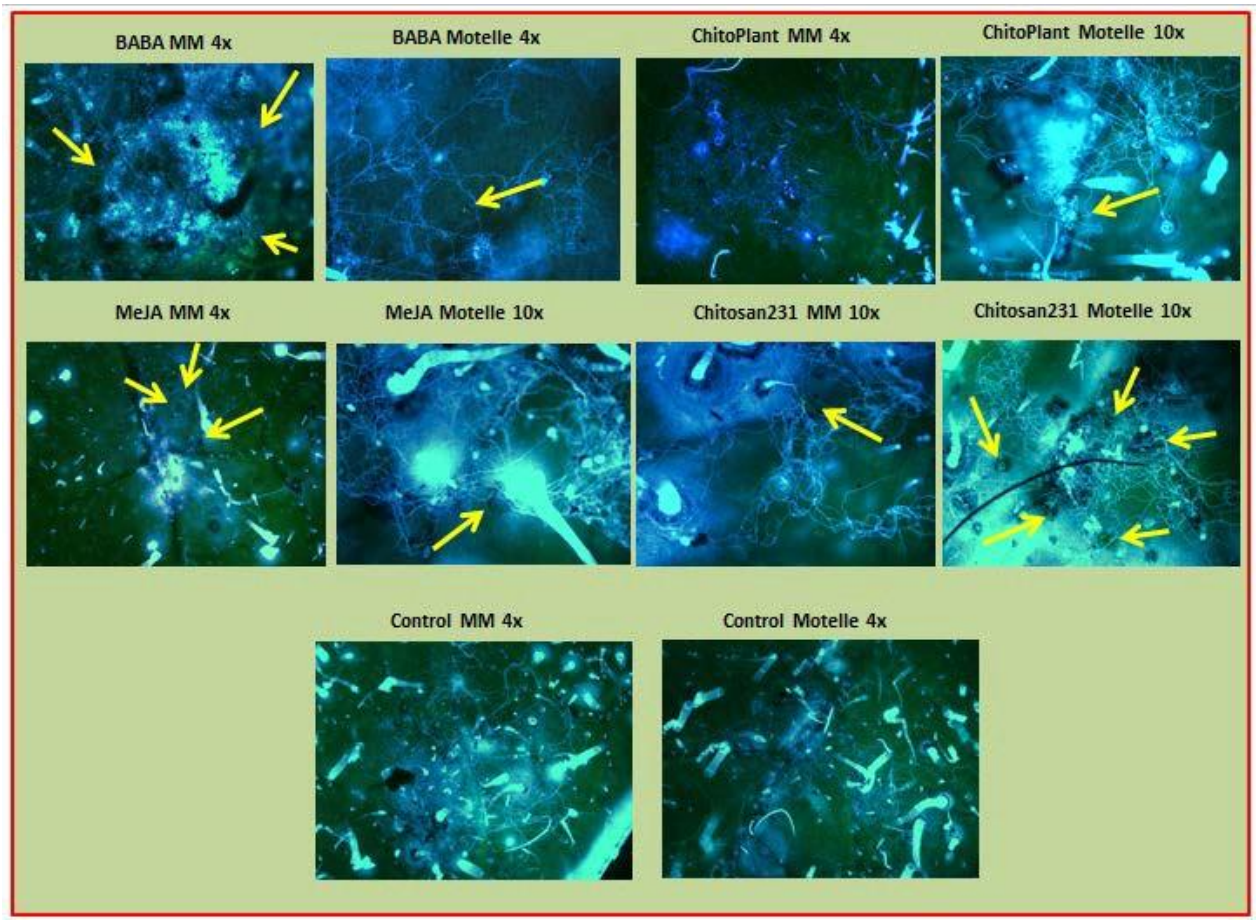


Figure 10. Callose deposition in tomato cv. Money-Maker and tomato cv. Motelle leaves after *B.cinerea* infection in Water-control, BABA, MeJA, ChitoPlant (ChiPro Germany) and Chitosan23(NC)-treated plants. 2 days post-inoculation leaves were harvested for Double Staining (Aniline Blue + Calcofluor) and then pictures were taken under fluorescence microscopy at 4x and 10x magnifications. Yellow arrows indicate callose papillae (greenish points) deposition surrounding pathogen mycelia (light blue colour) penetration sites into tomato epidermal surface.

Costs of elicitor-induced resistance

Elicitor-induced growth reduction in 2 tomato cultivars (Money-Maker and Motelle).

