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Sweet immunity in tobacco: Sugar priming against *Botrytis cinerea* infection.

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Summary

The need for alternatives to pesticide usage is imminent. Priming of the immune system allows a faster and stronger upregulation of defense responses in case of future biotic stresses. It has become clear that endogenous sugars are important signaling molecules in plant defense. Priming the plant with exogenous sugars, so called sweet priming, can increase resistance against pathogenic attacks. These observations have led to the sweet immunity concept in plants.

In this thesis, sweet priming has been performed on source leaves of *Nicotiana tabacum*, which were then challenged by *Botrytis cinerea* infection. To gain more insight into the physiological effects of priming, small sugar (glucose, fructose and sucrose) levels, reserve carbohydrates (starch) and acid invertase activity levels were measured.

Glucose and allose (a non-metabolizable variant), were compared as priming agents. Glucose priming was efficient in reducing fungal infection, while allose-primed leaves showed almost no reduction in lesion growth. The effect of inulin-type fructans (soluble fructose polymers: burdock fructooligosaccharides or BFO) was tested. A strong reduction in susceptibility to *B. cinerea* spores was apparent, but not for all experiments. Stronger disease resistance was generally linked to higher endogenous post-priming hexose levels, as well as higher cell wall invertase activities, except for BFO priming, where hexose levels remained low.

The results indicate that sweet priming is efficient against biotic stress. We have also proposed a concept in which the pre-priming status of the apoplastic environment may determine the physiological responses after priming.

Samenvatting

Wereldwijd wordt koortsachtig gezocht naar alternatieven voor pesticiden, vanuit het oogpunt van duurzame en ecologisch verantwoorde landbouw. Priming van het immuunsysteem laat de plant toe om sneller en beter te reageren bij een pathogene aanval. Het belang van endogene suikers als signaalmoleculen in deze context werd reeds aangetoond. Priming met endogene suikers, zogenaamde “sweet priming”, kan de resistentie van de plant verhogen: dit is de kern van het “sweet immunity” concept.

Sweet priming werd toegepast op “source” leaves van tabak (*Nicotiana tabacum*). Vervolgens warden deze bladeren blootgesteld aan *Botrytis cinerea* infectie. Om de fysiologische aspecten van priming te bestuderen werden de gehaltes aan glucose, fructose en sucrose, alsook zetmeelinhoud en invertase enzymactiviteit opgemeten.

De effecten van glucose en allose (een niet metaboliseerbaar hexose) priming werden vergeleken. Glucose priming bleek fungale infectie tegen te werken, maar dit werd niet vastgesteld voor allose. Soms werd ook een verhoogde resistentie waargenomen na priming met inuline-type fructanen (fructose-gebaseerde suikerpolymeren van burdock: BFO). Een hogere resistentie ging meestal samen met een hogere concentratie aan hexosen en een hogere celwandinvertase activiteit. Bij BFO priming daarentegen bleef het hexose gehalte laag.

Uit de resultaten leiden we af dat sweet priming effectief kan zijn als verdediging tegen biotische stress. Verder stellen we ook een concept voor waarbij de pre-priming status van de apoplast een rol kan spelen in de fysiologische veranderingen die optreden zowel tijdens als kort na priming (pre-infectie) periode.

List of abbreviations

A/NI	Alkaline/Neutral Invertase
ABA	Abscisic Acid
ABI	ABA Insensitive
ACC	1-AminoCyclopropane-1-Carboxylic acid
ALD1	ADP ribolysation factor GTPase-activating protein-Like Defense protein 1
AMP	Adenosine MonoPhosphate
ATP	Adenosine TriPhosphate
Avr gene	Avirulence gene
AzA	Azelaic Acid
BABA	β-AminoButyric Acid
BAK1	Brassinosteroid insensitive 1-Associated receptor Kinase
BFO	Burdock FructoOligosaccharides
BIK1	Botrytis-Induced Kinase 1
BR	Brassinosteroid
bZIP	basic leucine Zipper domain
CaM	Calmodulin
CC	Coiled-Coil
CDPK	Calcium-Dependent Protein Kinase
CEBiB	Chitin Elicitor Binding Protein
CERK1	Chitin Elicitor Receptor Kinase 1
CK	Cytokinin
CW	Cell Wall
CWI	Cell Wall Invertase
DA	DehydroAbietinal
DAMP	Damage-Associated Molecular Pattern
DIR1	Defective in Induced Resistance 1
DP	Degree of Polymerization
dpi	days past infection
Ecp6	Extracellular protein 6
EF-Tu	Elongation Factor Tu
EFR	EF-Tu Receptor
EGF	Epidermal Growth Factor
EIN	Ethylene InSensitive
ET	Ethylene

ETI	Effector-Triggered Immunity
ETS	Effector-Triggered Susceptibility
FEH	Fructan ExoHydrolase
FLS2	Flagellin Sensing 2
Fru	Fructose
G3P	Glycerol-3-Phosphate
GA	Gibberellin
GABA	γ-AminoButyric Acid
GDP	Guanine DiPhosphate
Glc	Glucose
G6P	Glucose-6-Phosphate
HAMP	Herbivory-Associated Molecular Pattern
HPAEC-PAD	High-Performance Anion Exchange Chromatograph with Pulsed Amperometric Detection
HR	Hypersensitive Response
HXK	Hexokinase
IAA	Indole-3-Acetic Acid
Ile	Isoleucine
ISR	Induced Systemic Resistance
JA	Jasmonic Acid
JAZ	Jasmonate ZIM domain
LEA	Late-Embryogenesis Abundant
LRR	Leucine-Rich Repeat
LYK5	Lysin motif receptor Kinase 5
LysM	Lysin Motif
MAMP	Microbe-Associated Molecular Pattern
MAPK/MPK	Mitogen-Activated Protein Kinase
MAPKK/MPKK/MKK	MAPK Kinase
MAPKKK/MPKKK/MKKK	MAPK Kinase Kinase
MeSA	Methyl Salicylate
NADPH	Nicotinamine Adenine DiNucleotide Phosphate
NBS	Nucleotide Binding Site
NINJA	Novel Interactor of JAZ
NLS	Nuclear Localization Signal
NO	Nitric Oxide
NPR	Nonexpressor of Pathogenesis-Related proteins
ODT	Optimal Defense Theory

OG	Oligogalacturonide
PAL	Phenylalanine Ammonia Lyase
PAMP	Pathogen-Associated Molecular Pattern
PCD	Programed Cell Death
PDF	Plant Defensin
PGPR	Plant Growth-Promoting Rhizobacteria
PIP5K9	PhosphatidylInositol monophosphate 5-Kinase 9
PR	Pathogenesis-Related
PRR	Pattern Recognition Receptor
PTI	PAMP-Triggered Immunity
PUB12/13	Plant U-Box 12/13
Rac GEF1	Ras-related C3 botulinum toxin substrate Guanine nucleotide Exchange Factor 1
RBOH	Respiratory Burst Oxidase Homologs
RFO	Raffinose-Family Oligosaccharides
RIN4	RPM1 Interacting Protein 4
RLCK	Receptor-Like Cytoplasmic Kinase
RLK	Receptor-Like Kinase
RLP	Receptor-Like Protein
ROS	Reactive Oxygen Species
RPM1	Resistance to *Pseudomonas syringae* 1
RPS2	Ribosomal Protein S2
RRS1	Regulator of Ribosome Synthesis 1
R gene	Resistance gene
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SNF1	Sucrose Non-Fermenting 1
SnRK	SNF1-Related Kinase
SPS	Sucrose Phosphate Synthase
Suc	Sucrose
SuSy	Sucrose Synthase
SUT	Sucrose Transporter
SWEET	Sugars Will be Eventually Effluxed Transporter
TF	Transcription Factor
TIR	Toll/Interleukin-1 Receptor
TOR	Target Of Rapamycin
T6P	Trehalose-6-Phosphate

UDP	Uridine DiPhosphate
VI	Vacuolar Invertase
WAK	Wall-Associated Kinase
WT	Wild type

Literature study

1. Introduction

In the last decades, the human population has been prone to an exponential growth and it is predicted that the worldwide population will double in the next 50 years, so a rise in food production must be achieved to sustain these increasing numbers. In this aspect, the use of marginal lands will become important as more food will need to come from drought- and salt-inclined areas. Increasing crop productivity in these less favorable conditions has become a major point of research (Munns, 2011). In all rural lands, plants face at least some constraints, either of biotic or abiotic origin, thereby limiting their production (Cramer *et al.*, 2011). This phenomenon, when actual yields are lower than the maximal theoretical potential yield due to suboptimal conditions, is called the yield gap. For wheat, rice and maize, some of the most important food crops worldwide, the yield gap is respectively 40, 75 and 30% (Atkinson *et al.*, 2014). As a consequence, roughly 826 million people worldwide are undernourished, 95% of which live in the developing world (Arora *et al.*, 2012).

Besides diminutive yields, crop loss is an important issue. Crop loss can be due to the presence of non-native crop species, as this causes significant losses in countries like Brazil and the United Kingdom (Pimentel *et al.*, 2000). Abiotic factors, such as excess water and flooding, can also lead to losses, in part by increasing susceptibility to diseases. A large part of cultivated land has become saline, mainly caused by land clearing and irrigation (Munns, 2011; Voesenek & Bailey-Serres, 2015). Climate change will affect crop growth in multiple ways. For example, changes in the hydrological cycle will cause frequent floods and storms, while rising temperature and precipitation will alter the biogeographic distribution of pathogens and pests (Atkinson *et al.*, 2014).

Besides abiotic stresses, biotic pests can cause major crop losses. Climate change can bring upon severe insect infestations by improving population characteristics such as reproduction and growth rate (Delcour *et al.*, 2015). Crop production in new lands may expose them to new pathogens to which they are susceptible, or may allow co-evolved pathogens to catch up with their host because of the new conditions, thereby causing major crop losses. Disease emergence goes hand in hand with globalization and intensive trading of plants and seeds (Evans & Waller, 2010; Arora *et al.*, 2012). The history of farming has led to less resistant crop plants, as compared to wild varieties, by selecting for so-called “soft growth” characteristics, such as hyper-hydrated tissues, and favorable appearance and taste, by selecting against high toxic metabolite content (Cassels & Rafferty-McArdle, 2012).

Because of the need for improved crop yields or expansion to marginal soils, different strategies have been introduced, often with setbacks. The use of genetically improved

cultivars with promising higher yields and increased disease resistance is still under relentless debate, while the use of synthetic pesticides can be detrimental, both for human health and for the environment (Arora *et al.*, 2012). Legislation limits the scope of pesticides to be used. However, developing countries often lack the resources and the correct management of pesticide use, as there are still risks for the local population (Handford *et al.*, 2015). It is predicted that climate change may have an effect on pesticide use, although the exact outcome is unknown (Delcour *et al.*, 2015).

New mechanisms, like biological control, have been investigated in light of elevated resistance to pathogens (Cassels & Rafferty-McArdle, 2012). Plant growth-promoting rhizobacteria (PGPR) can antagonize phytopathogens by means of nutrient competition, physical displacement, synthesis of antibiotics or induction of plant immune response (Arora *et al.*, 2012). Use of toxic compounds can be circumvented by microbes, since they possess ABC (ATP-binding cassette) or MSF (major facilitator superfamily) transporters as the first line of defense. For example, efflux pumps are implemented in tolerance of the fungus *Botrytis cinerea* to resveratrol, a phytoalexin of grapevine (*Vitis vinifera*) (Bardin *et al.*, 2015). Crops can also be primed by chemical plant activators of natural origin, like stress signals such as Salicylic acid (SA) and analogues. A good example of such an analogue is BTH (benzothiadiazole), which is a strong inducer of plant resistance and has been commercialized near the end of the last century (Görlach *et al.*, 1996). Application of these compounds induces a more rapid protection compared to PGPRs. Priming of plant defenses is less restricted through legislation (Cassels & Rafferty-McArdle, 2012), but may come at the expense of growth. However, there is no strict consensus on this definition, as others define priming as a mechanism without trade-offs in growth (Conrath, 2009). For centuries crops have been bred for optimization of growth-related traits, at the cost of their defense-related features (Huot *et al.*, 2014).

To sustain future food demands, we must reduce our dependency on chemical control and engineer broad-spectrum disease resistant crops. A major goal in the 21st century is to increase our understanding of the plant's immune system and defense responses, and how they are manipulated by pathogens. This way, making transgenic crops with durable resistance and increased yields can be accomplished (Piquerez *et al.*, 2014).

2. Plants are prone to different stress stimuli

Plants, unlike animals, are sessile organisms, making it impossible to escape exposure to stress. Therefore, they evolved a broad array of mechanisms to protect their homeostasis. Contrary to animals, plant cells possess a cell wall (CW), which encompasses the cell membrane and, besides other functions, forms a primary barrier against intruding pathogens. A network of cellulose microfibrils, composed of hydrogen-bonded β-(1,4)-glucans, is

synthesized by plasma membrane-resident multimeric complexes containing cellulose synthase activity (Blanco-Ulate *et al.*, 2014; Trouvelot *et al.*, 2014). Cellulose interacts with hemicelluloses, polysaccharides with a β-(1,4)-linked backbone of mannose, glucose (Glc) or xylose. In primary CWs, this matrix is embedded in pectin polysaccharides. They are secreted into the CW, where they are de-esterified by pectin methylesterase (PME) (Malinovsky *et al.*, 2014). Homogalacturonans and rhamnogalacturonans are the major pectins in dicots and non-graminaceous monocots (Blanco-Ulate *et al.*, 2014). Lignin is a phenolic polymer in the secondary CW, forming a water-impermeable layer and providing increased strength. It consists of monolignols, synthesized from coumaryl CoA, incorporated into a complex structural network (Van Baarlen *et al.*, 2007; Malinovsky *et al.*, 2014). The CW also harbors proteins such as Hydroxyproline-Rich GlycoProteins (HRGPs) and Glycine-Rich Proteins (GRPs) (Asselbergh *et al.*, 2007).

Plants contain molecules involved in both local and systemic regulation of cell processes. These phytohormones, which are present in low concentrations, play a central role in plant growth and development. Some of them are key players in defense against biotic and/or abiotic stresses. Abscisic acid (ABA) has a broad range of biological functions, such as regulation of stomatal closure, seed germination, and homeostasis under abiotic stress. Apart from these, ABA plays a main role during stress responses in general (Baxter *et al.*, 2014). Auxins and cytokinins (CKs), such as trans-zeatin, are central regulators of plant growth and development, functioning through opposing concentration gradients throughout the plant. The most prominent auxin is indole-3-acetic acid (IAA), which is transported throughout the plant in an unidirectional manner (Huot *et al.*, 2014). Salicylic acid (SA) and Jasmonic acid (JA) are the central hormones involved in plant defense against different pathosystems. They antagonize each other's biosynthesis and gene regulation through intensive cross talk. SA synthesis occurs mainly through the isochorismate pathway, while the phenylalanine pathway plays only a minor role in SA production during biotic stress resistance (SAR) (Chen *et al.*, 2009; Fu & Dong, 2013). Ethylene (ET) is a small volatile phytohormone that, besides being involved in senescence, fruit ripening, and flowering, works in synergism with JA during biotic stress (Han *et al.*, 2010). Gibberellins (GAs) and brassinosteroids (BRs) are the other main phytohormones. The most important steps in the biosynthesis of these hormones are summarized in addendum Figure 12 (Wasternack & Hause, 2013; Vidhyasekaran, 2015).

2.1. Abiotic stress

Water deficit or drought is one of the major abiotic stresses worldwide, causing a rapid decline in cell expansion and growth in the plant, as well as changes in growth priorities (Roitsch, 1999; Redondo-Gomez, 2013). One of the first mechanisms when facing drought

stress is the closure of the stomata through induction of ABA, which, through ROS and Ca^{2+} signaling, leads to membrane depolarization, causing an efflux of K^+ and other ions. Through turgor reduction, this causes water efflux out of the guard cells, thereby closing the stomata (Redondo-Gomez, 2013; Osakabe *et al.*, 2014). Reduction of CO_2 uptake by the leaves causes an inhibition of photosynthesis (Ruan *et al.*, 2010). Plants harbor different strategies to protect themselves against drought. Turgor is maintained through accumulation of compatible solutes such as polyols and soluble carbohydrates, but mainly proline and glycine betaine (Redondo-Gomez, 2013). CAM (crassulacean acid metabolism) and C4 photosynthesis are well-known strategies to reduce water loss while maintaining photosynthesis (Bohnert *et al.*, 1995). An excess of water, however, can be just as detrimental. Flooding can cause a major shortage in CO_2 and O_2. Main strategies to sustain flooding are either an escape strategy, characterized by elongation of aerial organs, or a quiescence strategy, by reducing growth until the stress stimulus disappears (Voesenek & Bailey-Serres, 2015).

Heat and cold stresses can also cause a reduction in the availability of water. Heat stress induces the upregulation of heat-shock proteins. High temperatures decline the rate of photosynthesis and affect membrane stability. Among plant defenses to heat stress, the heat shock response plays a central role (Kotak *et al.*, 2007; Redondo-Gómez, 2013). Cold stress causes a decrease in membrane fluidity and metabolic rates and, in the case of chilling, production of ice crystals. Production of sugars and other compatible solutes provides a major defense against cold stress (Jeon & Kim, 2013). Sugars also provide an important antioxidant mechanism for the plant against abiotic stresses. For example, fructans may be involved in ROS-scavenging in the vacuole, while raffinose family oligosaccharides (RFOs) detoxify ROS in chloroplasts (Van den Ende & Valluru, 2009; Keunen *et al.*, 2013). More recent literature strongly confirms that hydroxyl radical scavenging by sugars is a central part in oxidant homeostasis, by studying the fate of an artificial sucrose (Suc) analog *in vivo* (Matros *et al.*, 2015).

High concentrations of salts and heavy metals can induce toxic effects in the plant. The plant can prevent salt and metal uptake by the roots. If these compounds accumulate, they can be stored in the vacuole or excreted, for example through specialized salt glands (Munns, 2011; Van den Ende & El-Esawe, 2014). While a shortage of oxygen can be detrimental, an excess, in the form of ROS, can induce major toxic effects to the cell. Thus, plants have a broad array of antioxidant defenses to regulate ROS levels, as H_2O_2 also plays an important signaling role, especially under abiotic/biotic stress (Dat *et al.*, 2000).

2.2. Biotic stress

Biotic stress can come in different forms, depending on the type of organism that interacts with the plant. Herbivory can cause major mechanical damage to the plant, while pierce-sucking herbivores or cell content feeders inflict little physical damage (Heil, 2009). Upon recognition of herbivore-associated molecular patterns (HAMPs) or damage-associated molecular patterns (DAMPs), a herbivore-induced immunity (HTI) is activated. The wound induction itself can also stimulate a wound-induced resistance (WIR) (Wasternack & Hause, 2013). In tomato, at the local site of attack, prosystemin is cleaved to an 18 amino acid peptide, systemin, which is transported throughout the plant as a DAMP. Systemin causes induction of JA biosynthesis, thereby priming the entire plant against a future attack (Conrath, 2009). Many secondary metabolites, either present or induced, are important in herbivory resistance, as they can be toxic to the herbivore (Keane, 2012). Several nematode species are important plant parasites with the ability to lower biomass production, either ecto- or endoparasitic in the rhizosphere (Kerry, 2000).

Pathogens have a much closer relation with the host plant than herbivores. The plant can be tolerant to these pathogens by enduring the infection, the disease mechanisms, or both. Major plant phytopathogens include fungi, oomycetes, and bacteria (Scharte *et al.*, 2005). They can be classified as above- or below-ground pathogens, depending on the tissue they attack (Berger *et al.*, 2007). Another major classification method divides them into biotrophs or necrotrophs. Necrotrophs obtain nutrients from dead plant tissue, whereas biotrophs develop an intimate relation with the host, keeping it alive. Necrotrophs are more virulent, killing of plant tissue in a rapid way. Another class is the hemibiotrophs, which have a transient lifecycle, at first keeping the host alive, but becoming necrotrophic after some time (Struck, 2006). Plants must defend themselves against these different types of pathogens in different ways. The JA/ET-pathway is more important against necrotrophs, while the SA-pathway seems to be more effective against biotrophs. Since long, it has been noted that the amount of sugars present in the plant can make them more vulnerable to certain pathogens, while being more resistant to others. Hence, pathogens were classified as low sugar diseases and high sugar diseases, infecting plants with low and high sugar levels respectively (Horsfall & Dimond, 1957). Although this initial hypothesis was proven to be often incorrect, sugars tend to play an important role in infection. It is known that apoplastic sugars may indicate the presence of infection, as upregulation of apoplastic invertases results in increased Glc and fructose (Fru) concentrations (Roitsch, 1999). However, this response is strongly dependent on the pathosystem. For example, in Arabidopsis a repression in invertase expression is observed during infection with *Pseudomonas syringae,* while infection of Arabidopsis with *Albugo candida* causes an induction of invertase activity (Tauzin & Giardina, 2014).

2.2.1. Plant pathogenic fungi

In establishing a fungal infection, spores must adhere to the plant surface. Important factors determining the site of infection are water availability (fungi like a moist environment), and nutrient availability, especially for necrotrophs. Once fungal spores attach to the cuticle, a germ tube is formed on the surface that will develop an appressorium (Muthamilarasan & Prasad, 2013). Germination of the spores is only possible on an appropriate substrate. The adhesion process involves the release of glycoproteins, after which an extracellular matrix is often produced to facilitate the infection process. An appressorium is usually a dome-like structure, reinforced with high levels of melanin. This structure builds up a large turgor pressure through glycerol accumulation, thereby providing physical forces that rupture the cuticle and CW of the plant. During penetration of the plant surface, an infection peg (penetration hypha) grows into the leaf tissue. While the use of appressoria is quite common, most fungi mainly depend on CW-degrading enzymes that can affect the plant cuticle and CW, thereby facilitating penetration (Fitt *et al.*, 2006; Struck, 2006). However, this degradation process may at the same time stimulate the plant's immune system. For example, the degradation of homogalacturonan results in the production of oligogalacturonides (OGs), potent inducers of defense responses. Some fungi, such as *Puccinia striiformis* specifically grow towards stomata, where they enter the host, forming infection hyphae and haustoria (Allègre *et al.*, 2009; Lazniewska *et al.*, 2012). Biotrophs are in need of a more intimate relationship with the host to obtain the necessary nutrients, and hence, they form more specialized structures, haustoria, within the host cells (Keane, 2012).

2.3. Exposure to multiple stresses

As of late, there has been research on the combinatorial effect of multiple stresses on the plant, since this appears to differ from the additive effect of the independent stresses. For example, when drought stress causes closure of the stomata, ozone cannot enter the plant to exert its toxic effects (Suzuki *et al.*, 2014). Combinations of biotic and abiotic stresses can also occur. The effect of drought stress on pathogen stress has recently been studied. Pathogens that favor dry soils, like *Fusarium* species, display a more aggressive pathogenesis during drought conditions, while others need a more humid environment (Pandey *et al.*, 2014). From the interaction between biotic and abiotic stresses, it follows that ABA also influences the plant's immune system. Depending on when biotic stress occurs and if there is a simultaneous abiotic stress stimulus, ABA can antagonize defense signaling, or activate the defense response through stimulation of different pathways (Asselbergh *et al.*, 2008; Rejeb *et al.*, 2014).

3. Plant immune system

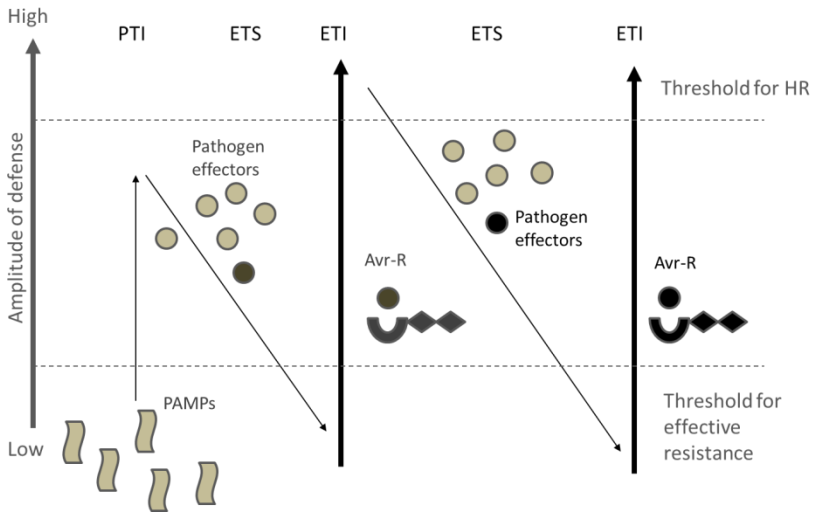

Figure 1: Zigzag model for plant innate immunity. In the first phase, PAMPs from the pathogen are recognized by PRRs, thereby leading to PTI induction, providing the plant with basal resistance. Many pathogens make use of effector molecules that turn down PTI-triggered responses in the plant. This is called ETS. Some plants have evolved receptor proteins to recognize these effectors, so-called R proteins, and will thereby induce a stronger immune response through ETI. This evolutionary arms race between host and pathogen may go even further, as the pathogen will evolve and use different effectors to make the host more susceptible. This can possibly be followed by a countermeasure in the host in the form of novel R proteins to recognize these effectors. *Abbreviations: Avr, Avirulence protein or effector; ETI, Effector-Triggered Immunity; ETS, Effector-Triggered Susceptibility; HR, Hypersensitive Response; PAMP, Pathogen-Associated Molecular Pattern; PTI, PAMP-Triggered Immunity; R, Resistance protein.* Adapted from Jones & Dangl, 2006.

Plants, much like animals, possess an innate or native immune system to fight off intruding and virulent organisms. However, this innate immunity is different from animals in several ways. For example, the mechanisms of production of antigen-specific receptors through somatic recombination do not exist in plants, and they do not contain specialized cells, such as macrophages and neutrophils, that are transported by vascular systems (Nürnberger *et al.*, 2004). Plants contain a non-host immunity and a host immunity. Non-host immunity is an evolutionarily ancient mechanism utilized against most pathogens. It is associated with the recognition of molecules derived from the pathogen or endogenous elicitors formed during the plant-pathogen interaction. These pathogen-derived molecules are called pathogen-associated molecular patterns (PAMPs) or, since they are also present in non-pathogenic microbes, microbe-associated molecular patterns (MAMPs). These molecules are recognized by plant receptors, giving rise to a downstream signaling cascade involving MAPK (mitogen-activated protein kinase) activation, ROS production, and transcriptional reprogramming. This first layer of plant defense is also called PAMP-triggered immunity (PTI) (Chrisholm *et al.*, 2006; Morkunas & Ratajczak, 2014). Host resistance is specific to certain genotypes within a susceptible host species. It thus acts within a species, controlled by R genes or resistance genes. R proteins can recognize effectors, or avirulence proteins (Avr proteins), from the pathogen, hence, this is called effector-triggered immunity (ETI) (Heath, 2000; Sanabria *et al.*, 2010). To bring PTI and ETI together, the 'zigzag' model has been proposed (Figure 1). In the first phase, PAMPs, either pathogen- or host-derived, are recognized by plant pattern recognition receptors (PRRs), resolving to PTI and blocking pathogen infection. However, successful pathogens secrete effectors which, once inside the plant, inhibit the PTI response. This mechanism is called effector-triggered susceptibility

(ETS) (second phase). The host plant has evolved, in some occasions, proteins to recognize these effectors, causing ETI, thereby acquiring disease resistance (third phase) (Jones & Dangl, 2006; Cassels & Rafferty-McArdle, 2012).

PTI and ETI differ not only in their mechanisms of pathogen recognition, but also in their responses to overcome infection. ETI preferentially operates at low temperatures (10-23°C), while PTI is typically more effective at higher temperatures (23-32°C) (Cheng *et al.*, 2013). When PAMPs are recognized the downstream responses occur within hours, while ETI can require up to two days past infection (dpi). However, ETI causes a visible response to fend off the pathogen through localized programmed cell death (PCD), forming a necrotic zone. This hypersensitive response (HR) is not observed in PTI. Nevertheless, the line between PTI and ETI is not always this clear, as some PAMPs, such as bacterial harpins, do induce a HR in *Arabidopsis thaliana*. Examples of a weak ETI response are also existing, such as the recognition of a *P. syringae* AvrRps4 effector by the RPS4 receptor (Thomma *et al.*, 2011; Stotz *et al.*, 2014). Although the plant innate immunity is very different from immunity in mammals, certain similarities exist, such as the use of LRR (leucine-rich repeat) domains for the extracellular reception of PAMPs and the use of MAPK cascades in downstream signaling (Nürnberger *et al.*, 2004). Recently, a new model has been proposed to characterize plant immune responses, including the fact that plants may modulate their defense responses based on the feeding behavior of the pathogen. This circular model contains an immunity activation component, involving pathogen recognition and response, and an immunity modulation component, fine-tuning defense induction via hormone crosstalk (Andolfo & Ercolano, 2015).

