UV-B priming for disease resistance: The use of UV-B light to reduce susceptibility of lettuce plants to downy mildew disease

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Plant Pathology

at Massey University, Manawatū,
New Zealand.

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2019

Acknowledgements

My PhD journey has been an exciting and fulfilling one. I am lucky to have landed a project that I am truly passionate about. For that, I have my supervisor, Jason Wargent, to thank. Jason has encouraged me since my undergraduate study to pursue research that is innovative and worthwhile. Jason has been incredibly helpful as a bank of photobiology knowledge with an infectious eagerness to discuss findings and future steps. I am extremely grateful to Jason for the skills, independence and confidence that I have gained as a researcher under his supervision.

My co-supervisor Julian Heyes has also been a valuable member of the team. Julian provided much perspective on new ways to look at data and implications for results outside of my own field. Thank you Julian for all the enjoyable thought-provoking discussions.

I would also like to express my gratitude to BioLumic and Massey University for their financial support of my PhD and access to staff and resources.

On a personal note, thank you to my friends and family for the support you have given me over the last three years. A special thanks to my husband Tim, who pulled me through the challenging times with constant positivity and encouragement. And when words of support were not enough, a thank you to my friend Keiran, who instinctively knew when only a glass of wine would fix a failed experiment.

Abstract

Biotrophic disease is one of the largest causes of decreased yield in horticulture. Integrated Pest Management (IPM) systems are required to control disease in a manner which is effective and sustainable, yet there are still a limited number of new approaches available. Pretreatments of UV-B light (280-320 nm) have been previously observed to reduce plant susceptibility to disease, and may be a potential disease control tool to use as part of an IPM approach. Here, I characterised the capability of UV-B LED technology to reduce susceptibility of a range of lettuce (*Lactuca sativa*) cultivars to downy mildew disease caused by the obligate biotroph *Bremia lactucae*. Reductions in disease susceptibility of UV-B-pretreated plants was observed as: delayed disease incidence, reduced visual disease rating and lower *B. lactucae* conidia count. UV-B-induced reductions to conidia counts were sufficient to reduce the infectivity of the diseased plant. Secondary infections caused by UV-B-pretreated plants exhibited yet further reduced disease severity. UV-B light has been observed to induce a similar gene expression profile to that of disease defence in plants. To determine the mechanism of a UV-B-induced disease defence, similarities between UV-B and disease defence pathways were identified. Analysis of previously published gene expression data revealed similarities in flavonoid-related gene expression between exposure to UV-B light in *Arabidopsis thaliana*, and resistance to downy mildew (*Hyaloperonospora arabidopsidis*). The specific role of flavonoids in UV-induced defence was further investigated, with *B. lactucae* conidia counts of lettuce plants negatively correlated with flavonoid level in a UV-B-dependent manner. LC-MS was used to identify metabolic features which contribute to this correlation, and of these, quercetin 3-O-(6″-O-malonyl)-β-D-glucoside had the strongest negative correlation with *B. lactucae* conidia count. The direct effect of quercetin 3-O-(6″-O-malonyl)-β-D-glucoside was tested through infiltration into lettuce leaves followed by subsequent downy mildew infection. Decreased *B. lactucae* conidia count was observed in two lettuce cultivars infiltrated with quercetin 3-O-(6″-O-malonyl)-β-D-glucoside concentrations similar to those induced by a UV-B-treatment. It was concluded that UV-B-pretreatments can decrease disease susceptibility to downy mildew in lettuce, and that this defence is underpinned in part by UV-B-induced phenolics. These findings highlight the opportunity for UV-B morphogenesis to be exploited in the development of next-generation, sustainable disease control tools.

Abbreviations

Lactuca sativa cultivars

CL Calicel

CS Casino

DS Desert Storm

ED El Dorado

FL Falcon

GW Greenway

IB Iceberg

LB La Brilliante

PD Pedrola

PV Pavane

SL Salinas

CA Chlorogenic Acid (5-Caffeoylquinic acid)

CTR Controlled temperature room

DCQA 3,5-dicaffeoylquinic acid

DoI Degree of infection

DPI Days post-inoculation

GH Glasshouse

Hpa *Hyaloperonospora arabidopsidis*

JA Jasmonic acid

LC-MS Liquid chromatography – mass spectrometry

LED Light-emitting diode

PAR Photosynthetically active radiation

PCA Principal component analysis

Q Quercetin 3-O-(6″-O-malonyl) -β-D-glucoside

ROS Reactive oxygen species

SA Salicylic acid

UV-B Ultraviolet B

UVR8 UV-B RESISTANCE LOCUS 8

Contents

Chapter 1

Introduction

Plant disease is a major threat to the horticultural industry. The world is facing a global food shortage in which increases to food production are unmatched by the exponential population growth (Chakraborty and Newton, 2011). Although this balance can in part be mitigated by increasing crop production, this is limited by a fixed amount of available land (Mahmuti et al., 2009). Increased efficiency of crop production is therefore required. Improved disease control will help maximise crop production efficiency by reducing both losses of quality and yield. Plant disease reduces the efficiency of crop production by decreasing potential yield by an estimated 16% (Oerke, 2006), with greater potentials for loss in different crops and production systems. Reducing loss through control of disease is a difficult task as current methods of disease control; primarily chemical sprays and breeding, place high selection pressure on the evolution of resistant pathogens (Strange and Scott, 2005). It is therefore important to continue to develop and improve plant disease control tools for reduction of potential yield loss from disease, and address the issue of food security. UV-B is an emerging technology which can be used to induce an increased defence against disease. Research into the UV-B response in plants is not as well studied as pathology; however, is rapidly becoming better understood. UV-B is a short wavelength, high energy light that acts as a signal to induce a protective response in plants (McDonald, 2003). Plants exposed to UV-B tend to be compact with thick leaves and high levels of polyphenols such as flavonoids (Jansen, 2002). The high tolerance phenotype of UV-B-exposed plants may decrease susceptibility to biotic stress such as pathogens and insects (Mewis et al., 2012; Demkura and Ballare, 2012). Although there are very few studies which examine the effect of UV-B on plant disease resistance, by reviewing

the two topics of the disease response and UV-B response in plants, overlaps between the pathways become apparent, suggesting possible theories on how UV-B may alter a plant's susceptibility to disease.

1.1. Plants have an arsenal of defences against pathogens

1.1.1. Plant pathogens are organisms which can induce disease

Micro-organisms are an essential part of all ecosystems for plants. The plant microbiota is essential for a number of plant functions including nitrogen-fixing, nutrient uptake as well as biotic stress protection (Pérez-Jaramillo et al., 2018; Schlaeppi and Bulgarelli, 2015; del Carmen Orozco-Mosqueda et al., 2018). However, some microbes form pathogenic relationships with plants, where they extract nutrients from the plant without returning any benefits. There are three key aspects which label these microbes as plant pathogens (Oku, 1994):

1. The ability to enter a plant
2. The ability to overcome host resistance
3. The ability to induce disease

Plant pathogens can cause disease; however, the disease itself is a pathological process of the plant (Oku, 1994). Agrios (2005) defines plant disease as "Any malfunctioning of host cells and tissues that results from continuous irritation by a pathogenic agent or environmental factor and leads to development of symptoms". This is a very broad term for disease that not only includes damage caused by pathogens but also environmental factors such as wind. In the plant pathology discipline, the term pathogen focuses on fungi, viruses, bacteria, nematodes and mycoplasma (Oku, 1994). Of these, filamentous pathogens, which include fungi and oomycetes such as *Bremia lactucae*, cause the greatest damage to plants (Oku, 1994).

1.1.1.1. Pathogens are classified as biotrophic or necrotrophic depending on their lifestyle

Plant pathogens are commonly classified based on their mode of infection (Oliver and Ipcho, 2004). Filamentous plant pathogens are classified as biotrophic or necrotrophic and in few cases hemibiotrophic (Oliver and Ipcho, 2004). In general, biotrophic pathogens feed on living plant tissue whilst necrotrophic pathogens feed on dead plant tissue (Glazebrook, 2005)). Hemibiotrophic pathogens have two life stages; initially, they infiltrate the plant in a similar way to that of a biotrophic pathogen (Oliver and Ipcho, 2004). Once established, a hemibiotrophic pathogen will switch to a necrotrophic lifestyle by releasing toxic compounds and enzymes to kill the host plant (Oliver and Ipcho, 2004). Biotrophic and necrotrophic pathogens must have different pathogenic lifestyles as biotrophic pathogens must maintain the plant host's life whilst a necrotrophic pathogen must kill the plant for nutrients (Glazebrook, 2005). Biotrophic pathogens are often obligate (must have living plant tissue to survive), produce haustoria, produce reduced lytic enzyme and result in reduced damage to the host (Oliver and Ipcho, 2004). However, these aspects can vary with the disease system. To respond to these two lifestyles, plants must have different pathways depending on the type of pathogen they are presented with. It is possible to also identify the nature of a pathogen based on the defence it elicits. Biotrophic pathogens are usually controlled in a gene for gene manner and will induce a hypersensitive response (Oliver and Ipcho, 2004). This is sensible as by inducing cell death, living plant tissue in the vicinity of the pathogen is killed thus removing the pathogens food source (Glazebrook, 2005). Glazebrook (2005) suggests that these groupings are not absolute and that an overlap between pathways thought for necrotrophic or biotrophic pathogens often occurs. The pathogen used in this project, *B. lactucae*, is an obligate biotrophic oomycete, and so biotrophic pathogens and defences are the focus of this review.

1.1.2. Plants have inbuilt mechanisms to combat biotrophic disease

Plants have mechanisms in place to try to survive potential pathogen attack. These can be considered as part of one of three processes; avoidance, resistance or tolerance (Bingham and Newton, 2009). Disease *avoidance* or escape refers to the ability of the plant to reduce the spread of pathogen spores through the canopy (Bingham and Newton,

2009). By avoiding the inoculum, plants remove the potential for disease. If spores reach a plant, *resistance* reduces the pathogen's infection or growth (Brown and Handley, 2006). *Tolerance* will often occur alongside resistance (Brown and Handley, 2006). Tolerance is the ability of the plant to exhibit less disease damage (and maintain yield) when infected (Brown and Handley, 2006). The key difference being; resistance describes processes that restrict pathogen growth within the plant, whereas, tolerance describes the plant's ability to grow normally despite an infection.

In order to defend against biotrophic pathogens, plants firstly attempt to prevent entry of the pathogen (Heath, 2000). If this fails, and the pathogen is recognised, innate immunity is induced (Jones and Dangl, 2006). Most commonly in biotrophic pathogens, this involves induction of the hypersensitive response, which kills cells in the area of the pathogen in order to limit growth (Jones and Dangl, 2006).

1.1.3. External barriers prevent entry of a pathogen

Prevention of penetration is of extreme importance to the disease response as part of disease avoidance. It is a major contributor to non-host resistance (Heath, 2000). Epidermal cells as the outermost layer of the plant have trichomes and a wax layer which can trap pathogens and prevent them from reaching the cell surface (Carver and Gurr, 2007). Pathogens which reach the cell surface are confronted with the cell wall. The cell wall acts as both a physical and chemical barrier as well as generating signals to recruit a PTI (pattern triggered immunity) response. Lignification of cell walls is a major contributor to the antimicrobial properties of the cell wall (Section 1.3.7.6).

The barrier is strengthened through the formation of cell wall appositions (papillae). Plants recognise the presence of a pathogen through pressure (Hardham et al., 2007), or perception of invasion patterns such as pathogen associated molecular patterns (PAMPs) (Macho and Zipfel, 2014) and cell wall products degraded by enzymes involved in pathogenesis (damage-associated molecular patterns (DAMPs) (Malinovsky F.G, 2014) by pathogen recognition receptors (PRRs). Upon recognition, the host cell undergoes cytoskeleton rearrangement (Hardham et al., 2007). At the point of recognition, actin filaments are re-organised, antimicrobial secretary products are transported, and callose syntheses are activated (Hardham et al., 2007). Together these result in the formation of the papillae.

Papillae tend to contain callose, phenolic compounds including lignin, proteins such as peroxidases and inhibitory enzymes and toxic compounds such as phytoalexins and H_2O_2 (Malinovsky F.G, 2014; Huckelhoven, 2007; Aist, 1976; Hardham et al., 2007; Zeyen et al., 2002). Formation of papillae has been associated with successful non-host resistance, such as resistance of *Arabidopsis thalina* to grass powdery mildew (*Blumeria graminis f. sp. hordei*) (Collins et al., 2003). Prevention of penetration is important to not only halt the pathogen infection but also reduce the induction of more costly defensive measures such as the hypersensitive response or induction of defensive gene expression.