The costs BABA-IR, Chitosan23-IR, ChitoPlant-IR and MeJA-IR on plant fitness, such as seedling growth were analysed by relative growth rate (RGR). Every other day after elicitor treatment, height measurements were obtained during the treatment week in both tomato cultivars. In 1- to 2-week-old plants, defence induction by BABA lead to statistically

significant reductions in RGR in both cultivars (Figure 11a). In contrast, ChitoPlant and MeJA RGR did not significantly differ from the control plants. Chitosan23 RGR was significantly higher than the control plants (Figure 11a) although plants under this treatment were creating thinner stems and less secondary leaves development (Figures 12 and 13, see appendix). BABA-growth repression was 32% in Money-maker (MM) and 45 % in Motelle, suggesting again that Motelle is more susceptible to BABA-IR. Significant differences between the 2 tomato varieties were also seen in the RGR (Figure 11a) verifying that every cultivar responds differently to elicitor-induced resistance and elicitor-induced fitness costs (Figures 11b, 12, 13, 14 and 15, see appendix).

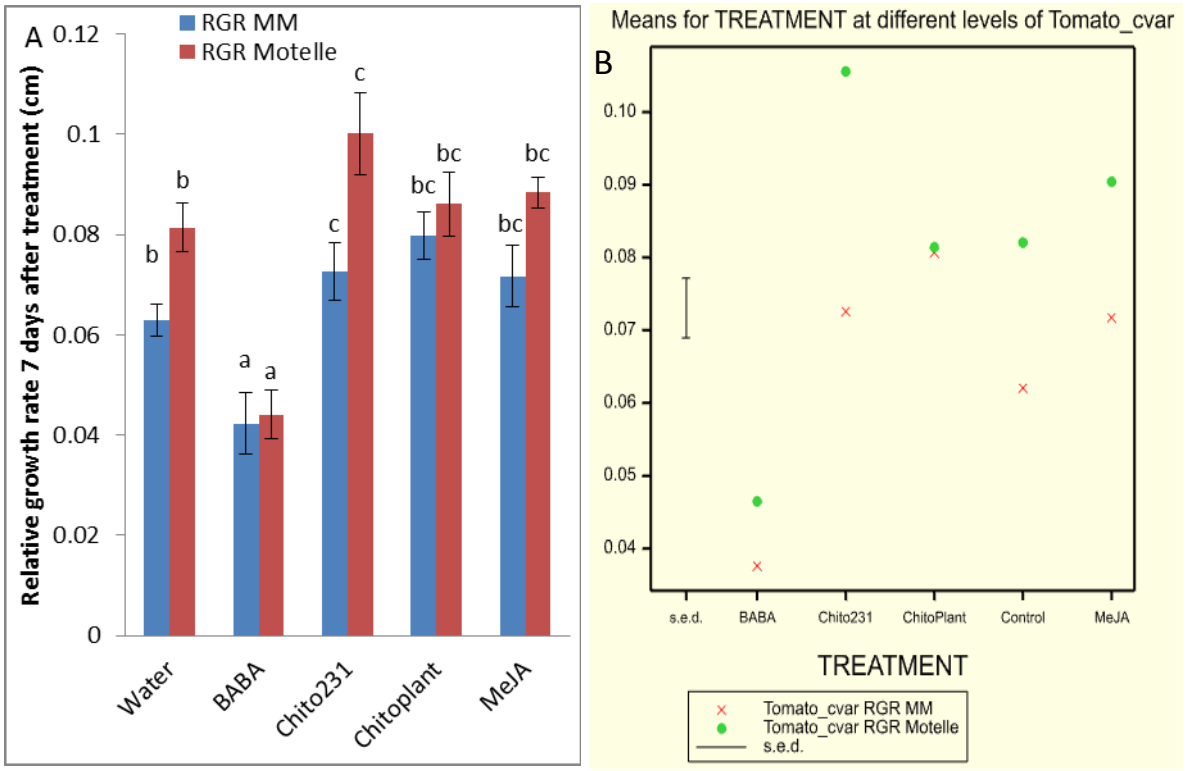


Figure 11. A. Quantification of relative growth rate of 2 tomato cultivars after elicitor treatment, during 7 consecutive days. Values presented are means ± SEM. Different letters indicate statistically significant differences (Fisher's least significant difference (LSD) test $P<0.001$, $\alpha=0.05$). 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).

Discussion

JA and SA are two of the most studied plant hormones and it is well known their key role in plant defence. Both hormones are involved in tomato defence against *Botrytis cinerea*. In addition, it is clear that there is cross-talk between both pathways and they can be neutral, synergistic, or in most cases antagonistic (Mur et al., 2006). This SA-JA cross-talk can be manipulated by some pathogenic fungi in their own benefit, such as *Botrytis cinerea*, in order to enhance its host susceptibility and cause disease (El Oirdi et al., 2011a). However, it is not necessary 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 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 elicitors, known to trigger JA or SA-dependent defence genes, were used in order to investigate gene expression through 3 different time points.