3.1. PTI: first tier in plant innate immunity

PAMPs are perceived at the plasma membrane by PRRs. PAMPs are molecules that are usually present in a broad range of pathogens and important for the pathogen's survival, like flagellin for bacterial motility and chitin in the cell wall of fungi. Different PRRs exist to recognize these different molecules. Receptor-like kinases (RLKs) or receptor-like proteins (RLPs) are localized to the cell membrane, possessing an extracellular ligand binding domain. RLKs contain an intracellular kinase domain for the activation of a downstream signaling cascade, while RLPs lack a kinase domain and require the association with other proteins to activate a phosphorylation cascade (Figure 2; Monoghan & Zipfel, 2012; Böhm *et al.*, 2014). Most kinases involved in PTI are of the non-RD domain type, having a cysteine or glycine (instead of an arginine) preceding the catalytic aspartic acid residue (Thomma *et al.*, 2011). A transmembrane domain connects the extracellular PAMP recognition domain with the intracellular kinase domain. The PAMP recognition domain typically consists of a LRR or a LysM (lysine motif) domain, but some carry other domains, such as an EGF-like ectodomain (Trouvelot *et al.*, 2014). This LRR is a tandem repeat of 20-30 amino acids, with

4 conserved leucine residues. The *Arabidopsis* genome contains 610 RLKs and 56 RLPs, but only a limited number is known to be involved as immune receptors (Sanabria *et al.*, 2010). LysM domain proteins recognize N-acetylglucosamine-containing molecules, such as chitin and peptidoglycan. Peptidoglycan is recognized by LYM1 and LYM2 in *A. thaliana*, and CERK1 in *Oryza sativa* (Tena *et al.*, 2011; Gust *et al.*, 2012). Interestingly, plant LysM receptors are also involved in the recognition of symbiotic micro-organisms, such as mycorrhizal fungi and rhizobia bacteria. It is hypothesized that the Nodulation factor receptors in the Brassicaceae have evolved from the chitin perceiving LysM receptors (Kombrink *et al.*, 2011).

Well-known examples of bacterial PAMPs are elongation factor Tu (EF-Tu), the most abundant bacterial protein, and Xoo, an elicitor of *Xanthomonas oryzae* pv. *oryzae*, sensed by the LRR-RLKs EFR (EF-Tu receptor) in Arabidopsis and XA21 in rice, respectively (Kawano & Shimamoto, 2013; Macho & Zipfel 2014). The best-studied bacterial PAMP receptor is FLS2, which recognizes a conserved 22 amino acid epitope of flagellin, flg22. In the absence of flg22, FLS2 associates with receptor-like cytoplasmic kinases (RLCKs), including BIK1 in Arabidopsis. Upon flg22 binding, FLS2 heterodimerizes with BAK1, a RLK without a PAMP recognition domain, which works as a signal amplifier. BAK1, through the associated PUB12/13 E3 ligases, causes BIK1 to dissociate from FLS2, forwarding the immune signal to downstream pathways (Denoux *et al.*, 2008; Henry *et al.*, 2013; Bigeard *et al.*, 2015). Activated FLS2 is removed through endosomal trafficking, preventing a prolonged activation of plant immune responses (Teh & Hofius, 2014).

Chitin is the major fungal PAMP recognized by plant cells at the plasma membrane. Chitin is a linear polymer of β-(1,4)-linked N-acetylglucosamine in the CW of fungi. Plants secrete chitinases which degrade the chitin into small fragments (Bueter *et al.*, 2013; Ökmen & Doehlemann, 2014). In Arabidopsis, chitin oligomers (DP 7-8) bind to the LysM-RLK AtCERK1. However, the binding affinity of this receptor for chitin is low, and more recently a new receptor has been proposed as the primary chitin receptor in Arabidopsis, namely lysine motif receptor kinase 5 (LYK5) (Cao *et al.*, 2014). LYK5 can subsequently activate CERK1, thereby activating the expression of chitin-responsive genes, such as WRKY transcription factors (TFs) and PR (Pathogenesis-Related) proteins (Eckardt, 2008; Hamel & Beaudoin, 2010). In rice, chitin recognition occurs through heteromultimerization. The LysM-RLP OsCEBiP homodimerizes to bind chitin oligomers. Since OsCEBiP has no intracellular kinase domain it forms a complex with OsCERK1. Two downstream signaling pathways are known. After chitin binding, OsRacGEF1 induces ROS production, phytoalexin accumulation, lignin production and gene expression of defense-related genes. Alternatively, an OsRLCK is activated, thereby activating defense responses through a MAPK pathway (Kawano & Shimamoto, 2013; Macho & Zipfel, 2014).

So far, pathogen-derived molecules have been discussed. However, as pathogens degrade the plant CW enzymatically, certain DAMPs are sensed by receptor kinases present in the pectin fraction of the plant CW. As predicted by the damaged-self hypothesis, this sensing can trigger plant immunity (Heil *et al.*, 2012). The best-studied DAMPs are OGs, which bind a wall-associated kinase (WAK) receptor. This receptor contains an EGF-like domain instead of a LRR or LysM domain. As WAKs are also involved in the maintenance of the CW, a different downstream pathway is activated depending on the DP of the OGs, involving MAPK3 and MAPK6 (Ferrari *et al.*, 2007; Kohorn & Kohorn, 2012; Ferrari *et al.*, 2013).

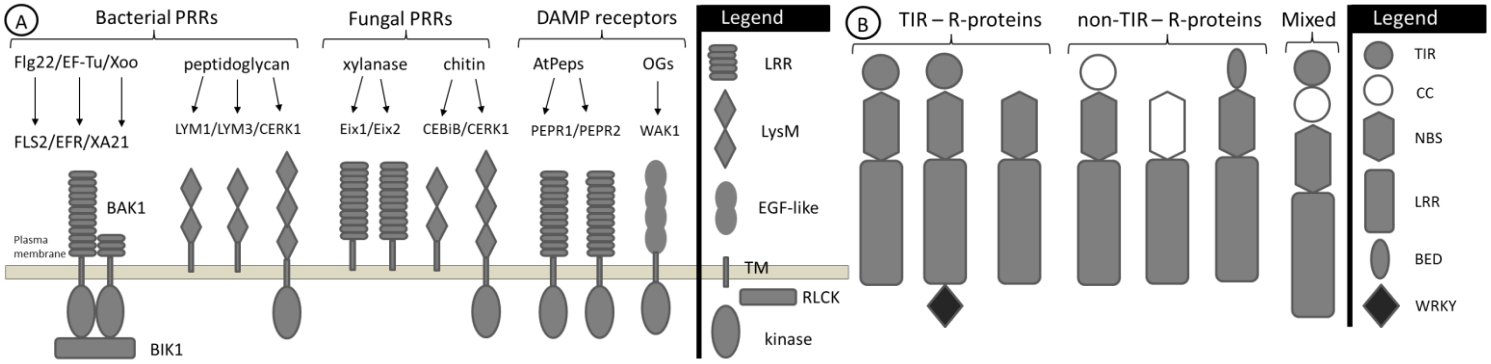

Figure 2: Variation in the domain structure of PRRs involved in PTI (A) and resistance proteins involved in ETI (B). A) Localization and structure of the major PRRs involved in PTI. Different domains are indicated in the Legend. PRRs are divided according to the origin of the PAMP/MAMP that binds the receptor. B) Known domain structure of resistance gene products. Different domains are indicated in the Legend. In the absence of a third domain, TIR-containing and non TIR-containing R proteins have respectively a TIR of CC domain associated with their NBS. *Abbreviations: BAK1, Brassinosteroid insensitive 1-associated receptor kinase 1; BED, Drosophila proteins BEAF/DREF; BIK1, Botrytis-induced kinase 1; CC, Coiled coil; CEBiB, Chitin elicitor binding protein; CERK1, Chitin elicitor receptor kinase; DAMP, Damage-associated molecular pattern; EFR, Elongation factor Tu receptor; EF-Tu, Elongation factor Tu; EGF, Epidermal growth factor; FLS2, Flagellin sensing 2; LRR, Leucine-rich repeat; LYM, LysM protein; LysM, Lysine motif; NBS, Nuclear binding site; OG, Oligogalacturonic acid; PRR, Pattern recognition receptor; RLCK, Receptor-like cytoplasmic kinase; TIR, Toll/interleuking-1 receptor; TM, Transmembrane domain; WAK1, Wall-associated kinase1; Xoo,* Xanthomonas oryzae pv. oryzae. Adapted from Glowacki *et al.*, 2010 an Monoghan & Zipfel, 2012.

3.2. ETI: second tier in plant innate immunity

ETI depends on the interaction between an Avr protein (effector protein) produced by the pathogen and an R protein from the host. The first model that was proposed is the gene-for-gene model, wherein the products of R genes are receptors that bind Avr proteins directly. If the Avr protein is recognized and bound by the R protein, host resistance occurs. However, in several cases no direct interaction has been found, leading to the guard model for ETI. This model hypothesizes that the R protein guards the intracellular targets of the pathogen effector and when the target gets modified, the R protein is activated. Still, in some cases, the guarded target does not play an important role in the absence of a receptor and the interaction with the effector does not induce virulence. This means the effector does not increase the pathogen's fitness. To explain these phenomena, more recently a third model has been proposed, the decoy model. Proteins similar to those targeted by pathogen effectors are present in the plant cell, and their only function is to bind the effector and

mediate the interaction with the R protein. As the decoy binds the effectors, it can lower their interaction with the operative targets, thereby reducing the pathogen's virulence. Moreover, the decoys do not enhance the pathogen's fitness in the absence of a functional R protein (Van der Hoorn & Kamoun, 2008; Glowacki *et al.*, 2010). Selection is an important driving force in ETI mechanisms, causing an arm's race between the pathogen and its host. For example, when an Avr protein's frequency drops in the pathogen population, plants might be selected to lose the corresponding R gene, as R genes may have a fitness cost for the plant (Jones & Dangl, 2006).

The major class of R proteins has a nucleotide binding site (NBS) and a LRR domain. At the N-terminus, they generally contain a coiled-coil (CC) domain or a Toll-interleukin-1 receptor (TIR) domain. In Arabidopsis, over 150 proteins are predicted as NBS-LRR proteins (Chrisholm *et al.*, 2006). TIR-NBS-LRR proteins seem to be absent in monocots. In the case of the RRS1 R protein, there is an additional WRKY DNA-binding domain at the C-terminus (Sanabria *et al.*, 2010). Receptors containing both a TIR domain and a CC domain have also been found in poplar (Figure 2; Glowacki *et al.*, 2011). Binding of a pathogen effector can induce conformational changes in the R protein through ADP/ATP exchange at the LRR domain. A conformational change in the N-terminus (TIR, CC) enables the transmission of the signal towards downstream signaling targets. It is predicted that they can be transported into the nucleus, since some R proteins contain a nuclear localization signal (NLS) (Hou *et al.*, 2013).

Effector proteins must be secreted into the host cell in order to inhibit the plant's defense signaling. Bacterial effectors are mainly injected into the host cell through a type III secretion system (TTSS). *P. syringae* produces several effectors. AvrRpm1 is known to be localized to the plant cell's plasma membrane, where it modifies RIN4. When the plant RPM1 R protein is present, HR is induced. Another effector, AvrRpt2, also targets RIN4 at the plasma membrane, causing increased virulence in the absence of the RPS2 R protein (Jones & Dangl, 2006). AvrRps4 activates the TGN/EE complex (trans Golgi network/early endosome complex), thereby causing the removal of FLS2 from the plasma membrane (Teh & Hofius, 2014). Another well-studied pathogen is the fungus *Cladosporium fulvum*. Two effectors, Ecp6, and Avr4, are involved in the inhibition of chitin recognition by the plant. Avr4 binds to chitin in the fungal CW, inhibiting the action of plant chitinases, while Ecp6 sequesters chitin oligomers degraded by plant enzymes, thereby preventing the binding to the corresponding PRR (Kombrink *et al.*, 2011; Thomma *et al.*, 2011).

3.3. Downstream signaling leading to disease resistance

In order to upregulate the plant's defense responses, after binding of a PAMP to a PRR or binding of an Avr gene product to an R gene product, a downstream signaling pathway must

be activated, ultimately leading to induction of the plant's immune response. Among the earliest events, a rise in cytosolic [Ca^{2+}] and production of ROS are important for the activation of components further downstream. The most well-characterized phosphorylation cascade involves MAPK components. Finally, through activation of TFs gene expression is altered (Stael *et al.*, 2015).

As one of the earliest responses, occurring within 0.5-2 min after recognition of the pathogen, an influx of H^+ and Ca^{2+}, together with an efflux of K^+, causes membrane depolarization (Savatin *et al.*, 2014). This Ca^{2+} is mainly taken up from the apoplastic environment, however, it has been suggested that intracellular organelles such as the vacuole and the endoplasmatic reticulum can also be a source of Ca^{2+} during pathogen infection, as is the case in animals (Xu & Heath, 1998; Lecourieux *et al.*, 2006). The plant distinguishes between different Ca^{2+}-dependent signals through so-called calcium signatures, characterized by the magnitude, duration and number of spikes in cytosolic [Ca^{2+}]. The deciphering of these signatures is achieved by different groups of Ca^{2+}-binding proteins. The major group of EF-hand-containing proteins can be subdivided into sensor relays and sensor responders. Sensor relays do not contain a known enzymatic function, but rather bind with other proteins after Ca^{2+}-binding. Calmodulin (CaM) and CaM-like proteins (CMLs) belong to this category. Calcium-dependent protein kinases (CDPKs) are the most important sensor responders involved in plant innate immunity (Reddy *et al.*, 2011). Different TFs are activated upon Ca^{2+}-signaling, and some of them are specifically induced in response to biotic stress. CAMTA3 (CaM-binding transcription activator 3), several WRKY and TGA TFs and CBP60 (Calmodulin Binding Protein 60) are all involved in modulation of the plant's immune response (Tena *et al.*, 2011). Ca^{2+}, through CaM, activates the production of ROS and nitric oxide (NO) (Vidhyasekaran, 2014c).

Oxygen, as the most abundant element in living organisms, is a powerful oxidizer and relatively stable molecule. However, energy input can increase its reactivity, and ROS are formed. The major forms of ROS are $O_2^{-\bullet}$ (superoxide anion), H_2O_2 (hydrogen peroxide) and $OH^\bullet$ (hydroxyl radical). H_2O_2 is the most stable form, with a lifetime of less than 1 s due to detoxifying enzymes (Demidchik, 2015). $OH^\bullet$ is the most reactive ROS and thus the most toxic, interacting with proteins, DNA and membrane lipids. Oxidation of lipids causes a chain reaction, leading to the collapse of membrane functions and disintegration of cellular organelles (Farmer & Mueller, 2013). However toxic they are, ROS also constitute a major signaling role in the plant. To tightly regulate ROS levels, several antioxidant mechanisms are present, such as catalases (CAT), superoxide dismutases (SOD), and non-enzymatic proteins such as the tripeptide glutathione (GSH) (Mittler, 2002; Yun *et al.*, 2012). During biotic stress, NADPH oxidase RBOHs (respiratory burst oxidase homologs) in the plasma membrane are the major source of ROS, responsible for the first ROS burst, thereby

potentiating defense mechanisms (Baxter *et al.*, 2014). The second burst in ROS production induces secondary defenses, such as phytoalexin production (Dat *et al.*, 2000; Lyon *et al.*, 2004). Propagation of ROS signals in the plant can be achieved through ROS waves, as it has been hypothesized that they are important in systemic signaling (Gilroy *et al.*, 2014). Early research suggested that necrotrophs, contrary to biotrophs, can withstand ROS production at the site of infection. However, a timely hyperinduction of H_2O_2-dependent defenses was shown to be effective against the necrotroph *B. cinerea* (Asselbergh *et al.*, 2007). ROS, together with NO, has been implicated in the HR. In plants, NO is thought to be produced through an L-arginine-dependent pathway and a nitrite-dependent pathway, by NO synthase and nitrite reductase, respectively (De Stefano *et al.*, 2005; Rasul *et al.*, 2012). It has been shown that, besides being involved in the downstream signaling, MAPKs play a role in the regulation of NO synthesis in *Nicotiana benthamiana* (Bellin *et al.*, 2013).

A MAPK kinase kinase (MPKKK or MKK) receives a signal from upstream receptors and activates a MAPK kinase (MPKK) by phosphorylating serine/threonine residues. The activated MPKK, in turn, phosphorylates a downstream MAPK on a threonine and tyrosine in the TXY activation motif (Han *et al.*, 2010). MAPK signaling is involved in several biotic stress responses, such as ethylene induction, SA accumulation and NO biosynthesis (Tena *et al.*, 2011). The Arabidopsis genome encodes 20 MAPKs, 10 MAPKKs and 60 MAPKKKs, however, only for a selected few the role in plant defense has been (partly) elucidated. One pathway that has been characterized consists of MKK4/5 activating MPK6 and MPK3. For example, binding of flg22 to FLS2 activates MPK3/6, which phosphorylates ACC synthases ACS2/6, causing ethylene induction (Bigeard *et al.*, 2015). *B. cinerea* infection leads to the production of plant defensins, including PDF1.1 and PDF1.2, mediated through MPK3/6-induced ERF1/6 activation. However, MPK3 and MPK6 do not play equivalent roles, as MPK3 is more important in PTI, whereas MPK6 plays a more pronounced role in ETI (Galletti *et al.*, 2011; Meng *et al.*, 2013). *Nicotiana tabacum* WIPK and SIPK are orthologous to Arabidopsis MPK3 and MPK6, respectively (Mao *et al.*, 2011). It has been shown that G proteins might play a role in connecting the activation of RLKs with the downstream MKK1/2-MPK4 pathway, which is involved in phytoalexin biosynthesis, among other plant defense responses (Vidhyasekaran, 2014b; Nitta *et al.*, 2015).

3.4. Systemic acquired resistance & induced systemic resistance

As the host plant recognizes the pathogen's Avr gene product through a compatible R protein, resistance to the pathogen is acquired, usually with the involvement of HR. Concurrent with this HR, a secondary resistance mechanism can be induced systemically in uninfected tissues, called systemic acquired resistance (SAR). SAR can provide the plant with a long-lasting resistance against a future pathogen attack (Mou *et al.*, 2003). It is said

that the systemic tissues are primed, as they show enhanced upregulation of defense responses after contact with an infection. A second phenomenon, called induced systemic resistance (ISR), coincides when the plant is associated with beneficial rhizobacteria, such as *Pseudomonas fluorescens*, resulting in a systemic upregulation of defense responses (Smith *et al.*, 2009). Contrary to SAR, which is effective against biotrophs, ISR primarily confers resistance against necrotrophs and the underlying mechanisms involve the upregulation of SA and JA, respectively (Pieterse & Dicke, 2007). Nevertheless, it has been observed that infection of tobacco with *B. cinerea* can induce SAR, not only towards the biotroph *P. syringae*, but even to a subsequent *B. cinerea* infection (Frías *et al.*, 2013).

For priming to occur systemically, a mobile signal must exist that travels from the primary infection site towards the systemic tissues. How this works is still not fully understood. However, some candidates have been proposed. In tobacco, the first mobile signal described to be essential in SAR was methyl salicylate (MeSA) (Liu *et al.*, 2011; Henry *et al.*, 2013). Interestingly MeSA is not essential for systemic SA production in Arabidopsis, since most of it vaporizes into the atmosphere (Attaran *et al.*, 2009). Other candidates have since been proposed. After induction of ETI, NO and ROS catalyze the oxidation of unsaturated fatty acids into azelaic acid (AzA), which consequently triggers glycerol-3-phosphate (G3P) accumulation (Gao *et al.*, 2015). Both require DIR1, encoding a putative lipid transfer protein, for transport. Since AzA accumulation occurs only several hours after infection, it is doubtful to be the initial SAR signal. The diterpenoid dehydroabietinal (DA) and the non-protein amino acid pipecolic acid (Pip) are also known to be involved, with the former also requiring DIR1 for transport (Fu & Dong, 2013; Aranega-Bou *et al.*, 2014).

Concentrations of SA vary between the site of infection and the surrounding tissue. At the infection site, high levels of SA induce PCD, causing necrosis. 2 proteins, NPR3, and NPR4, are involved in the degradation of NPR1, the SA receptor. Surrounding the infection site, defense responses are induced in the presence of low SA levels through induction of gene expression by NPR1 (Fu & Dong, 2013; Veloso *et al.*, 2014; Janda & Ruelland, 2015). In the infected leaf production of G3P, DA, AzA and Pip are upregulated, together with MeSA. These mobile signals are then transported to systemic tissues, with the involvement of DIR1 in the case of DA, AzA and G3P. MeSA can be demethylated, yielding SA. G3P and DA can stimulate this reaction. It has been hypothesized that G3P can negatively regulate the methyltransferases responsible for MeSA production. DA, AzA, and Pip can induce SA biosynthesis indirectly through a positive feedback loop involving FMO1 (Flavine MonoOxygenase 1) and ALD1. Higher levels of SA activate NPR1, which leads to activation of SAR through gene expression activation (Návarová *et al.*, 2012; Shah & Zeier, 2013).

ISR does not require SA, but uses JA and ET instead. Not only PGPRs have been found to induce ISR. For example, colonization of tomato roots by mycorrhizal fungi protects the

plant against future infection by *Phytophthora parasitica*. There is no immediate induction of defense responses, however, as the cells have been primed for a faster upregulation in case of an infection. The volatile JA analog, MeJA seems to be the mobile signal throughout the plant, together with ET (Beckers & Conrath, 2007; Conrath, 2009). The role of ET has been well established, as a higher ACC-converting capacity is observed in ISR-induced plants (Conrath *et al.*, 2002; Pieterse *et al.*, 2007) and interaction with arbuscular mycorrhizal fungi has been shown to induce higher MeJA levels in the plant (Nair *et al.*, 2015). Recently, it has been discovered that the progeny of plants in which SAR or ISR has been induced also exhibit a higher resistance to infections. In the same line, it has been observed that new somatic cells formed during mitosis are readily primed through ISR (Henry *et al.*, 2013). This transgenerational resistance has also been detected in Arabidopsis plants treated with the SAR-inducing beta-amino-butyric acid (BABA) (Slaughter *et al.*, 2012). Epigenetic changes, such as chromatin or histone modifications, and alterations in DNA methylation patterns, must be involved in this process. Small interfering RNAs (siRNAs) have also been reported to play a role (Cassels & Rafferty-McArdle, 2012; Muthamilarasan & Prasad, 2013).

3.4.1. Salicylic acid and jasmonic acid, central regulators of plant immunity

The regulation of plant defense responses is achieved mainly by SA and JA signaling. However, other phytohormones influence this mechanism through extensive crosstalk. NPR1, the SA receptor, controls over 2000 defense-related genes in Arabidopsis. The receptor contains 2 protein-protein interaction domains, a BTB (Broad-complex, Tramrack, and Bric a brac) domain and an Ankyrin repeat domain, and a NLS (Mou *et al.*, 2003). SA does not interact with its receptor directly, but through redox changes in the cell. NPR1, present in the cytoplasm in an oligomeric form, is released upon changes in cysteine residues upon pathogen attack and translocates to the nucleus as a monomer. There, it physically interacts with redox sensing TGA TFs to form a transcription activating complex. NPR1 also induces several WRKY TFs as positive regulators of SA-mediated resistance (Moore *et al.*, 2011). Superimposed on redox-dependent disulphide bond formation, NO-mediated S-nitrosylation on the cysteine residues of NPR1 seems to positively affect oligomerization and inactivation. Upon SA accumulation, redox changes induce the reduction of the disulphide bonds in a biphasic trend (Yun *et al.*, 2012). NPR1 turnover is regulated by NPR3 and NPR4, two adaptor proteins for the CUL3 E3 ligases that target NPR1 for proteasome degradation. SA accumulation breaks the NPR1-NPR3 interaction, whereas it elevates the binding between NPR1 and NPR4 for NPR1 degradation. Both NPR3 and NPR4 are SA-binding proteins, but NPR3 has a lower binding affinity (Moreau *et al.*, 2012; Fu *et al.*, 2012). SA itself can be synthesized upon infection, however, it can be readily available in the cell as inactive SA glucosides (Fu & Dong, 2013).

JA, in order to activate downstream signaling, must be conjugated to isoleucine. In a resting state, JA-Ile levels are low and JA-responsive genes are repressed by a complex involving MYC2, a basic helix-loop-helix domain-containing TF. MYC2 interacts with JAZ proteins, through a JAZ interaction domain (JID), NINJA and TPL, causing repression of gene transcription. Upon JA-Ile accumulation, the repressor complex dissociates and binding of JA-Ile to JAZ targets the protein for degradation by the proteasome, hence JA responses are induced. MYC2 associates with the Mediator complex subunit MeD25 for gene transcription activation (Kazan & Manners, 2013; Wasternack & Hause, 2013). JA usually works in an antagonistic way to SA signaling, as SA seems to have transcriptional control over JA responses, even when JA levels are high (Caarls *et al.*, 2015). However, the antagonism may be weaker in certain plants, such as in rice. Here, JA, involved in defense against necrotrophs, provides protection against hemibiotrophs (Takatsuji & Jiang, 2014).

As already has been mentioned, other phytohormones may affect the SA-JA pathways through crosstalk. The trade-off between growth and defense plays a central role herein, as growth-promoting hormones such as auxin and GA negatively regulate SA and JA responses (Huot *et al.*, 2014; Takatsuji and Jiang, 2014). The crosstalk between both defense signaling pathways allows the plant to have a flexible network to fine-tune its defense response according to the pathogen. However, pathogens can take advantage of this network. The phytotoxin coronatine, produced by *P. syringae*, is a JA analog, thereby suppressing SA-dependent responses. This promotes susceptibility of the host plant (El Oirdi *et al.*, 2011). Some fungi are not only able to regulate ABA signaling in the plant, but also produce ABA themselves. Since ABA is known to inhibit SA biosynthesis, the immune response of the plant is weakened (Asselbergh *et al.*, 2008). Interestingly, ABA, which is important in abiotic stress signaling, can also work in a synergistic manner with the SA pathway in early stages of infection, thus allowing a fine-tuning of plant response to a combination of stresses (Atkinson *et al.* 2014).

3.4.2. Priming of plant defense responses

After induction of SAR or ISR, the systemic plant tissues are said to be primed for future attack. A primed state is achieved by a priming agent that increases the ability of the plant to cope with succeeding stresses. This can be attained not through direct induction of defense responses but by improved perception and amplification of response-inducing signals. For example, MPK3 and MPK6 are found to be accumulated after priming, without displaying activity (Conrath, 2009; Aranega-Bou *et al.*, 2014). Recently, it has been proposed that a chromatin assembly factor, CAF-1, represses plant defense at the level of DNA and transcription in the absence of biotic stress (Mozgová *et al.*, 2015). The primed state can be promoted by chemical inducers, microorganisms or constitutive priming through mutations in

defense-repressive genes. A reorganization in metabolic pathways occurs as sugar and amino acid anabolic and catabolic pathways are modified and production of mobile signals and secondary metabolites is induced (Gamir *et al.*, 2014).