1.1.4. Plants can recognise extracellular invasion patterns

Whilst the pathogen is in the extracellular space attempting to overcome barriers to enter the cell, the plant induces the first stage of induced defence. Traditionally, this first line of defence has been labelled pattern-triggered immunity (PTI) (Dodds and Rathjen, 2010). The PTI response is induced through recognition of PAMPs (Postel and Kemmerling, 2009). PAMPs are epitopes of molecules in pathogens that are essential for the pathogen's survival (Schwessinger and Zipfel, 2008). As they are essential for fitness, it is unlikely the pathogen will lose these patterns in evolution, making them an ideal target for host recognition (Postel and Kemmerling, 2009). To avoid recognition of self-molecules, epitopes which are present in both the host plant and pathogen are not recognised as PAMPs (Zipfel, 2014).

Recognition of PAMPs by plants occurs through PRRs on the surface of the plant cell (extracellular) (Macho and Zipfel, 2014) (Figure1.1). PRRs fall into two main categories; receptor-like kinases (RLK) which enable intracellular signalling through a ligand-binding ectodomain and intracellular kinase domain or receptor-like proteins (RLP) which lack this signalling domain (Macho and Zipfel, 2014). In order to induce signalling, an RLP must associate to a RLK, which often occurs as a result of PAMP binding (Macho and Zipfel, 2014).

Plants have a plethora of PRRs with different recognition site, in order to perceive a large range of PAMPs (Zipfel, 2014). PAMPs are highly specific. For example, the PRR; FLS2, recognises bacterial PAMPs from flagellum (flg22) but not oomycete PAMPs from the cell wall peptide Pep13 (Schwessinger and Zipfel, 2008). This specificity is caused by a binding domain that will only recognise the corresponding PAMP (Postel and Kemmerling, 2009). Once bound, the PRR undergoes a formational change, such as dimerization or

phosphorylation, to activate intracellular signalling (Macho and Zipfel, 2014). The activated receptor complex is translocated into the cell through endocytosis (Geldner and Robatzek, 2008). Endosomes carrying active receptor complexes can be used as a signalling platform to enhance the PAMP signal (Geldner and Robatzek, 2008). To activate downstream components such as gene expression of defence proteins and the resulting PTI, secondary messengers are required (Vidhyasekaran, 2014). Mitogen-Activated Protein Kinase (MAPK) cascades are thought to be the major signal system (Figure 1.1) (Pitzschke et al., 2009). Signal cascades are aided by messaging systems involving Guanosine triphosphate (GTP)-binding protein, the calcium ion, and reactive oxygen species (ROS) to cause a PTI response (Vidhyasekaran, 2014). PTI signalling results in a mild increase of a range of general defence genes (see 1.1.10).

Plants can also enhance a PTI response through the recognition of DAMPs (Boller and Felix, 2009). DAMPs are endogenous molecules to the host plants which are released upon injury or infection (Yamaguchi and Huffaker, 2011). This includes oligosaccharide fragments, ROS and protein fragments (Yamaguchi and Huffaker, 2011). DAMPs are either the by-products from attacks by pathogen derived enzymes (Boller and Felix, 2009), such as attack of the cell wall (Malinovsky F.G, 2014), or are produced by the host plant to amplify the PTI signal (Yamaguchi and Huffaker, 2011). In *Arabidopsis*, *PROPEP* are key DAMP genes which are up-regulated upon pathogen challenge (Ross et al., 2013). PROPEP proteins have an elicitor-active epitope (pep) which is recognised by cell surface receptors (PEPR) to result in increased amplification of the PTI response (Ross et al., 2013).

Extracellular recognition of PAMPs results in a response which can stop most non-adapted pathogens making it a very successful line of defence (Schwessinger and Zipfel, 2008). However, adapted pathogens possess effectors or toxins which suppress this innate immunity (Schwessinger and Zipfel, 2008). For example, in oomycetes, effectors commonly interfere with plant process such as enzyme inhibitors (Stassen and Van den Ackerveken, 2011). These pathogens successfully cause disease unless a strong specific response is induced. This is traditionally labelled effector-triggered immunity (ETI) is induced (Schwessinger and Zipfel, 2008).

Figure 1.1.: A pathogen recognition receptor (PRR) such as a receptor like kinase (RLK) recognises a pathogen associated molecular pattern (PAMP) from the pathogen and induces a signal cascade such as MAPK. The signals cause a change in expression of defence and regulation genes resulting in a PAMP triggered immunity (PTI) response. Figure from Bent and Mackey (2007)

1.1.5. Resistance genes recognise intracellular receptors

Pathogens have evolved alongside their host plants resulting in an 'arms race' of defence. Pathogens which can overcome the PTI response do so through the use of effectors (Schwessinger and Zipfel, 2008). However, a resistant host plant can recognise effectors resulting in ETI (Dangl et al., 2013). As an ETI response tends to be more intense than PTI, it is highly regulated as to carefully control resources (Jones and Dangl, 2006).

Effectors, from pathogen *AVR* (Avirulence) gene loci (Dangl and Jones, 2001) enter the plant cell and are thus recognised by intracellular receptors (Chiang and Coaker, 2015). These receptors are known as R (Resistance) proteins and can recognise the effector either directly or indirectly (Figure 1.2) (Bent and Mackey, 2007). Direct recognition is achieved by forming a physical association with the effector (Figure 1.2) resulting in a conformational change thus activating the R protein (Chiang and Coaker, 2015). Indirect recognition involves recognition of a plant protein that has been modified by the effector, usually a virulence target (guard) or mimic of a target (decoy) (Figure 1.2) (Dodds and Rathjen, 2010). The binding event is very specific, so for resistance to occur, a plant host must have the R protein which recognises the infecting pathogens AVR protein in order for induction

of ETI (Dangl et al., 2013).

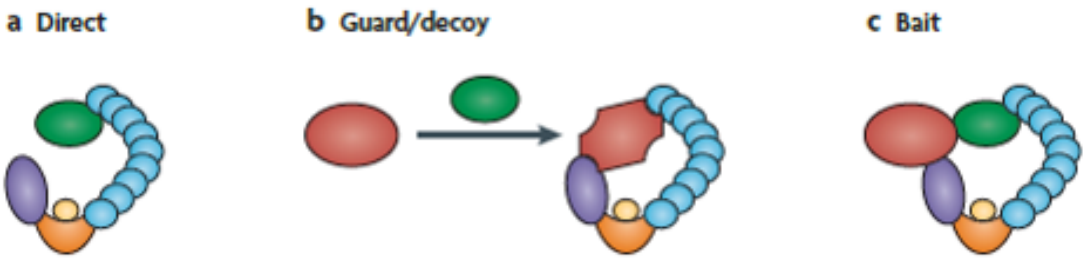


Figure 1.2.: Recognition of the effector can be achieved directly or indirectly. In the direct approach (a), the receptor binds directly to the effector (green). In an indirect event (b & c), the receptor recognises a plant protein (red) that is modified by an effector (green). Indirect recognition can also occur by facilitation of binding by an accessory protein (c). Adapted from Dodds and Rathjen (2010).

The majority of R proteins are nucleotide binding-leucine-rich repeat (NB-LRR) proteins (Wu et al., 2014) (Figure 1.3). In the absence of a pathogen, the C-terminus of the LRR domain binds to the NB domain resulting in a closed or inactive state (Takken and Goverse, 2012). However, when the LRR domain senses a pathogen, it undergoes a conformational change, exposing the NB region (open state) (Takken and Goverse, 2012). The exposed binding site allows recognition of an effector as described above. Once activated the N-terminal region can be used to signal activation of ETI response (Takken and Goverse, 2012), or for R proteins with a C-terminal extension, are transported to the nucleus and directly influence gene expression (Wu et al., 2014). In both cases a full ETI response in launched.

ETI stimulates many of the same genes as a PTI response, but in a much more amplified way, which can result in a hypersensitive response (Dodds and Rathjen, 2010). ETI can also induce systemic acquired immunity (SAR), with the aid of defence hormone salicylic acid (SA), which prepares non infected plant parts for potential attack (Dodds and Rathjen, 2010). Together, this response provides such an effective defence it can deem a plant completely resistant to a pathogen containing the *AVR* gene which induced it.

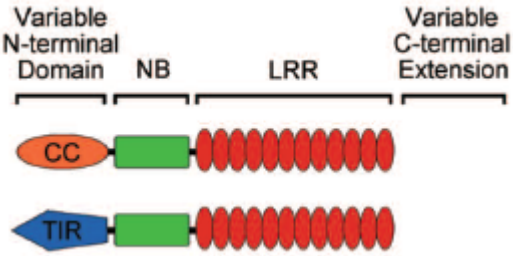


Figure 1.3.: The majority of R proteins are NB-LRR proteins. These are grouped as CC (coiled-coil) or TIR (toll/interleukin 1 receptor like) depending on the N-terminal group. Some R proteins also have an additional group at the C-terminal for their biological function. Adapted from Wu et al. (2014).

1.1.6. Traditional plant pathology models have some limitations

The current plant pathology model (ZigZag model) is as described in Sections 1.1.4 and 1.1.5 in which there are two lines of defence. PTI acts as a first line of defence by producing a weak response through recognition of extracellular PAMPs (Jones and Dangl, 2006; Dangl and Jones, 2001). If PTI is overcome by the use of effectors, and the host plant is able to recognise the pathogen's intracellular effector, a stronger ETI response is induced which commonly results in HR and race-specific protection of the host plant (Jones and Dangl, 2006; Dangl and Jones, 2001). More recent publications (Cook et al., 2015; van der Burgh and Joosten, 2019; Kanyuka and Rudd, 2019) suggest that this model has several limitations:

- An exclusion of DAMPs,
- PTI and ETI can induce a similar response type and magnitude
- Several effectors are conserved across pathogens indicating a lack of specificity
- PTI has the ability to induce HR
- PRs and intracellular receptors can have similar structures
- Effectors can be recognised by extracellular receptors

These studies propose that plant immunity is too broad to be described in such strict terms, and instead a 'Spatial Invasion model' which characterises immunity based on the localization of pathogen reception as either intra- or extra- cellular (Kanyuka and Rudd, 2019). Pathogen reception is achieved through recognition of invasion patterns (IP) or danger signals which may be pathogen or plant derived (Cook et al., 2015; Kanyuka and

Rudd, 2019; van der Burgh and Joosten, 2019). Both intra- and extra- cellular responses can be weak or strong, narrow or specific and may induce a HR (Kanyuka and Rudd, 2019). Although the Spatial Invasion Model is still a relatively recent suggestion, it is likely to replace the ZigZag model in future due to the simplicity of the model and the ability to describe such as broad range of pathogen types and invasion patterns.

1.1.7. SAR prepares non-infected plant parts for pathogen attack

SAR is a broad-spectrum resistance which provides prolonged (up to several months) protection in uninfected plant tissue (Kachroo and Robin, 2013). Protection is transgenerational with immune 'memory' continued in subsequent generations (Kachroo and Robin, 2013). Pathogen recognition (commonly by NB-LRR receptors) induces the generation of mobile signals in the area of infection (Kachroo and Robin, 2013). The mobile signal translocate, possibly via the phloem, to uninfected tissues (Gao et al., 2014) and stimulates accumulation of SA (Henry et al., 2013). As described in Section 1.1.9.1, SA is a key hormone in regulating defence genes, in particular, those for combating biotrophic pathogens (Henry et al., 2013). Expression of these defence genes results in preparation of the plant tissue for attack. In most cases, these prepared tissues will resist infection when confronted with a pathogen (Henry et al., 2013).

1.1.8. The hypersensitive response results in programmed cell death of pathogen threatened cells

The HR is a localised, controlled cell death at the site of infection (Kombrink and Schmelzer, 2001). As biotrophic pathogens extract nutrients from living tissue, the HR is particularly effective against biotrophic attacks. Through killing the host cell, the nutrient supply is removed, causing the biotrophic pathogen to starve (Morel and Dangl, 1997). The HR may also have a role in protection against necrotrophic pathogens; however, this is not strongly supported. As necrotrophic pathogens reside primarily in the extracellular space, nutrients are gained from the extracellular matrix (Morel and Dangl, 1997), and the death of a cell may provide helpful to the necrotrophic lifestyle (Johal et al., 1995). It has been suggested that upon cell death due to HR, toxins, such as phytoalexins, may be released creating an antimicrobial environment, which could provide some protection against necrotrophic as well as biotrophic pathogens (Morel and Dangl, 1997). In most incompatible interactions, the HR prevents biotrophic infections or slows the infection

process, allowing more time for other disease responses to be induced (Camagna and Takemoto, 2018).