In general, activation of SA-dependent tomato PR-1 was expectedly induced by the SA functional analogue BTH and by β-Aminobutyric Acid (BABA) which also triggers the SA-pathway. Also, JA-dependent PI I was highly induced by the positive control MeJA. However, PI I was also expressed after BABA and water-treated control plants at 24 hours, presumably because methyl jasmonate, when applied to surfaces of tomato plants, has been shown to induce the synthesis of defensive PIs in the treated plants and in nearby plants as well (Farmer & Ryan, 1990). Further qRT-PCR experiments will be conducted in order to investigate this SA/JA crosstalk.

Callose accumulation has proved to be an effective induced resistance mechanism against *B. cinerea* (Kravchuk et al., 2011). The primary objective of this study was to evaluate the robustness and reproducibility of a widely used system in plant induced resistance research, i.e., detached-leaves assay after *B. cinerea* challenge in elicitor-induced plants. Furthermore, it was intended to evaluate the robustness and reproducibility of a popular system in *Arabidopsis thaliana* and relatively novel system in tomato quantification activity of plant immunity, i.e., elicitor-induced callose deposition in cotyledons of tomato seedlings before and after pathogen inoculation.

The secondary objective was to assess the differences in the variety defence response to pathogen attack and the differences in the plant fitness costs. It is well known the divergence in the immune defence pathways within plant species (Achuo et al., 2004; Vicedo et al., 2009). However, little is known about the difference in the resistance among varieties of the same plant species. In tomato, some varieties respond better to some

elicitor treatments, such as hexanoic acid-IR, where treatment with this elicitor resulted in an enhanced callose deposition in most of the tomato back- grounds studied, although this effect seems to be cultivar dependent because tomato cv. Castlemart plants displayed Hx- IR with no accumulation of callose (Vicedo et al., 2009). Thus, hexanoic acid effects in tomato resistance against *B.cinerea* are cultivar-dependent (Kravchuk et al., 2011).

Here we show that, in mock plants, treatment with MeJA triggered the deposition of callose in tomato cotyledons in tomato cv. Money-Maker whereas BABA triggered callose in Motelle. After pathogen challenged, both MeJA and BABA-treated Money-Maker plants deposited more callose surrounding the mycelia penetration holes in the tomato epidermis. In cv. Motelle, only Chitosan23 induced callose around the growing hyphae of the pathogen, mainly around the penetration sites. Thus, there is a cultivar effect on callose deposition, as previous results seen in elicitor-induced callose deposition by Kravchuk et al., 2011 and Vicedo et al., 2009.

In both experiments, callose was found in tomato cv. Money-Maker in samples of MeJA-treated plants (Figure 4, Figure 10) which correlates with the lesion size significant reduction of the same treatment. As seen in Figure 4 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 10).This result can be an indirect effect on the plant, due to priming of the JA-signalling pathway triggered by this elicitor. MeJA-IR decreases and slows down the pathogen infection, thus allowing more time to the plant to activate its defences, including cell wall defence throughout callose-rich papillae deposition. Further experiments need to be done in order to assess the reproducibility of this result.

It is widely known that there are costs and trade-offs associated with induced resistance (van Hulten et al., 2006), however few studies have examined it in detail in crop systems (Redman et al., 2001). Costs are a traditional topic in ecological research on plant resistance to biological threats, but have only been recently considered in detail in studies of systemic acquired resistance (SAR) (Walters & Heil, 2007). In this study, I assessed the cost associated with plant growth after treatment with various elicitors which can successfully induce long-lasting resistance. It has been clearly demonstrated that some of these elicitors, such as BABA, highly reduces plant growth in both varieties. It however seems that the magnitude of the growth suppression is cultivar-dependent, because tomato cv. Motelle is less tolerant to BABA-induced growth reduction.

Induced resistance bioassays showed that, although not significant, there was a general trend in the cultivar immune response to *B. cinerea*, as Money-Maker resistance to the

pathogen was higher than Motelle. Moreover, all elicitor's treatments with the exception of BABA, resulted in a more pronounced long-lasting induced resistance in MM plants compared to Motelle, were BABA-IR was higher (Figure 3b). This result can be correlated with the relative growth rate results of both varieties, where Motelle is shown to be less tolerant to BABA-induced growth reduction in comparison with Money-Maker. Thus, tomato cv. Motelle perceives BABA in a higher level than MM.