Priming can be induced by application of chemical inducers to the plant. GABA (γ-aminobutyric acid) is a non-proteinogenic amino acid, of which the concentration increases in the apoplast during infection (Shelp *et al.*, 2006). GABA has been shown to prime plants against osmotic stress and pathogen infection, although this is pathosystem-dependent. GABA-treated plants contain higher sugar levels, indicating the involvement of GABA in sugar metabolism (Dimlioglu *et al.*, 2015; Vijayakumari & Puthur, 2015). It plays, however, a signaling role in the plant, for which ALMT, an aluminium-activated malate transporter, is a receptor (Ramesh *et al.*, 2015). Polyamines, small polycationic molecules, play important roles in many physiological processes such as organogenesis and floral initiation. Spermine, a tetraamine polyamide, is ubiquitous in plants and can produce GABA as a result of oxidation pathways in the peroxisome. It is known that spermine, when applied exogenously to tobacco, can activate plant defense responses through MAPK cascades. It can also enhance endocytosis, which can increase uptake of sugars (Tiburcio *et al.*, 2014; Sagor *et al.*, 2015). BABA, another non-proteinogenic amino acid, can also prime plants against abiotic stress, but also protects plants against different pathogens, including *B. cinerea* and *P. parasitica*. Application of low amounts of BABA can induce the primed state without a major reduction in growth rate. However, higher doses affect growth and seed production (Conrath, 2009). In Arabidopsis, BABA potentiates a SA-dependent signaling response to restrict *B. cinerea* growth, independent of JA and ET (Zimmerli *et al.*, 2001), while in pea it causes an upregulation of phytoalexin production after infection with *Uromyces pisi* (Barilli *et al.*, 2015). Seed priming with BABA can be of great value in agriculture, as it enhances the plant's resistance to pathogens without supplementary growth reduction (Walters *et al.*, 2013). Other compounds, such as menadione sodium bisulphite (MSB), probenazole, and several SA analogs, have also been found to induce a primed state (Walters *et al.*, 2013; Borges *et al.*, 2014). Natural compounds like oligosaccharides, vitamins, and aromatic compounds tend to be tolerated better by the plant than synthetic ones. The lower toxicity and fewer concerns for human health make them more applicable in the field (Aranega-Bou *et al.*, 2014).

3.5. Plant defenses against biotic stress

After the induction of PTI or ETI, followed by downstream signaling pathways, a broad array of plant defenses can be upregulated to restrict further infection or wounding. Besides these inducible responses, the plant has several constitutive defenses. The first line of defense are the physical barriers, only, these can be breached by most pathogens. The second line of

defense is provided by constitutively available antimicrobial compounds. The diversity of these plant defenses is relatively limited in comparison to the diverse biotic stresses at hand (Cassels & Rafferty-McArdle, 2012; Rojas *et al.*, 2014).

3.5.1. Constitutive defenses

The two main physical barriers against pathogens are the cuticle and the CW. The cuticle is the external surface of the epidermis, which is hydrophobic due to the presence of cutin and waxes. Some pathogens use natural openings, such as stomata, hydathodes, or wounds to enter the host plant. However, most pathogens can penetrate the physical barriers, either by mechanical ruptures or secretion of hydrolytic enzymes, like cutinases and pectinases. Nevertheless, hydrolysis can stimulate plant defenses, since breakdown products of the cuticle and the CW can stimulate the immune system, as proposed by the damaged-self hypothesis (Chrisholm *et al.*, 2006; Chassot *et al.*, 2008; Bigeard *et al.*, 2015).

Apart from these physical barriers, plants possess an array of structurally diverse chemical compounds that can be toxic to the pathogen or the herbivore. The distribution of these chemicals throughout the plant is not random, but possibly in function of the fitness of the organs, or the risk of exposure to biotic stress. These predictions are confined in the optimal defense theory (ODT). However, these predictions are sometimes violated, as older leaves have higher defense levels in Arabidopsis. This phenomenon is better explained by the growth-differentiation balance hypothesis. Both models are based on the fact that defenses must be costly to produce, otherwise they would be present in high amounts throughout the entire plant (Barto & Cipollini, 2005; Radhika *et al.*, 2008). A possible downside to the ODT is the assumption that similar defenses with similar costs are used throughout the plant for the protection of different tissues (McCall & Fordyce, 2010).

Since defenses are costly for the plant and chemical defenses can be toxic to the plant itself, it may be preferred to induce their synthesis only during infection or wounding. However, this can be risky if the initial attack is severe (Wittstock & Gershenzon, 2002). Antimicrobial compounds that are constitutively present in the plant are called phytoanticipins. Saponins are glycosides with amphipathic characteristics and the ability to disrupt membranes. The structurally related glycoalkaloid α-tomatine is produced in tomato, acting as a potent antifungal compound, specifically interacting with sterols in membranes. Some fungi, like *B. cinerea*, are able to detoxify this secondary metabolite by sugar glycolysis (Van Baarlen *et al.*, 2007). A common strategy to reduce toxic effects for the plant is the storage of inactive precursor metabolites. One example is the glucosinolates, mainly present in the Brassicales. Infection causes a cellular rupture, thereby releasing hydrolyzing enzymes, myrosinases, that result in the release of the toxic isothiocyanates (Moore *et al.*, 2014). Cyanogenic glucosides are more widespread, but follow the same principle, as they

are inactive in the absence of biotic stress. After exposure, they are hydrolyzed, yielding hydrogen cyanide, a toxic compound that inhibits cellular respiration (Piasecka *et al.*, 2015).

3.5.2. Inducible defenses

When constitutive defenses are insufficient to block infection, the plant immune system will be triggered through PTI and/or ETI, and through downstream signaling lead to the induction of new defenses. This process causes major changes in metabolism. Blocking infection causes a strong energy demand, leading to an upregulation of energy-producing pathways, such as glycolysis and the pentose phosphate pathway. Amino acids can be redirected into energy-generating pathways, such as the TCA (tricarboxylic acid) cycle. Concerning nitrogen, the plant may actively mobilize it away from the infection site, thereby depriving the pathogen of this nutrient. However, nitrogen can also be directly involved in the defense response, as NO is a central player in the downstream signaling (Delledonne *et al.*, 1998; Scharte *et al.*, 2005; Bolton, 2009). The accumulation of certain amino acids may play an important defensive role, as higher proline levels are associated with the onset of HR (Qamar *et al.*, 2015). Photosynthesis, together with energy-storing pathways, such as starch and lipid metabolism, are downregulated during infection (Rojas *et al.*, 2014).

An improvement of the CW strength is usually observed, as lignin synthesis is upregulated during infection, with the involvement of ROS. Another well-characterized response is the deposition of callose at the site of infection, forming reinforced structures termed papillae (Malinovsky *et al.*, 2014). Callose is a β-1,3-glucan polymer, synthesized by callose synthases at the cell membrane. It is not only involved in biotic stress defenses, since it is produced during cytokinesis, after which it is quickly replaced in the newly formed CW by cellulose (Nedukha, 2015). GSL5 (Glucan Synthase-Like 5) is responsible for the formation of the papillae (Ellinger & Voigt, 2014). Callose production may be triggered by ROS, for example after recognition of flg22 (Hou *et al.*, 2013; Muthamilarasan & Prasad, 2013). SA seems also to be involved, as the direct application of SA to Arabidopsis induces callose deposition, leading to the closure of plasmodesmata (Wang *et al.*, 2013).

When ETI is activated, a strong antimicrobial mechanism is induced around the site of infection. This HR, first described over 100 years ago, forms a necrotic zone, surrounding the infection site and thereby halting the pathogen (Ghannam *et al.*, 2005). HR is a mechanism of PCD, showing some similarities to apoptosis in animals, such as cytoplasmic contortions by cytoskeletal rearrangements. A swelling of the mitochondria can be observed, as mitochondrial function is lost. Contrary to apoptosis, ATP generation is badly sustained during HR. Vacuolar processing enzymes seem to play an important role in the induction of this response. Other important players are ROS, with NADPH oxidases as a major source. Studies in tobacco have shown that NO and H_2O_2 play overlapping signaling roles (Mur *et al.*,

2008). While HR provides the plant with an important defense against biotrophs, necrotrophs seem to be more tolerant. Moreover, it has been shown that necrotrophs, such as *B. cinerea*, actually grow and spread faster when HR is induced (Govrin & Levine, 2000).

While many plants possess phytoanticipins, induction of secondary metabolite accumulation is widespread, hence these are termed phytoalexins. An illustrative example is the upregulation of PAL (Phenylalanine ammonia lyase), catalyzing an important regulatory point for the production of lignin, flavonoids, stilbenes and many more (Huang *et al.*, 2010). A well-characterized phytoalexin is camalexin, found in members of the Brassicaceae, including Arabidopsis. Its biosynthesis is regulated by MAPK pathways, sensitive to PAMP recognition. Camalexin function involves cell membrane damage and it provides resistance to both necrotrophs and hemibiotrophs. However, some fungi can transport camalexin out of their cells, or detoxify it through metabolization. Major phytoalexins in tobacco include scopoletin and capsidiol (Ahuja *et al.*, 2012). Another interesting class is the stilbenes, whose biosynthesis requires the presence of only one additional enzyme, stilbene synthase (Chong *et al.*, 2009).

Biotic stress not only causes the accumulation of secondary metabolites, but it also induces several antimicrobial proteins. These are called pathogenesis-related (PR) proteins. They can also be induced by abiotic stresses or priming and have been characterized in a broad range of plants. Originally, only five classes of PR proteins were known. However, many more have recently been discovered, although the exact function is not always known (Stintzi *et al.*, 1993; Van Baarlen *et al.*, 2007). In tobacco, 33 PR proteins have been described, predominantly localized in the leaves. PR-1 is the most abundant, making up to 2% of the leaf protein content. Several of these proteins are enzymes, such as β-1,3-glucanases (PR-2), chitinases (PR-3, PR-4, and PR-11) and peroxidase-like enzymes (PR-9). Other classes contain proteins without inherent enzymatic function, such as thionins (PR-13), osmotins (PR-5) and defensins (PR-12). Several classes have antimicrobial properties, and it is known that PR1-5 inhibit fungal growth (Sudisha *et al.*, 2012; Nawrot *et al.*, 2014). SA and JA/ET each induce a different set of PR proteins. For example, in Arabidopsis, expression of PR-1, PR-2 and PR-5 is primarily controlled by SA, while defensins and thionins are more readily induced by JA and ET (Thibaud *et al.*, 2004). Sugars, such as Glc and Suc, can also stimulate PR gene expression. PR protein induction thus provides one of many links between sugar metabolism and plant defense, which have led to the concept of sweet immunity (Bolouri Moghaddam *et al.*, 2012).

4. Sugar dynamics

One of the main characteristics ascribed to plants is the fact that they can converse light energy into carbon-containing carbohydrate molecules through the process called photosynthesis. This not only provides themselves with the means to use these sugars for their growth and differentiation, but also the many heterotrophic life forms, including bacteria, fungi, and animals. Energy captured during the photosynthetic process, involving photosystem I and II and the associated electron transport chain, is used to incorporate carbon from CO_2 into organic molecules, in the form of triose phosphates. These can be exported from the chloroplasts to the cytosol, where they can be transformed to organic acids in glycolysis to feed the Krebs cycle, or transformed into sugar phosphates for Suc synthesis and subsequent export from the leaf (Rolland *et al.*, 2006). Through the intermediate fructose-1,6-biphosphate, several important hexoses are formed. Glc-6-phosphate (G6P) can be directed to glycolysis via hexokinase (HXK), a 38-68 kDa enzyme. Alternatively, it can feed the pentose phosphate pathway for the biosynthesis of nucleotides, aromatic amino acids, and phenylpropanoids, or provide a source for UDP- and GDP-sugars. UDP-Glc is important in the biosynthesis of CW polysaccharides such as cellulose and glycosylation reactions of hormones or metabolites. Moreover, UDP-Glc and G6P are involved in the formation of trehalose-6-phosphate (T6P), an important sugar signaling molecule for plant growth (Claeyssen & Rivoal, 2007; Granot *et al.*, 2013; Ruan, 2014). G6P can also be redirected to the production of sugar alcohols, such as myo-inositol, which has important signaling and regulatory functions (Valluru & Van den Ende, 2011). Excess of energy acquired during the day will be stored in the form of starch when assimilation exceeds the Suc demand of the plant, partly in the form of long storage starch in amyloplasts, partly as transitory starch. The latter will be used at night, when there is a negative carbon balance (Osuna *et al.*, 2007; Ramon *et al.*, 2007). The degradation products Glc and maltose can be transported to the cytosol to enter glycolysis through HXK. Maltose cleavage by a transglucosidase yields a second Glc molecule (Claeyssen & Rivoal, 2007).

UDP-Glc plays an important role in Suc biosynthesis. Suc is the main transported sugar molecule in the plant kingdom. The Suc transport is important for the allocation of energy from sources to sinks. Sources are the photosynthetically active organs that provide energy for their own metabolism, while allocating the rest to sink organs or long-term storage. The phloem, consisting of sieve elements and companion cells, is central in this transport (Lalonde *et al.*, 1999). Suc synthesis in sources can be achieved by Suc-phosphate synthase (SPS) followed by Suc-phosphate phosphatase, while its degradation in the sinks depends either on SuSy or on invertases (Claeyssen & Rivoal, 2007; Ruan, 2014). G6P and inorganic phosphate can respectively activate or inhibit SPS allosterically, while phosphorylation on conserved serine residues provides an additional layer of regulation, integrating sugar

availability and osmotic stress (Winter & Huber, 2000). SuSy is localized in the cytosol, either free or attached to membranes, and catalyzes Suc degradation, with the production of UDP-Glc (Claeyssen & Rivoal, 2007; Ruan, 2014). SuSy activity is regulated by phosphorylation/ dephosphorylation, according to the level of available sugars in the plant (Koch, 2004). On the other hand, invertases catalyze an irreversible Suc hydrolysis, yielding Glc and Fru. Phloem loading from sources usually involves Suc transporters, using the proton gradient established by H^+/ATPases, but in some species, symplastic loading can occur (Lemoine *et al.*, 2013). SWEETs (Sugars Will be Eventually Effluxed Transporters) are more recently discovered and constitute another class of transporters involved in phloem loading (Ruan, 2014). Phloem unloading may involve sucrose transporters (SUTs) or import by monosaccharide transporters (MSTs), after being cleaved to Glc and Fru by cell wall invertases (CWIs) (Claeyssen & Rivoal, 2007). CWI is particularly important when no symplastic route is present, in other words, no plasmodesmata are existent for the Suc transport, or when at least some Suc arrives apoplastically (Koch, 2004). Suc can be translocated to the vacuole for storage, where it can be degraded by vacuolar invertases (VIs), an important source for hexoses during tissue expansion (Rolland *et al.*, 2006). Sink strength depends on the ability to enact a benign hydrostatic pressure gradient in the phloem to drive water flow (Ruan *et al.*, 2010; Lemoine *et al.*, 2013; Ruan, 2014). Phloem rate can also be modified by several phytohormones. It is known that auxin and CK can increase flow rate, while ABA downregulates flow rate (Lalonde *et al.*, 1999). During plant development, a progression is seen in the change from sink to source. Sink initiation and expansion involves the use of invertases for Suc cleavage, which can maintain a high hexose/Suc ratio, while transition to source dynamics, including storage and maturation, causes a switch to SuSy activity and a possible decline in hexose/Suc ratio (Koch, 2004; Claeyssen & Rivoal, 2007; Ruan, 2014).

Although Suc is the prevalent transport sugar, several plants also utilize different carbohydrates, such as polyols and RFOs. RFO-producing plants, such as legumes, grapes, and grains, use these molecules as main transport compounds. According to the polymer trap model, Suc diffuses to the bundle sheath cells via plasmodesmata where they are converted into RFOs, which due to their large size cannot diffuse back through plasmodesmata and are then transported into the phloem (Turgeon & Medville, 2004). Besides their role in transport, they can scavenge ROS and function as reserve compounds, for example in seeds. They can be accumulated to high concentrations in some plant species and are osmotically more flexible as starch, since their DP can readily change. This characteristic is also shared with another class of Suc-extended sugars, termed fructans (Valluru & Van den Ende, 2011; Van den Ende, 2013; Elsayed *et al.*, 2014). Fructans are present in about 15% of angiosperms, synthesized by linking a Fru to a hydroxyl group of

Suc. From the basic trisaccharides 1-kestose, 6-kestose and neokestose, fructosyl transferases elongate the chain by adding fructofuranosyl units via a β-2,1- or β-2,6-glycosidic bond. Inulin-type fructans consist of β-2,1-linkages, while levan-type representatives contain β-2,6-bonds. Levans, together with graminan-type fructans containing both glyosidic linkages, are found in grasses, while inulins are present in some dicot species. More complex fructans have been found in some species, such as oat, and these are termed neo-inulins and neo-levans. Fructan degradation involves removal of terminal Fru moieties by fructan exohydrolases (FEHs). Fructans have several roles besides carbon storage, including cold stress resistance through membrane stabilization and ROS scavenging. Possibly, plants have evolved these diverse sugars to cope with stressful environments, as they have a different solubility and membrane integration properties, in comparison to starch. Other differences with starch include ongoing biosynthesis at low temperatures and faster degradation due to the necessity of less enzymatic steps (Van den Ende *et al.*, 2004; Valluru & Van den Ende, 2008; Van den Ende, 2013; Tarkowski & Van den Ende, 2015).

4.1. Sugar signaling

As autotrophic organisms, sugars play a prominent role in their lifecycle. Presence or absence of abundant carbon sources thus has a major impact on their growth and differentiation. To regulate growth patterns according to the sugars at hand, signaling pathways involving sugars themselves play a central role. When less carbon is available and sugar levels decline, growth is reduced, while catabolism of organic acids, lipids, and proteins is upregulated to provide the necessary energy. Only basic metabolism is sustained under these circumstances, with an induction of photosynthesis. When sugars are once again abundant, processes such as glycolysis and nitrogen assimilation are induced after 3 h, while amino acid synthesis has an even longer delay. To minimize carbon deprivation, plants must be able to sense and respond to changing sugar levels. In this role sugars themselves can be involved as signaling molecules (Roitsch, 1999; Gibson, 2005; Rolland *et al.*, 2006; Osuna *et al.*, 2007).

T6P, present in low concentrations in plants, has an important role in sugar status signaling, thereby controlling growth and development. Its synthesis involves trehalose phosphate synthase, using G6P and UDP-Glc to yield T6P. Conversion to trehalose is managed by T6P phosphatase (Rolland *et al.*, 2006). T6P synthase senses Suc availability, thereby producing T6P for an upregulation of growth, starch accumulation and CW biosynthesis (Avonce *et al.*, 2004; Lunn *et al.*, 2006). The main network that responds to the energy status and nutrient availability involves SNF1-related kinase 1 (SnRK1), homologous to the animal AMP-activated protein kinase and the yeast sucrose non-fermenting 1 (SNF1),

and target of rapamycin (TOR) kinase. SnRK1 kinase activity is repressed by Glc, thereby inhibiting growth, while TOR kinase is activated by Glc, leading to growth stimulation (Lastdrager *et al.*, 2014; Sheen, 2014). Glc-induced activation of TOR kinases provides the plant with a central modulator linking photosynthetic rate to growth through transcriptional regulation (Xiong *et al.*, 2013). SnRKs are serine/threonine kinases, of which 3 different classes have been described in plants. SnRK2 and SnRK3 subfamilies are known to be involved in osmotic stress and ABA signaling (Lu *et al.*, 2007). SnRK1 functions in a trimeric complex consisting of a catalytic subunit and 2 regulatory subunits. The catalytic subunit is highly conserved and known as KIN10/11 in Arabidopsis (Ramon *et al.*, 2007). Contrary to their animal and yeast counterparts, they are not allosterically regulated by AMP/ATP (Ghillebert *et al.*, 2011). SnRK1 regulates the induction of genes involved in catabolic processes and the repression of biosynthetic pathways in conditions of low sugar availability. T6P is known to inhibit SnRK1 activity. Vice versa, SnRK1 regulates T6P production through bZIP11 TF, which is closely connected to SnRK1 gene expression regulation (Cramer *et al.*, 2011; Delatte *et al.*, 2011; Valluru & Van den Ende, 2011).

Besides TOR and SnRK kinases, the second main branch in Glc signaling is HXK, which phosphorylates not only Glc, but also Fru, mannose, and galactose. Besides this catalytic function, it also acts as a sugar sensor in plants, analogous to other organisms (Jang *et al.*, 1997; Moore *et al.*, 2003). HXK activity is mainly associated with the mitochondria and overexpression of HXK1 in Arabidopsis causes inhibition of seedling development and expression of photosynthetic genes under high Glc conditions (Granot *et al.*, 2013). There is, however, redundancy in the plant genome, as multiple isoforms of this enzyme are present, differing in subcellular localization and kinetic properties (Claeyssen & Rivoal, 2007). Binding of sugars to HXK causes a conformational change, thereby allowing protein-protein interactions for the activation of a downstream signaling cascade (Sheen *et al.*, 1999). Sugar signaling pathways have long been classified according to the involvement of HXK, discerning a HXK1-dependent pathway, a glycolysis-dependent pathway involving HXK1 catalytic activity, and HXK1-independent pathways. HXK1-dependent pathways involve repression of photosynthetic gene expression and a downregulation of KIN10/11 from SnRK1, while Glc-induced upregulation of PAL and CHS and induction of TOR kinase are known to be HXK1-independent (Price *et al.*, 2003; Price *et al.*, 2004; Rolland *et al.*, 2006; Xiong *et al.*, 2013; Sheen, 2014). Interestingly, it seems only Glc that is transported into the cell triggers these pathways (Smeekens, 1998; Kunz *et al.*, 2015).

Besides Glc, other sugars can play key roles in signaling. For example, a Suc-specific regulatory pathway has been proposed, causing repression of photosynthetic genes and induction of sink-specific enzymes (Ehness *et al.*, 1997; Sinha *et al.*, 2002). However, Suc-induced signaling can also result from its breakdown products Glc and Fru, which may act as

the direct signals, rather than Suc itself (Jang & Sheen, 1997). Nevertheless, the specific players in this possible signaling network still need to be elucidated. Ca^{2+} may be one of the main upstream signaling components to induce Suc-mediated signaling (Furuichi *et al.*, 2001). However, in animals, it is known to be downstream of G6P, which regulates the accumulation in the cytosol (Cole *et al.*, 2012). Sugar signaling may regulate a wide array of TFs in the plant, including ABI4, EIN3, and bZIP11, the latter of which can be directly inhibited by Suc (Hanson *et al.*, 2008). Since ABI4 and EIN3 are involved in phytohormone signaling pathways, important crosstalk can be expected with sugar signaling cascades, and the general strategies to dissect these pathways usually involved the generation of sugar-responsive mutants (Gibson, 2000; Arenas-Huertero *et al.*, 2000). The best-characterized crosstalk involves the participation of ABA in Glc signaling, as Glc induces expression of several ABA signaling components, including ABI3, ABI4, and ABI5. In this way, both ABA and Glc can partly induce gene expression of common genes, such as LEA protein encoding genes. LEA proteins are important in the response to abiotic stresses, especially osmotic stresses, by preventing protein aggregations (León & Sheen, 2003; Dekkers *et al.*, 2008). Crosstalk with ABA regulates seed development, while the interplay of Glc signaling with auxin, CK and ET inhibits seedling development (Finkelstein & Gibson, 2002; Ramon *et al.*, 2007). More recently, it has also been discovered that sugars are the initial regulators to control apical dominance, contrary to auxin, which was believed for a long time to be the primary signal (Mason *et al.*, 2014, Van den Ende, 2014). New research also places Suc as a positive regulator of DELLA protein stability, thereby enhancing anthocyanin biosynthesis (Li *et al.*, 2014; Ljung *et al.*, 2015). DELLA proteins are central repressors of GA pathways, thereby inhibiting plant growth processes. In the last decade, a more prominent role has been proposed, as DELLA may be a primary converging point for several hormone pathways, including GA, SA, and JA, thereby regulating growth and defense trade-offs (De Bruyne *et al*., 2014). Moreover, Suc not only plays a role as a substrate in fructan biosynthesis, but also acts as a signal, determining fructan accumulation, for example, through induction of fructosyltransferases, as discovered in barley (Valluru & Van den Ende, 2011; Van den Ende & El Esawe, 2014).

Sugars can provide the plant with another defensive strategy against abiotic stresses, as has already been pointed out repeatedly above. However, it seems that sugars may also aid the plant in the protection against biotic stress, through stimulation of the plant immune system. Such observations have led to the establishment of the sweet immunity concept (Bolouri Moghaddam & Van den Ende, 2013).

4.2. Sugars in plant immunity

A plethora of observations derived from different plant-pathogen interactions strongly supports a critical role for soluble sugars in the context of plant immunity. In a way, they can support the pathogen's proliferation inside the plant, but these sugars can be used by the plant as well for the biosynthesis of both structural and chemical defenses. Several sugars even have a direct immune-stimulating role (Morkunas & Ratajczak, 2014; Trouvelot *et al.*, 2014). In tobacco, application of Glc, Fru, or Suc leads to accumulation of PR proteins. Application of Suc in lupin leads to increased resistance against *Fusarium oxysporum*, and invertases, as key modulators of sugar metabolism, probably occupy a central role during infections. Suc/hexose ratios mediate the induction of PAL and anthocyanin production in tobacco. Induction of some PR proteins by soluble sugars has been observed both in Arabidopsis and tobacco, with the involvement of SA signaling (Herbers *et al.*, 1996; Couée *et al.*, 2006; Rojas *et al.* 2014). Other small sugars, such as raffinose, and the disaccharides trehalose and galactinol, accumulate to high concentrations in syncytia during nematode infections. Disturbances in source-sink equilibria during biotic stress is known to stimulate raffinose synthesis. Even D-allose, a rare sugar present in some plant species, has been observed to induce resistance to *Xanthomonas oryzae* in rice, through induction of ROS and PR proteins (Bolouri Moghaddam & Van den Ende, 2012; Kano *et al.*, 2013; Tauzin & Giardina, 2014).

It can be deduced that sugars may have a dual, but controverting role during pathogen infection. Firstly, sugars are not only important to the plant, they can be a potential carbon and energy source for the pathogen. Pathogens often possess sucrolytic enzymes such as invertases, since Glc appears to be the main acquired carbohydrate (Berger *et al.*, 2007). As this creates an additional sink that will compete with other sinks in the plant, the central role of invertases is unambiguous (Bolton, 2009; Lemoine *et al.*, 2013). CWIs of both the plant and the pathogen increase Suc degradation at the site of infection and may cause a sugar deficiency, which can result in decreased crop yields (Morkunas & Ratajczak, 2014). Pathogens also affect sugar transporters in the plant to enhance sugar supply and a novel class of transporters, SWEETs, are often targeted. 17 SWEETs have been identified in Arabidopsis, used for transport of neutral sugars, such as Suc, Glc, and Fru. Secondly, while the role of host sugars for the pathogen is clear, changes in sugar dynamics can trigger plant defense responses, as has been illustrated in the previous paragraph (Lastdrager *et al.*, 2014; Ruan, 2014; Chen *et al.*, 2015).

Sweet immunity lays the focus on sweet, endogenous sugars that can play an important role in innate immunity. Suc may potentially work as a priming molecule for plant defenses, due to sudden increases in apoplastic concentrations during infection, freezing and other stresses that may cause cell rupture. As Suc is readily hydrolyzed by CWIs, the produced

sweet signals may boost immune responses of the plant. In this fashion, CWIs can be considered PR proteins that can be induced by endogenous sugars, as by microbial elicitors and JA. However, the pathogen may also profit from these sweets, as mentioned earlier. Several research papers suggest that a possible sweet immunity signaling uses downstream MAPK signaling pathways, corresponding to the cascades used in classical immunity (Bolouri Moghaddam & Van den Ende, 2013; Bolouri Moghaddam *et al.*, 2015). From this perspective, exogenous application of sweets to plants may prime the immune system for future infections. The β-1,3-glucan laminarin from brown algae stimulates the expression of PAL, chitinases, SA and PR proteins in tobacco, as well as the production of the phytoalexin resveratrol in grapevine (Aziz *et al.*, 2003). Also, components of fungal CWs, such as chitosan, the deacetylated derivative of chitin, elicits defense responses in plants. Exogenous application to plants has been shown to control fungal diseases in multiple crop species (Sharp, 2013; Iriti & Varoni, 2015). Chitosan itself also harbors antifungal and antioxidant properties (Amborabé *et al.*, 2008; El Hadrami *et al.*, 2010). OGs, as described by the damaged-self hypothesis, can signal the presence of biotic stresses to the plant. Their DP, as well as methyl esterification and acetylation, are crucial for the induction of defense responses. OGs with a DP between 7 and 10 are usually the most active oligomers (Trouvelot *et al.*, 2014). In Arabidopsis, OG-triggered immunity involves CDPKs to induce defense responses against *B. cinerea*, including ET biosynthesis (Gravino *et al.*, 2015). Exogenous spraying with fructans may also prime the plant's immune system. In tomato, an induction of PR1-3 and phenolic compound biosynthesis has been observed after priming (Wang *et al.*, 2009). Application of burdock fructooligosaccharides (BFOs) to tobacco induced expression of defense genes after inoculation with *B. cinerea* (Van den Ende & El Esawe, 2014 and references therein). BFO application induces both NO and ROS production, triggers PR protein accumulation and increases SA levels through upregulation of PAL and ICS. The latter is in strong discordance with the main line of thought, as JA is usually involved in defense against necrotrophs (Guo *et al.*, 2013).