Although the HR has been induced by a PPR type receptor response, it is much more commonly, and effectively induced by intracellular NB-LRR receptors (Naito et al., 2007; Taguchi et al., 2003). These induced immune responses result in the production of Reactive Oxygen Intermediates (ROIs), as well as SA, which acts as signals to induce defence responses (Camagna and Takemoto, 2018; Morel and Dangl, 1997). If these defence signals reach a certain threshold (dependent on the disease-plant system), the HR is induced (Kombrink and Schmelzer, 2001). These signals are very rapid, with the HR response launching when a pathogen is detected, but has not yet penetrated the cell membrane. HR begins by migration of the nucleus toward the pathogen (Freytag et al., 1994) (Figure 1.4). If cell wall barriers prevent entry of the pathogen, this action is reversible, and the nucleus will return to normal when the threat is over (Freytag et al., 1994). However, if the pathogen penetrates the cytoplasm, an irreversible programmed cell death begins (Freytag et al., 1994). Organelles and the nucleus condense, and granules form followed by shrinkage of the cytoplasm and eventual collapse of the cell resulting in cell death (Chen and Heath, 1991) (Figure1.4).

Figure 1.4.: The Hypersensitive response process. Upon recognition of a pathogen by a cell, the nucleus moves towards the potential penetration site (a-b). If the pathogen successfully penetrates the cell, the organelles and nucleus condense and cytoplasmic granules form (c-d). The nucleus and vacuoles rupture and eventual collapse of the protoplast occurs resulting in the final stage of cell death (e-f). Diagram from Camagna and Takemoto (2018).

1.1.9. Phytohormones are induced as a response to pathogen infection

Confrontation with a pathogen results in the induction of a number of hormones (Bari and Jones, 2009). SA, jasmonic acid (JA), and ethylene (ET) are thought to be the key hormones which contribute to defence signalling (Bari and Jones, 2009). These are usually induced downstream of PTI or ETI induction (Pieterse et al., 2012). SA primarily aids in defence against biotrophic pathogens through induction of pathogenesis-related (PR) proteins and SAR (Grant and Lamb, 2006). JA and ET are more involved in protection against necrotrophic pathogens and herbivorous insects as well as a range of other growth responses (Lorenzo and Solano, 2005). JA provides defence through direct and indirect induction of defensive genes (Lorenzo and Solano, 2005). JA and SA pathways are mutually antagonistic; however, there has been recent evidence that suggests they may work synergistically in some scenarios (Bari and Jones, 2009).

1.1.9.1. SA induces PR proteins

As well as inducing a SAR, SA induces expression of defence genes, including PR proteins (Section 1.1.10.1) which aid in disease resistance (Loake and Grant, 2007). SA is synthesised

through the shikimate pathway (Vlot et al., 2009) (Figure 1.5). SA can be converted into a number of forms, including methylated SA (MeSA) (Kumar, 2014). MeSA can cross into the cytosol of the plant to induce disease resistance in that cell or recruit a mobile signal to transport to distal cells (Kumar, 2014). The mobile signal then increases production of SA in the distal cells as part of a SAR response (Figure 1.5) (Gao et al., 2014).

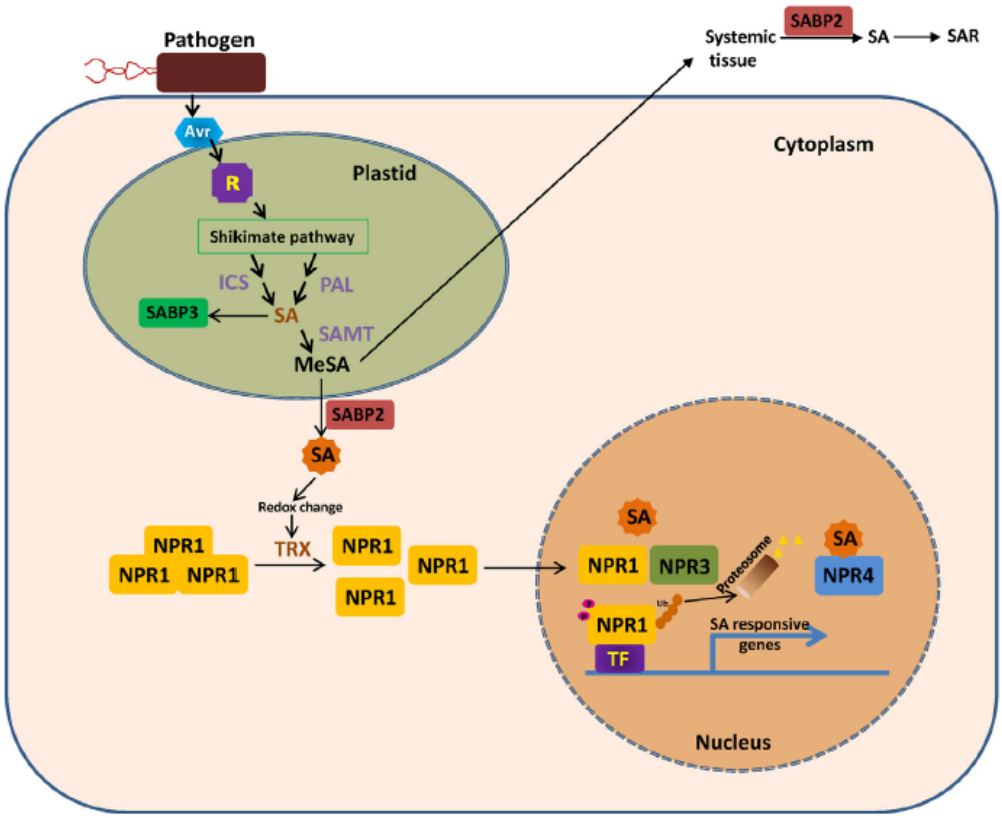


Figure 1.5.: A model of salicylic acid (SA) signalling in a plant cell from Kumar (2014). Pathogen attack induces increased production of SA. SA is converted to methylated SA (meSA); the mobile form. MeSA can be transported to other cells to cause a systemic acquired repsonse.. SA can also cause a redox change which allows the messenger, Non-expressor of PR1 (NPR1), to enter the nucleus and up-regulate pathogen defence genes. Avr = avirulence protein, R = resistance protein, ICS = isochorismate synthase, PAL = phenylalanine ammonia-lyase , SAMT = SA-methyl transferase, SABP2 = SA-binding protein 2, NPR3 = Nonexpressor of PR3.

As SA cannot directly move into the nucleus to alter gene expression of defence genes, it uses a signal protein; Nonexpressor of PR1 (NPR1) (Mou et al., 2003). NPR1 exists in an inactive state as an oligomer, with monomers held together by disulphide bonds (Mou et al., 2003). Upon activation of immunity, accumulation of SA causes a redox change in the cell (Mou et al., 2003). This reducing environment causes reduction of the disulphide bonds, releasing NPR1 into an active monomer state (Mou et al., 2003). As

a monomer, NPR1 translocates into the nucleus where it associates with transcription factors to activate transcription of defence genes including *PR* genes (Figure 1.5) (Mou et al., 2003). Expression of these genes results in increased disease defence with particular effectiveness against biotrophic pathogens.

1.1.10. Induced immunity results in increased production of antimicrobial compounds

The PTI, ETI and hormone responses activate many defensive genes. These cause the production of a range of proteins and metabolites that act to inhibit growth or kill the pathogen (Freeman and Beattie, 2008).

1.1.10.1. Pathogenesis-related proteins protect plants against biotrophic pathogens

PR genes are a major group of genes that are activated to defend against biotrophic pathogens (Van Loon and Van Strien, 1999). Most commonly these genes encode enzymes that degrade molecules specific to non-plant organisms such as chitinases; however, some PR families have an unknown function (Stintzi et al., 1993). PR proteins possess anti-fungal activity and are often associated with SAR and the HR (Van Loon and Van Strien, 1999). Antimicrobial peptides, as indicated by the PR families under 10kDA, such as defensins, have more recently been added to the list of PR families (Sels et al., 2008). PR proteins usually act as blockers or inhibitors of the biological activity of the invading pathogen (Sels et al., 2008). This includes inhibiting protein translation, enzymes, or ion channels as well as a range of other essential processes depending on the peptide (Carvalho and Gomes, 2009). Although the structure and mechanism vary depending on the PR protein, inhibition is commonly achieved by binding to the microbial target (Carvalho and Gomes, 2009). Once bound, the microbial target can no longer carry out normal function resulting in reduced virulence of the pathogen (Carvalho and Gomes, 2009). The use of PR proteins in protection against oomycetes, such as *B. lactucae*, will heavily depend on the PR protein target. For example, many PRs (PR3,4,8,11) target chitin in fungal cell walls (Sels et al., 2008); however, oomycete cell walls are composed primarily of cellulose (Beakes et al., 2012), resulting in a lack of effectiveness of these PRs.

1.1.10.2. Defensive secondary metabolites strengthen and protect the host plant

A number of secondary metabolites, called phytochemicals, have a role in increased protection against disease (Bednarek, 2012). These usually contribute to strengthening

physical barriers to prevent entry of the pathogen, such as lignins, or have antimicrobial properties, such as phytoalexins. Phytochemicals are most commonly phenolics and are derived from shikimic acid as part of the phenylpropanoid pathway (Figure 1.6) (Bennett and Wallsgrove, 1994).

The key example for strengthening of barriers to prevent entry is lignin. Lignins are an important part of the cell wall in plants (Bhuiyan et al., 2009) and are produced by the phenylpropanoid pathway (Vance et al., 1980). Upon induction of the disease response, monolignol biosynthesis and accumulation into cell walls is increased (Vance et al., 1980). The increased lignin content of the cell wall provides an increased resistance to the mechanical pressure from penetration structures of invading fungi(Bhuiyan et al., 2009). Lignin also increases the hydrophobic nature of the cell wall, resulting in reduced efficiency of the pathogen released cell wall degrading enzymes, thus further preventing entry (Bhuiyan et al., 2009). Lignin precursors have also been suggested to be toxic to pathogens by binding to the pathogen cell wall restricting growth through inhibition of water and nutrient uptake (Lattanzio et al., 2006). Other structural phenolic compounds are used to plug wound sites to prevent entry of more opportunistic pathogens (Bennett and Wallsgrove, 1994).

The host plant also produces a number of antimicrobial secondary metabolites. These tend to disrupt pathogen metabolism or cellular structurein a plant species specific manner (Freeman and Beattie, 2008). The plant produces some antimicrobial secondary metabolites as part of normal growth (phytoanticipins) and store these in specialised tissues, to be used upon attack (Bednarek and Osbourn, 2009). Phytoanticipins are often stored in an inactive form in the epidermal cell cytoplasm or vacuole, then activated through cleavage upon pathogen recognition (Lattanzio et al., 2006). Others may be present in active form, but are on the plant surface, such as in waxes, as a deterrent to pathogens (Lattanzio et al., 2006). Phytoanticipins are often simple phenols, phenolic acid, flavonoids and dihydrochalcones (Lattanzio et al., 2006).