Picture 1. 2-week-old tomato cv. Motelle and Money-maker 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.

Plant survival in the presence of necrotrophic pathogens needs the activation of the plant hormones JA and ET-signalling pathways (Ton & Mauch-Mani, 2004; Zimmerli, Métraux, & Mauch-Mani, 2001). However, depending on the plant-pathogen system the plant hormone SA-immune pathway can be also beneficial for the plant. The SA functional analogue BTH is effective against *B.cinerea* but it does not protect against *Oidium neolycopersici* in tomato. However, it induced resistance against this biotrophic pathogen in tobacco but had no effect on *B. cinerea* (Achuo et al., 2004), which reflects the variance in the resistance pathways within plant species. In the present study, We showed that in response to a high aggressive pathogen strain, BTH can provide significant lesion reduction just at an early stage of the infection (3 d.p.i.) in both varieties. However, the combination of BTH + MeJA did not provide further significant resistance at any time point after inoculation. The lack of synergistic effect of BTH and MeJA against *B. cinerea* can be due to the hormone concentration used as it may be different and/or specific for every pathogen, as previous studies suggested that the outcomes of JA-SA interactions could be specifically adapted to pathogen attack by the relative concentration of each hormone (Mur et al., 2006).

Furthermore, in the SA/JA cross-talk, it seems that the defence signalling network activated and utilized by the plant may be also dependent on the nature of the pathogen and its mode of pathogenicity (Bari & Jones, 2009). However, there is evidence of synergistic interactions between both plant hormones when applied at low concentrations (Mur et al., 2006). Further SA-JA co-treatment experiments will be conducted in order to investigate their potential synergistic effects on the tomato-*B.cinerea* model system.

Picture 2. Damage response in the cotyledons of chitosan-treated plants (commercial formulation, ChitoPlant)

Conclusions

- Long-lasting defence provided by the defence inducers BABA, MeJA, Chitosan 23 and ChitoPlant can significantly reduce necrotic lesion expansion at both time points and in the tomato varieties. This effect differs between tomato cultivars.
- ChitoPlant induces callose deposition in tomato cotyledons before pathogen challenge.
- JA-triggering elicitors (i.e. MeJA-IR and chitosan-IR) are able to provide long-lasting defence and limit *B. cinerea* infection progress in both cultivars.
- BABA is more effective in cv. Motelle than in Money-Maker. Significant differences between Motelle and Moneymaker were found in BABA-IR against *B.cinerea*.
- Long-lasting BABA-IR in tomato against *B. cinerea* can be observed depending on the pathogen inoculum concentration and the infection virulence.
- There is cost in plant fitness after elicitor-induced resistance. BABA caused the greatest growth inhibition of all elicitors.

- There are differences in the tomato variety response to elicitor-IR and elicitor-induced fitness cost. MeJA-induced callose deposition was higher in Moneymaker than Motelle and tomato cv. Motelle perceives BABA at a higher level than Money-Maker.
- SA-signalling pathway is involved in basal defence of tomato against *B. cinerea*, however its effect and efficacy in long-lasting resistance is still unclear. Further experiments with SA-IR elicitors (i.e. BTH) will be needed.
- It has been demonstrated that both SA and the JA-signalling pathways are involved in tomato resistance against *B. cinerea.* However, their efficacy may vary depending on the pathogen strain, tomato cultivar and infection time point.

Knowledge and Technology Transfer

Seminars:

De Vega Perez, D. “Integrated protection of horticultural crops through enhancing their endogenous defence mechanisms”. Department of Plant and Animal Science, University of Sheffield, 12th June 2014.

De Vega Perez, D. Long lasting elicitor-Induced resistance in tomato against the necrotrophic fungus *Botrytis cinerea*. First Year PhD Transfer talk, The James Hutton Institute, 29th July 2014.

De Vega Perez, D. “Hexanoic acid protects tomato plants against *Botrytis cinerea* by priming defence responses and reducing oxidative stress”. Fungal Lab meeting, The James Hutton Institute, 19th August 2014.

Reports:

De Vega Perez, D. “Integrated crop protection”. Six Month PhD Report, 31st March 2014.

De Vega Perez, D. Integrated protection of horticultural crops through enhancing their endogenous defence mechanisms. Eleven months Transfer report, 29th July 2014.

Posters:

De Vega Perez, D. “Integrated Crop Protection” Poster, JHI annual Postgraduate competition 20th March 2014.

De Vega Perez, D. “Integrated crop protection of S*olanum lycopersicum* against *Botrytis cinerea* through enhancing its endogenous defence mechanisms”. BSPP Annual Presidential Conference: “Some like it hot”, 1-2 September 2014, BSPP J Colhoun Poster Competition.