4.2.1. Invertases

Invertases are classified into two classes based on their subcellular localization and pH optimum (Valluru & Van den Ende, 2011). The first class contains the acid invertases, belonging to the GH32 family. Both CWI and VI have an acidic pH optimum, with CWI in the apoplast and VI localized in the vacuole. They have a high sequence homology, similar biochemical properties and are both glycoproteins. CWIs have a high isoelectric point and are ionically bound to the CW. Nevertheless, in maize 2 CWIs have been characterized to be unbound from the CW in the apoplast (Kim *et al.*, 2004). They play a central role in Suc partitioning, regulation of seed development, and most importantly in immunity, as they

function in response to pathogen infection. VIs are involved in several metabolic processes, such as osmoregulation, control of sugar composition in storage organs and fruits, and response to several abiotic stresses. The second class of invertases is alkaline/neutral invertases (A/NIs), belonging to the GH100 family. Their localization is more diverse, as they are found in the cytosol, mitochondria, chloroplasts and even nuclei. Due to their role as Suc/hexose ratio regulators present in multiple cell compartments, invertases are involved in growth and development. During infection in tobacco and Arabidopsis, an increase in activity is measured, correlated to the increase in CWI activity (Koch, 2004; Roitsch & González, 2014; Tauzin & Giardina, 2014). In wheat, A/NIs may act as a negative regulator of disease resistance to *Puccinia striiformis* by increasing cytoplasmic hexose concentrations (Liu *et al.*, 2015). Both CWI and VI are stable due to their glycan chains, thus, they require inhibitor proteins to block their activity. These inhibitors, with a molecular mass of 15 to 23 kDa are localized in the vacuole or the CW, where they form a stable complex with their respective invertases. An increase in CWI activity by 45-60% was observed after silencing of invertase inhibitor 1, showing that a high proportion of this invertase is post-translationally regulated. During pathogen attack, the inhibitor will be repressed, leading to an upregulation of CWI activity. For A/NIs the presence of these inhibitors is unknown (Bonfig *et al.*, 2010; Ruan *et al.*, 2010; Ruan, 2014). Their stability *in vivo* may be increased owing to interaction with other proteins, such as 14-3-3 proteins and PIP5K9 (Vargas & Salerno, 2010; Gao *et al.*, 2014).

5. *Botrytis cinerea* and the interaction with the host plant

Botrytis species are commonly known as gray moulds, with some members being rather saprophytic, others truly pathogenic. The genus belongs to the Sclerotiniaceae within the Ascomycetes, which are characterized by septate mycelia and asci bearing reproductive spores, usually eight. A common trait of this family is the formation of sclerotia, structures produced in the vegetative phase that enable the fungi to survive under restrictive conditions (Horst, 2001). All *Botrytis* species are pathogens, but only *B. cinerea* exhibits a broad host range of over 200 dicot species. Its biogeographical distribution ranges from tropical to cold areas, dependent on the presence of its natural hosts, including many crop species. Others are more specific, such as *B. tulipae*, which infects tulips (Elad *et al.*, 2007; Van Baarlen *et al.*, 2007; Leyronas *et al.*, 2015). The lifecycle of *B. cinerea* consists of both sexual and asexual reproductions. Macroconidia germinate on host substratum and these conidia will differentiate in one or two germ tubes for the penetration. Germ tube protrusion is turgor-driven and simultaneously a mucilaginous sheath of polysaccharides is formed. Hence, a primary lesion is observed, followed by a second invasive growth phase. Hereafter, the soft rot lesion can form macroconidia asexually, or sexual reproduction may yield microconidia and sclerotia. In the latter case, asci will be formed containing new ascospores that will

develop into macroconidia. One such infection cycle, from adhesion to the host until conidiation, may be completed within 3-5 d (Tenberge, 2007; Schumacher & Tudzynski, 2012). It is clear that the conidia are the main dispersal unit of *B. cinerea*. They are released from the conidiophore (ascus) in a mechanical way, caused by changes in relative humidity (Holz *et al.*, 2007). *B. cinerea* also forms appressoria, however, physical pressure alone is not effective since there is no septum sealing off the germ tube. Consequently, this fungus relies primarily on enzymes for penetration (Van Kan, 2006). The genome of *B. cinerea* contains several hemicellulose-modifying enzymes, polygalacturonases, and glucosidases (Blanco-Ulate *et al.*, 2014).

The fungus also produces several plant hormones, such as ABA and CK. ET enhances spore germination and high auxin levels have been observed in infected plants (Sharon *et al.*, 2007; Gimenez-Ibanez & Solano, 2013). Infection of tomato plants showed the induction of the SA pathway, thereby contradicting the SA-JA discrepancy for defense against biotrophs and necrotrophs respectively. Exopolysaccharides from Botrytis induce NPR1 activation and repression of JA-dependent gene expression, thus making the plant less resistant to the infection (El Oirdi *et al.*, 2011). In Arabidopsis, however, it has been shown that SA confers resistance to Botrytis infection (Ferrari *et al.*, 2003). Also, effects of SA on *B. cinerea* were assessed using a proteomics approach, showing an inhibitory effect on the fungus that may be connected to ROS accumulation, CW remodelling, and several other metabolic processes. This fungus produces components that are toxic to the host plant, such as botrydial, a sesquiterpene that induces chlorosis and cell disintegration, and botcinic acid in the more aggressive strains (Choquer *et al.*, 2007, Rossi *et al.*, 2011). Botrydial induces HR in both Arabidopsis and tobacco plants and initiates SA- and JA-dependent pathways. Fungal oxalic acid is involved in pathogenesis as well, since it inhibits plant defensive enzymes and induces PCD (Rossi *et al.*, 2011; Nakajima & Akutsu, 2013).

In general, young plants are less susceptible to infection than older ones (Dik & Wubben, 2007). In tobacco, resistance is cultivar-dependent. *N. tabacum* cv. Petit Havana is a resistant cultivar that accumulates high amounts of PR proteins and the secondary metabolite scopoletin, as compared to *N. tabacum* cv. Xanthi, a more susceptible cultivar (El Oirdi *et al.*, 2010; El Oirdi *et al.*, 2011). Fungal infection involves a reorganization of plant sugar metabolism. Powdery mildews, biotrophic pathogens, increase Glc uptake rate in infected tissues to 140%, and an increase in invertase activity is also observed (Sutton *et al.*, 2007). Botrytis, as a necrotroph, also contributes to higher CWI activity during grapevine infection. It has several hexose uptake systems to acquire hexoses from the plant (Lemoine *et al.*, 2013; Lemonnier *et al.*, 2014). Systemic sugar dynamics are changed during infection. While photosynthesis is downregulated during infection, it has recently been observed that this process is induced in inflorescences (Vatsa-Portugal *et al.*, 2015).

6. Aim

The aim of this Master's Thesis is to investigate the possibility of priming plant defenses with soluble sugars, in the research context of the emerging sweet immunity concept. Utilizing the knowledge from previous experiences performed in our research group, I will try to further improve sugar priming and infection techniques in the *N. tabacum* - *B. cinerea* pathosystem. This host plant was chosen because of the abundant literature available concerning plant pathology, especially regarding links to sugar dynamics. It has a relatively short growth period with high biomass production as well, thus making it an excellent choice for research purposes. As the interacting pathogen, *B. cinerea* was chosen because of the huge body of knowledge already available, as it is considered a model necrotrophic pathogen. It has the ability to infect tobacco as well as a number of other model plants and crops. This allows me to compare my results with those already present in literature. More specifically, the B05.10 strain will be used, proven to be highly infectious on the *N. tabacum* cv. Xanthi that is used in our lab.

Picking up were previous year's master thesis left off, I will continue working with the most promising sugar compounds in several priming and infection experiments, focusing on small sugars as well as fructans. Finding the optimal priming and infection duration will be part of these experiments. The second part of this thesis will focus on measuring essential parameters related to sugar metabolism in primed and infected tissues, more specifically sugar levels, starch levels and invertase activities. This includes refining some techniques for use on the employed pathosystem.

Accessory experiments will be performed to get a clearer view of the processes involved. These include the measurements of sugar content in leaves of an entire plant, useful to collect information on Suc/hexose ratios and sugar levels along the leaf gradient. Studying the fate of fructans inside the leaves after BFO priming may allow us to exclude fructan degradation. During the course of this thesis, we will try to incorporate some others points of interest within the research group into our sweet immunity investigation. The role of spermine, a polyamine, will be tested in combination with sugar priming. Also, in an attempt to gather some extra information, we have acquired *N. attenuata* plants with a knocked out CWI inhibitor gene. We will perform infection experiments on these mutants as well as sugar measurements, in light of investigating the importance of invertase enzymes prior to and during the interaction between tobacco and *B. cinerea*.

As such, we hope to establish solid proof for the sweet immunity concept, where application of sugars primes the plant's immune system, making them more resistant to prospective pathogen manifestation.

Materials & Methods

1. Biological material

1.1. Nicotiana species

The *N. tabacum* cv. Xanthi plants, kindly provided to our lab by Dr. Evelien De Waele (U Ghent), were grown under controlled conditions in the greenhouse, subjected to a fixed light/dark cycle (14h/10h) at 20±4°C. The soil contained the following NPK (Nitrogen, Phosphorus and Potassium) composition: 14-16-18 (in kg/m³). Seeds were sown in sowing plates, spread out in a homogeneous fashion. To optimize the germinating conditions the plates were covered with a glass panel, thus creating a humid environment. After approximately one week, when seeds had germinated and grown to a 2-leaf stage, the glass panel was removed. At the 4-leaf stage, the necessary amount of plants was selected and transferred to larger pots (15 cm diameter). Plants were watered 2 times every week, providing the plants with adequate water supply without creating exceedingly high humidity, as this might promote growth of fungi or mosses. 8 to 9 weeks after sowing, when in the 16^{th}-17^{th} leaf stage, plants of the same size were used for priming experiments.

N. attenuata plants were a kind gift of Prof. Ian T. Baldwin (Max-Planck Institute, Jena, Germany) and were grown as described in Krügel *et al.* (2002). After sterilization in 1.6% sodium hypochlorite, seeds were incubated in liquid smoke, complemented with GA3. Plants were sown in petri dishes containing B5 Gamborg medium with phytoagar (16h/8h light/dark cycle; 26/24°C), transferred in soil after approximately one week and cultured in the greenhouse.

1.2. Botrytis cinerea B05.10 strain

The fungus was grown in petri dishes on PDA (potato dextrose agar) medium. A patch of mycelium from the mother plate was transferred to these new plates and grown in a growth chamber under fixed conditions (14h/10h light/dark cycle; 24-16°C) for at least 2 weeks. Prior to the infection experiments, fungal spores were harvested from the plates using a 0.0001% Tween 20 solution. The suspension was then washed through a nylon membrane to filter out remaining pieces of mycelium. Spores were pregerminated in PDB (potato dextrose broth) medium, supplemented with ca. 10 mM of KH_2PO_4 (potassium dihydrogen phosphate) for approximately 2 h, before performing the infection. A final concentration of 1-2 x 10^5 spores/mL, calculated with the help of a Toma counting chamber and an optical microscope, was used.

1.3. Priming and subsequent infection experiments

8 to 9 weeks after sowing, when in the 16th-17th leaf stage, plants of the same size were selected for the priming experiments. For each plant, 3 source leaves were picked. Priming solutions were applied to these leaves by spraying homogeneously over the adaxial side of the leaves. Approximately 1 mL of solution was applied on each leaf by shortly spraying a few times across the leaf surface. Plants primed with one treatment were spatially separated from the other treatments to prevent direct contact. All priming solutions were prepared with 0.001 % Tween 20 solution. For the preparation of the hydrolyzed BFOs, a resin with immobilized FEH enzymes was added to a BFO solution (5 g/L) overnight to degrade all fructan content. The complete hydrolysis of the polymers was then verified through HPAEC-PAD (High-Performance Anion-Exchange Chromatograph with Pulsed Amperometric Detection) analysis.

In the infection experiment, primed leaves were cut off at the base of the leaf blade. Part of these leaves was submerged in liquid nitrogen and stored at -80°C. These were used for sugar and enzyme assays. The remainder of the leaves were placed on wet kitchen paper in petri dishes. Leaves were inoculated with 4 spots of 5 µL spore solution (1-2 x 10^5 spores/mL), after which the plates were sealed off with parafilm M®. The plates were placed in an incubator at 18°C in 'dusk-like' light conditions.

For disease scoring, lesion surfaces were measured using ImageJ 1.4.3.67 software (Abràmoff *et al.*, 2004) and converted to cm² values by using a 1 cm² reference. Using the data from 6 dpi, lesions were categorized into 4 different groups: 0 – 0.2 cm², 0.2 – 1 cm², 1 – 2 cm² and 2 - 5 cm² (see addendum Figure 13). An extra category (> 5 cm²) was added for lesions 10 dpi. This grouping was chosen starting from the drop size of the spore solution (± 0.1 cm²) applied during infection. For lesions larger than 1 cm² the distribution of the data was analyzed and category borders were determined based on these results.

2. Sugar and enzyme assays

Leaf material was stored at -80°C. Leaves were grinded in liquid nitrogen and 100 mg of grinded material was used for subsequent assays. After the assays, samples were loaded into a HPAEC-PAD for analysis, or stored at -20°C.

2.1. Sugar assay

Sugar extraction and measurement was performed as described in Vergauwen *et al.* (2000). Ultrapure water was added to the material (grinded in liquid N_2) in a 1:6 ratio and boiled for 15 min at 100°C to extract sugars and inactivate all metabolic enzymes present. After centrifugation (10 min at 14.000 rpm), 200 µL of the supernatant was applied to a mixed bed ion exchange column (Dowex® Ac^- and H^+ resins). 6 additional column volumes of ultrapure water were applied afterwards. 50 µL of the supernatant was diluted in 50 µL of 20 µM

mannitol solution (1:2) after an additional round of centrifugation (5 min at 14.000 rpm). Samples were loaded in a HPAEC-PAD and concentrations of Glc, Fru, and Suc were measured with the help of an external standard.

2.2. Starch assay

Ethanol (80% v/v) was added to the grinded material in a 1:6 ratio, boiled for 5 min at 80°C and subsequently centrifuged (5 min at 14.000 rpm). The supernatant was removed, after which this process was repeated an additional 2 times. The remaining pellet was then briefly boiled at 85°C to remove the remaining ethanol. This extraction removes the small sugars, while starch remains inside the pellet.

A mastermix was prepared containing 25 µL α-amylase (10 mg/mL) and 250 µL amyloglucosidase (10 mg/mL), diluted in 600 µL NaAc (50 mM, pH 4.5-5) to a total volume of 875 µL. Ultrapure water was added to the pellet after ethanol extraction in a 1:6 ratio, and boiled for 10 min at 100°C. After centrifugation (10 min at 14.000 rpm), 200 µL of supernatant was added to a reaction tube containing 15 µL NaAc (1M, pH 4.5-5) and 35 µL mastermix. Reaction tubes were placed in a hot water bath at 30°C. At different time points (0, 3 h and >12 h) 10 µL was diluted with 90 µL 20 µM mannitol (1:10) and boiled for 5 min at 100°C. Samples were loaded in a HPAEC-PAD and Glc concentration was measured. This protocol was adjusted from Smith & Zeeman (2006).

2.3. Invertase activity assay

Grinded material was crushed in a mortar with extraction buffer (50 mM NaAc, pH 4.5-5; 10 mM $NaHSO_3$; 2 mM β-mercaptoethanol; 0.05% v/v polyclar; 0.02% v/v sodium azide) in a 1:4 ratio. A small amount of phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, was added to inhibit protease activity. The cell lysate was centrifuged (10 min at 14.000 rpm), after which both fractions were separated.

The CWI, contained in the pellet, was washed 5 times with 600 µL NaAc (50 mM, pH 4.5-5), removing the supernatant after centrifugation. The pellet was resuspended in 350 µL NaAc (50 mM, pH 4.5-5), starting the reaction by adding 40 µL Suc (0.5 M). Samples were incubated at 30°C and 800 rpm in an Eppendorf Thermomixer Comfort®.

Ammonium sulfate ($(NH_4)_2SO_4$) was added to the supernatant, containing VI, for an 80% saturation. After precipitation and centrifugation (8 min at 14.000 rpm), the supernatant was removed and the pellet resuspended with 300 µL 80% $(NH_4)_2SO_4$ for an additional round of centrifugation. Subsequently, the pellet was resuspended with 100 µL NaAc (50 mM, pH 4.5-5) and 30 µL was transferred to a reaction tube with 60 µL NaAc (50 mM, pH 4.5-5) and 10 µL Suc (0.5 M). Samples were incubated at 30°C in a hot water bath.

At different time points (0 min, 30 min and 90 min) 10 µL was diluted in 90 µL 20 µM mannitol (1:10) and boiled for 5 min at 90°C. Samples were loaded into a HPAEC-PAD and Fru concentration was measured.

2.4. HPAEC-PAD

A High-Performance Anion-Exchange Chromatograph with Pulsed Amperometric Detection (Dionex ICS 3000) was used to measure sample sugar content. Samples (diluted in 20 µM mannitol) were transferred to glass vials and loaded into the well plate of the HPAEC-PAD. Through the addition of 90 mM NaOH, sugars in the sample were changed to an anionic state, then passing through an anion exchange column. Detection required an oxidation of the sugars, leading to a current measured by the detector. Concentrations of Glc, Fru, and Suc (GFS) were calculated based on a GFS standard (10 µM) ran together with the samples.

3. Experiment setup

3.1. Experiment 1

3 source leaves of each *N. tabacum* cv. Xanthi plant were primed through spray application on the adaxial side.

The following priming conditions were used:

- Negative control:
  - Ultrapure water
- Osmotic control:
  - Sorbitol (50 mM)
- Positive control:
  - OGs (1 g/L)
  - GABA (10 mM)
- Sugars:
  - Glucose (50 mM)
  - Allose (50 mM)
  - Fructose (50 mM)
  - BFOs (5 g/L)
  - Hydrolyzed BFOs (5 g/L)

A priming duration of 3 d was applied, after which the leaves were cut off at the base of the leaf blade. One leaf from each plant was treated with liquid nitrogen and stored at -80°C for molecular analyses. Both other leaves were used for infection (4 infection spots per leaf) and lesions were scored after 3 d of infection. Since the infection experiment failed molecular analyses were not performed for this experiment.

For one tobacco plant, leaves from base to apex were cut off and grinded in liquid nitrogen. Small sugar content was measured, thus providing a gradient across the entire length of the stem, characterizing a source-to-sink transition.

3.2. Experiment 2

First, a small scale infection experiment was set up to validate the performance of the pathogen on the tobacco leaves, as infection failed in the first experiment (no pesticides had since been used in the greenhouse). Different light conditions were tested, each with and without wounding of the leaf surface. Leaves of all treatments were infected properly by the pathogen, thus giving the green light for the main infection experiment.

Again, 3 source leaves of each *N. tabacum* cv. Xanthi plant were primed through spray application, homogeneously across the adaxial leaf surface. The same priming conditions were used as in the previous experiment.

After a priming period of 3 d, the leaves were cut off at the base of the leaf blade. One leaf from each plant was grinded in liquid nitrogen and stored at -80°C for molecular analyses. Both other leaves were used for infection (4 infection spots per leaf) and lesions were scored at 5, 6 and 7 dpi. The multiple time points provided the possibility to measure lesion growth rate after 5 dpi.

3.3. Experiment 3

3 source leaves of each *N. tabacum* cv. Xanthi plant were primed through spray application on the adaxial side of the leaf. An additional priming condition was added, using 12 week-old instead of 9 week-old leaves, to investigate if age would significantly increase lesion size. The same priming conditions as in the previous experiment were used, with the inclusion of water priming on 12 week-old leaves.

A priming duration of 3 d was applied, after which the leaves were cut off at the base of the leaf blade. One leaf from each plant was treated with liquid nitrogen and stored at -80°C for molecular analyses. Both other leaves were used for infection (4 infection spots per leaf) and lesions were scored after 6 d.

3.4. Experiment 4

3 source leaves of each *N. tabacum* cv. Xanthi plant were primed by spraying priming solutions on the adaxial side of the leaf. Spermine, a polyamine known to induce endocytosis (Sagor *et al.*, 2015), was added to some solutions.

Allose and Fru priming were left out and replaced by 50 mM Glc with spermine (100 µM) and BFO (5 g/L) with spermine(100 µM). Other priming conditions were the same as previous experiment.

A priming duration of 2 d was applied, to study treatment differences in a shorter time interval. Afterwards, the primed leaves were cut off at the base of the leaf blade. One leaf from each plant was treated with liquid nitrogen and stored at -80°C for molecular analyses. Both other leaves were used for infection (4 infection spots per leaf) and lesions were scored 3 and 6 dpi.

3.5. Experiment 5

3 source leaves of each *N. tabacum* cv. Xanthi plant were primed by spraying priming solutions on the adaxial side of the leaf. A spermine control treatment (100 µM spermine) was added, as well as 50 mM Fru with spermine (100 µM), while GABA priming was left out. Other priming conditions were the same as previous experiment.

A priming duration of 2 d was applied, after which the primed leaves were cut off at the base of the leaf blade. One leaf from each plant was treated with liquid nitrogen and stored at -80°C for molecular analyses. Both other leaves were used for infection (4 infection spots per leaf) and lesions were scored after 3, 6 and 10 dpi.

To study the fate of fructans, introduced in the plant through BFO priming, leaf samples were taken at different time points after BFO priming. After grinding in liquid nitrogen, samples were analyzed by sugar assay and run on the HPAEC-PAD, including a sample of the BFO priming solution (5 g/L) as a reference (diluted 70x for comparison with samples after sugar analysis).

3.6. Characterization of an **N. attenuata** *CWI inhibitor knock-out mutant*

We obtained seeds of this mutant from the lab of professor Ian T. Baldwin (Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Jena, Germany). These mutants show altered responses to biotic stress, such as insects, as described in Ferrieri *et al.* (2015). We investigated their reaction to *Botrytis cinerea* infection, thereby also characterizing sugar levels and invertase activity. After a growth period of 6 weeks, an infection experiment was performed on leaves of both mutant and WT plants and disease scoring was performed after 3 d of infection. Non-infected leaves (both mutant and WT) were sampled and grinded in liquid nitrogen for sugar and invertase activity assays. This allowed us to characterize the differences in Suc and hexose concentrations, as well as the activity of CWI and VI of this mutant compared to WT plants.

4. Statistical analysis

All graphs and figures were created in Microsoft Excel 10. All statistical analyses were performed with R v. 3.2.2, using package car 2.0-25 for the analysis of variances (ANOVAs), package multcomp 1.4-1 for parametric post hoc comparisons, package PMCMR 3.0 for nonparametric post hoc comparisons, package vegan 2.3-2 for principal component analysis (PCA) and package cluster 1.15.3 for cluster analysis. Before analysis, influential outliers were removed based on the cook's distance (D) criterion for model residuals. Normality of the data was assessed using Shapiro-Wilk normality test, while homogeneity of variances was examined with Levene's test. A one-way ANOVA was performed on normal data with equal variances, followed by a Tukey's multiple comparison test. If data showed no normal distribution, no equality of variances, or both, a nonparametric Kruskal-Wallis one-way ANOVA was performed, followed by pairwise comparisons using Tukey and Kramer (Nemenyi) tests. PCA analyses were performed on normal data, using scree plot and Kaizer criterion as a guideline to verify the optimal number of principal component axes. Concerning cluster analysis, after calculation of Bray-Curtis dissimilarities, the optimal clustering approach was verified through cophenetic correlation and Gower's distance. The number of clusters was selected based on silhouette plots. A p-value <0.05 was considered as significance level.

Results

1. Experiment 1

For the first experiment, several priming conditions were tested, including sugar treatments. After 3 d, primed source leaves of tobacco plants (cv. Xanthi) were infected with *B. cinerea* spores (strain B05.10). Disease scoring was performed after 3 d of inoculation, however only a very small amount of lesions was present. The fungal spores were inefficient in infecting tobacco leaves, since no change was present after 3 more d in the incubator. Due to problems with whitefly infestations in the greenhouse, insecticide sprays had been used. The most likely explanation of this incompatible interaction between plant and fungus seems to be the use of these insecticides, giving the tobacco immune system the upper hand.

As a side experiment, the sugar gradient was measured of leaves of a tobacco plant in a directional approach going from base (leaf 1) to apex (leaf 15) (see addendum Figure 14 for results) in order to collect background data about the physiological sugar levels of this tobacco variety. Results show a clear increase in hexose levels when going from base to apex, with a steep increase for the real sink leaves (leaves 11-13) and a clear decline for the smallest apical leaves (leaves 14-15). Suc shows a steady increase along the entire axis, visualizing a clear gradient. The Suc/hexose ratio shows this same trend, with the exception of leaves 11-13. To better visualize the source to sink transition, PCA and cluster analysis were performed. Here, source and sink leaves are clearly separated in distinct groups.

2. Experiment 2

After insecticide spraying was halted, a small-scale infection trial was performed to check the compatibility of the pathogen with untreated tobacco plants. The formation of lesions within 3 dpi strongly suggested to us that the insecticide spraying was the major disruption during the first experiment. However, the whitefly infestation was still existent in the greenhouse and can therefore influence the experiment outcome.

The setup of experiment 1 was repeated and lesions were measured after 5, 6 and 7 dpi. Differences in lesion size between these time points were minimal, thus, 6 dpi was chosen as a reference time point for future infections. As can be seen in Figure 3a, the lesion area of Glc-primed leaves is significantly smaller than that of water-treated leaves (p-value: 0.0333). As a positive control, OG priming shows a relatively small mean lesion area as compared to water treatment, assuming water can be considered as a valid negative control, which is questionable since it led to extremely low hexose levels. This suggests a specific physiological reaction of the leaves under this condition, possibly a hypo-osmotic shock. Fru and hydrolyzed BFO priming show the same mean lesion size, which was expected because the latter consists of Fru, degraded from an equimolar amount

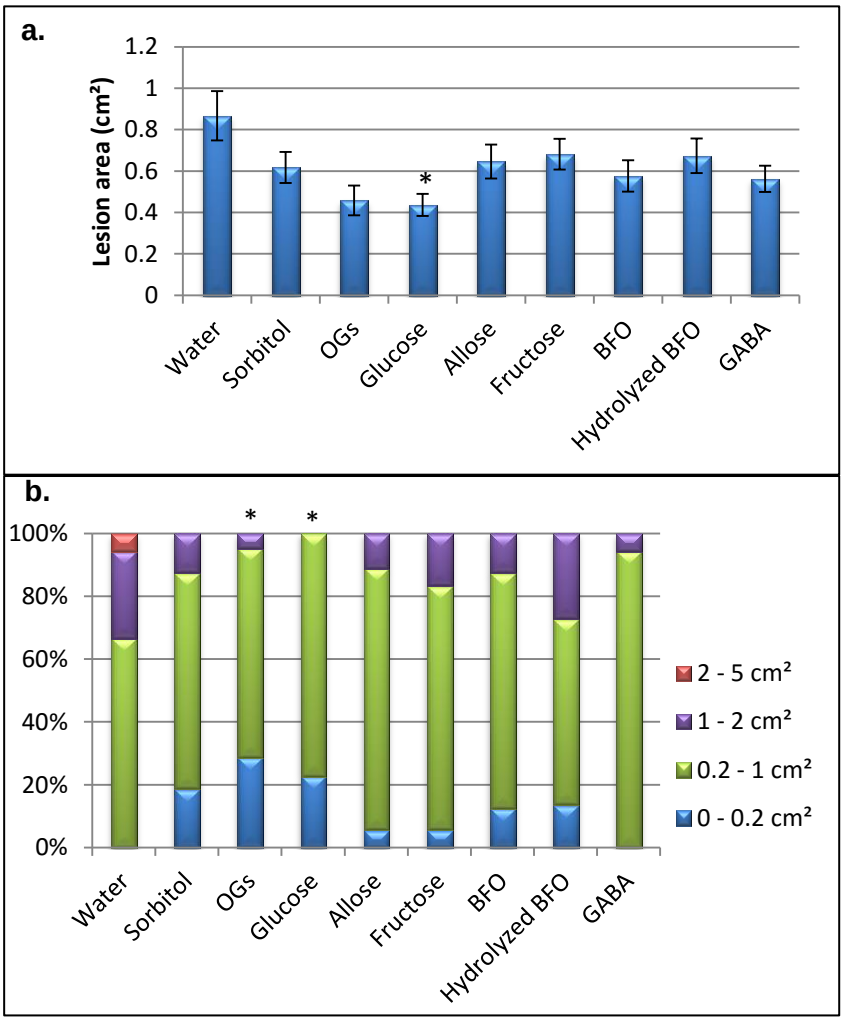

Figure 3: Experiment 2 disease scoring 6 dpi. Source leaves of *N. tabacum* cv. Xanthi were primed by spraying priming solutions on the adaxial side. The following priming conditions were used: ultrapure water, 50 mM sorbitol, 1 g/L OGs, 50 mM Glc, 50 mM allose, 50 mM Fru, 5 g/L BFO, 5 g/L hydrolyzed BFO and 10 mM GABA. After 3 d of priming, leaves were infected with *B. cinerea* spores. Disease scoring was performed 6 dpi **a.** Mean lesion areas 6 dpi, calculated based on a 1 cm² reference. **b.** Lesion categorization 6 dpi using the following categories: 0 – 0.2 cm², 0.2 – 1 cm², 1 – 2 cm² and 2 – 5 cm². Bars illustrate mean values ± standard errors. An asterisk indicates significance compared to the control water, unless denoted otherwise (*: <0.05; **: <0.005).

of BFO through enzymatic digestion with the use of resin-immobilized FEHs. After categorization of the lesions we see that, except for Glc-primed leaves, OG priming also shows differences as compared to the water treatment. The proportion of spots with no infection or very small lesion area is significantly higher in these treatments, while large lesions are less abundant.