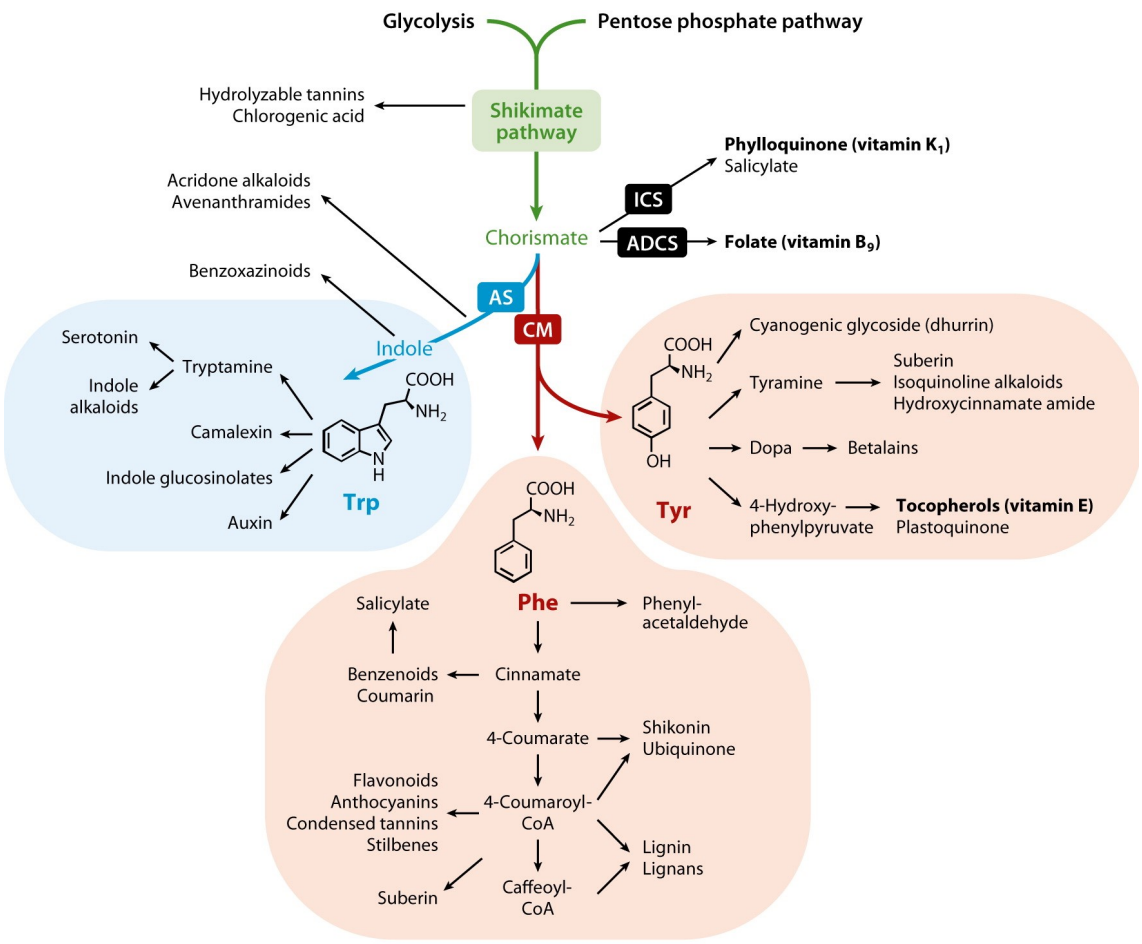


Figure 1.6.: Many secondary metabolites involved in defence against pathogens are derived from shikimic acid pathway. This includes defensive phytoalexins such as camalexin, defensive flavonoids, glucosinolates, lignin, and defensive phytohormone salicylic acid. Diagram from Maeda and Dudareva (2012). ICS = isochorismate synthase, ADCS = aminodeoxychorismate synthase, CM = Chorismate mutase, Phe = phenylalanine, Tyr = tyrosine or Trp = tryptophan.

Others phytochemicals which are synthesised as a response to microbial attack are labelled phytoalexins (Bednarek and Osbourn, 2009). These are usually pterocarpan, isoflavan, and isoflavanone-type compounds (Dixon et al., 2002). Camalexin, in the Brassicaceae family, is most the studied phytoalexin, which causes resistance to many necrotrophic pathogens and some biotrophic pathogens through disruption of the cell membrane (Ahuja et al., 2011). Other plant families (Table 1.1) have different phytoalexins with a range of resistance mechanisms. Although the range of phytoalexins are structurally diverse, they mostly tend to be a local response, with accumulation at the site of infection (Bennett and Wallsgrove, 1994). Phytoalexins are of extreme importance to the resistance of the plant with many strong correlations between rapid biosynthesis of phytoalexins, and pathogen resistance published (Graham et al., 1990; Fett and Jones, 1984; Snyder and Nicholson,

1990; Hahn et al., 1985).

Table 1.1.: Example phytoalexins in some plant families. Table adapted from Ahuja et al. (2011).

Plant family	Example phytoalexins
Brassicaceae	Brassinin, camalexin spirobrassinin, cyclobrassinin, rutalexin, rapalexin A and brassilexin
Fabaceae	Medicarpin and its isoflavone precursors, sativan, maackiain, luteone, wighteone, pisatin, gyceollins, resveratrol, arachidin-1, arachidin-2, arachidin-3, isopentadienyl-4,3′,5′-trihydroxystilbene, SB-1, arahypin-1, arahypin-2, arahypin-3, arahypin-4, arahypin-5, arahypin-6, arahypin-7, aracarpene-1 and aracarpene-2
Solanaceae	Scopoletin, capsidiol, resveratrol, viniferins, piceids and pterostilbene
Poaceae	Kauralexins, avenanthramides, momilactone A, momilactone B, phytocassane A – phytocassane E, sakuranetin and oryzalexin E, luteolin, apigenin and 3-deoxyanthocyanidins and zealexins

Glucosinolates (GL) are antimicrobial compounds found in the order Capparales (includes the Brassicaceae family), which although primarily associated with protection against insects, have been shown to protect plants against fungal pathogens also (Bednarek et al., 2009). GLs are non toxic in their native state, which reduces the risk of damaging the host cell (Manici et al., 1997). Upon detection of a pathogen or herbivore, a myrosinase enzyme hydrolyses the GL, releasing a toxic side chain called a Enzymatic Hydrolysis Derived Product (EHDP) (Manici et al., 1997). There is huge variation in GLs and their resulting EHDPs with different toxicities, and may provide local or systematic resistance depending on cultivar (Figure 1.7) (Redovnikovic et al., 2008). These include isothiocyantes, nitriles, thiocyanates and thiones (Gil and MacLeod, 1980; Uda et al., 1986a,b). The EHDP is more important for plant resistant to pathogens than the GL it is derived from (Buxdorf et al., 2013).

Figure 1.7.: Toxic side chains (Enzymatic Hydrolysis Derived Product) that could be derived from different types of glucosinolates. R group depends on the glucosinolate it is derived from. From Redovnikovic et al. (2008).

1.1.10.3. Oxidative burst acts as an antimicrobial agent as well as a danger signal

The oxidative burst is one of the early responses to pathogen attack in plants (Wojtaszek, 1997; Lamb et al., 1998). The oxidative burst leads to the production of ROS; mostly hydrogen peroxides (H_2O_2), through NAPPH oxidases in the cell membrane or peroxidases in the cell wall (Bindschedler et al., 2006). In most cases, the cell has antioxidents which reduce the number of potentially harmful ROS; however, during the oxidative burst, these protective mechanisms are overwritten, and a large amount of ROS accumulate within the cell (Wojtaszek, 1997). In response to a pathogen, ROS can act as both a protectant against the invading pathogen or as a signal to recruit other defence responses (Tenhaken et al., 1995). At the cell surface, H_2O_2 is directly toxic to microbes causing direct protection (Peng and Kuc, 1992). ROS has also been shown to be involved in inducing SA resulting in both SAR and the HR response (Chen et al., 1993; Tenhaken et al., 1995) as well as being associated with phytoalexin accumulation (Apostol et al., 1989).

1.2. Downy mildew is a major threat to the lettuce industry

1.2.1. Lettuce is a leafy crop produced in high volume

Cultivated lettuce (*Lactuca sativa*), from the family Asteraceae, is a rosette style crop plant, most commonly used uncooked in salads and sandwiches (Ryder, 1999). Lettuce plants have a thick, short taproot and shortened stem, giving them a leafy appearance (Whitaker, 1974). Crisphead lettuce (iceberg) is the most common lettuce type, making up 61% of total lettuce production in the US in 2009 (ERS, 2011). The leaf is the harvestable part of the lettuce; however, a head of leaves is often sold as a whole unit (Whitaker, 1974). New Zealand makes up only a very small (0.1%) of worldwide lettuce production (23 thousand metric tons), with the largest lettuce production occurring in China (12 million metric tons), and the USA (4 million metric tonnes) in 2009 (ERS, 2011).

Lettuce production systems vary between countries depending on labour costs, climate and landscape. In America, where labour can be inexpensive, lettuce is commonly direct seeded into the field for growth until harvest (Kerns et al., 1999; Turini et al., 2011; Whitaker, 1974). In other countries, such as New Zealand and Australia, lettuce is sown into trays, and seedlings are reared in a glasshouse before being transplanted into the field (Heisswolf et al., 1997). Although not as common, lettuce can also be produced hydroponically; however, this method is not considered appropriate for the production of iceberg type lettuce (Parkell et al., 2015).

1.2.2. *B. lactucae* is the causal agent of downy mildew in lettuce

B. lactucae is the causal organism of the disease downy mildew in lettuce (*Lactuca*) species (Lebeda et al., 2008). *B. lactucae* is an obligate biotrophic pathogen belonging to the oomycete class (Cavalier-Smith and Chao, 2006). Although oomycetes are not phylogenetically fungi, they are considered 'fungal-like' due to similar hyphal systems, modes of nutrition and ecological roles to fungi (Beakes et al., 2012) (Table 1.2). Oomycetes are often grouped with biotrophic fungi in the plant pathology discipline (Clark and Spencer-Phillips, 2004).

Lettuce within a nursery; such as prior to transplant, have the highest risk of infection. Nursery plants are grown in high density, providing more opportunity for spores to spread, and are a young age which is more vulnerable (Dickinson and Crute, 1974). Once outdoors,

key infection times are determined by climate; where wet periods, moderate temperatures and high humidity strongly encourage downy mildew infection thus increase risk of disease (Fall et al., 2015, 2016; Hovius et al., 2007; Kushalappa, 2001; Scherm and Bruggen, 1994a,b, 1995; Su et al., 2000, 2004; Wu et al., 2000). Near harvest is an economic risk point as disease can ruin an entire field resulting in wastage of resources up to the final stage. Current control systems are largely based on protection at these risk points.

Formation of sporangiophores is the most distinguishing symptom of downy mildew disease in lettuce. Sporangiophores are visible as a fluffy white growth usually on the undersides of leaves (Koike and Davis, 2012). The leaf becomes yellow and chlorotic on the upper side of the leaf in an angular area corresponding to sporangiophores on the underside (Koike and Davis, 2012). If the disease progresses, these spots often become brown necrotic lesions and can cause leaf death (Koike and Davis, 2012). Occurrence of any of these symptoms on a singular leaf will deem that leaf unmarketable and will need to be removed (Scheufele, 2017). In severe cases where symptoms have spread throughout the plant, this can result in the entire head, or in extreme cases the entire planting being left in the field (Scheufele, 2017). Downy mildew also can encourage post harvest disease by acting as portals for secondary infections by necrotrophic pathogens, commonly *Botrytis cinerea* (Raid, 2003). Plants which are weakened by downy mildew disease also have a higher risk of colonization by human pathogens such as *Escherichia coli* O157:H7 and *Salmonella enterica* (Simko et al., 2015).

Table 1.2.: Key similarities and differences between oomycetes and true fungi. Table based on information from Beakes et al. (2012); Latijnhouwers et al. (2003)

Characteristic	True fungi	Oomycete
Vegetative body	Mycelium formed from hyphal networks	
Principle cell wall component	Chiten	Cellulose
Lysine biosynthesis pathway	α-amino adipic acid pathway	α-ε-diaminopimelic acid pathyway
Hyphae	Septate	Non-septate
Mitochondria	Flattened cristae	Tubular cristae
Genome	Haploid or dikaryotic	Diploid

1.2.3. *B. lactucae* penetrates outer defences then colonizes sub-epidermal tissues

B. lactucae produces two types of inoculum: oospores from sexual reproduction and conidia from asexual reproduction (Fletcher, 1976). These both have roles in infection of a lettuce crop (Figure 1.8). Oospores generally act as a source of primary inoculum (Fall et al., 2016). Oospores are much larger than conidia, with a thicker wall and the ability to survive for a longer period giving them the ability to overwinter in soil and debris (Morgan, 1978). The oospores remain dormant in the soil with a low level of germination (Tommerup et al., 1974). In the presence of germinating lettuce seeds, the level of oospore germination increases to encourage infection (Morgan, 1978). Production of oospores occurs in a much lower abundance and with a lower germination rate than conidia (Fall et al., 2016). Conidia, although short-lived, have very effective aerial dispersal, allowing quick spread of infection to nearby plants (Fall et al., 2016). Conidia are a very effective form of secondary inoculum (Fall et al., 2016). The quantity of airborne conidia is a major factor in determining the risk of downy mildew development and the resulting loss of yield (Fall et al., 2016). Studies on the process of infection by conidia rather than by oospores are better documented. Experiments planned for this project use conidia as the form of inoculum to generate infected plants.