De Vega Perez, D. HDC Studentship Conference Poster, 16-17th September 2014.

Glossary

Technical terms are defined at first use in the text although. Molecular biology terminology used is standard for this field but is too broad to be covered in detail here. However it will be defined in basic molecular text books.

References

Achuo, E. A., Audenaert, K., Meziane, H., & Höfte, M. (2004). The salicylic acid-dependent defence pathway is effective against different pathogens in tomato and tobacco, 65–72. doi:10.1046/j.1365-3059.2003.00947.x

Adie, B. a T., Pérez-Pérez, J., Pérez-Pérez, M. M., Godoy, M., Sánchez-Serrano, J.-J., Schmelz, E. a, & Solano, R. (2007). ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. *The Plant Cell*, *19*(5), 1665–81. doi:10.1105/tpc.106.048041

Agrios GN (2005) Plant Pathology, Ed 5. Elsevier Academic Press, Bur- lington, MA

Ahmad, S., Gordon-weeks, R., Pickett, J., & Ton, J. (2010). Natural variation in priming of basal resistance : from evolutionary origin to agricultural exploitation, *11*, 817–827. doi:10.1111/J.1364-3703.2010.00645.X

Arie, T., Takahashi, H., Kodama, M., & Teraoka, T. (2007). Tomato as a model plant for plant-pathogen interactions. *Plant Biotechnology*, *24*(1), 135–147. doi:10.5511/plantbiotechnology.24.135

B. Williamson, B. Tudzynski, P.Tudzynski, J. K. (2007). Botrytis cinerea : the cause of grey mould disease, *8*, 561–580. doi:10.1111/J.1364-3703.2007.00417.X

Bardoel, B. W., van der Ent, S., Pel, M. J. C., Tommassen, J., Pieterse, C. M. J., van Kessel, K. P. M., & van Strijp, J. a G. (2011). Pseudomonas evades immune recognition of flagellin in both mammals and plants. *PLoS Pathogens*, *7*(8), e1002206. doi:10.1371/journal.ppat.1002206

Bari, R., & Jones, J. D. G. (2009). Role of plant hormones in plant defence responses. *Plant Molecular Biology*, *69*(4), 473–88. doi:10.1007/s11103-008-9435-0

Barnes, S. E., & Shaw, M. W. (2002). Factors affecting symptom production by latent Botrytis cinerea in Primula × polyantha, 746–754.

Cantu, D., Blanco-Ulate, B., Yang, L., Labavitch, J. M., Bennett, A. B., & Powell, A. L. T. (2009). Ripening-regulated susceptibility of tomato fruit to Botrytis cinerea requires NOR but not RIN or ethylene. *Plant Physiology*, *150*(3), 1434–49. doi:10.1104/pp.109.138701

Cohen, Y. R. (2002). β -Aminobutyric Acid-Induced Resistance Against Plant Pathogens, *86*(5).

Conrath, U. (2011). Molecular aspects of defence priming. *Trends in Plant Science*, *16*(10), 524–31. doi:10.1016/j.tplants.2011.06.004

Darras, A. I. (2011). Novel Elicitors Induce Defense Responses in Cut Flowers. Doares, S. H.,

Narvaez-Vasquez, J., Conconi, a., & Ryan, C. a. (1995). Salicylic Acid
Inhibits Synthesis of Proteinase Inhibitors in Tomato Leaves Induced by Systemin and Jasmonic Acid. *Plant Physiology*, *108*(4), 1741–1746. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=157556&tool=pmcentrez&rendert ype=abstract

El Oirdi, M., El Rahman, T. A., Rigano, L., El Hadrami, A., Rodriguez, M. C., Daayf, F., … Bouarab, K. (2011a). Botrytis cinerea manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *The Plant Cell*, *23*(6), 2405–21. doi:10.1105/tpc.111.083394

El Oirdi, M., El Rahman, T. A., Rigano, L., El Hadrami, A., Rodriguez, M. C., Daayf, F., … Bouarab, K. (2011b). Botrytis cinerea manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *The Plant Cell*, *23*(6), 2405–21. doi:10.1105/tpc.111.083394

Farmer, E. E., & Ryan, C. a. (1990). Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(19), 7713–6. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=54818&tool=pmcentrez&renderty pe=abstract

Finkers, R., Bai, Y., Berg, P., Berloo, R., Meijer-Dekens, F., Have, A., … Heusden, A. W. (2007). Quantitative resistance to Botrytis cinerea from Solanum neorickii. *Euphytica*, *159*(1-2), 83–92. doi:10.1007/s10681-007-9460-0

Finkers, R., van den Berg, P., van Berloo, R., ten Have, A., van Heusden, A. W., van Kan, J. a L., & Lindhout, P. (2007). Three QTLs for Botrytis cinerea resistance in tomato. *TAG.*

Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik, *114*(4), 585–93. doi:10.1007/s00122-006-0458-0

Flors, V., Ton, J., & Jakab, G. (2007). Abscisic Acid and Callose : Team Players in Defence Against Pathogens ?, *383*(2005), 377–383.