Leaves were sampled 3 d after priming, crushed and stored at -80 °C. Samples were analyzed on small sugar levels (Glc, Fru, and Suc), starch content and invertase activities. As compared to water priming, the Glc, Fru, and Suc levels are significantly higher in OGs- and Glc-primed leaves (Figure 4a). Both treatments showed the smallest lesion size in the infection experiment. Higher Glc levels are also measured in Fru- and GABA-treated leaves, while allose priming raised Fru content significantly. An increase in Suc is discernible in allose- and Fru-primed leaves. Even so, Suc content in sorbitol-primed leaves is significantly higher than in the control treatment (p-value 0.0054), suggesting that higher Suc levels may be due to osmotic adjustments in response to sorbitol. OGs- and Glc-primed leaves show a significantly higher total hexose as well as total sugar level (Figure 4b). The same trend is present in allose-primed leaves. Since sorbitol priming significantly increased Suc content, total sugar levels differ from the control treatment as well (p-value: 0.0342). Although BFO-primed leaves show high sugar content (Glc, Fru, and Suc), this is not significantly different. In Figure 4c it is clear that all priming solutions implement a significant decline in Suc to hexose ratio. However, since sorbitol is lower (p-value: 0.0018), this may be ascribed to osmotic effects, except for GABA, for which the ratio is significantly higher than for sorbitol.

Concerning invertase activity, we focused on CWI and VI measurements, the acid isoforms that are often associated with immune responses in literature. A significant increase in CWI activity was found in leaves primed with the hexoses Glc and Fru, as compared to water. VI activity decreased significantly after sorbitol priming. Since other priming conditions don't show this decrease in activity, it can be attributed to the effect of sorbitol itself, rather than an osmotic effect. Interestingly, BFO-primed leaves also have a significantly lower VI activity. Before invertase activity analysis, a linearity check was performed on a few samples, taking multiple time points between 0 and 90 min of reaction time. The results, as seen in addendum Figure 15, show that a linear relationship is present.

Glc levels were measured after starch degradation to compare leaf starch content. Overall a very low starch content is found, with the highest Glc concentrations in water-, Glc- and allose-primed samples, however with large standard errors. Starch content in sorbitol-treated leaves is significantly lower than in unprimed ones (p-value: 0.0351).

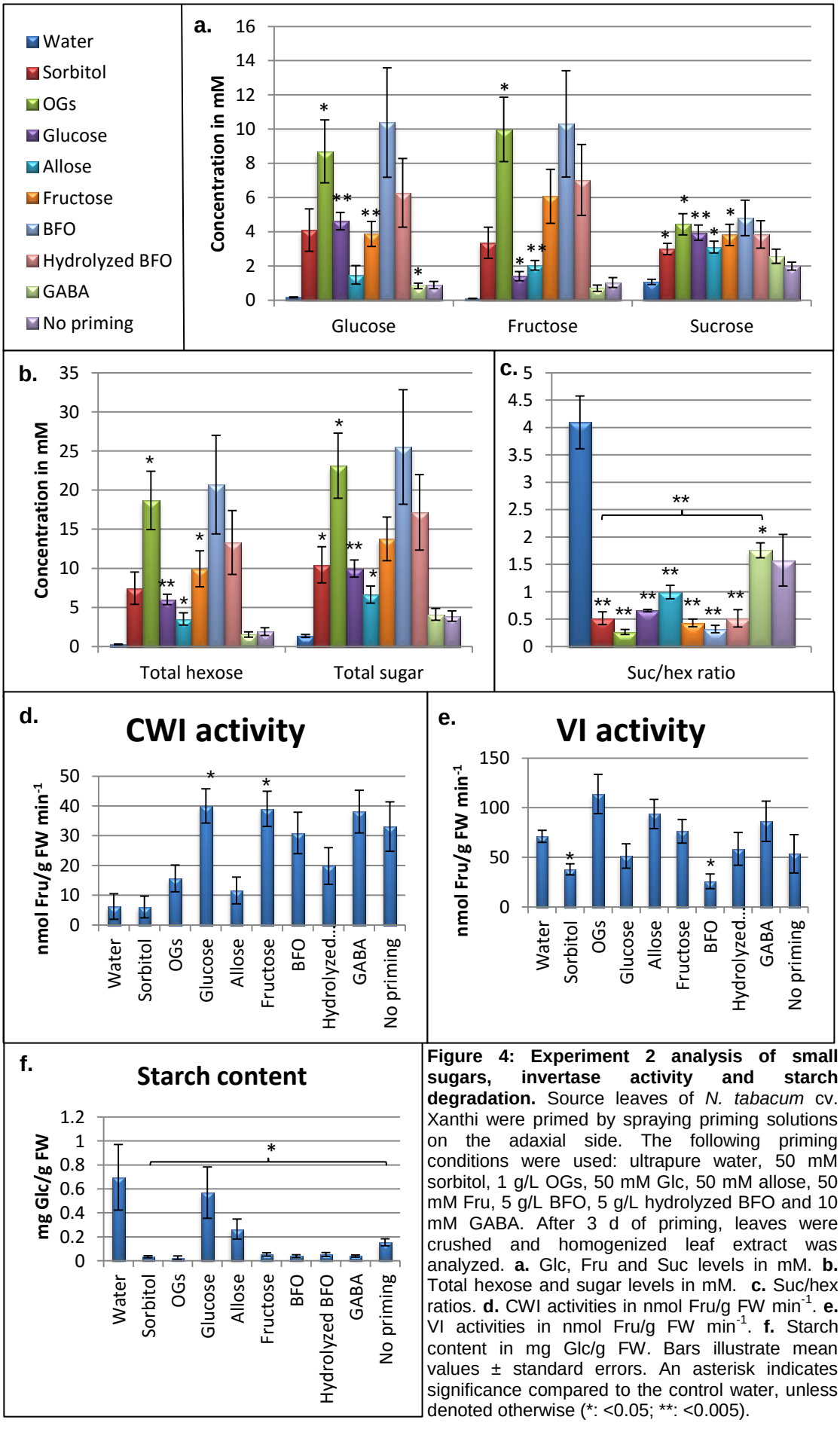

Figure 4: Experiment 2 analysis of small sugars, invertase activity and starch degradation. Source leaves of *N. tabacum* cv. Xanthi were primed by spraying priming solutions on the adaxial side. The following priming conditions were used: ultrapure water, 50 mM sorbitol, 1 g/L OGs, 50 mM Glc, 50 mM allose, 50 mM Fru, 5 g/L BFO, 5 g/L hydrolyzed BFO and 10 mM GABA. After 3 d of priming, leaves were crushed and homogenized leaf extract was analyzed. **a.** Glc, Fru and Suc levels in mM. **b.** Total hexose and sugar levels in mM. **c.** Suc/hex ratios. **d.** CWI activities in nmol Fru/g FW min^{-1}. **e.** VI activities in nmol Fru/g FW min^{-1}. **f.** Starch content in mg Glc/g FW. Bars illustrate mean values ± standard errors. An asterisk indicates significance compared to the control water, unless denoted otherwise (*: <0.05; **: <0.005).

3. Experiment 3

The same treatments as used in the previous experiment were sprayed on source leaves of *N. tabacum* to have a replicate of experiment 2. The white flies were still present in the greenhouse during this experiment. After 3 d of priming, spore solution of *B. cinerea* B05.10 was applied and lesions were measured 6 dpi. Leaves of older plants were water-primed and infected as well, in an effort to check the effect of age on pathogen susceptibility.

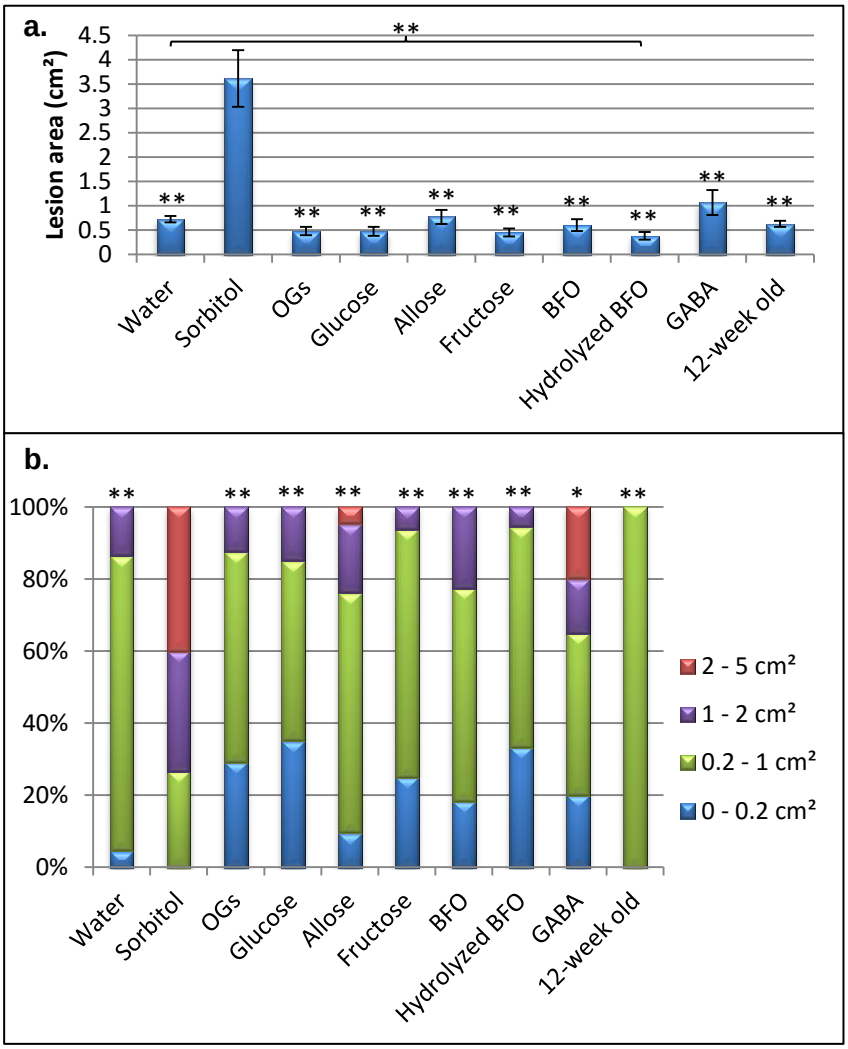

Figure 5: Experiment 3 disease scoring 6 dpi. Source leaves of *N. tabacum* cv. Xanthi were primed by spraying priming solutions on the adaxial side. The following priming conditions were used: ultrapure water, ultrapure water on 12 week old leaves, 50 mM sorbitol, 1 g/L OGs, 50 mM Glc, 50 mM allose, 50 mM Fru, 5 g/L BFO, 5 g/L hydrolyzed BFO and 10 mM GABA. After 3 d of priming, leaves were infected with *B. cinerea* spores. Disease scoring was performed 6 dpi **a.** Mean lesion areas 6 dpi, calculated based on a 1 cm² reference. **b.** Lesion categorization 6 dpi using the following categories: 0 – 0.2 cm², 0.2 – 1 cm², 1 – 2 cm² and 2 – 5 cm². Bars illustrate mean values ± standard errors. An asterisk indicates significance compared to sorbitol, unless denoted otherwise (*: <0.05; **: <0.005).

As can be seen in Figure 5a, sorbitol-primed leaves behave differently as compared to all other treatments. Many lesions grew to large proportions, as can be seen in Figure 5b, where the category of 2 – 5 cm² constitutes 40 % of total lesions. Visually it was clear that sorbitol-primed leaves showed earlier signs of senescence after infection. Interestingly, GABA shows quite high susceptibility to the pathogen as well. Fru- and hydrolyzed BFO-treated leaves seem to have performed better than Glc and OGs, contrary to the results of the previous experiment. Mean lesion area of hydrolyzed BFO-primed leaves is significantly lower than the control (p-value: 0.034). However, this difference is not apparent after categorization of the data. Water-primed leaves

from 4-week older plants seem to have the same susceptibility to the pathogen as younger ones, thus using older plants for infection experiments will likely not help to draw significant conclusions.

Concerning Glc, Fru, and Suc levels, the water-primed leaves behave divergent, not only against the other treatments in this experiment, but to the results for water-primed leaves in experiment 2 as well. Possibly, the plants were in a different physiological condition, allowing a hyperosmotic shock but preventing a hypo-osmotic shock (see below). For sorbitol Glc, Fru, and Suc concentrations were significantly lower. As can be seen in Figure 6a, GABA-treated leaves also contained significantly lower Glc levels (p-value: 0.0175). Glc and allose show very high hexose levels, like the control treatment, but with large standard errors. Fru- and hydrolyzed BFO-primed leaves have significantly higher hexose levels as compared to sorbitol. Sorbitol and GABA, having the lowest hexose levels, showed the highest susceptibility during the infection experiment. Suc levels were generally quite low and significantly lower than water in all treatments except Fru- and Glc-treated leaves. As Suc levels were low, total hexose and total sugar levels show the same trends as the hexose concentrations (Figure 6b), with sorbitol and GABA significantly lower than the control, whilst Glc-, Fru- and hydrolyzed BFO-primed leaves showed significantly higher levels than sorbitol. The control treatment has a Suc/hexose ratio of approximately 0.1, as can be seen in Figure 6c). Remarkably, the unprimed leaves have a significantly lower ratio than water (p-value: 0.009). Generally, standard errors were very high, thereby limiting the statistical differences between treatments. It is clear that this biological variability may hinder data interpretation.

Differences in CWI activity seem to be limited, as only one significant difference was found. CWI activity was significantly lower in sorbitol-primed leaves as compared to the control (p-value: 0.0181) (Figure 6d). Again, sorbitol- and GABA-treated leaves show the lowest values, as they also had the lowest hexose levels. For VI activity, mean values ranged from 27 for BFO to 50 nmol Fru/g FW min^{-1} for sorbitol (see addendum Figure 16). Fru- and hydrolyzed BFO-primed leaves also show lower activity. However, no significant differences were found for VI. In general, starch levels were very low, indicating almost no starch content was present in the leaves, even with enzymatic digestions performed overnight. Glc-primed leaves show some degradation, but water shows the highest Glc accumulation, much higher as compared to the unprimed leaves. Starch content was significantly lower in Fru- and GABA-primed leaves as compared to the control (Figure 6e).

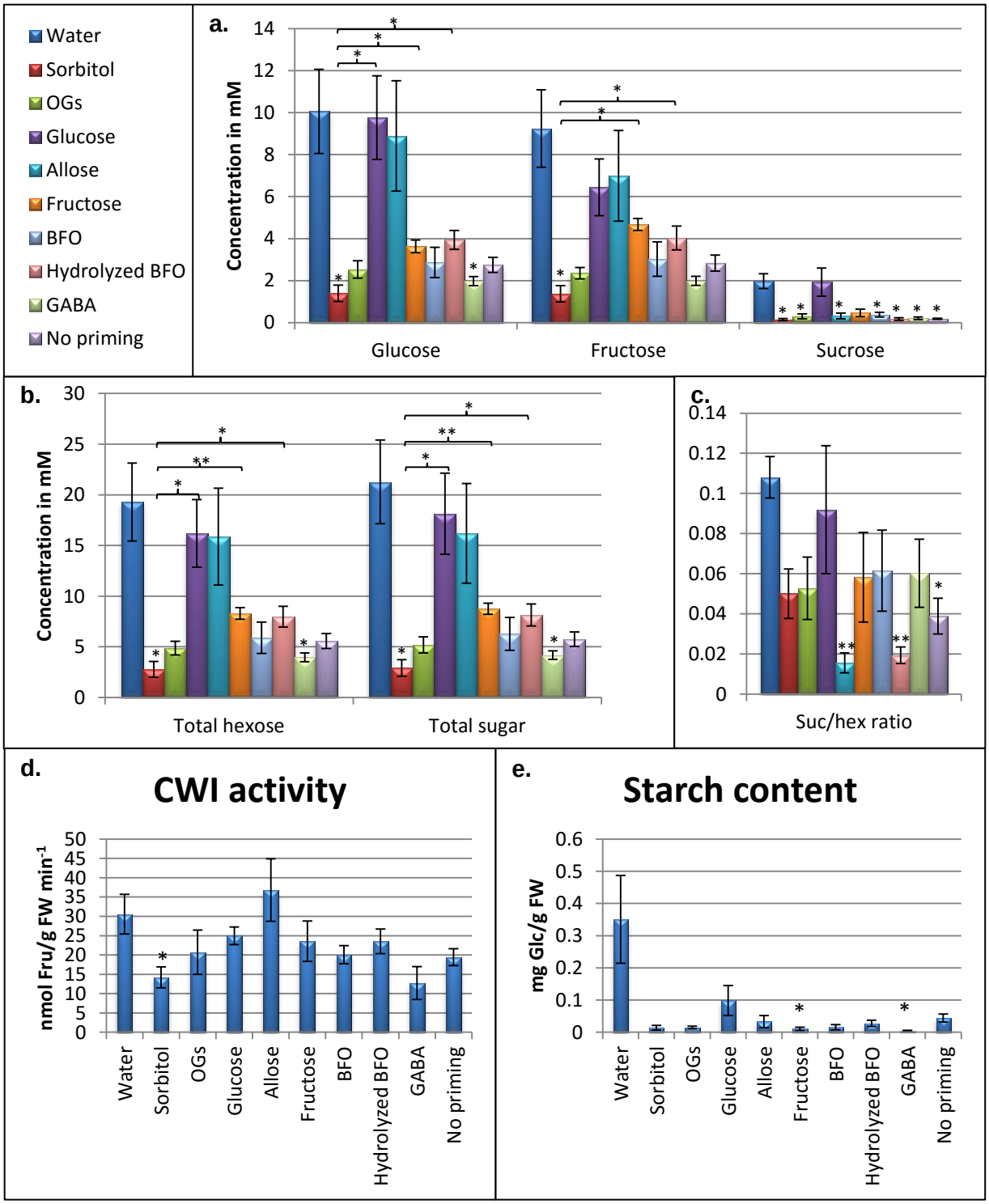

Figure 6: Experiment 3 analysis of small sugars, invertase activity and starch degradation. Source leaves of *N. tabacum* cv. Xanthi were primed by spraying priming solutions on the adaxial side. The following priming conditions were used: ultrapure water, 50 mM sorbitol, 1 g/L OGs, 50 mM Glc, 50 mM allose, 50 mM Fru, 5 g/L BFO, 5 g/L hydrolyzed BFO and 10 mM GABA. After 3 d of priming, leaves were crushed and homogenized leaf extract was analyzed. **a.** Glc, Fru and Suc levels in mM. **b.** Total hexose and sugar levels in mM. **c.** Suc/hex ratios. **d.** CWI activities in nmol Fru/g FW min^{-1}. **e.** Starch content in mg Glc/g FW. Bars illustrate mean values ± standard errors. An asterisk indicates significance compared to the control water, unless denoted otherwise (*: <0.05; **: <0.005).

4. Experiment 4

Before this experiment, the greenhouse was emptied and the white flies were eliminated within a period of 1 week, after which new plants were sown. A priming duration of 2 d was chosen in order to check if a shorter duration may help to let emerge significant differences between treatments and controls, as compared to the 3 d used until this point. For this experiment, spare tobacco plants from one of the lab technicians was used to provide me with the means to perform an additional infection experiment. However, these plants were grown in smaller pots, thereby influencing the morphology of the plant significantly. These plants were already in an early stage of senescence by the time the infection experiment was performed (2 d after priming), and as such differ strongly in terms of disease scoring and other metabolic measurements. As a result, these data (see addendum Figures 17, 18 and 19) will not be discussed in detail or compared with the other experiments.

5. Experiment 5

Since the polyamine spermine has interesting effects on endocytosis, for this last infection experiment we focused more on this molecule in combination with Glc and BFO priming. A spermine priming solution was also applied to control for the effects of spermine itself. A combination of Fru and spermine was chosen as well. As such we can verify if the trends are comparable to those found in combination with Glc or BFO. Also, a priming period of 2 d was chosen to see if the results differ from previous experiments, followed by 6 d of infection. No signs of senescence were present after 6 dpi, so the option to take a later time point was available. Hence a second disease scoring was performed 10 dpi. To optimize the lesion categorization, an extra category was added for the 10 dpi data.

Figure 7a and 7c show the results of the disease scoring 6 dpi. OG priming performed well, as our positive control and BFO-primed leaves performed equally well. Both treatments have significantly smaller lesion areas as compared to water. Water and sorbitol lesion areas are much more alike, in comparison to previous experiments where the difference was often substantial. Although not significant, Glc priming also shows a smaller mean lesion area, with or without addition of spermine. Spermine priming, however, shows similarly small lesions. BFO-treated leaves are significantly more resistant to the pathogen than BFO and spermine (p-value: 0.0384) and hydrolyzed BFO (p-value: 0.0176). For the categorized data, no significant differences were found, although it is clear from Figure 7c that OGs- and BFO-primed leaves are less susceptible to *B. cinerea* infection.

10 dpi the significant differences become more apparent (Figure 7b). Water and sorbitol clearly show much larger lesion areas than the other treatments. Interestingly, spermine, compared to the data 6 dpi, has much larger lesions, thus meaning that lesions of spermine-treated leaves have grown substantially from 6 to 10 dpi compared to other treatments. Hence, the mean lesion area of Glc and spermine is significantly lower than that of spermine alone. OGs-, Glc-, Glc and spermine-, BFO- and Fru and spermine-primed leaves all show significantly smaller lesions than the negative control water. BFO is also significantly lower than BFO with spermine and hydrolyzed BFO. The category with lesions larger than 5 cm^2 is clearly represented in water-, sorbitol- and spermine-primed leaves (Figure 7d). Here, OGs-, Glc and spermine-, and BFO-primed leaves performed significantly better than water as well. Comparable to the analysis of the lesion areas, categorization also shows a significant difference for BFO as compared to BFO and spermine (p-value: 0.0099) and hydrolyzed BFO (p-value: 0.0011).

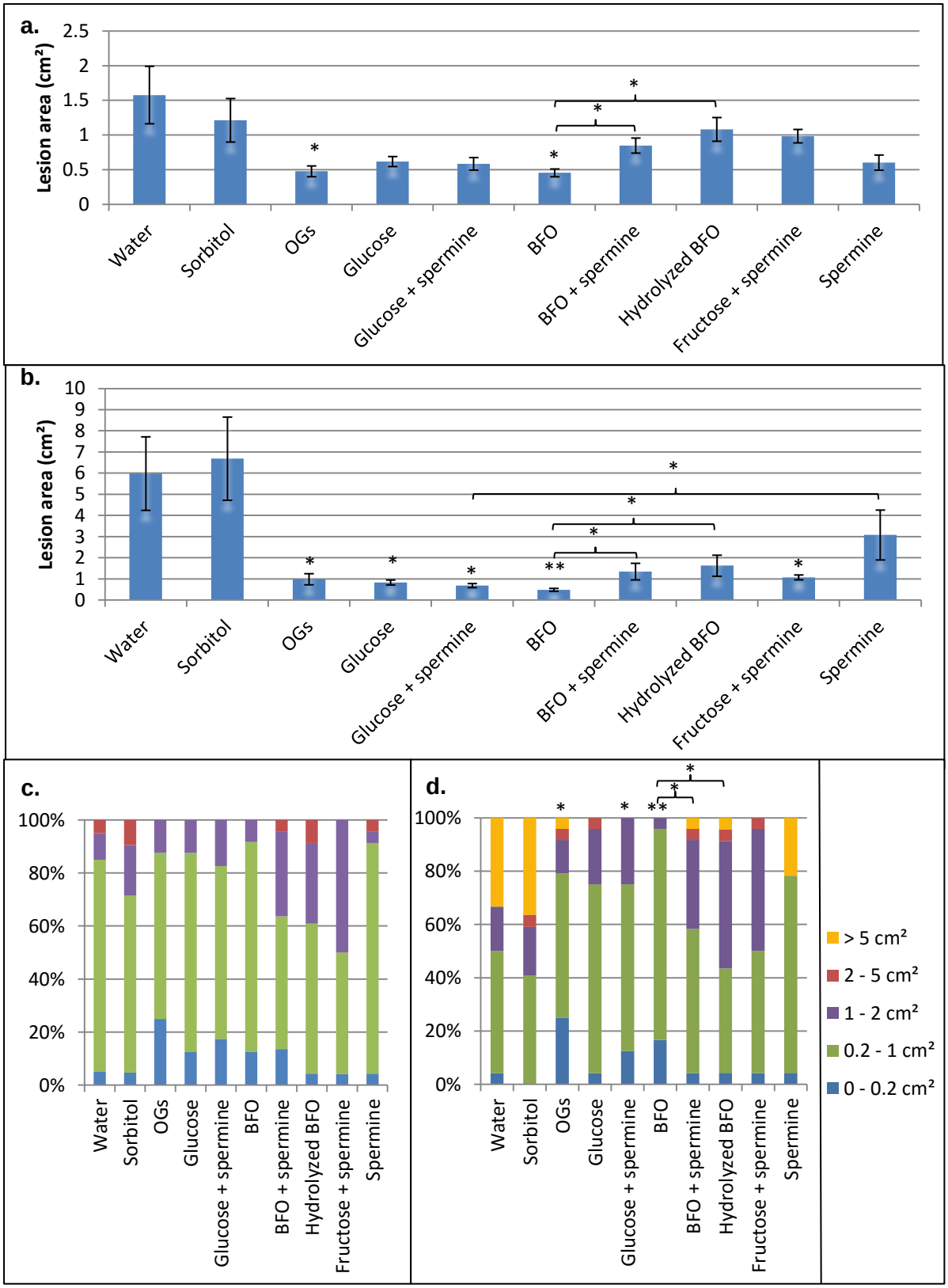

Figure 7: Experiment 5 disease scoring 6 and 10 dpi. Source leaves of *N. tabacum* cv. Xanthi were primed by spraying priming solutions on the adaxial side. The following priming conditions were used: ultrapure water, 50 mM sorbitol, 1 g/L OGs, 50 mM Glc, 50 mM Glc and 100 µM spermine, 5 g/L BFO, 5 g/L BFO and 100 µM spermine, 5 g/L hydrolyzed BFO, 5 g/L hydrolyzed BFO and 100 µM spermine and 100 µM spermine. After 2 d of priming, leaves were infected with *B. cinerea* spores. Disease scoring was performed 6 and 10 dpi **a.** Mean lesion areas 6 dpi, calculated based on a 1 cm² reference. **b.** Mean lesion areas 10 dpi, calculated based on a 1 cm² reference **c.** Lesion categorization 6 dpi. **d.** Lesion categorization 10 dpi. The following categories were used: 0 – 0.2 cm², 0.2 – 1 cm², 1 – 2 cm² ,2 – 5 cm² and >5 cm². Bars illustrate mean values ± standard errors. An asterisk indicates significance compared to water, unless denoted otherwise (*: <0.05; **: <0.005).