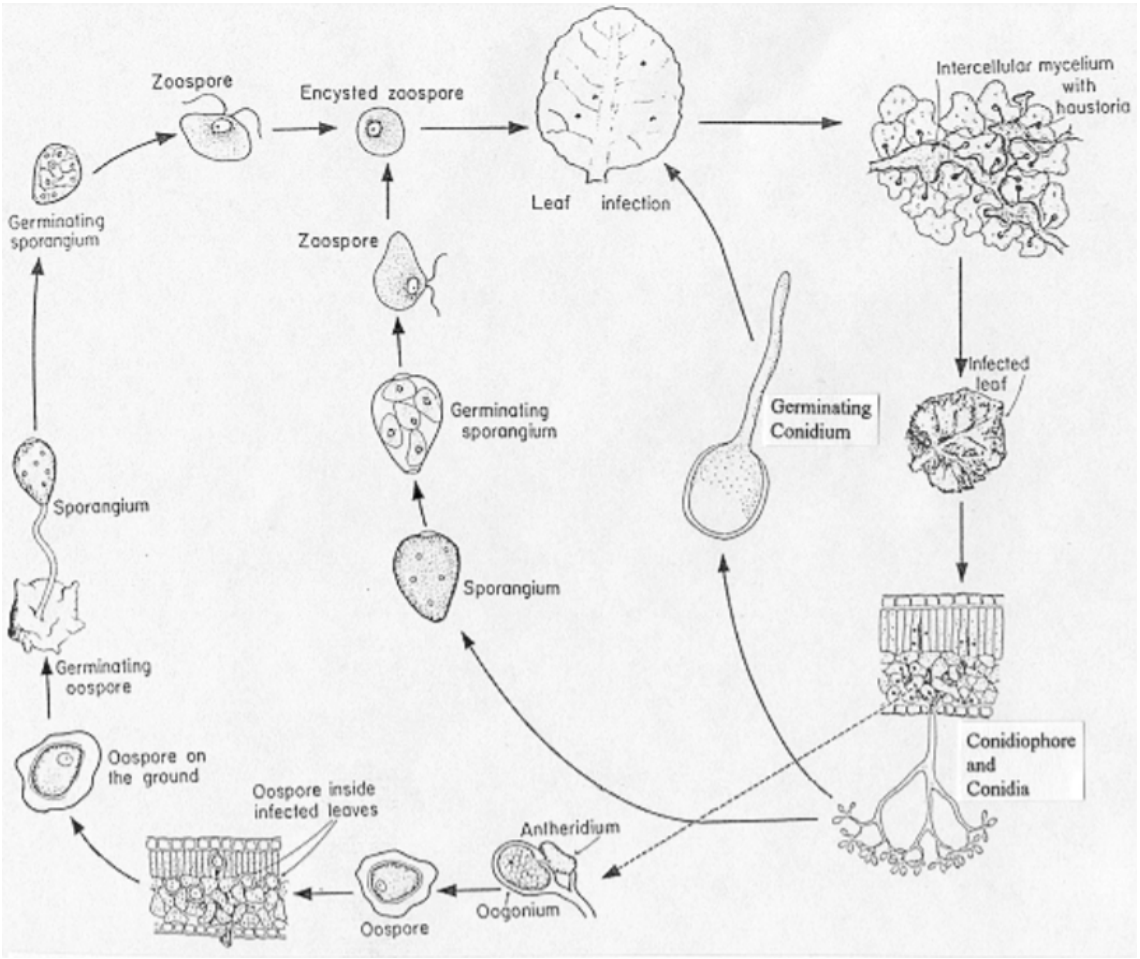


Figure 1.8.: The lifecycle of *Bremia lactucae* as described by (Agrios, 1988). *B. lactucae* produce sexual spores [oospores] as well as asexual spores [conidia]. Conidia are able to directly germinate upon a host plant to attempt to initiate an infection (Agrios, 1988). Oospores germinate by producing sporangium in favourable conditions. These sporangium form and release zoospores which can then infect a susceptible host plant.

The process of infection by *B. lactucae* conidia is summarised in Figure 1.9. Once a conidium has been spread to a lettuce leaf (inoculation), it undergoes an activation phase in which the endoplasmic reticulum, dictyosomes, and mitochondria activate and lipid droplets are dispersed (Duddridge and Sargent, 1978). The germ tube begins to develop, simultaneously with increases in enzymatic activity in the mitochondria and vacuoles (Duddridge and Sargent, 1978). The germ tube swells to form an appressorium over the junction of two epidermal cells (Sargent et al., 1973). The appressoria then forms penetration pegs (Sargent et al., 1973). Penetration pegs pierce the periclinal cell wall and allow entry of *B. lactucae* into the leaf (Lebeda et al., 2008). In most cases, penetration occurs through the cuticle but in 1-5% of germ tubes penetration will occur through the stomata (Lebeda et al., 2008). This process is aided by the release of degradative enzymes,

including polygalacturonases, esterases and proteases. These degradative enzymes attack the cuticle and cell wall to create an entry point (Lebeda et al., 2008).

Once past the external barriers, *B. lactucae* invaginates the host's plasma membrane to form the primary vesicle (Maclean and Tommerup, 1979). The cytoplasm relocates from the conidia and germ tube into this vesicle and callose blocks the end of the penetration peg (Sargent et al., 1973). A secondary vesicle forms from the primary vesicle (Sargent et al., 1973). Hyphae grow from the secondary vesicle, as well as haustoria in some cases (Lebeda et al., 2008). Haustoria are hyphal side branches that remain outside of the host protoplast, and exchange signals which are thought to help with nutrient uptake between *B. lactucae* and the host (Lebeda et al., 2008). *B. lactucae* spreads through the plant using extracellular hyphae. Once established, reproductive growth is induced (Lamour and Kamoun, 2009).

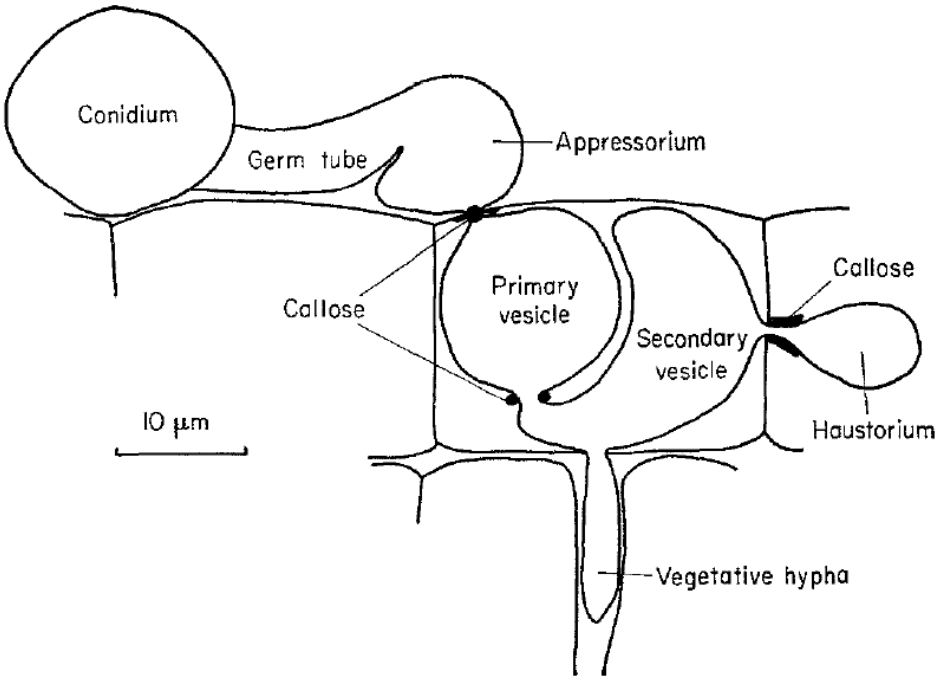


Figure 1.9.: Upon landing on a susceptible lettuce cultivar, *B. lactucae* conidia germinate by forming a germ tube. The germ tube forms an appressorium, allowing penetration of the leaf and formation of primary and secondary vesicles and in some cases haustoria. Figure and description adapted from Sargent et al. (1973).

Asexual reproduction occurs through differentiation of hyphal tips into sporangiophores (Lamour and Kamoun, 2009), which then emerge through stomatal openings (Su et al., 2004). Sporangia directly germinate as germtubes so are labelled conidia. Conidia develop on the conidiophores (sporangiophores) (Lamour and Kamoun, 2009). Once mature, the

conidiophores release conidia for dispersal to start new infections (Lamour and Kamoun, 2009).

1.2.4. *Dm* genes are the main cause for resistance against downy mildew in lettuce

Resistant cultivars of lettuce can launch a very effective defence against *B. lactucae*. The resistance may occur in a number of forms. Some pathogens cannot infect a species of plant (Mysore and Ryu, 2004). As the plant is not a viable host to that pathogen, this is called non-host resistance (Mysore and Ryu, 2004). In the case of *L. sativa*, many cultivars are susceptible to *B. lactucae* so non-host resistance does not occur (Lebeda et al., 2008). Instead, lettuce cultivars may display host-resistance in which the cultivar is resistant to one or more strain of *B. lactucae* (Lebeda et al., 2008). Host resistance against *B. lactucae* may occur as either race-specific, non-race-specific or field resistance (Lebeda et al., 2008). Race-specific resistance is the best understood. In race-specific resistance, the presence of R genes, known as downy mildew resistance (*Dm*) genes, provide resistance against *B. lactucae* strains with the corresponding effector gene in a gene-for-gene manner (Shen et al., 2002).

In race specific resistance to downy mildew, induced defence is strongly associated with the HR (Lebeda et al., 2008). The extent of cell death and its timing in the infection progress is dependent on the *Dm /AVR* genes present (Mauch-Mani, 2002). For example, lettuce carrying *Dm2* or *Dm5/8* induced HR during expansion of the *B. lactucae* primary vesicle; however, lettuce with *Dm1* and *Dm3* did not induce a HR until the formation of a secondary vesicle (Mauch-Mani, 2002). Regardless of the timing, *Dm* genes result in a HR only in cells penetrated by *B. lactucae* (Crucefix et al., 1984).

In field resistance, and nonspecific resistance (as well as host resistance), a number of defensive phytochemicals and proteins are induced upon pathogen recognition. As with general defence against biotrophic pathogens, lettuce possess a number of physical barriers to prevent entry of *B. lactucae* with an emphasis on the trichomes and cuticle (Lebeda et al., 2008). If barriers cannot prevent entry, they increase the time for the host lettuce plant to induce a defence response (Mauch-Mani, 2002). The key to a successful disease response has been identified as the induction of phenolic compounds; including strengthening of the cell wall and phytoalexins, the phytohormone SA and corresponding response and a number of PR proteins (Lebeda et al., 2008; Mauch-Mani, 2002).

In initial infection, preformed phenolics in the vacuole are transferred to the cell wall to add to an immediate defence (Sedlarova and Lebeda, 2001). Peroxidases and other enzymes facilitate phenolic transport (Sedlarova and Lebeda, 2001). High peroxidase activity could increase the polymerization of phenolics at the site of penetration thus increasing resistance (Mauch-Mani, 2002). The importance of peroxidases in this process was highlighted by Reuveni et al. (1991) in which increases in peroxidase activity correlate with field resistance in lettuce. As the disease progresses, the plant increases production of new phenolic compounds as part of the HR as well as strengthening cell walls (Sedlarova and Lebeda, 2001). These compounds include quercetin, rutin, caffeic acid and chlorogenic acid (Lebeda et al., 2008). It is interesting to note, in other species, some of these secondary metabolites are up-regulated by UV-B light also (see 1.3.3).

Phytoalexins; in particular lettucenin A, has been noted to be important in lettuce defence (Mauch-Mani, 2002). In a study by Bennett et al. (1994), the levels of Lettucenin A significantly increased after inoculation and closely reflected the number of cells undergoing HR. At a concentration of 5.0µg mL^{-1}or higher, lettucenin A totally inhibited spore germination (*B. lactucae* races TV, V0/11, CL9W) (Bennett et al., 1994). Although the exact mode of action of lettucenin A is unknown, it is thought to be involved in restricting growth or preventing secondary infections (or both) of *B. lactucae* (Mauch-Mani, 2002).

1.2.5. Downy mildew disease is commercially controlled by breeding or chemicals

Chemical control is the currently the most effective and economical method to control downy mildew on non-resistant, or partially resistant lettuce cultivars (Gisi, 2002). Phenylamide (PA) (Gisi, 2002), dithiocarbamate and strobilurin (Barriere et al., 2014) fungicides are often used in lettuce downy mildew control. For effective control of *B. lactucae* on lettuce, a foliar spray is applied several times per season (Gisi, 2002). Although some models have been developed to improve timing of spraying (Fall et al., 2016, 2015), most horticulturists implement spraying on a calendar basis.