Giovannoni, J. J. (2004). Genetic Regulation of Fruit Development and Ripening, *16*, 170–181. doi:10.1105/tpc.019158.Fruit

Häffner, E., Karlovsky, P., Splivallo, R., Traczewska, A., & Diederichsen, E. (2014). ERECTA, salicylic acid, abscisic acid, and jasmonic acid modulate quantitative disease resistance of Arabidopsis thaliana to Verticillium longisporum. *BMC Plant Biology*, *14*(1), 85.

doi:10.1186/1471-2229-14-85

Heil, M., & Ton, J. (2008). Long-distance signalling in plant defence. *Trends in Plant Science*, *13*(6), 264–72. doi:10.1016/j.tplants.2008.03.005

Hein, I., Gilroy, E. M., Armstrong, M. R., & Birch, P. R. J. (2009). The zig-zag-zig in oomycete – plant interactions, *10*, 547–562. doi:10.1111/J.1364-3703.2009.00547.X

Hématy, K., Cherk, C., & Somerville, S. (2009). Host-pathogen warfare at the plant cell wall. *Current Opinion in Plant Biology*, *12*(4), 406–13. doi:10.1016/j.pbi.2009.06.007

Hückelhoven, R. (2007). Cell wall-associated mechanisms of disease resistance and susceptibility. *Annual Review of Phytopathology*, *45*, 101–27. doi:10.1146/annurev.phyto.45.062806.094325

J. Kars, G. Krooshof, L. Wagemakers, R. Joosten, J. B. and J. va. K. (2005). Necrotizing activity of five Botrytis cinerea endopolygalacturonases produced in Pichia pastoris.

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature*, *444*(7117), 323–9. doi:10.1038/nature05286

Kan, J. A. L. Van. (2002). The Role of Ethylene and Wound Signaling in Resistance of Tomato to Botrytis cinerea, *129*(July), 1341–1351. doi:10.1104/pp.001453.1

Kan, J. A. L. Van. (2005). Infection Strategies of Botrytis cinerea, 77–90.

Kravchuk, Z., Vicedo, B., Flors, V., Camañes, G., González-Bosch, C., & García-Agustín, P. (2011). Priming for JA-dependent defenses using hexanoic acid is an effective mechanism to protect Arabidopsis against B. cinerea. *Journal of Plant Physiology*, *168*(4), 359–66. doi:10.1016/j.jplph.2010.07.028

Kunkel, B. N., & Brooks, D. M. (2002). Cross talk between signaling pathways in pathogen defense. *Current Opinion in Plant Biology*, *5*(4), 325–331. doi:10.1016/S1369-5266(02)00275-3

Laird, J., Armengaud, P., Giuntini, P., Laval, V., & Milner, J. J. (2004). Inappropriate annotation of a key defence marker in Arabidopsis: will the real PR-1 please stand up? *Planta*, *219*(6), 1089–92. doi:10.1007/s00425-004-1355-x

Loon, L. C. Van, Bakker, P. A. H. M., & Pieterse, C. M. J. (1998). SYSTEMIC RESISTANCE INDUCED.

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B., & Ton, J. (2011). Callose deposition: a multifaceted plant defense response. *Molecular Plant-Microbe Interactions:*

MPMI, *24*(2), 183–93. doi:10.1094/MPMI-07-10-0149

Mur, L. A. J., Kenton, P., Atzorn, R., Miersch, O., & Wasternack, C. (2006). The Outcomes of Concentration-Specific Interactions between Salicylate and Jasmonate Signaling Include Synergy , Antagonism , and Oxidative Stress Leading to Cell Death, *140*(January), 249–262. doi:10.1104/pp.105.072348.was

Newton, A. C., Fitt, B. D. L., Atkins, S. D., Walters, D. R., & Daniell, T. J. (2010). Pathogenesis, parasitism and mutualism in the trophic space of microbe-plant interactions. *Trends in Microbiology*, *18*(8), 365–73. doi:10.1016/j.tim.2010.06.002

Pappas, a. C. (1997). Evolution of fungicide resistance in Botrytis cinerea in protected crops in Greece. *Crop Protection*, *16*(3), 257–263. doi:10.1016/S0261-2194(96)00096-8