The addition of spermine to Fru shows striking differences in disease scoring. When added to BFO, spermine makes leaves more vulnerable to *B. cinerea* than BFO alone, while it seems to enhance resistance in combination with Fru as compared to Fru alone, although the latter difference is not significant. Priming with Glc and spermine as compared to Glc alone shows no real difference in lesion size.

Glc, Fru, and Suc levels of leaves sampled after 2 d of priming are shown in Figure 9a-c. Water and unprimed leaves clearly show the lowest concentrations. Hexose levels are higher in sorbitol-treated leaves, but never significantly. BFO-primed leaves have relatively low hexose and Suc concentrations compared to other treatments, and this was also apparent in previous experiment. Glc-, spermine-, Fru and spermine-, and hydrolyzed BFO-primed leaves have significantly higher Glc and Fru levels. Although OG priming induced high hexose content, this difference was only significant for Fru levels. Interestingly, while Glc, as well as spermine, shows high hexose content, combining Glc with spermine significantly lowers concentrations of both Glc and Fru. Hydrolyzed BFO, a control for BFO priming, has significantly higher hexose content as compared to BFO. From Figure 9a it is clear that no significant differences were found for Suc levels, indicating a tight regulation of its concentration independent of the priming agent. Total hexose and total sugar content shows the same trends as Glc and Fru levels. OGs, Glc, spermine, hydrolyzed BFO, and Fru and spermine all have significantly higher sugar content (Glc, Fru, and Suc) than water in general. Again, concentrations in hydrolyzed BFO-treated leaves were significantly higher as compared to BFO-primed ones, while combining Glc and spermine shows a significant decline in content in comparison to Glc and spermine separately. All treatments have a lower Suc to hexose ratio than water- and unprimed leaves, with a significant difference for hydrolyzed BFO and Fru and spermine priming (Figure 9c).

As in previous experiments, invertase activity levels were measured, with a focus on CWI and VI. Like before, activity levels are very similar between treatments (Figure 9d-e). For CWI, activities were significantly higher for OGs- and BFO and spermine-treated leaves as compared to water. All data range around 100 nmol Fru/g FW min^{-1} for VI activity, with the exception of Glc and spermine (p-value: 0.0281). Compared to Glc and spermine primed separately, the combination of both provides a significantly higher VI activity level. Besides invertase activity and small sugars, starch content was measured as well, with the results shown in Figure 9f. Starch degradation was generally low, with the exception of OGs, hydrolyzed BFO, and Fru and spermine. However, only hydrolyzed BFO is significantly higher (p-value: 0.0272).

To study the fate of BFOs after priming, samples were taken from BFO primed leaves at different time points and compared to a sample of the BFO priming solution. Figure 8 shows part of the chromatogram of these samples on the HPAEC-PAD from 28 to 36 min, with peaks from DP 10 to 15, accompanied by an F-series (chains of Fru moieties). At time 0, before priming, no fructan content is present (blue line), however after 4 h of priming a rise in fructan content is apparent. Notably, no further increase is found after longer priming durations. Peaks of primed leaf samples are much smaller as compared to the priming solution itself, since only 100 mg FW is used for each sample, while the priming solution of 5 g/L is sprayed across the entire leaf. Leaf FW was measured with a mean of approximately 5.8 g, thus a factor 55 – 60 must be used to convert the peak area from a 100 mg sample to an entire leaf. Multiplying the peak areas by this factor compares to the areas of the corresponding peaks from the BFO priming solution. Thus a concentration of 5 g/L BFO is present inside the leaves already after 4 h of priming, with no further increase thereafter. No change in fructan pattern, increase/decrease of certain DPs, is observed. Also, hexose levels as well as Suc levels remained roughly unchanged.

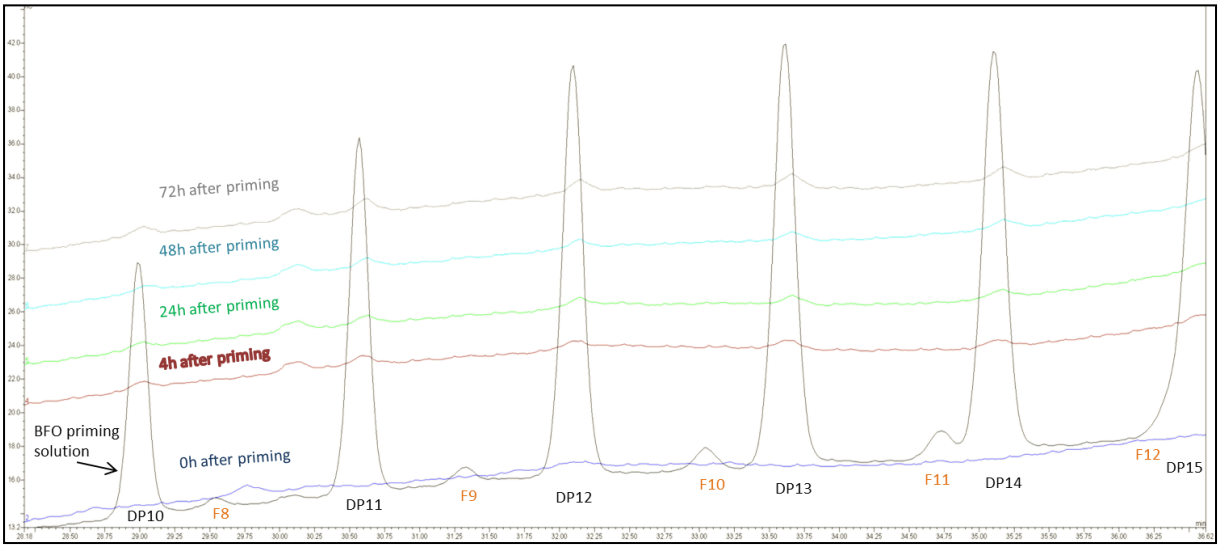

Figure 8: Fructan content after BFO priming. Leaves of 4 *N. tabacum* cv. Xanthi plants were primed with BFO (5 g/L) and leaves were sampled at different time points: 0, 4, 24, 48 and 72 h after priming. A sampl e fo the BFO priming solution was analysed together with the samples as a reference. DPs are indicated for fructan peaks between 28 and 36 min on the HPAEC-PAD, F-series of fructans (Fru chains) are indicated in orange. Taking into account that only 100 mg FW was analyzed of total leaf content, multiplying sample peak areas with a factor 55 – 60 (mean leaf FW), corresponds to peaks of the priming solution.

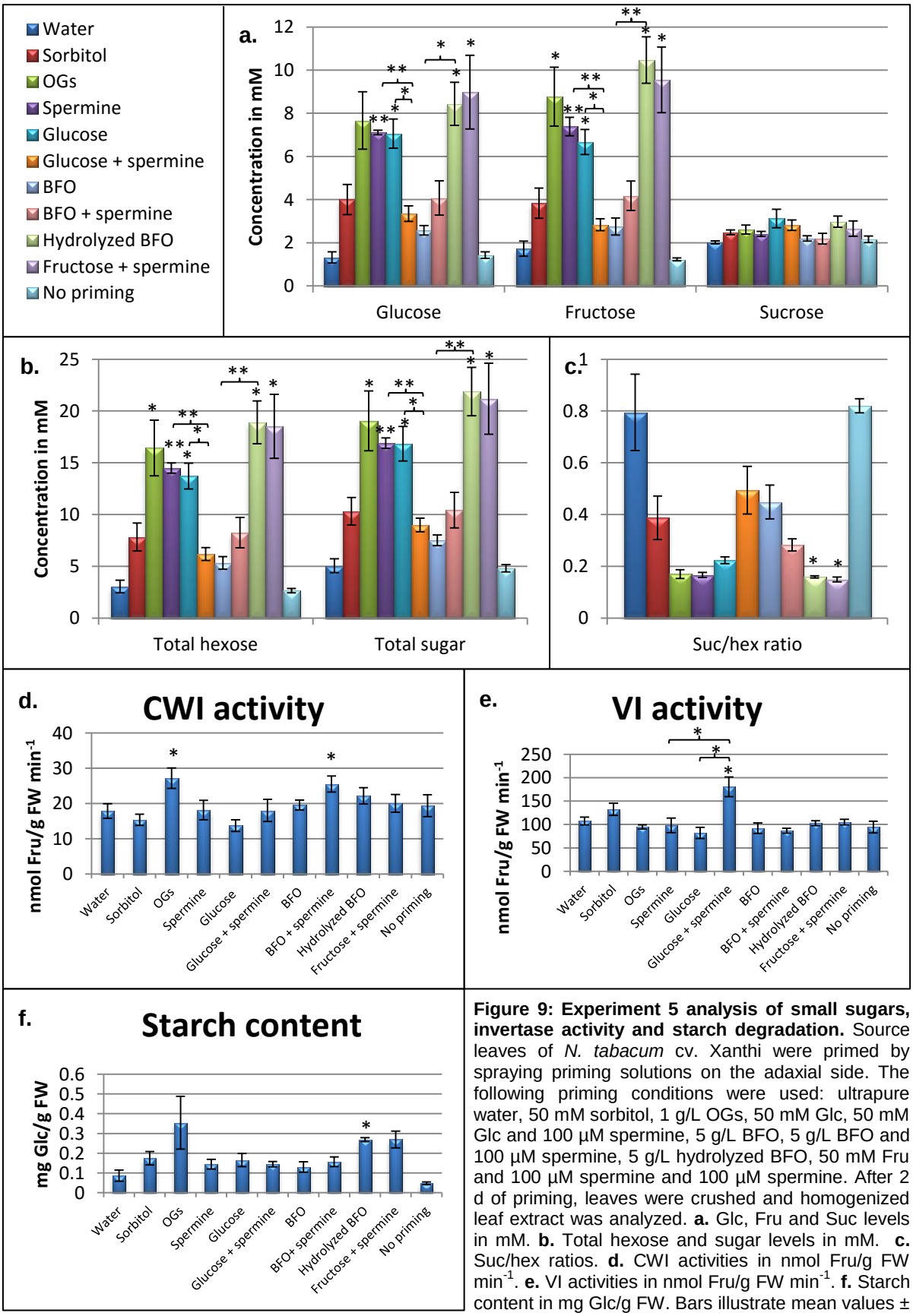

Figure 9: Experiment 5 analysis of small sugars, invertase activity and starch degradation. Source leaves of *N. tabacum* cv. Xanthi were primed by spraying priming solutions on the adaxial side. The following priming conditions were used: ultrapure water, 50 mM sorbitol, 1 g/L OGs, 50 mM Glc, 50 mM Glc and 100 µM spermine, 5 g/L BFO, 5 g/L BFO and 100 µM spermine, 5 g/L hydrolyzed BFO, 50 mM Fru and 100 µM spermine and 100 µM spermine. After 2 d of priming, leaves were crushed and homogenized leaf extract was analyzed. **a.** Glc, Fru and Suc levels in mM. **b.** Total hexose and sugar levels in mM. **c.** Suc/hex ratios. **d.** CWI activities in nmol Fru/g FW min^{-1}. **e.** VI activities in nmol Fru/g FW min^{-1}. **f.** Starch content in mg Glc/g FW. Bars illustrate mean values ± standard errors. An asterisk indicates significance compared to the control water, unless denoted otherwise (*: <0.05; **: <0.005).

6. Characterization of an *N. attenuata* CWI inhibitor knock-out mutant

N. attenuata plants with a CWI inhibitor knock-out were grown in the greenhouse. Source leaves of both mutant and wild type (WT) plants were sampled and infected with *B. cinerea* spores. Disease scoring was performed 3 dpi (Figure 10). Contrary to infection of *N. tabacum* cv. Xanthi, lesions were well developed after 3 d. As the results point out, the mutant seems to be more resistant to infection, although the difference with WT plants is not significant.

A change in the Suc to hexose balance is apparent after analyzing Glc, Fru, and Suc concentrations of mature source leaves of mutant and WT plants. In mutant plants, this ratio was significantly higher than in WT plants (p-value: 0.0391) (Figure 11c). Figure 11a shows somewhat smaller hexose levels, while Suc concentration is significantly higher as compared to the wildtype (p-value: 0.0071). No significant difference was found for total hexose levels and total sugar levels were the same for both genotypes (Figure 11b).

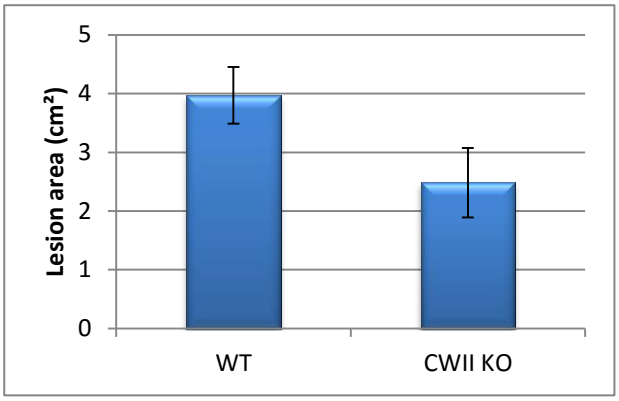

Figure 10: *N. nicotiana* disease scoring 3 dpi. *N. nicotiana* CWI inhibitor knock-out mutant source leaves were infected with *B. cinerea* spores. A comparison was made with wildtype plants. Disease scoring was performed 3 dpi. Bars illustrate mean values ± standard errors.

Since a CWI inhibitor gene is knocked out in this mutant, analyzing invertase activity should provide us with differences as compared to the wildtype (Figure 11 d-e). Strangely, CWI activity was not significantly different, although a clear trend is present. VI activity is significantly lower in mutant plants (p-value: 0.0431).

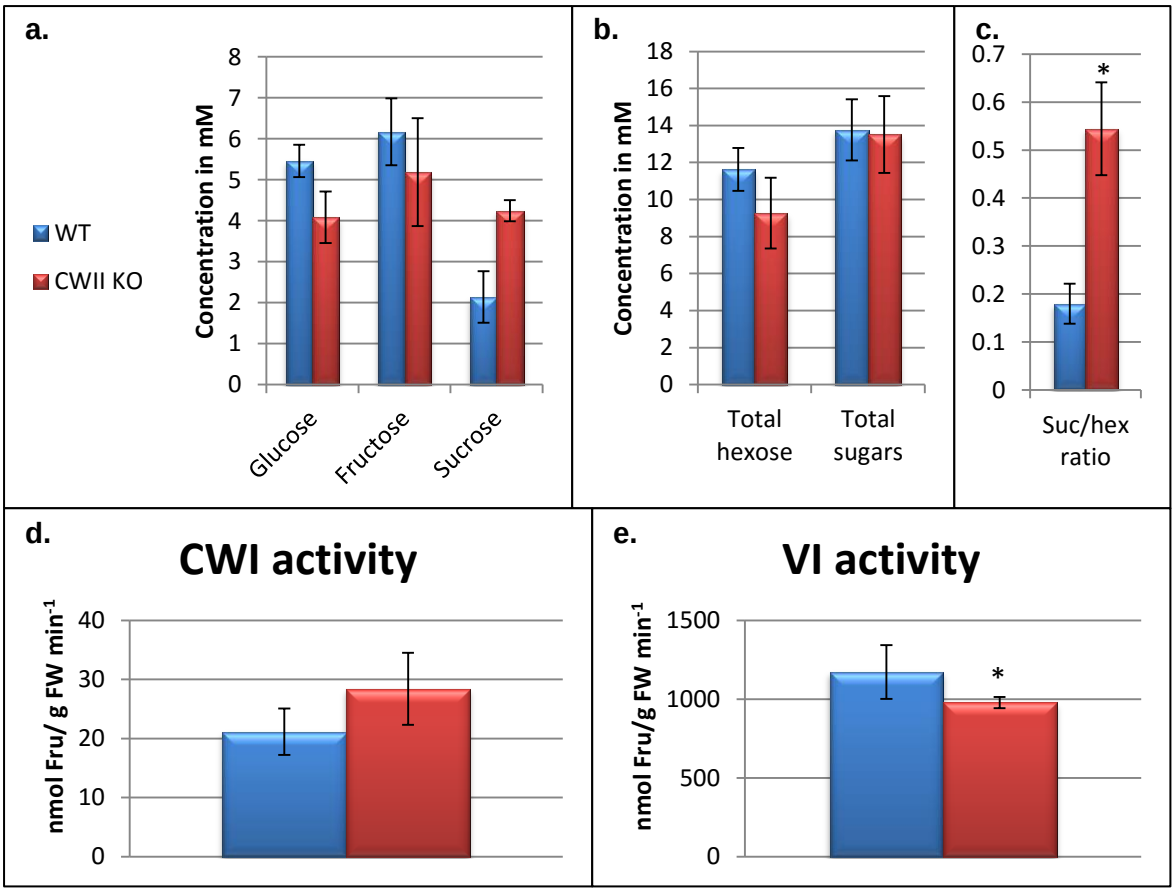

Figure 11: *N. attenuata* analysis of small sugars and invertase activity. Source leaves of *N. attenuata* CWI inhibitor knock-out mutants and wildtype plants were crushed and homogenized leaf extract was analyzed. **a.** Glc, Fru and Suc levels in mM. **b.** Total hexose and sugar levels in mM. **c.** Suc/hex ratios. **d.** CWI activities in nmol Fru/g FW min^{-1}. **e.** VI activities in nmol Fru/g FW min^{-1}. Bars illustrate mean values ± standard errors. An asterisk indicates significance compared to the wildtype (*: <0.05; **: <0.005).

Discussion

Building further upon previous knowledge, the *B. cinerea – N. tabacum* pathosystem was used to gain more insight into the sweet immunity concept. Tobacco cv. Xanthi was chosen because of its higher susceptibility to infection, as compared to the cv. Petit Havana, which had been used in the lab earlier on (El Oirdi *et al.*, 2010; El Oirdi *et al.*, 2011). As Xanthi grows faster, the growth-defense trade-off may be shifted more towards growth as compared to Petit Havana, thus lowering defensive capacities. A more susceptible variety is preferable in order to obtain clear differences in disease resistance between treatments.

B. cinerea, a necrotrophic fungus, was chosen because of the large amount of knowledge present in the literature and prior use of the tobacco - *B. cinerea* pathosystem to study plant immune responses. It is known that this fungus has a very broad host range, thus being able to infect most plant species, including tobacco (Van Baarlen *et al.*, 2007). A more virulent strain of the pathogen, B05.10, was used to ensure lesion formation.

The need for testing alternative strategies to pesticide usage is imminent. Pathogens account for a large part of crop losses, and although pesticide use is the most common procedure, it comes with many problems and side-effects for ecosystems worldwide (Handford *et al.*, 2015). Plants themselves have a scope of defensive mechanisms against biotic stresses, including physical barriers, defensive compounds, and ROS. However, these often don't suffice to withhold the pathogen (Arora *et al.*, 2012). Priming of the plant's immune system can increase the efficacy against future infection (Beckers & Conrath, 2007). Sweet immunity presents the importance of sugars in the defense against pathogens. It is known that sugar metabolism changes during infection, as the pathogen will create a new sink, often accompanied by an increase in CWI. The increase in hexose sugars by the pathogen can, however, provide a signal for the plant to induce immune responses (Berger *et al.*, 2007). In this thesis, further evidence for the role of sugars in plant immune responses was tested through sweet priming, with the focus on some more promising sugar compounds.

1. Experiment 1

Some of the more promising treatments (results of previous infection experiments done in the lab) were used for the first experiment. The hexoses Glc and allose were chosen based on previous results, while BFO was included to test the effect of fructans against *B. cinerea* infection. Sun *et al.* (2013) showed a clear increase in fungal resistance in postharvest grapes after BFO spraying. They found that BFO priming activates the SA-dependent signaling pathway and upregulates expression of PR1, PAL, and NPR1 among others. As a

control to the BFO priming, hydrolyzed BFOs (obtained by using resin-immobilized FEH) were used as a priming solution, thus allowing us to verify if the effects of BFO priming are due to the fructans as such, or dependent on possible degradation into Fru. A Fru priming solution was used to control for possible contamination of BFO extract with immune system-activating compounds, compared to the hydrolyzed BFO treatment for differences. Water-primed leaves were taken as a negative control, while sorbitol priming was used to account for any effects induced through osmotic stimuli as such. As a positive control, OGs was chosen. Previous studies have shown OGs to be an effective DAMP, thus inducing a range of defense responses in the plant (Aranega-Bou *et al.*, 2014). In Aziz *et al.* (2004), exogenous application of OGs protects grapevine against *B. cinerea infection*. GABA, a non-proteinogenic amino acid, has been shown to be involved in plant defenses against several stress conditions, and was used for priming as well (Kinnersley & Turano, 2000).

Priming was performed through spray application to minimalize the effect of leaf manipulation itself, as other strategies would be more invasive. A priming duration of 3 d was chosen to allow the priming of plant defenses. For infection, cut-off leaves were placed in petri dishes. To optimize the spore germination and lesion formation a humid environment was maintained by sealing of the petri dishes during incubation, as this generally favours fungal growth. Temperature, however, is known to be the most important environmental factor. For *B.* cinerea, a rather low temperature (18-20 °C) is the most effective (Thomas *et al.*, 1988; Lahlali *et al.*, 2006). Importantly, temperature also influences plant immune responses, as described in Cheng *et al.*, 2013.

As a result of unfortunate events, no clear lesion formation was found after 3 and 6 dpi as it seems that the fungus was unable to penetrate the leaf. The most logical explanatory factor is the use of insecticide spraying in the greenhouse, as other conditions were the same as in previously performed experiments within the lab. Due to problems with whitefly infestations, insecticides were used as a last resort, since other strategies failed to improve the situation. However, this consequent spraying may have tremendous effects on the ability of *B. cinerea* to infect these leaves, as the insecticide can affect plant physiology and thus defense responses. Since the fungus fails to penetrate the leaf surface, a strengthening of the physical barriers may be such a consequence of insecticide use. It is known that certain insecticide classes can induce salicylic acid-associated defense responses in *A. thaliana* (Ford *et al.*, 2010).

To have a good background on sugar levels and source-sink dynamics in tobacco, leaves from one plant were analyzed in a gradient-like fashion. An increase in the hexose sugars Glc and Fru was observed when going from base to apex, but only at the transition from source to sink. For Suc, concentrations steadily increased when going towards the apex, showing a nice gradient throughout the plant. Interestingly, whereas Glc and Fru decline in

the smallest apical leaves, Suc still increases. Suc thus seems to be tightly regulated, as source leaves must provide the necessary carbon source for sink leaves to develop. Sugars are involved in regulating these dynamic processes by signaling the energy status within the plant (Roitsch, 1999). Invertases are important players in this context, as they degrade the bulk of Suc in sink leaves (Roitsch & González, 2004). Addendum Figure 14 shows a clear distinction between source and sink leaves when taking into account hexose and Suc levels, as well as total sugar levels and Suc/hexose ratios. During infection, *B. cinerea* will become a new sink for the plant's sugars, with Glc being the major carbohydrate to be taken up by the pathogen (Lemoine *et al.*, 2013).

2. Experiment 2

Insecticide spraying was halted since the last infection experiment failed. The white flies, however, were still present in the greenhouse and affected the tobacco plants by feeding on the abaxial sides of the leaves. This can lead to recognition of HAMPs or DAMPs, which may trigger plant defense mechanisms that could interfere with the infection experiment (Mithöfer *et al.*, 2005). Since herbivory-specific defense pathways would be induced, one can predict the impact on *B. cinerea* to be small, as HAMPs upregulate JA signaling, while resistance to *B. cinerea* is conferred through SA signaling (Howe & Jander, 2008; Sun *et al.*, 2013). Nevertheless, an infection trial was performed to ensure our predictions were correct. After 3 d, lesions were apparent, thus confirming that *B. cinerea* infects tobacco plants after whitefly infestation. From this trial, we can conclude that our suspicions are rectified and that insecticide spraying strongly affects plant immunity in this context.

The same treatments as used in experiment 1 were used during priming. 3 dpi, lesions were still very small, thus, a later time point (6 dpi) was chosen as a standard for the disease scoring. Figure 3 shows that mean lesion areas were still small, especially when compared to the total leaf area. A first indication for sweet immunity is apparent, since lesion areas were significantly smaller after Glc priming. The results show that our positive control is reliable, as OG-primed leaves appear more resistant. After categorization, OGs differ significantly from the negative control, thereby confirming the results of previous reports (Aranega-Bou *et al.*, 2014). BFO priming proved to be less effective, although there is a noticeable difference with hydrolyzed BFO lesions.

The variation in hexose levels between treatments was much higher as compared to Suc levels, indicating Suc concentrations to be more strongly regulated. The negative control shows very low Glc, Fru, and Suc levels, but a high Suc/hexose ratio, resembling a sink rather than a source leaf. Sugar levels (Glc, Fru, and Suc) were high after OG and Glc priming, which indicates that high sugar content may be important for pathogen resistance. Even so, BFO-primed leaves show high hexose and Suc levels as well, although

susceptibility to *B. cinerea* was not significantly lower. A significant rise in Glc, Fru, and Suc was found after GABA priming, although the concentrations were much lower in comparison to the positive control. This rise is in accordance with Vijayakumari and Puthur (2015), reporting an increase in total sugar level after GABA priming in *Piper nigrum*.

To avoid any problems with the linearity of the response for invertase activities, a linearity check was performed, hoping to minimalize the deviation from linearity for invertase activity for different time points through careful handling of the samples and minimization of pipetting errors. Results (addendum Figure 15) show that the response is more or less linear. VI activity shows somewhat higher R^2 values for the corresponding linear trend lines as compared to CWI (CWI measurement is more complex).

Starch concentrations were extremely low for most treatments. Less than 0.2 mg Glc/g FW was produced through starch degradation reactions. The highest starch content was found in water-primed leaves, although Glc and allose priming show some considerable content as well. One hypothesis may be that priming with most solutions depletes the energy reserves of the leaf. Application of Glc, as a favorable energy source, may provide the plant with the necessary extra energy for priming. Through lugol staining of stock solution (stored in the freezer) from samples of the different treatments before starch degradation, we obtained the same results, thus indicating that the starch measurement was performed correctly.

A clear link between starch content and pathogen susceptibility seems improbable, since water and Glc priming gave the highest starch content, while the latter was significantly more resistant as compared to the other during the disease scoring. A correlation with sugar levels and invertase activity is less far-fetched, as sugars are known to play a role in plant immune responses. Recently, it has been hypothesized that Suc/hexose ratios are important determinants of the cellular responses, and these ratios are strongly influenced by invertases (Bolouri Moghaddam & Van den Ende, 2012). Previous papers have reported enhancement of plant resistance to fungal pathogens through high sugar levels, by increasing lignification, flavonoid synthesis, and enhancement of oxidative burst (Morkunas & Ratajczak, 2014). Our results show the same trends, as OG and Glc priming show high total sugar levels, accompanied with increased pathogen resistance.

Water-primed leaves, show much lower Glc, Fru, and Suc levels than sorbitol-treated leaves, as well as low CWI and VI activity, although the variability is quite high for CWI. Glc and OG priming show higher invertase activity. Thus, plant susceptibility may not only be increased by low sugar levels, but by low invertase activity as well. It is known that CWI activity often increases during pathogen interaction, while the reports on VI are inconclusive (Tauzin & Giardina, 2014). Allose-primed leaves show low Glc, Fru, and Suc levels and CWI activity, although VI activity is quite high. Although allose priming induced resistance to

Xanthomonas oryzae in rice by triggering ROS generation (Kano *et al.*, 2013) and promising results were found in previous experiments performed in the lab, allose performed less well during disease scoring. Possibly, this may be cultivar-specific, as previous experiments were mainly performed on tobacco cv. Petit Havana.