Although effective, chemical control alone is becoming an issue of debate. Fungicides can have a high impact on the environment through alteration of natural oomycete populations, as well as public concern for consumer and applicator health (Barriere et al., 2015). To address this public concern, there are increasingly stricter policies on the levels of fungicide residue acceptable on foods (Barriere et al., 2014). The development of fungicide resistance

is also a concern (Gisi, 2002). Spraying applies a strong selection toward resistant strains, which over generations can result in a completely resistant population. Resistance risk is higher for PA fungicides (Gisi, 2002), which have one mode of action than dithiocarbamates (Barriere et al., 2014), which have multiple. Together these have prompted a shift to move away from chemical control of *B. lactucae*. Many governments, including New Zealand, are actively seeking alternatives to the use of fungicides such as those used to control lettuce downy mildew (DEFRA, 2013; Douglas, 2000).

Breeding for lettuce cultivars that are resistant to downy mildew can be used with chemical control or as an alternative (Barriere et al., 2015). Although breeders have attempted to integrate qualitative or field resistance, most resistant cultivars have *Dm* genes integrated to provide specific race resistance (Grube and Ochoa, 2005). This approach provides effective resistance against strains containing the corresponding *AVR* genes. However, race-specific resistance can also easily be overcome by virulent strains (Grube and Ochoa, 2005). Effectors, unlike PAMPs, are not essential for survival of the pathogen (Chiang and Coaker, 2015) so can undergo mutations without disrupting its fitness. Therefore, under the selection pressure of ETI, pathogens can quickly evolve new or modified effectors which are no longer recognised by an R protein (Bent and Mackey, 2007). Pathogens can evolve new effectors to overcome the integrated R genes over generations limiting the long term effectiveness of race specific breeding.

Although chemical control and breeding are the major methods in protection of lettuce against downy mildew, a number of other techniques are also used (Lebeda et al., 2008). Techniques include implementing rotations, solarisation, and bio-fumigation to the soil prior to planting lettuce (Barriere et al., 2014). Once planted, biocontrol such as introduction of natural enemies, and high hygiene can also act as preventative or curative measures (Barriere et al., 2014). An alternative to fungicides is the use of elicitors to induce a broad spectrum, long-lasting quantitative resistance (Thakur and Sohal, 2013; Walters et al., 2005). Elicitors may be of plant, pathogen, or abiotic origin (Thakur and Sohal, 2013). The elicitor is recognised by the plant's innate immune systems to induce an immune response, thus taking advantage of the plant's inbuilt defences (Walters et al., 2005). These techniques are usually not effective enough alone to combat downy mildew and must be used in an integrated approach (Barriere et al., 2014).

There are many limitations to the current methods used to control downy mildew in

lettuce (Table 1.3). Horticulture is starting to move away from the use of chemical controls, and so it is imperative to develop new effective technologies to combat disease. Although still in early stages of development, a promising new technology is the use of UV-B-pretreatments (Biolumic.com). In lab conditions, lettuce plants treated with UV-B prior to disease infection can have reduced susceptibility to downy mildew disease (Wargent et al., 2006). The New Zealand based company; Biolumic, currently use UV-B-pretreatments to induce a stress-resistance phenotype, including observed increases to disease tolerance in the field (J. Wargent, pers comm). Further research is required to investigate the potential of UV-B priming as an effective control solution to lettuce downy mildew.

Table 1.3.: Current control options available for lettuce downy mildew control have several limitations (Barriere et al., 2014; Crute, 1984; Gisi, 2002; Kerns et al., 1999; Koike and Davis, 2012; Maor and Shirasu, 2005; Morgan, 1984; Norwood et al., 1983; Turini et al., 2011; Walters, 2009; Wicks et al., 1993; Wright, 2007)

Method	Application stage of crop cycle	Limitations
Breeding	Crop development	• Only protects against specific strains • Takes many years to develop new cultivars • New resistance genes must be found in wild accessions • Resistance can develop over time
Soil Sterilization	Pre-planting	• Removes beneficial microbes • Doesn't protect against airborne spores • High labour costs
Chemical Sprays	In field	• Health and safety concerns • Resistance can develop over time • High labour and chemical costs
Biocontrol	In field	• Not effective alone • Very few developed options available

1.3. UV-B can reduce susceptibility of a plant to disease

1.3.1. Plants use UV-B as a signal to induce a range of protective responses

Sunlight is a major regulator of growth and development in plants. Sunlight provides plants with energy for photosynthesis and can act as an environmental cue for important processes such as flowering, competition reactions, and other responses to adapt to a changing environment (Aphalo et al., 2012). Natural sunlight is composed of a spectrum

of wavelengths, which differ in relative activity and biological potential (Bjorn, 2015) (Table 1.4). The spectrum of 400 to 700nm contains wavebands (e.g. blue, red) active in signalling and as a whole are utilized by plants as photosynthetically active radiation (PAR) (McDonald, 2003). UV-B light (280-315nm) contains short wavelengths that have a high energy per photon (McDonald, 2003). Early research into UV-B light responses investigated high doses of UV light that often resulted in damage to the plant such as DNA damage (Ambler et al., 1975; Green and Fluhr, 1995; Krizek, 1975; Mackerness et al., 1999; Manning and v. Tiedemann, 1995). Research from the mid-1980s onwards was carried out to investigate the consequences of a depleting ozone layer; however, the doses used were much more extreme than actual UV light levels reaching the earth's surface. Due to the unrealistic nature of these doses, the responses recorded in these publications can be considered a general stress response rather than a UV-B-specific response. More recently, balanced treatments of UV which, respect our recent understanding of the composition of ambient sunlight, have been used to investigate the UV-B plant response (Qian et al., 2016; Wang et al., 2016; Wargent et al., 2015; Willing et al., 2016). At a more realistic dose, it was found that UV-B can also act as a signal to induce a number of morphological changes revolving around protection against stress as well as shade avoidance (Wargent and Jordan, 2013).

Table 1.4.: Colours associated with light wavelength ranges (Aphalo et al., 2012).

Colour	Wavelength (nm)	Frequency (THz)
UV-C	100 - 280	3000 - 1070
UV-B	280 - 315	1070 - 950
UV-A	315 - 400	950 - 750
violet	390 - 455	770 - 660
blue	455 - 492	660 - 610
green	492 - 577	610 - 520
yellow	577 - 597	520 - 502
orange	597 - 622	502 - 482
red	622 - 770	482 - 390
near IR	770 - 3000	390 - 100
mid IR	3000 - 50000	100 - 6
far IR	50000 - 10^6	6 - 0.3

1.3.2. UV-B exposure results in tougher, more compact plants

Growth of the seedlings is altered as a response to exposure to UV-B light. UV-B exposed plants tend to have a reduced leaf area, increased leaf thickness (Wargent et al., 2011), leaf curling (Fierro et al., 2015; Jansen, 2002), and inhibition of hypocotyl elongation (Biever

et al., 2014; Jansen, 2002). UV-B has also been observed to increase axillary branching in plants (Ziska et al., 1993). Growth architecture of a plant is strongly controlled by the phytohormone auxin with increased levels of auxin promoting vertical growth (Smet and Jurgens, 2007; Zhao, 2010). UV-B, however, inhibits auxin biosynthesis (Hayes et al., 2014), resulting in shortened plants. This correlation between decreased auxin and decreased plant size as a response to UV-B was explored by Hectors et al. (2012). At a low dose of 0.56KJ $m^2$of UV-B light, a decrease in leaf area (23% reduction compared to visible light only) was accompanied by a decrease in free auxin (Hectors et al., 2012). The UV-B-induced decrease in leaf area was increased in auxin biosynthesis (*mit1-3*), and transport (*axr4-1*) knock out mutants, strengthening the hypothesis that auxin down-regulation is involved in the UV-B plant phenotype (Hectors et al., 2012). It is interesting to note that UV-B also increases flavonoids which have been suggested to interfere with auxin transport, thus further enforcing the shortened plant architecture (Jansen, 2002). Although smaller as a result of UV-B exposure, plants also tend to have a higher stress tolerance. Increased hardiness was observed by Wargent et al. (2011), in which lettuce plants that received UV-B light appeared more compact as seedlings; however, following transplantation into the more stressful environment of a field, UV-B-pretreated plants had a greater yield at harvest.

1.3.3. UV-B exposure increases plant phenolic levels

The best described UV-B response is an up-regulation of UV-absorbing pigments, especially flavonoids (Bidel et al., 2015; Lee et al., 2014; Singh et al., 2014). Due to the high energy nature of UV-B light, it is understandable that plants up-regulate compounds which can absorb UV-B light thus protecting the plant from damage (Agati et al., 2013). UV-absorbing pigments are most commonly accumulated in the epidermal layer of the plant in order to protect UV-vulnerable macromolecules, such as photosynthetic machinery (Burchard et al., 2000). Changes to flavonoid levels are species and dose specific (Jordan, 2017). However, in general, if given an appropriate dose of UV-B, plants tend to up-regulate flavonoid levels; most commonly: querticin, rutin and kaempferol flavonoids (Reifenrath and Muller, 2007; Winter and Rostas, 2008; Hectors et al., 2012; Cvetkovic et al., 2011; Kuhlmann and Muller, 2010; Fraser et al., 2017), as well as anthocyanins (Inostroza-Blancheteau et al., 2014; Wargent et al., 2011; Takshak and Agrawal, 2016; Cominelli et al., 2008; Zhang et al., 2013b). Often studies will measure the “total phenolics” or

"total UV-B-absorbing compounds" as an indication of flavonoid increase due to UV-B radiation (Lee et al., 2014; Demkura et al., 2010; Duval et al., 1999). As well as flavonoids, phenolic acids (Inostroza-Blancheteau et al., 2014; Giuntini et al., 2008), syringyl type lignin (Demkura and Ballare, 2012; Jansen et al., 1998) and possibly SA (Section 1.3.7.4) have been increased by UV-B-induced up-regulation of the phenylpropanoid pathway. UV-B-induced compounds can often cause quality changes to the plant such as altered taste and colour (Wargent and Jordan, 2013). For example, in red lettuce cultivars, UV-B induces increased production of anthocyanins resulting in a more favourable, redder crop (Klaudia et al., 2016).

The phenylpropanoid pathway is an extremely diverse pathway resulting in a number of secondary metabolites including flavonoids, anthocyanins, lignin, and phytoalexins (Vogt, 2010; Ferrer et al., 2008). Although there are a few entry points into the phenylpropanoid pathway, the shikimate pathway is the primary source of precursor compounds (Figure 1.6) (Vogt, 2010). It is now widely acknowledged that UV-B up-regulates one of the most important enzymes in the flavonoid branch of the phenylpropanoid pathway; chalcone synthase (CHS)(Frohnmeyer et al., 1997; Fuglevand et al., 1996; Hartmann et al., 1998; Ruhland and Day, 2000; Stratmann, 2003; Weisshaar and Jenkins, 1998). Other enzymes in this pathway up-regulated by UV-B light include Phenylalanine Ammonia Lyase (PAL), Chalcone Isomerase (CHI), Dihdroflavonol Reductase (DHFR) and Flavanone 3-Hydroxylase (F3H) (Kalbin et al., 1997; Liu et al., 2013; Hua et al., 2013; Huang et al., 2010; Kim et al., 2008a). UV-B up-regulation of these genes likely results in the increases to the many phenolic secondary metabolites which accumulate as a response to UV-B irradiation.

UV-B up-regulates a number of other secondary metabolites with primary roles in protection against biotic or abiotic stresses. Examples include carotenoids (Becatti et al., 2009), xanthophylls (Tohidi-Moghadam et al., 2012; Takshak and Agrawal, 2016), GLs (Mewis et al., 2012; Schreiner et al., 2 05; Reifenrath and Muller, 2007; Tohidi-Moghadam et al., 2012) and alkaloids (Ramani and Chelliah, 2007; Binder et al., 2009). GLs are described in further concerning their role in defence in Section 1.3.7.6. These metabolites are again often species-specific, for example, GLs are only found in Capparales (Bednarek et al., 2009).

1.3.4. Plants have multiple UV-B response pathways

Plants appear to use different pathways to respond to a low and high dose of UV-B light

(Figure 1.10) (Jenkins and Brown, 2007). A high dose, characterised by shorter wavelengths, higher fluence rates or longer duration, tends to induce a non-specific UV-B response pathway which is generally associated with stress or wounding. (Jenkins, 2009). At a lower doses (longer wavelength, lower fluence rate, shorter duration), a specific photomorphogenic responses occurs (Jenkins, 2014). Although there is some overlap of responses between pathways, it is generally accepted a UV-B-specific response occurs via a-UV-B-specific receptor (Jenkins, 2014). UV RESISTANCE LOCUS 8 (UVR8) is a known UV-B-specific photoreceptor which has been linked to a number of responses in plants such as inhibition of hypocotyl elongation and auxin signalling (Hayes et al., 2014; Mazza and Ballare, 2015).