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., & Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annual Review of Cell and Developmental Biology*, *28*(April), 489–521. doi:10.1146/annurev-cellbio-092910-154055

Pluskota, W. E., Qu, N., Maitrejean, M., Boland, W., & Baldwin, I. T. (2007). Jasmonates and its mimics differentially elicit systemic defence responses in Nicotiana attenuata. *Journal of Experimental Botany*, *58*(15-16), 4071–82. doi:10.1093/jxb/erm263

Powell, a L., van Kan, J., ten Have, a, Visser, J., Greve, L. C., Bennett, a B., & Labavitch, J. M. (2000). Transgenic expression of pear PGIP in tomato limits fungal colonization. *Molecular Plant-Microbe Interactions : MPMI*, *13*(9), 942–50. doi:10.1094/MPMI.2000.13.9.942

Redman, A. M., Jr., D. F. C., & Schultz, J. C. (2001). Fitness costs of jasmonic acid-induced defense in tomato, Lycopersicon esculentum. *Oecologia*, *126*(3), 380–385. doi:10.1007/s004420000522

Romanazzi, G., Feliziani, E., Santini, M., & Landi, L. (2013). Effectiveness of postharvest treatment with chitosan and other resistance inducers in the control of storage decay of strawberry. *Postharvest Biology and Technology*, *75*, 24–27. doi:10.1016/j.postharvbio.2012.07.007

Scranton, M. a, Fowler, J. H., Girke, T., & Walling, L. L. (2013). Microarray analysis of tomato's early and late wound response reveals new regulatory targets for Leucine aminopeptidase A. *PloS One*, *8*(10), e77889. doi:10.1371/journal.pone.0077889

Sellal, Z., Dahmani, J., Benkirane, R., Touhami, A. O., & Douira, A. (2013). Pathogenic Capacity of Botrytis cinerea on Leaves of Pyrus mamorensis, an Endemic Tree of Mamora Forest in Morocco. *Atlas Journal of Biology*, *2*(2), 125–129. doi:10.5147/ajb.2013.0083

Song, W., Ma, X., Tan, H., & Zhou, J. (2011). Abscisic acid enhances resistance to Alternaria

solani in tomato seedlings. *Plant Physiology and Biochemistry : PPB / Société Française de Physiologie Végétale*, *49*(7), 693–700. doi:10.1016/j.plaphy.2011.03.018

Sowley, E. N. K., Dewey, F. M., & Shaw, M. W. (2009). Persistent, symptomless, systemic, and seed-borne infection of lettuce by Botrytis cinerea. *European Journal of Plant Pathology*, *126*(1), 61–71. doi:10.1007/s10658-009-9524-1

Stahmann, K., Monschau, N., Sahm, H., Koschel, A., Gawronski, M., Conrad, H., … Kopp, F. (1995). Structural properties of native and sonicated Botrytis cinerea, *266*, 115–128.

Sticher, L., Mauch-Mani, B., & Métraux, J. P. (1997). Systemic acquired resistance. *Annual Review of Phytopathology*, *35*(197), 235–70. doi:10.1146/annurev.phyto.35.1.235

Stukenbrock, E. H., & McDonald, B. a. (2009). Population genetics of fungal and oomycete effectors involved in gene-for-gene interactions. *Molecular Plant-Microbe Interactions : MPMI*, *22*(4), 371–80. doi:10.1094/MPMI-22-4-0371

Sun, J.-Q., Jiang, H.-L., & Li, C.-Y. (2011). Systemin/Jasmonate-mediated systemic defense signaling in tomato. *Molecular Plant*, *4*(4), 607–15. doi:10.1093/mp/ssr008

Ten Have, A., Dekkers, E., Kay, J., Phylip, L. H., & van Kan, J. a L. (2004). An aspartic proteinase gene family in the filamentous fungus Botrytis cinerea contains members with novel features. *Microbiology (Reading, England)*, *150*(Pt 7), 2475–89. doi:10.1099/mic.0.27058-0

Ten Have, A., Espino, J. J., Dekkers, E., Van Sluyter, S. C., Brito, N., Kay, J., … van Kan, J. a L. (2010). The Botrytis cinerea aspartic proteinase family. *Fungal Genetics and Biology : FG & B*, *47*(1), 53–65. doi:10.1016/j.fgb.2009.10.008

Ten Have, A., van Berloo, R., Lindhout, P., & van Kan, J. a. L. (2006). Partial stem and leaf resistance against the fungal pathogen Botrytis cinerea in wild relatives of tomato. *European Journal of Plant Pathology*, *117*(2), 153–166. doi:10.1007/s10658-006-9081-9