BFO shows Glc, Fru, and Suc levels similar to OGs, however, BFO-treated leaves were more susceptible to *B. cinerea*. Strongly differing is the low VI activity found after BFO priming. Hydrolyzed BFO and Fru generally behaved in the same way, showing similar pathogen susceptibility in the disease scoring, as well as matching sugar levels (Glc, Fru, and Suc). These data confirm that hydrolyzed BFO contains only Fru. Strangely, CWI activity is higher for Fru than for hydrolyzed BFO. Even so, we can conclude both treatments to be very similar. Interestingly, both Glc and Fru priming show significantly higher CWI activity than the negative control. We would expect that the strong presence of hexose sugars Glc and Fru slows CWI activity, thereby halting the further production of these hexoses. Research on *saccharomyces cerevisiae* pointed out that Glc inhibits mRNA production and translation, but no direct catabolite inhibition of invertase was found (Elorza *et al.*, 1977). Zhang & Yongzhang (2002) found a role for Glc in acid invertase inhibition through post-translational effects. Alternatively, exogenous hexoses may resemble the hexose increase during pathogen infection, which is known to stimulate CWI activity.

3. Experiment 3

Given the unclear results of my first trial, a second experiment was performed using the same treatments as before, in the hope to draw some main conclusions. Because we lacked statistical significance during the previous infection experiment, water priming was also performed on source leaves that were 4 weeks older, to see whether age influences susceptibility. Several studies report a decrease in susceptibility in function of age in different pathosystems, but results may differ for the tobacco – *B. cinerea* system (Reuveni *et al.*, 1986; Bonde *et al.*, 2012). Figure 5 indicates that mean lesion area is clearly similar to younger water-primed leaves. The use of older leaves than the ones used here may present us with a difference in susceptibility in this pathosystem, although most literature points in the other direction. The use of younger leaves in not optional, since the plants must contain large and mature source leaves for the infection experiment.

When looking at the results in Figure 5, sorbitol stands out with a considerably higher mean lesion area compared to all other treatments, and as such behaving very different from previous experiment. Coincidentally, leaves chosen for sorbitol priming may have progressed more into a senescing state before treatment, or because of a sorbitol priming effect itself. Wether sorbitol is a good osmotic control in this context can be doubted, especially when its effect on the plant varies between experiments. Possibly, sorbitol itself can exert certain

effects on the plant, despite simply osmotic effects. Members of certain plant families, like Rosaceae, can synthesize sorbitol as an end product of photosynthesis, which is often reported in the adaptation to abiotic stresses such as chilling and drought (Escobar Gutiérrez & Gaudillère, 1996). However, so far no sorbitol receptor or sorbitol-specific signaling pathway has been discovered in plants, so we can assume the effects of sorbitol priming to be through osmotic changes.

Leaving out sorbitol, we can see that mean lesion areas for OGs and Glc are relatively low against the control, as they were in the last experiment. Allose and GABA show no decrease in susceptibility at all. Interestingly, Fru and hydrolyzed BFO behave in a similar way, as we would expect, but lesion area is smaller than that of BFO-primed leaves. Lesion categorization allows us to verify where the main differences can be found in the distribution of lesion sizes. GABA shows some resemblance to sorbitol priming, with a relatively high proportion of large lesions. The results of both experiments combined give some doubt about the repeatability of using GABA as priming agent.

When looking at the results of the physiological parameters measured, a large difference can be observed compared to results from previous experiment when looking at water and sorbitol. Strangely, water-primed leaves show very high levels of both hexoses and Suc, while sorbitol has the lowest concentration of these sugars. Water-primed leaves had very low hexose and Suc content in the previous experiment. The Suc/hexose ratio for water remains within the same range, however, but a higher variability is observed for this parameter as compared to previous results. Glc again shows high sugar content (Glc, Fru, and Suc) in general, while the concentrations in OG-treated leaves are considerably lower this time. Concerning allose, hexose levels are within the same range as for Glc, but allose differs notably in Suc concentration.

Glc, Fru, and Suc concentrations for BFO-primed leaves are generally low. Although in great contrast with the previous experiment, this connects better with the hypothesis that higher sugar levels confer resistance against the pathogen. Glc mean lesion area was relatively low, accompanied by overall high hexose and Suc levels. Although OGs show a similar resistance, as a DAMP, it can exert its effects through different signaling pathways to activate plant immune responses (Ferrari *et al.*, 2013). Water, BFO, and GABA show relatively low hexose levels, going with higher mean lesion areas after infection. Although allose shows similar hexose levels to Glc, leaves were more susceptible to *B. cinerea* infection. This may indicate the importance of not only hexoses, but Suc as well. During infection, Suc transporters may be upregulated, causing Suc accumulation in the apoplast. Invertases can then degrade the Suc into hexoses, which are readily taken up by the pathogen (Gómez-Ariza *et al.*, 2007; Tauzin & Giardina, 2014).

By becoming more experienced in performing invertase activity measurements, samples are manipulated more accurately, thus lowering the variability seen in the previous experiment. Interestingly, sorbitol and GABA, which had the highest susceptibility during disease scoring, show the lowest CWI activity. Thus, we can hypothesize more clearly from these results that high sugar levels, together with higher CWI activity, confer better resistance against infection. Results from starch degradation assays are clearly similar for both experiments, although sugar levels, invertase activity, and lesion areas varied more thoroughly. After 3 d of priming, leaves were sampled in the morning, when starch reserves are generally low, explaining the generally low Glc accumulation during the breakdown reaction. Nevertheless, water, Glc, and allose show considerably higher starch content as compared to the other treatments. For Glc priming, this can be explained, as Glc is the main energy source used by the plant, thus possibly increasing energy storage during the day and decreasing starch breakdown overnight (Bolton, 2009). During infection, a decrease in starch content is often observed, since starch degradation can provide another energy source for the pathogen (Tauzin & Giardina, 2014). However, this energy source may be used to fuel plant defense responses as well. Since priming of the leaves and presence of exogenous sugars may signal a similar situation as during pathogen infection, this may explain the generally low starch content in our samples. Assuming water spraying doesn't prime the plant's immune system, we can hypothesize starch content to remain relatively high.

We previously stated the diverging behavior of sorbitol-primed leaves during the disease scoring. However, physiological measurements of sugars and invertase activity resemble those found in the last experiment. Taking this into account, another hypothesis is that the problem may be situated in the water-primed leaves, rather than the sorbitol-primed ones. We would expect water-treated leaves to have considerably lower sugar levels (Glc, Fru, and Suc) and less invertase activity in order to be comparable to previous results as well as unprimed leaves. Mean lesion area, however, is expected to be larger. Possibly, the water priming solution may have been contaminated. If these contaminants have an immune system-priming effect, this could explain the relatively high sugar content and CWI activity. It may also explain the large difference between water and sorbitol during disease scoring. Without these contaminants, mean lesion area of water-primed leaves may have been larger.

4. Experiment 4

Before the startup of this experiment, in the absence of any plants, the greenhouse was sprayed with insecticides to remove all whiteflies. Results obtained in the following experiments may thus differ from previous experiments, since this stress factor has been removed. By focusing mainly on disease scoring results, we chose to leave out the allose treatment, because of the higher susceptibility of allose-primed leaves compared to other

treatments. Since Fru and hydrolyzed BFO showed very similar results, Fru priming was left out as well. BFO, although not performing well in the previous experiments, was maintained as an important treatment, because of its promising results in lettuce (L. Tarkowski, personal communication).

In addition to the other treatments, Glc, and BFO, now our main point of focus for sweet priming, were combined with the polyamine spermine. Although spermine is not necessary for normal growth in plants, several functions have been described for this compound. Many evidence indicates a role in the control of ion channels and receptors. Spermine, like other polyamines, is directly involved in the plant's carbon- and nitrogen metabolism, starting from ornithine. During pathogen attack, accumulation of spermine in the apoplast causes the upregulation of defense-related genes and often a hypersensitive response (Takahashi & Kakehi, 2010). In tobacco, activation of wound-induced protein kinase and SA-induced protein kinase trigger downstream defense responses through a spermine-signalling pathway (Sagor *et al.*, 2015). Spermine priming and presence of exogenous spermine may thus resemble a situation similar to pathogen infection.

Because of the morphological differences with plants from the other experiments, the results of this batch are not discussed in detail. Since these plants were grown in much smaller pots, leaves were smaller and signs of senescence were present. Many reports in the literature have focused on the prominent effect of pot size, not only on plant growth rates, but on physiological parameters as well (Ray & Sinclair, 1998; Kasai *et al.*, 2012). Clearly, results obtained from this experiment cannot be compared with previous results. However, they clearly illustrate the importance of maintaining the same conditions as best as possible. Changing one of the parameters, like pot size, can have an enormous effect on the plants, indicating their high sensitivity and plasticity.

5. Experiment 5

For this last experiment, we focused strongly on spermine in combination with Glc and BFO priming. Both treatments were already introduced in experiment 4, however, spermine was now added as a separate treatment to take into account any effects caused by the polyamine itself. Also, because previous results pointed out a notable difference in the effect of spermine on Glc and BFO, we combined spermine with Fru. This allows us to conclude whether the effect of spermine combined with Fru differs from the other treatments. Since the effectiveness of GABA is somewhat doubtful from our previous results, we left out this treatment, focusing more on spermine. Based on previous results, we shortened the priming duration from 3 to 2 d. A shorter window for priming before infection allows us to investigate the earlier differences between treatments, as these may be greater as compared to later time points.

After 6 dpi, our positive control OGs has significantly lower lesion area than the control. Interestingly, BFO-primed leaves are now significantly more resistant as well, while this wasn't clear in previous experiments. This is in accordance with previous results found in the literature (Guo *et al.*, 2012; Guo *et al.*, 2013). Unlike in previous infection trials, leaves were still in a good state after 6 d, showing no signs of senescence, thus we opted to take a later time point for disease scoring at 10 dpi, in order to look for more apparent differences between treatments. It is clear that the difference between water-primed leaves and other treatments increases after a longer duration of infection. Besides OGs and BFO, treatments involving Glc now show significantly lower susceptibility to *B. cinerea*. The effect of spermine, when combined with Glc, seems minimal in terms of lesion size. Spermine itself, as a control, is not significantly different from the negative control.

Looking back at the results from experiment 3, we saw that sorbitol was much more susceptible to infection than all other treatments. Here, results show that sorbitol behaves in the same manner. However, no difference with water-primed leaves is found here. This may strengthen our suspicions that something went wrong with the water priming during experiment 3. sorbitol-primed leaves show very similar hexose levels to the ones obtained in experiment 2, while water shows higher sugar levels (Glc, Fru, and Suc) as compared to this first experiment. As seen earlier on, water-primed leaves show a relatively high Suc/hexose ratio, while this is lower for sorbitol. Water and sorbitol show the same invertase activity levels as well as similar starch content. Interestingly, starch levels are relatively higher when compared with previous experiments, with OGs, hydrolyzed BFO, and Fru and spermine showing higher starch content. It seems Fru priming increases starch accumulation in the plants. Overall, higher starch degradation was measured, but this may be explained by the timing of the experiments. Leaves of previous experiments were sampled in November – December, while this experiment was performed in March – April. This means the days were longer and light conditions may be more in favor of starch accumulation.

The results of the sugar analysis show similarities with what we observed before. OGs- and Glc-primed leaves have higher hexose levels, in accordance with experiment 2, while BFO priming causes relatively low concentrations, comparable to what we found in experiment 3. Hydrolyzed BFO hexose levels, as found earlier on, were higher than in BFO-treated leaves. Suc/hexose ratios are generally low, in accordance with previous findings. Interestingly, the results found for water- and unprimed leaves are very similar, while this was less the case in previous experiments. Suc concentrations show very low variation between treatments, which clearly indicates that these levels are tightly regulated by the plant, even in case of plant immune system priming. This tight regulation was already apparent in the results of the sugar gradient measured in experiment 1, where an almost perfect gradient was found throughout the plant.

Across the different treatments, invertase activity levels are very similar. For CWI, OGs and BFO with spermine show significantly higher activity. Interestingly, Glc-, and especially BFO-primed leaves showed higher resistance to *B. cinerea*. Glc and spermine shows an interesting rise in VI activity, thus indicating that spermine acts on these enzymes in a certain way, but only when combined with Glc. The effect of spermine is quite clear when looking at sugar levels, although the effect differs depending on the priming solution it was combined with. Spermine itself causes an increase in hexose levels, comparable to the effect of Glc priming. However, when the two are combined, the concentrations of Glc and Fru decline dramatically. Even though the difference is less substantial, adding spermine to BFO increases hexose levels. No real difference can be found when combined with Fru as compared to the hydrolyzed BFO treatment. Although it is quite clear that we find higher hexose levels in treatments that have smaller mean lesion areas after infection, when combined with spermine, this trend seems less clear and even going in the opposite direction. Glc with spermine shows lower hexose levels and smaller lesion areas when compared to Glc priming, while BFO with spermine shows higher hexose levels accompanied with larger lesions. In summary, higher hexoses make the plant more susceptible to the pathogen when combined with spermine.

Figure 8 shows part of the chromatogram received after running BFO-primed leaf samples. As a control, the BFO priming solution used during priming experiments can be seen as well. Clearly, *N. tabacum* contains no fructans before the priming, which is in accordance with what we expected, since tobacco is no fructan accumulator. Although peaks are generally very small, an increase in fructan content is apparent 4 h after priming for all DPs, including the ones shown in the figure. Since BFO is prepared from burdock (*Arctium lappa*), these are inulin-type fructans (Wang *et al.*, 2009). The DP-series on the chromatogram thus presents a 1-kestose series (inulin-type fructans), while the F-series represents an inulobiose-series of fructans, containing only Fru moieties (no terminal Glc moiety present) (Van den Ende, 2013). Of course, samples present fructan content for 100 mg of total leaf content, whereas the priming solution's concentration is for the entire source leaf. Thus, peaks are a factor of 55-60 smaller, meaning that F-series peaks are not measurable for these samples. 4 h after priming the concentration of 5 g/L BFO has already been reached, with no further increase at later time points. Interestingly, there is no sign of BFO degradation, especially since Fru levels don't increase towards later time points (data not shown). This confirms that no FEH activity is present in tobacco leaves and that all effects obtained after priming are due to BFO itself and not its degradation products.

6. Sweet immunity, sugars and invertase – conceptualization

Up until this point, our findings, and certain anomalies within these findings have been discussed from a more methodological point of view. However, it is important to synthesize these results from a plant physiology perspective as well. Repeatability has been a major issue throughout all experiments, with the main problems in our control treatments. These differences can be explained from a physiological standpoint, since all human manipulations, as well as the plant's history in the greenhouse and the physical conditions, like temperature and light, are summed up in the results we have obtained. We hypothesize a concept in which the apoplastic environment of the leaf can be a major regulator, leading to the differences we observed.

It is important to keep in mind that all experiments were done on major source leaves. These leaves must provide energy to all sinks in the plant, transported in the form of Suc. The results in addendum Figure 14 show a steep increase in Suc concentrations when going from base (source leaves) to apex (sink leaves). In other words, for source leaves to load the Suc into the phloem against this strong concentration gradient probably requires a lot of energy. The hexose levels point out that the situation is somewhat more complex than previously anticipated. The very smallest leaves (leaf 14-15) behave differently as compared to the real sink leaves 11-13, as can be seen for Glc and Fru levels, as well as Suc/hexose ratios. We can hypothesize that sink leaves can be categorized into 2 groups. The smallest leaves, growing through cell divisions, follow the expected Suc/hexose ratio, and the other sink leaves are characterized by very high hexose levels. These high concentrations of Glc and Fru might be explained through an upregulation of VI activity, assuming growth in these leaves is dominated by cell elongation, for which VI is known to play an important role (Wang *et al.*, 2010). These results stress the importance of Suc to hexose ratios, as these are linked to hexose and Suc levels as well as invertase activities.

Because of the steep Suc gradient that source leaves need to overcome for phloem loading and the correlated energetic costs to do so, we hypothesize that the apoplast can play an important role. The apoplast has certain osmotic properties for which phloem loading may occur optimally. However, this apoplastic balance can change depending on internal and external changes and disturbances. There may be a certain range between which this osmotic balance can change without affecting apoplastic processes, which can be seen as a tolerance zone. If the apoplast would shift away from balance and out of this tolerance zone, this could affect phloem loading processes and cause changes in Suc and hexose levels in source leaves. Spraying the leaves with priming solutions can change the apoplastic balance, either increasing or decreasing osmolality depending on the solution, and thus disturb the physiology in the leaf.

A hypo-osmotic apoplastic shock (experiment 2, water priming) may cause sugars/osmolytes to fall below a certain threshold. This may signal increased sink strength (and thus Suc export) to other parts of the plant, stimulating anabolic pathways for Suc, starch and protein synthesis from hexose skeletons. This may explain the higher starch content measured in water-primed leaves. Since the apoplastic balance was situated near the lower border of the tolerance zone, sorbitol priming, as well as the other priming solutions, may have caused an increase in solute concentration without reaching the upper border. Thus, perhaps sorbitol would be more a reliable control than water in experiment 2.

When we look at the results of experiment 3 (Figure 5a), it is clear that sorbitol shows a very high susceptibility to infection. Also, hexose and Suc levels are low when compared to other treatments (comparable to the water priming in experiment 2). In this case, we can speculate that sorbitol priming causes an imbalance in the apoplastic environment through hyperosmotic shock, which is plausible when osmolality was already closer to the upper boundary of the tolerance zone. It is known that such osmotic stress can induce ABA biosynthesis, thus increasing ABA signaling (Xiong & Zhu, 2003) and massive synthesis of osmotic compounds (e.g. proline) from hexoses. As a consequence, defense-related pathways will be downregulated, since ABA negatively regulates SA signaling. A shift will probably be made towards abiotic stress resistance, for which ABA is known to be a central player (Spoel & Dong, 2008). As a result, leaves have a low biotic stress response, which is clear from the large lesion areas found. Water priming in this experiment would have caused a decline in osmolality, but without crossing the lower boundary of apoplastic balance, not triggering the anabolic pathways described above. Thus, a higher sugar (especially hexose) balance is maintained. Unfortunately, hexose and Suc levels were very large after water priming (Figure 6a). Despite the fact that other sugar priming solutions have the same osmolality as the sorbitol priming solution, no evidence has been found for a similar hyperosmotic shock in the apoplast. The main difference is that in most plant species sorbitol cannot be taken up by the cells and stay in the apoplast. Glc, for example, is readily taken up by the plant by MSTs or SWEETs when concentrations rise in the apoplast (Slewinski, 2011). Given the steep Suc gradient from base to apex in the plant, it is likely that the range in osmolality to be tolerated is relatively narrow in tobacco. Presumably, this tolerance zone is larger in lettuce, since fewer problems have occured for water and sorbitol priming in our lab. However, some abnormalities have been found in this crop as well.

From these reasonings it is clear that the balance in the apoplastic environment will be very sensitive to any external factors. This again stresses the importance of working in a controlled environment with a minimal in differences for the plants between experiments. Clearly, the effect of smaller pot size caused an earlier induction of leaf senescence and transition to flowering in experiment 4. Comparing this with experiment 5, leaves showed no

signs of senescence even after 10 dpi, indicating that these plants have grown in more constant and optimal conditions. We can presume from this that the apoplastic balance was more or less centered within the tolerance zone. For experiments 2 and 3, the whitefly problem may be one the external factors to shift the balance towards the boundaries of the tolerance zone.

7. Characterization of an *N. attenuata* CWI inhibitor knock-out mutant

A knock-out of a CWI inhibitor gene will cause an increase in CWI activity in the plant. In our previous results, it was clear that a higher CWI activity is accompanied with a higher resistance to *B. cinerea* infection. Although the difference is not significant, this trend is clearly visible in Figure 10. This confirms the importance of CWI in priming and biotic stress tolerance. Keeping in mind that this disease scoring was performed already after 3 dpi, it is clear that *N. attenuata* is much more susceptible to our B05.10 strain than *N. tabacum*. Of course, this higher CWI activity cannot be compared with the increased activity after priming. Priming may induce a shock in the apoplastic environment thus leading to changing CWI activities, however, 2 or 3 d after priming it is unlikely that a new balance has already been established. The *N. attenuata* mutants have had a higher CWI activity throughout their entire growth, thus having more than enough time to find a new apoplastic balance.

Interestingly, the results in Figure 11 clearly show a different balance as compared to WT plants. At first glance, a higher Suc to hexose ratio may seem shocking, given that a higher CWI activity would cause more Suc degradation into the hexoses Glc and Fru. Moreover, Figure 11d proves that there is a higher CWI activity in these plants, although the difference is not significant. This is to be expected, since only one CWI inhibitor gene has been knocked out while a large family of inhibitors is known to exist in plants. Also, this inhibitor seems to be specifically involved in response to herbivory, thus in case of a fungal attack it may not play an important role (Ferrieri *et al.*, 2015). Knocking-out this CWI inhibitor gene may induce an increase in production of other inhibitors, thus minimalizing the increase in CWI activity. Nevertheless, a higher activity is present, accompanied with a higher Suc to hexose ratio, which can be the result of a new apoplastic and cellular balance that has been established. Even though a higher invertase activity is present, hexose levels became lower, indicating that hexoses are used for synthesis (Suc, antimicrobial compounds). The higher energy flux towards biotic stress tolerance may explain the tendency of lower susceptibility to pathogen infection.

Conclusions and future prospects

The main goal of this thesis was to provide evidence for the sweet immunity concept. Using our *N. tabacum* - *B. cinerea* pathosystem, it has become clear that sugar priming can have a profound effect on biotic stress tolerance. Mainly, Glc and BFO priming showed the most promising results, being even more effective than our positive control OGs at times. Reflecting on the results obtained from sugar measurements and invertase activities, a positive relation with pathogen resistance is clear. Higher Glc and Fru concentrations, as well as higher CWI activities markedly increase resistance to *B. cinerea* infection, as significantly smaller mean lesion areas have been measured. This fits quite well with the sweet immunity concept, thus, we can conclude that the role of sugars in biotic stress tolerance may definitely be non-negligible. The use of spermine in one of our experiments gave some unexpected but intriguing results, as it seems that the effect on the plant depends tremendously on the sugar it is combined with. A further focus on the role of this polyamine in sweet priming could provide some more answers as to how these differences in disease resistance and physiological parameters are caused.

Perhaps of equal importance are the things we learned about our control treatments and the sensitivity of the plants. Repeatability has been the major issue when summarizing all experiments, though mostly because our negative and osmotic controls caused problems. Thinking about these problems, we came up with a concept involving disturbances of the apoplastic balance through priming, which provides us with the possible means to explain at least some of the observed differences. For the future, it will be important to learn more about the apoplast through extraction of apoplastic fluid, a technique for which the efficiency is now tested in our lab. Knowing the situation in the apoplast at the moment of priming may allow us to correct for the variation of osmolality and apoplastic balance. Moreover, a new negative control, characterized by an osmolality equal to that of the apoplast and pH-buffering action, can be used as a more accurate control treatment.

Some of the smaller experiments performed, like measuring the sugar gradient across leaves of an entire plant, also provided the necessary information to understand certain observations. The notion that a steep Suc gradient exists in *N. tabacum* plants is important background information to understand physiological processes. Measuring invertase activity levels in a gradient-like fashion may provide us with even more insights. After a few days of priming, it is unclear at which stage we are in terms of reaching new physiological balances. Using shorter priming periods may allow us to exclude long term effects like the high susceptibility of sorbitol-primed leaves in experiment 3, in which ABA is presumably involved. However, trying different durations of priming before performing infection experiments may allow us to better understand how these processes evolve and when a new balance is

established. Measuring key enzymes will give more insights as to how energy fluxes are directed to different cellular processes.

Finally, the effect of external parameters, thinking about insecticide spraying, whitefly infestations, or smaller pot sizes or less controllable factors such as temperature, is enormous. This has become clear on several occasions. To conclude, in order to obtain consistent results, it is very important that we are able to control these factors, thus keeping all parameters constant. This requires optimal infrastructural facilities as well as consistent handling of the plant material by the researcher.

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Addendum

Risk Assessment

Lab safety rules were provided at the start of this thesis. Lab coat and nitrile disposable lab gloves were used for all laboratory procedures. Thermal protective gloves were utilized when working with liquid nitrogen. To avoid contamination, manipulations of *B. cinerea* were performed in a laminar flow cabinet. Infected leaves and disposable materials that came into contact with the fungal mycelia or spores were disposed off in a biowaste bin. The working bench was disinfected with 70% ethanol. Refillment of the liquid nitrogen tank and replacement of pressurized helium tanks were performed by lab technicians.

Before using any chemicals present in the lab, the S- and R-sentences were first evaluated in order to enact proper safety measures. Some chemicals, such as ammonium sulphate, β-mercaptoethanol and PMSF are irritating to eyes and/or skin and were thus always handled with lab gloves and potentially safety glasses. Sodium hydroxide, β-mercaptoethanol and PMSF can also cause burns. In case of contact, eyes must be rinsed with water using a lab eye cleaner. Since ethanol is highly flammable, close contact with fire was avoided. An emergency shower is present in the lab in case of emergency. B-mercaptoethanol, stored in the fridge, was handled carefully to avoid dropping and spilling this concentrated solution, and preferably opened using a fume hood. Sodium azide was used in all solutions as antimicrobial agent. This chemical is also very toxic to humans in high concentrations or amounts, and was utilized with great care. Sodium hydroxide waste is collected in a separate waste container.