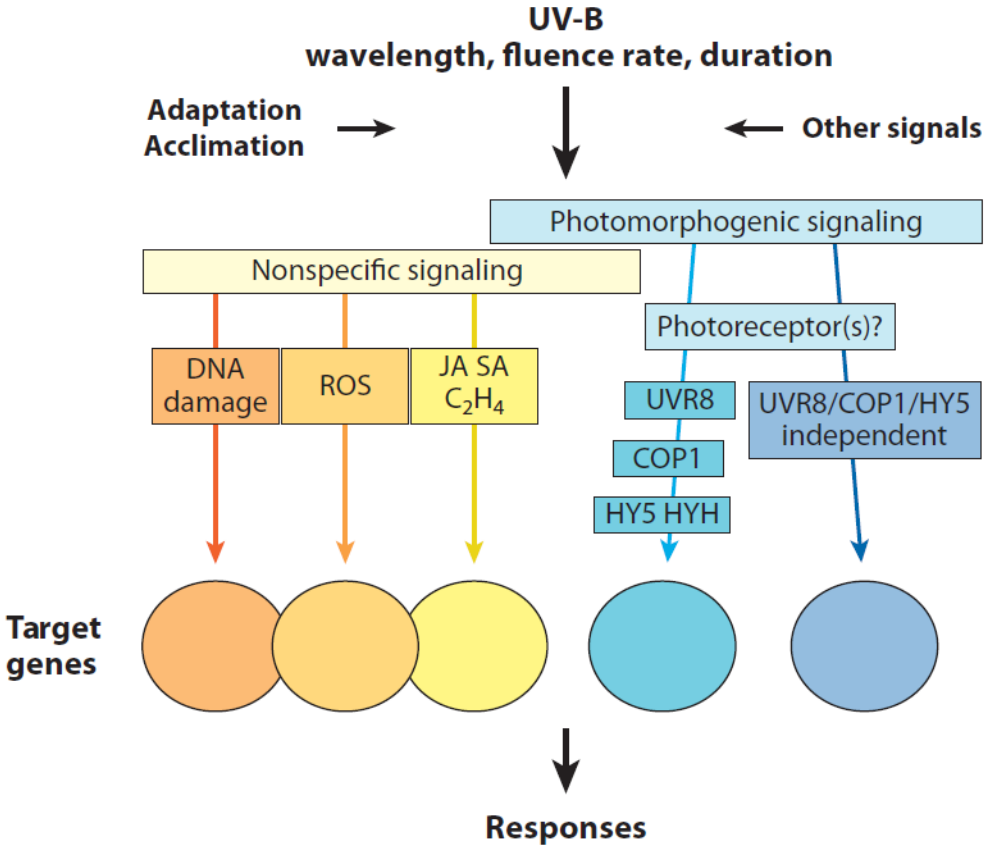


Figure 1.10.: The dose of UV-B light; including the UV-B wavelength, fluence rate and duration, influence which signalling pathway is induced. At high doses, a non-specific protection pathway is induced which attempts to repair damage caused from the high UV-B energy. At lower doses, photomorphogenic signalling is induced through reception of UV-B through a photoreceptor such as UVR8. This induces morphogenic and metabolic changes in the plant to adapt to an elevated UV-B environment. Figure from Jenkins (2009). ROS = reactive oxygen species, JA = jasmonic acid, SA = salicylic acid, UVR8 = UV RESISTANCE LOCUS8, COP1 = CONSTITUTIVELY PHOTOMORPHOGENIC1, HY5 = ELONGATED HYPOCOTYL5, HYH = HY5 HOMOLOG.

1.3.5. UVR8 induces UV-B-specific genes through a signal cascade

1.3.5.1. UVR8 is a UV-B photoreceptor

UVR8 exists in a base state as a dimer with sequence similarity to Regulator of Chromatin Condensation 1 (RCC1) (Kliebenstein et al., 2002). Each monomer forms a seven-bladed β-propeller fold protein (Wu et al., 2012) (Figure 1.11). The monomers are held together in dimer form through salt bridges between complementary charged amino acid residues at the dimer interface (Wu et al., 2012). Arginine-Aspartate bonds, primarily Arg 286 and Arg 338 (Huang et al., 2014), are thought to be of key importance to maintaining the dimer due to the formation of stronger double hydrogen bonds (Jenkins, 2014).

UVR8 differs from other plant photoreceptors as it lacks an external cofactor as a chromophore (Wu et al., 2012). Instead, UVR8 is believed to sense UV-B light through a tryptophan pyramid (Miyamori et al., 2015). Tryptophan (Trp) is an aromatic amino acid which can absorb UV-B light with a maximum absorption of 280-300nm (Rizzini et al., 2011). When UV-B light is applied, the key tryptophan, W285, as well as W337 and W233, become excited and cause a dissociation of the UVR8 dimer, creating an active monomer which can induce UV-B response genes (Voityuk et al., 2014).

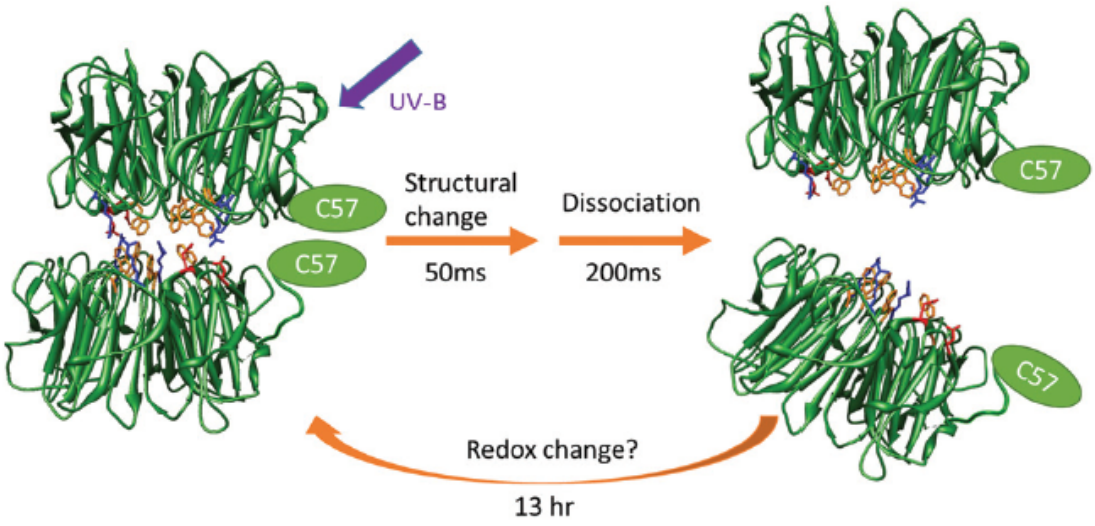


Figure 1.11.: UV-B light excites tryptophan residues on the UVR8 dimer. This causes a structural change resulting in dissociation of the dimer into a monomer. The UVR8 monomer has an exposed C terminus which allows it to bind to other proteins to induce a UV-B response (active state). Figure adapted from Miyamori et al. (2015)

1.3.5.2. UVR8 monomers use COP1 to turn on UV-B response genes

The role of UVR8 as the main UV-B photoreceptor is now well understood and has been reviewed multiple times (Heijde and Ulm, 2012; Jenkins, 2014; Lee, 2016; Parihar et al., 2015; Ulm and Jenkins, 2015; Yang et al., 2015). Upon reception of UV-B light, UVR8 dissociates to form two active monomers (Lee, 2016). The monomer is the active form of UVR8 due to an exposed C terminus allowing it to bind to downstream proteins such as COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1) (Figure 1.12)(Lee, 2016).

The relationship between COP1 and UVR8 is far from understood. COP1 is a known E3 ubiquitin ligase which works with SPA1 (SUPPRESSOR OF PHYA) to target many photomorphogenesis promoting transcription factors for degradation such as HY5 (ELONGATED HYPOCOTYL5) (Zhu et al., 2008). HY5 is a key transcription factor in the expression of a large number of UV-Bresponse genes (Heijde and Ulm, 2012). Although, COP1 promotes degradation of HY5, in *cop1* knock out mutants, expression of HY5 regulated genes is decreased indicating COP1 is essential for HY5 function (Oravecz et al., 2006).

A suggested hypothesis of the role of COP1 in UVR8 function is that binding of the UVR8 monomer to the COP1-SPA1 complex inactivates the ubiquitinase activity and results in lower amounts of COP1 in the nucleus (Jenkins, 2009). Therefore, degradation of HY5 as well as the closely related protein HY5 HOMOLOG (HYH) in the nucleus is reduced (Jenkins, 2009). HY5 accumulates and induces expression of a large number of UV-B-related genes (Jenkins, 2009). However, this does not explain why the presence of COP1 is required for HY5 function. In the presence of UV-B light, COP1 may instead positively regulate HY5 through an unknown manner (Jenkins, 2009). Regardless of mechanism, binding of UVR8 to COP-SPA1 results in up-regulation of the HY5 transcription factor which activates a large number of UV-B-response genes (Jenkins, 2009) (Figure 1.12).

HY5 induces a number of UV-B-response genes in a UVR8 dependent manner (Jenkins, 2009). HY5 also regulates expression of repressors of the UVR8 dependent responses; RUP1 and RUP2 (REPRESSOR OF UV-B PHOTOMORPHOGENESIS), to provide a balanced response (Heijde and Ulm, 2013). RUP1/2 proteins share a high level of conservation in the WD-40 repeat domains with COP1 and SPA proteins (Gruber et al., 2010). This domain is the binding site of COP1-SPA to the C terminus of the UVR8 monomer (Cloix et al., 2012). It was therefore hypothesised, that the RUP1/2 proteins bind to the UVR8 monomer at the

same location as the COP1-SPA complex (Jenkins, 2009). Upon reception of UV-B light, RUP1/2 are increased in abundance, resulting in displacement of COP1-SPA complex as the RUP proteins compete to bind to the UVR8 monomers (Figure 1.12) (Jenkins, 2009). Once bound, RUP1/2 facilitates the redimerization of UVR8 into base dimer form (Lee, 2016). RUP proteins create a negative feedback loop in order to ensure an excessive UV-B response is not elicited.

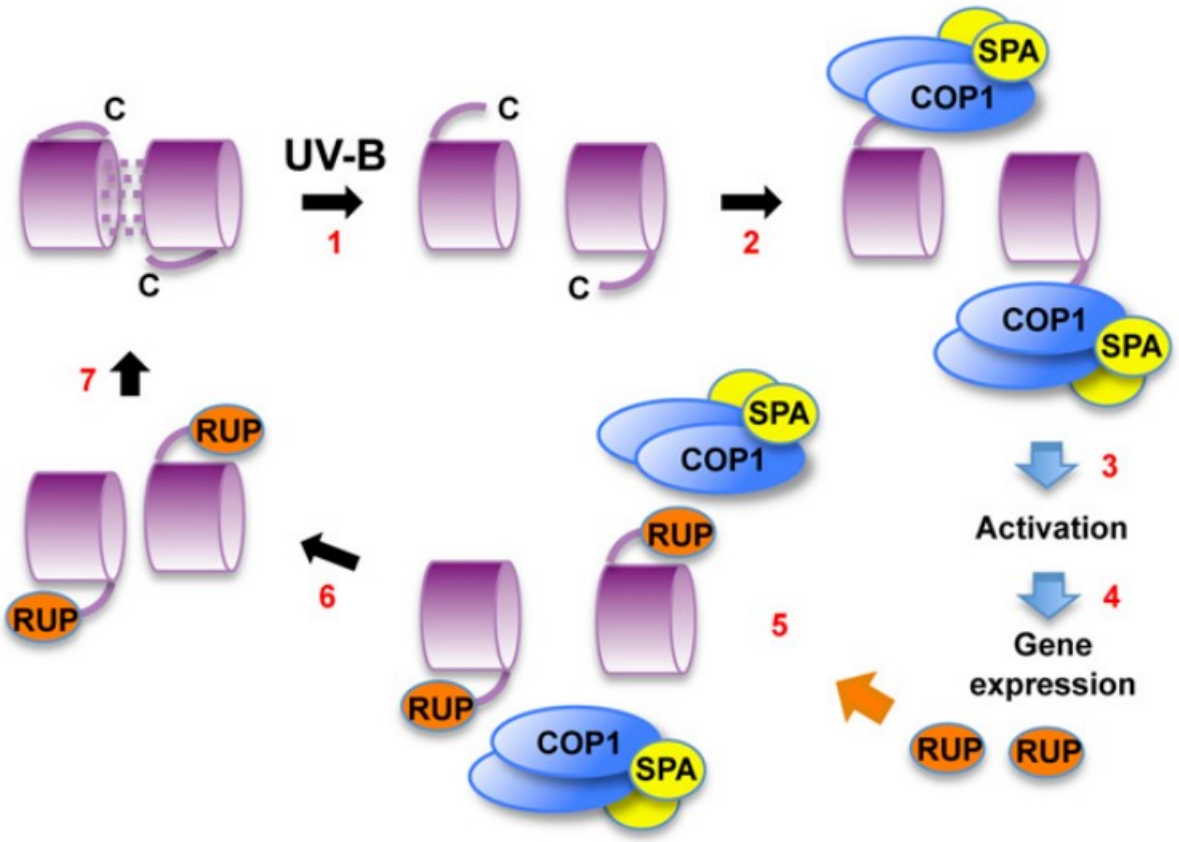


Figure 1.12.: Upon reception of UV-B light, the UVR8 photoreceptor dissociates to form active monomers (1). The active monomers associate with a COP1-SPA1 complex (2) to induce promotion on the transcription factor HY5 (3). HY5 up-regulates a number of UV-B response genes (4). Two of these genes includes RUP1 and RUP2 which are negative regulators of the UV-B response. These bind to UVR8 to dissociate it from the COP1-SPA1 complex (5 and 6) and facilitate the return of UVR8 to dimer form (7). Figure from (Jenkins, 2014).COP1 = CONSTITUTIVE PHOTOMORPHOGENIC 1, SPA = SUPPRESSOR OF PHYA, RUP = REPRESSOR OF UV-B PHOTOMORPHOGENESIS, UVR8 = UV RESISTANCE LOCUS 8.