Terry, L. (2004). Elicitors of induced disease resistance in postharvest horticultural crops: a brief review. *Postharvest Biology and Technology*, *32*(1), 1–13. doi:10.1016/j.postharvbio.2003.09.016

Thakur, M., & Sohal, B. S. (2013). Role of Elicitors in Inducing Resistance in Plants against Pathogen Infection: A Review. *ISRN Biochemistry*, *2013*, 1–10. doi:10.1155/2013/762412

Ton, J., Ent, S. Van Der, Hulten, M. Van, & Pozo, M. (2009). Priming as a mechanism behind induced resistance against pathogens , insects and abiotic stress, 3–13.

Ton, J., & Mauch-Mani, B. (2004). Beta-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. *The Plant Journal : For Cell and Molecular Biology*, *38*(1), 119–30. doi:10.1111/j.1365-313X.2004.02028.x

Van der Does, D., Leon-Reyes, A., Koornneef, A., Van Verk, M. C., Rodenburg, N., Pauwels, L., … Pieterse, C. M. J. (2013). Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59. *The Plant Cell*, *25*(2), 744–61. doi:10.1105/tpc.112.108548

Van Hulten, M., Pelser, M., van Loon, L. C., Pieterse, C. M. J., & Ton, J. (2006). Costs and benefits of priming for defense in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(14), 5602–7. doi:10.1073/pnas.0510213103

Van Kan, J. a, van't Klooster, J. W., Wagemakers, C. a, Dees, D. C., & van der Vlugt-Bergmans, C. J. (1997). Cutinase A of Botrytis cinerea is expressed, but not essential, during penetration of gerbera and tomato. *Molecular Plant-Microbe Interactions : MPMI*, *10*(1), 30–8. doi:10.1094/MPMI.1997.10.1.30

Van Wees, S. C., Luijendijk, M., Smoorenburg, I., van Loon, L. C., & Pieterse, C. M. (1999). Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene Atvsp upon challenge. *Plant Molecular Biology*, *41*(4), 537–49. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10608663

Vicedo, B., Flors, V., de la O Leyva, M., Finiti, I., Kravchuk, Z., Real, M. D., … González-Bosch, C. (2009). Hexanoic acid-induced resistance against Botrytis cinerea in tomato plants. *Molecular Plant-Microbe Interactions : MPMI*, *22*(11), 1455–65. doi:10.1094/MPMI-22-11-1455

Voigt, C. a. (2014). Callose-mediated resistance to pathogenic intruders in plant defense-related papillae. *Frontiers in Plant Science*, *5*(April), 168. doi:10.3389/fpls.2014.00168

Walters, D., & Heil, M. (2007). Costs and trade-offs associated with induced resistance. *Physiological and Molecular Plant Pathology*, *71*(1-3), 3–17. doi:10.1016/j.pmpp.2007.09.008

Weiberg, A., Wang, M., Lin, F.-M., Zhao, H., Zhang, Z., Kaloshian, I., … Jin, H. (2013). Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science (New York, N.Y.)*, *342*(6154), 118–23. doi:10.1126/science.1239705

Williams, J. C. (2011). New EU pesticide legislation – the view of a manufacturer, 269–274.

Zhang, W., Fraiture, M., Kolb, D., Löffelhardt, B., Desaki, Y., Boutrot, F. F. G., … Brunner, F. (2013). Arabidopsis RECEPTOR-LIKE PROTEIN30 and Receptor-Like Kinase SUPPRESSOR OF BIR1-1/EVERSHED Mediate Innate Immunity to Necrotrophic Fungi. *The Plant Cell*, *25*(10), 4227–41. doi:10.1105/tpc.113.117010

Zhong, Y., Wang, B., Yan, J., Cheng, L., Yao, L., Xiao, L., & Wu, T. (2014). DL-β- aminobutyric

acid-induced resistance in soybean against Aphis glycines Matsumura (Hemiptera: Aphididae). *PloS One*, *9*(1), e85142. doi:10.1371/journal.pone.0085142

Zimmerli, L., Métraux, J. P., & Mauch-Mani, B. (2001). beta-Aminobutyric acid-induced protection of Arabidopsis against the necrotrophic fungus *Botrytis cinerea*. *Plant Physiology*, *126*(2), 517–23. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=111145&tool=pmcentrez&rendertype=abstract

Zipfel, C. (2014). Plant pattern-recognition receptors. *Trends in Immunology*, *1*(Figure 1), 1–7. doi:10.1016/j.it.2014.05.004

Appendices

None included but the full records are contained in laboratory notebooks.