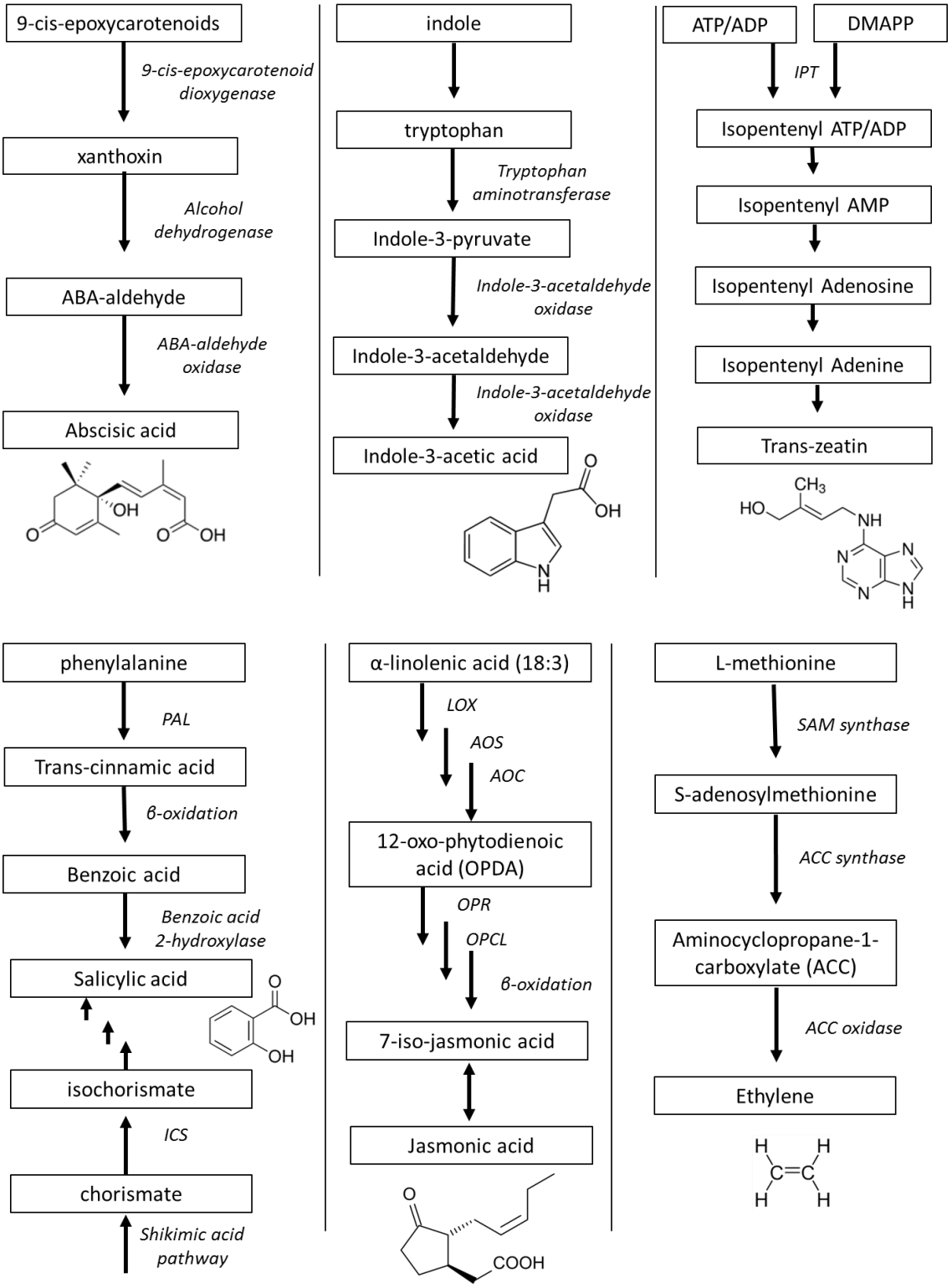

Figure 12: Main steps in the biosynthetic pathways of the major plant phytohormones (except for gibberellins and brassinosteroids. The most important intermediates in the biosynthesis are shown, with the corresponding enzymes shown in italics. Structures of the phytohormone end products are shown. *Abbreviations: ABA, abscisic acid; AMP, adenosine monophosphate; AOC, allene oxide cyclase; AOS, allene oxide synthase; ATP/ADP, adenosine triphosphate/adenosine diphosphate; DMAPP, dimethylallyl pyrophosphate; ICS, isochorismate synthase; IPT, isopentenyltransferase; LOX, lipoxygenase; OPCL, 3-oxo-2(-2-pentenyl)cyclopentane-1-octanoic acid-8:0-CoA ligase; OPR, OPDA reductase; PAL, phenylalanine ammonia lyase; SAM, S-adenosyl methionine.*

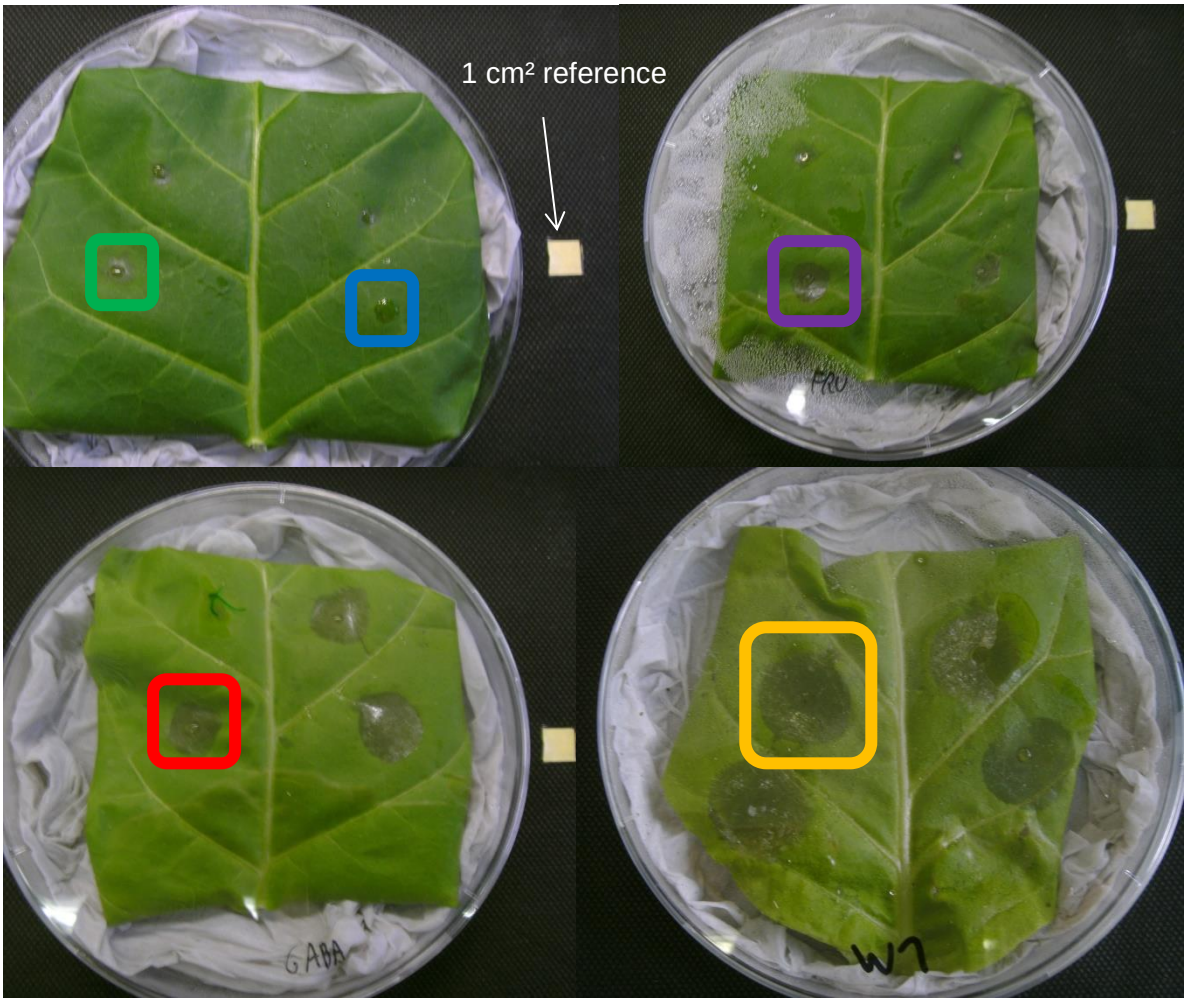

Figure 13: Disease scoring and lesion categorization. Pictures of *N. tabacum* source leaves, 6 and 10 dpi, taken from different experiments. Squares indicate the 5 categories used for lesion categorization with following code: dark blue = 0 – 0.2 cm², green = 0.2 – 1 cm², purple = 1 – 2 cm², red = 2 – 5 cm² and orange = >5 cm². The dark blue square shows a droplet of spore solution without visible lesion formation.

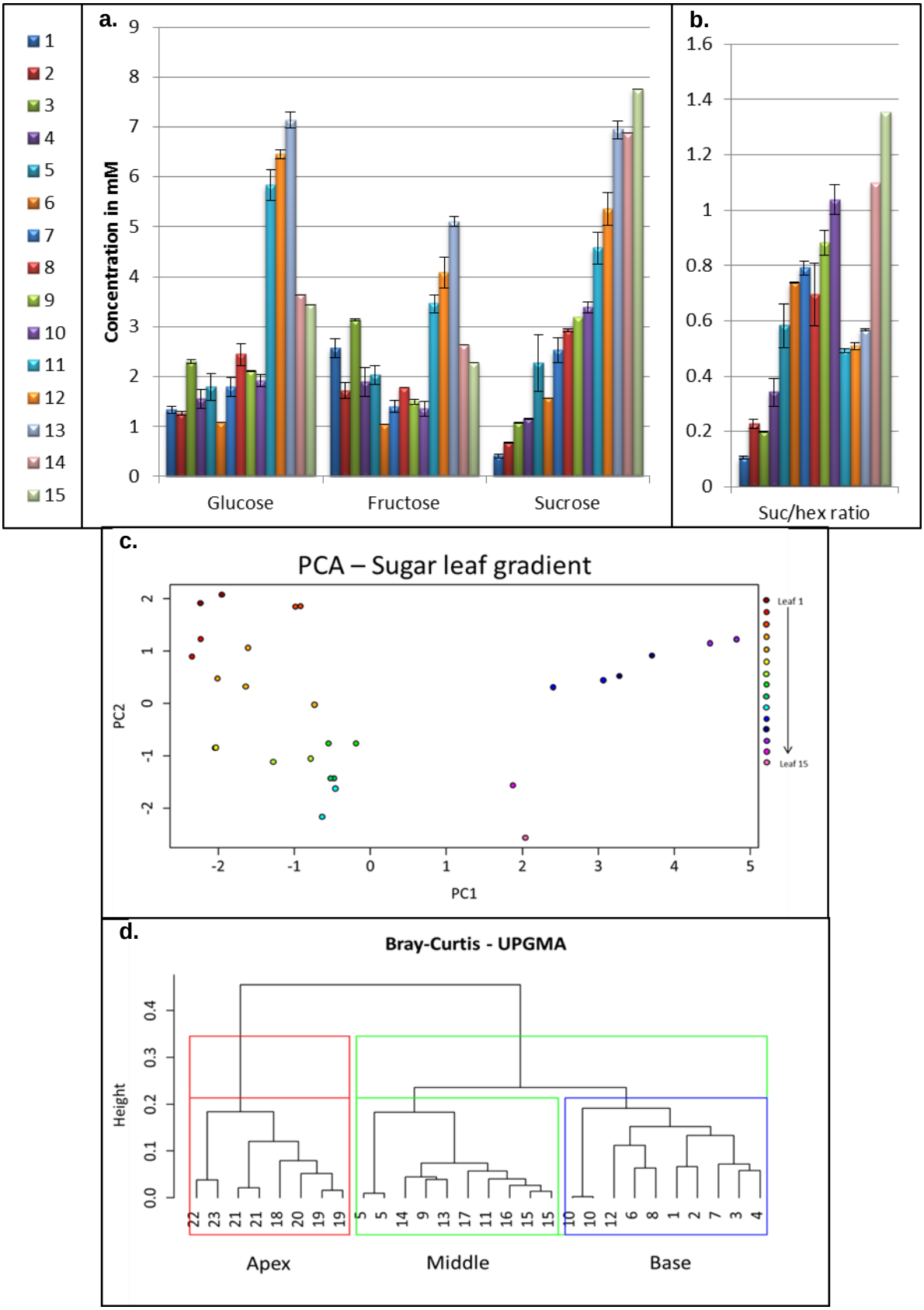

Figure 14: Experiment 1 sugar gradient. 15 leaves from one *N. tabacum* cv. Xanthi plant were sampled in a directional manner along the axis of the stem. Going from base to apex, leaves are numbered 1 through 15. **a.** Concentrations of Glc, Fru and Suc in mM. **b.** Suc to hexose ratios. **c.** PCA analysis using 2 principal components (PCs). **d.** Cluster analysis using an UPGMA clustering method on Bray-Curtis calculated distances for the data. Glc, Fru and Suc levels, as well as Suc to hexose ratios were used to do PCA and clustering. Bars represent mean values ± SE.

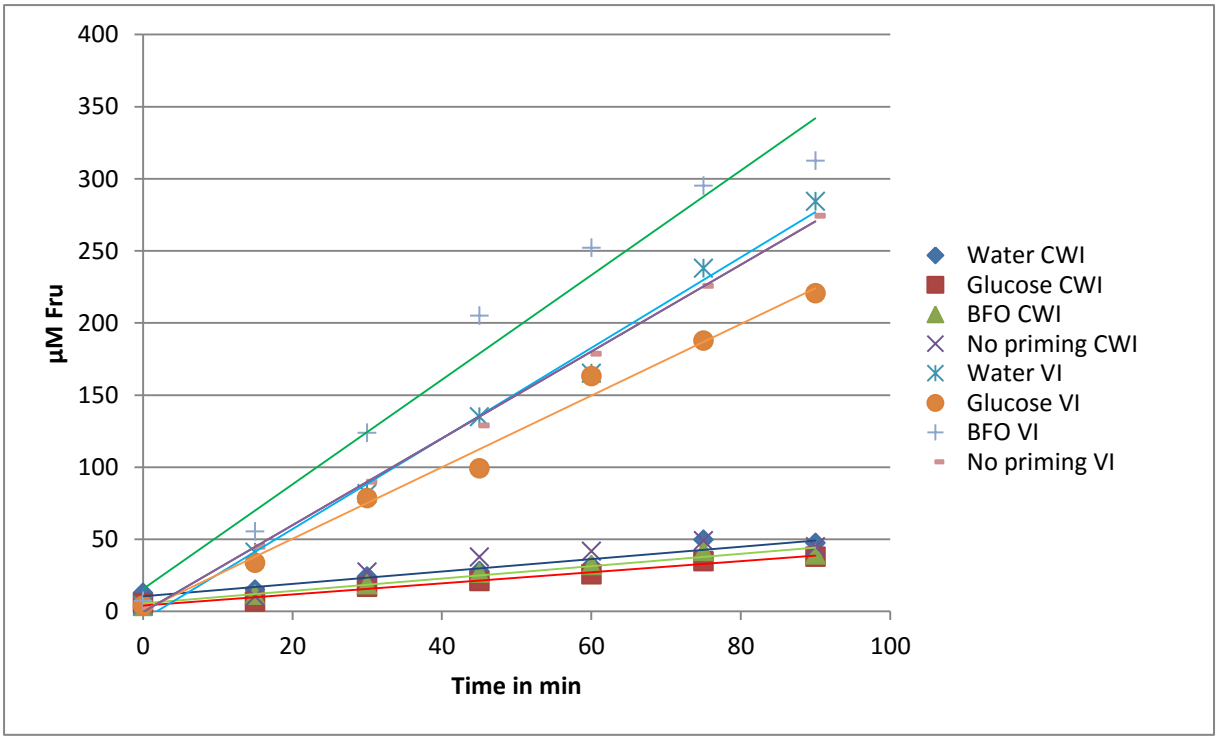

Figure 15: Time lineraity check for CWI and VI activity. Invertase activity was measured in Fru concentration for several different time points. 1 sample was used of each of the following treatments: Water-, Glc-, BFO- and unprimed. The following time points were taken: 0', 15', 30', 45', 60', 75' and 90'. Linear fits are plotted for each series of time points. R^2 values for linear fit CWI activity: 0.9327, 0.9794, 0.9613 and 0.8909 respectively. R^2 values for linear fit VI activity: 0.9909, 0.9895, 0.9735 and 0.9988 respectively.

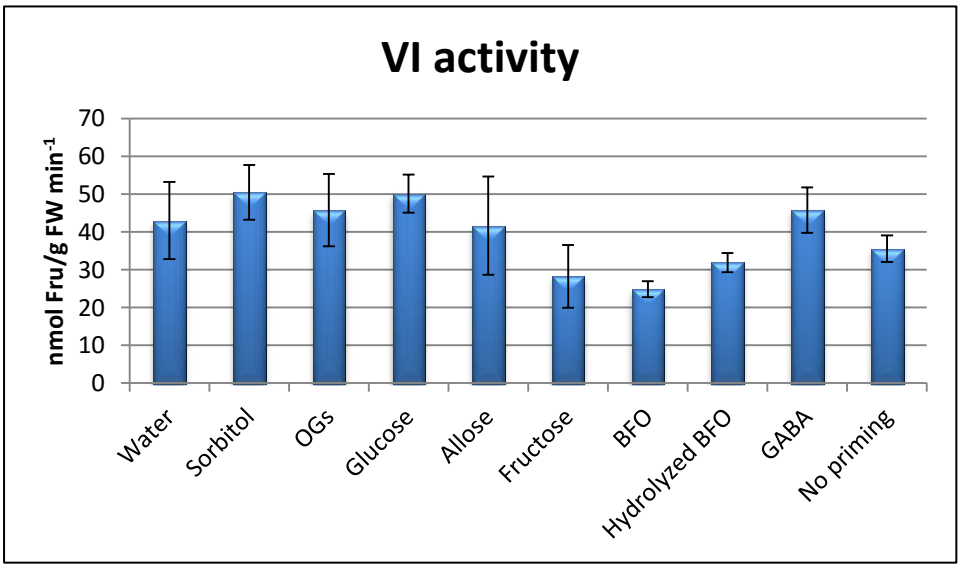

Figure 16: Experiment 3 VI activity. Source leaves of *N. tabacum* cv. Xanthi were primed by spraying priming solutions on the adaxial side. The following priming conditions were used: ultrapure water, 50 mM sorbitol, 1 g/L OGs, 50 mM Glc, 50 mM allose, 50 mM Fru, 5 g/L BFO, 5 g/L hydrolyzed BFO and 10 mM GABA. After 3 d of priming, leaves were crushed and homogenized leaf extract was analyzed. VI activities are shown in nmol Fru/g FW min-1. Bars illustrate mean values ± standard errors. An asterisk indicates significance compared to the control water, unless denoted otherwise (*: <0.05; **: <0.005).

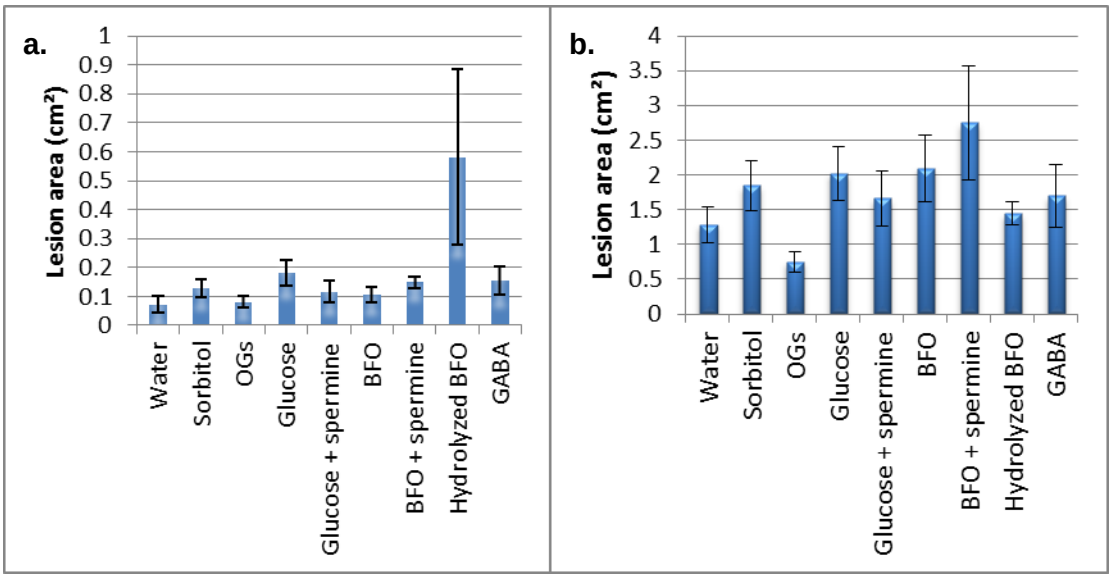

Figure 17: Experiment 4 disease scoring 3 and 6 dpi. Source leaves of *N. tabacum* cv. Xanthi were primed by spraying priming solutions on the adaxial side. The following priming conditions were used: ultrapure water, 50 mM sorbitol, 1 g/L OGs, 50 mM Glc, 50 mM Glc + 100 µM spermine, 5 g/L BFO, 5 g/L BFO + 100 µM spermine, 5 g/L hydrolyzed BFO and 10 mM GABA. After 2 d of priming, leaves were infected with *B. cinerea* spores. Disease scoring was performed 3 dpi. **a.** Disease scoring 3 dpi. **b.** Disease scoring 6 dpi. Mean lesion areas are shown, calculated based on a 1 cm² reference. Bars illustrate mean values ± standard errors. An asterisk indicates significance compared to water, unless denoted otherwise (*: <0.05; **: <0.005).

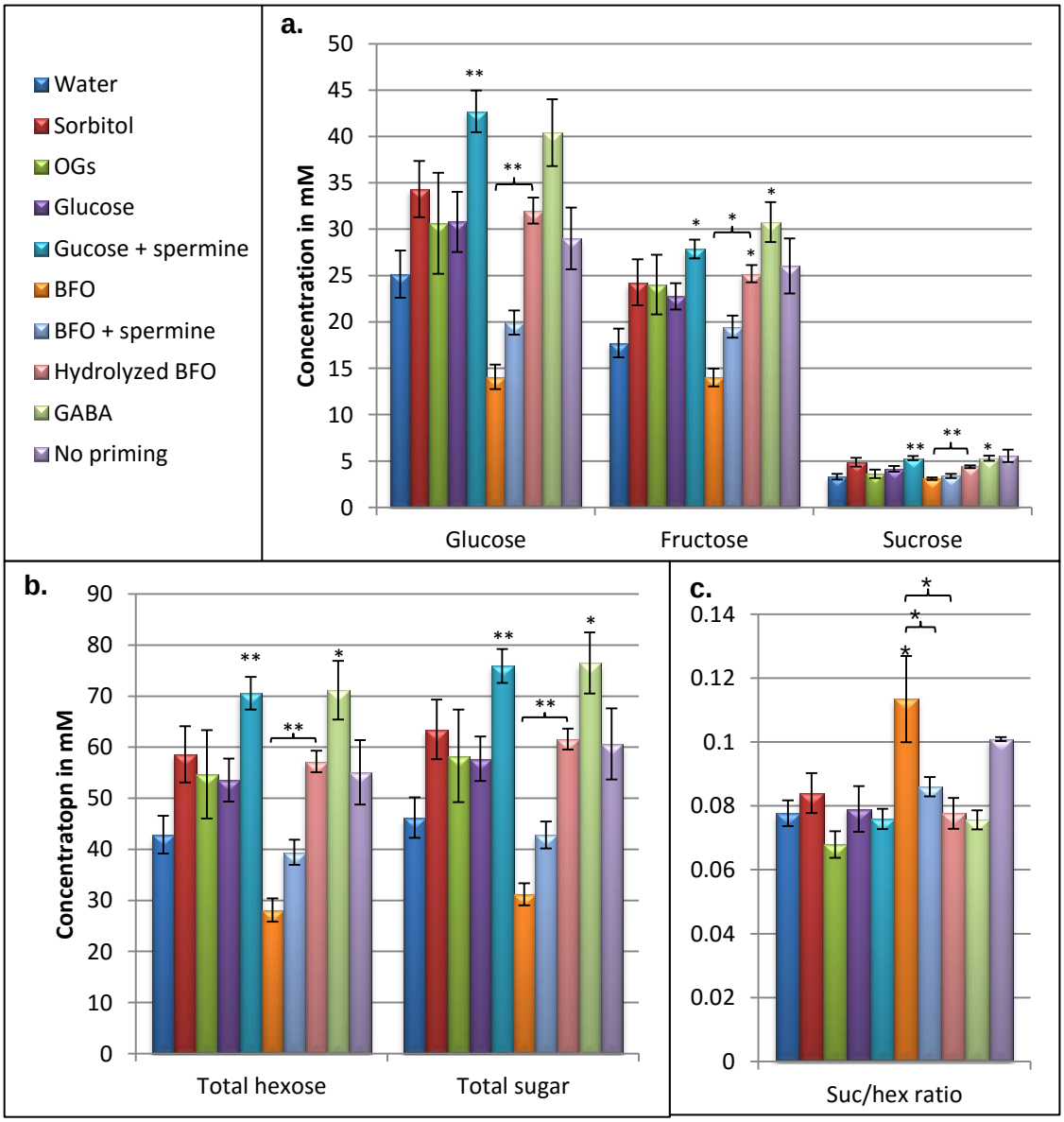

Figure 18: Experiment 4 analysis of small sugars. Source leaves of *N. tabacum* cv. Xanthi were primed by spraying priming solutions on the adaxial side. The following priming conditions were used: ultrapure water, 50 mM sorbitol, 1 g/L OGs, 50 mM Glc, 50 mM Glc and 100 µM spermine, 5 g/L BFO,, 5 g/L BFO and 100 µM spermine, 5 g/L hydrolyzed BFO and 10 mM GABA. After 2 d of priming, leaves were crushed and homogenized leaf extract was analyzed. **a.** Glc, Fru and Suc levels in mM. **b.** Total hexose and sugar levels in mM. **c.** Suc/hex ratios. Bars illustrate mean values ± standard errors. An asterisk indicates significance compared to the control water, unless denoted otherwise (*: <0.05; **: <0.005).

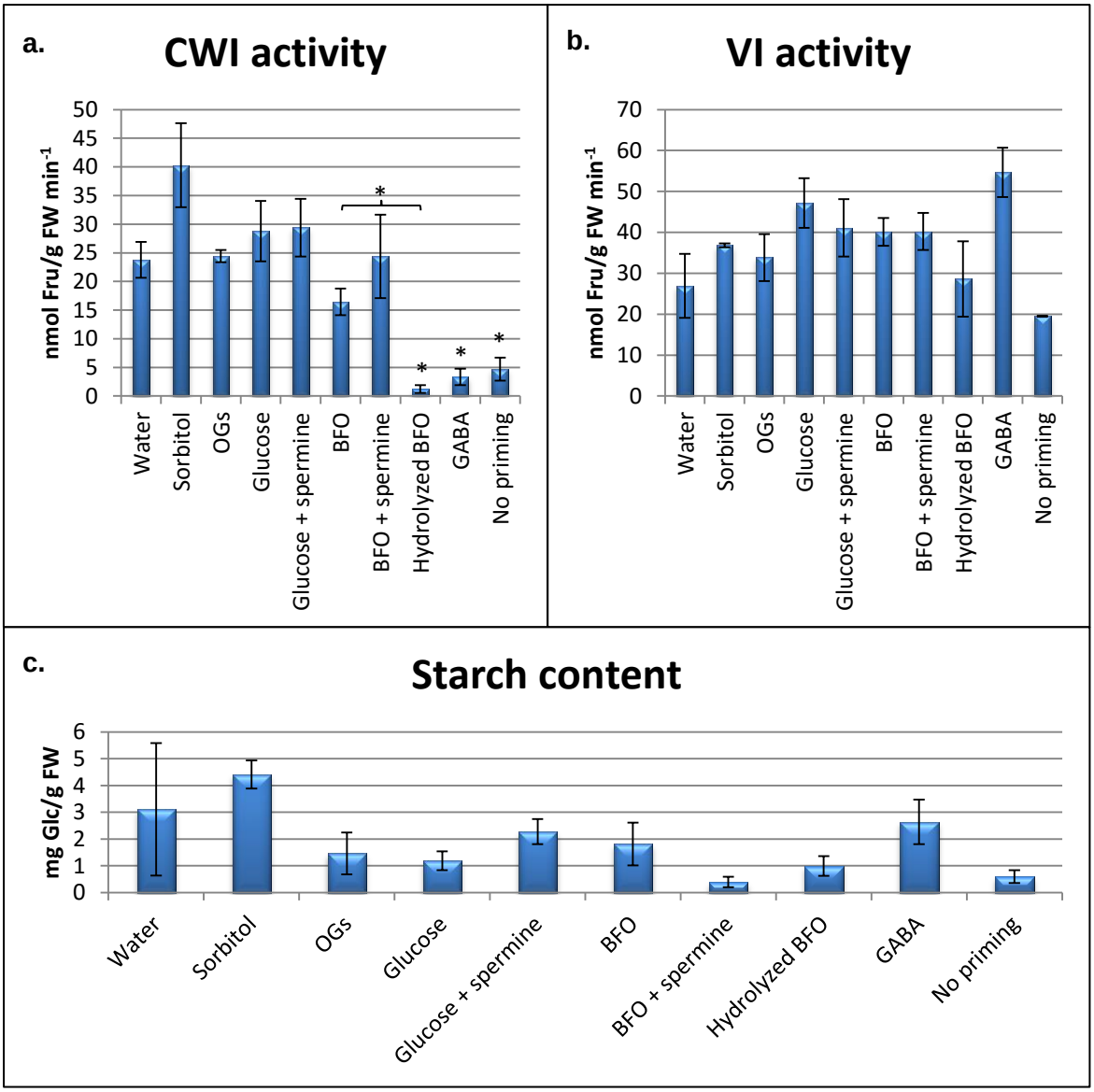

Figure 19: Experiment 4 invertase activity and starch degradation. Source leaves of *N. tabacum* cv. Xanthi were primed by spraying priming solutions on the adaxial side. The following priming conditions were used: ultrapure water, 50 mM sorbitol, 1 g/L OGs, 50 mM Glc, 50 mM Glc and 100 µM spermine, 5 g/L BFO,, 5 g/L BFO and 100 µM spermine, 5 g/L hydrolyzed BFO and 10 mM GABA. After 2 d of priming, leaves were crushed and homogenized leaf extract was analyzed. **a.** CWI activity in nmol Fru/g FW min^{-1}. **b.** VI activity in nmol Fru/g FW min^{-1}. **c.** Starch content measured in mg Glc/g FW. Bars illustrate mean values ± standard errors. An asterisk indicates significance compared to the control water, unless denoted otherwise (*: <0.05; **: <0.005).

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