1.3.6. UVR8 independent pathways contribute to the UV-B phenotype

Although only UVR8 has been identified as a UV-B-specific receptor, other receptors may also induce UV-B responses (Heijde and Ulm, 2012). It is suggested that phototropin can

also sense UV-B light and aid in induction of a photomorphogenic response (Vanhaelewyn et al., 2016). As well as photoreceptors, direct absorption of UV-B by cell components such as DNA can induce photomorphogenic responses. Biever and Gardner (2016) suggest direct absorption of UV light by DNA may play a role in UV-B signalling of emerging seedlings. As seeds germinate in the soil, they have very little flavonoids due to a lack of solar exposure. As the seedling emerges into sunlight, it lacks protection against UV-B light. Whilst UVR8 will sense this UV-B light and induce the production of flavonoids and other protective compounds, this takes time, resulting in UV-B-induced DNA damage until flavonoids form. UV-B damaged DNA (and other compounds) signalling at this stage likely work in tandem with UVR8 to promptly reduce plant growth and induce a UV-B response in order to protect the plant from further damage (Biever and Gardner, 2016).

1.3.6.1. Stressors can activate signalling pathways

At ambient sunlight levels, UV-B acts as a "eustress", which is an activating or stimulating stress that promotes health and growth, rather than permanent damage to result in plant acclimation to UV-B (Hideg et al., 2013). These responses tend to be mediated by the UVR8 dependent pathway (Section 1.3.5). However, if a plant is exposed to a high dose of UV-B, or the plant lacks sufficient protection against UV-B, damage occurs and causes a "distress" (Gonzalez-Besteiro and Ulm, 2013). Whether a plant suffers eustress or distress depends on not only UV-B dose and duration but also background light, plant acclimatisation and genotype (Hideg et al., 2013).

DNA can directly absorb UV-B light resulting in the formation of photodimers (Biever and Gardner, 2016). Dimerization occurs through the binding of adjacent pyrimidine bases creating pryrimidine-4,6-pyrimidione (6,4PPs) or more commonly in the case of UV damage; cyclobutane pyrimidine dimers (CPDs) (Biever and Gardner, 2016). These dimers can be repaired by nucleotide excision repair (NER) or by light-activated photolyases (photreactivation) (Biever and Gardner, 2016). UV-B up-regulates CPD photolyase in a UVR8 dependent manner (Brown et al., 2005). Gonzalez-Besteiro and Ulm (2013) suggest that these pyrimidine dimers, in particular CPDs, can act as a signal to trigger MAPK activation resulting in a signal cascade that induces the expression of UV-B protective genes. This was shown by an increased activation of MPK3 and MPK6 (important in MAPK cascades) in photolyase knockout mutants (Gonzalez-Besteiro and Ulm, 2013). Photolyase knockout mutants result in a decrease in repair of photodimers, allowing them to

accumulate. Increased accumulation of photodimers could result in an amplified activation of the MAPK pathway. Gonzalez-Besteiro and Ulm (2013) support, although could not identify, a pyrimidine dimer upstream of the MAPK pathway.

As well as inducing a signalling pathway, UV-B-induced DNA damage can also cause an arrest in the cell cycle to prevent replication of damaged DNA (Jiang et al., 2011; Biever et al., 2014). A halt in the progression of cell replication would result in fewer cell numbers and may help explain the commonly observed reduced plant growth under UV-B light (Jiang et al., 2011; Biever et al., 2014). Biever and Gardner (2016) argue that DNA damage may also contribute to the photomorphogenic UV-B response, independently of UVR8, through induction of specific UV-B-response genes. However, compared to the role of UVR8 in a photomorphogenic response, it seems unlikely DNA damage would be a major UV-B-specific pathway in seedlings and mature plants.

UV-B induces the production of ROS; however, at ambient sunlight levels, the production of antioxidants, which remove ROS, are also produced through the UVR8 pathway (Hideg et al., 2013). At high UV-B levels or insufficient protection, the plant produces more ROS than antioxidants, resulting in an imbalance called an oxidative burst (Hideg et al., 2013). A membrane-bound NADPH oxidase produces the ROS; superoxide ion ($O_{2^{-}}$)in a UV-B-induced manner into the apoplast (Kalbina and Strid, 2006). The superoxide ion rapidly transforms into hydrogen perioxide (H_2O_2) (Kalbina and Strid, 2006). Both the superoxide ion and hydrogen peroxide have been suggested to act as signals for abiotic and biotic stress responses (Choudhury et al., 2016). In terms of UV-B response, these ROS can activate MPKs (Mitogen-Activated Kinases). For example; UV induced ROS activate UV specific LeMPK3 and general stress response LeMPK1 and LeMPK2 in tomato (Holley et al., 2003). This results in the activation of a MAPK signal cascade to induce the expression of UV-B-protective genes.

1.3.6.2. MAPK cascades relay signals to induce UV protective responses.

Many signalling networks use MAPK cascades to respond to a wide range of environmental and developmental signals (Rodriguez et al., 2010). These cascades are therefore tightly regulated. Although only DNA damage and ROS production are mentioned in Section 1.3.6.1, there are likely a range of UV-B associated stress signals which can induce a MAPK cascade (Gonzalez-Besteiro and Ulm, 2013). In general, an external stimuli activates a receptor, or a MAP kinase kinase kinase (or higher level of kinase), which is phosphorylated

to activate a MAP kinase kinase, which is phosphorylated to activate a MAP kinase in a cascading manner (Rodriguez et al., 2010). At the end of the cascade, MAPKs target other proteins such as; enzymes, transcription factors, or other kinases, in the nucleus or cytoplasm to elicit a response (Rodriguez et al., 2010). Different MAP Kinases have different substrate preferences (Rodriguez et al., 2010). MKP3 and MKP6 activated by MKP1 are identified in *Arabidopsis thaliana* to control signal cascades related to UV-B stress with a primary role in above ground tissue (Gonzalez-Besteiro and Ulm, 2013).

Stressful UV-B doses tend to induce MAPK-dependent UV-B pathways. Hideg et al. (2013) compared expression of photomorphogenic UV-B response genes following low UV-B exposure to ROS treatment (MAPK pathway) in wildtype and a *uvr8* knockout mutant. All photomorphogenic response genes at a low dose appeared to be UVR8-dependent with no overlap to ROS-induced expression, indicating that genes expressed under ambient UV-B are distinct from the general MAPK stress response (Hideg et al., 2013). However, when comparing against a high dose, there was some overlap between ROS-induced and UV-B-induced genes (Hideg et al., 2013).This separation of pathways is further confirmed by Kalbina and Strid (2006), which show up-regulation of PR and defensin genes at high but not low levels of UV-B in a ROS-MAPK dependent manner. Kalbina and Strid (2006) suggest that MAPK can also induce a photomorphogenic UV-B response though MAPK up-regulation of UV-B photomorphogenic marker; CHS.

1.3.7. Increased disease resistance may be a response to UV light in plants

1.3.7.1. UV-B-induced disease resistance has been recognised in the literature

Multiple studies support the ability of UV light (UV-B and UV-C) to induce amplified disease defence in plants. However, there have been few published investigations into the mechanism that causes this phenomenon (Demkura and Ballare, 2012; **?**).

The first instance of a UV-B-induced disease defence reported a decrease in downy mildew disease in lettuce cultivar 'Rex' (Wargent et al., 2006). Experiments involved treatment of lettuce plants with UV-B doses between 1 and 12 kJ m-2 day-1 in a growth chamber followed by inoculation with *B. lactucae* conidia (Wargent et al., 2006). The conidia were then washed off and counted. Conidia counts showed the number of conidia per plant produced decreased with increasing UV-B-pretreatment dose (Wargent et al., 2006). Therefore UV-B-treated lettuce plants have an increased ability to limit *B. lactucae*

growth and development or release of conidia.

A UV-B-induced disease defence was also observed in *A. thaliana* infected with the necrotrophic fungal pathogen *B. cinerea* by Demkura and Ballare (2012). *Arabidopsis* plants were treated with UV-B in a glasshouse followed by inoculation with a droplet of *B. cinerea* (Demkura and Ballare, 2012). At 48 h post-infection, the UV-B-pretreated *Arabidopsis* leaves had a reduced lesion area indicating a greater resistance, and tolerance to the necrotrophic pathogen (Demkura and Ballare, 2012).

Increased resistance to *B. cinerea* has also been witnessed in *Pelargonium* plants pretreated with UV-C irradiation (Darras et al., 2015). *Pelargonium* × *Hortorum* L.H. Bail (zonal geranium) plants were pretreated with eight pulses of UV-C doses between 0.5 and 10 KJm-2 over eight weeks (once a week) then artificially infected with *B. cinerea* (Darras et al., 2015). UV-C pulse treatment resulted in a 47% reduction in lesion diameter indicating an increased tolerance to *B. cinerea* (Darras et al., 2015). The observed tolerance was only effective at 24 h with a similar progression of disease to control plants after this time (Darras et al., 2015). Although UV-B is a lower energy light, with longer wavelengths than UV-C, pretreatment resulted in a similar reduction in *B. cinerea* lesion size (Darras et al., 2015; Demkura and Ballare, 2012). Demkura and Ballare (2012) suggest a UV-B-induced resistance to *B. cinerea* is UVR8-dependent. However UV-C light is not known to be recognised by UVR8. Therfore induced tolerance to *B.cinerea* in these two systems may use different pathways. Darras et al. (2015) believe that the UV-C induced tolerance is due to increases in secondary metabolites which inhibit fungal growth. Many of the secondary metabolites suggested, such as flavonoids, phenolic compounds as well as PR proteins are also up-regulated by UV-B radiation. Therefore, although different pathways are used, a similar UV-B phenotype is created resulting in a similar increased *B. cinerea* tolerance. UV-C induced resistance has been noted in a number of other studies including induced resistance to *B.cinerea* of lettuce (Vasquez et al., 2017), carrots (post-harvest) (Mercier et al., 1993) and tomatoes (post-harvest) (Charles et al., 2008d,c,b,a, 2009) as well as resistance to *Hyaloperonospora parasitica* by *A. thaliana* (Kunz et al., 2008). As the UV-B-induced disease response is a fairly recent discovery, the mechanism by which it is caused is largely unknown. Demkura and Ballare (2012) observed that the UV-B-induced defence against *B. cinerea* was lost in the *uvr8-6* knock-out mutant indicating that this response is dependent on the UVR8 photoreceptor.

These publications strongly support the idea that UV-B may induce an increased resistance to filamentous pathogens. However, the width of this response is not very well understood. The resistance may be a direct or indirect response of UV-B light, have a broad or narrow pathogen range, and provide partial or full resistance. The potential of a UV-B-induced disease defence as a tool in horticultural crop protection is significant, so it is of high interest to examine this response further and determine the underlying mechanisms.

1.3.7.2. UV-B mediates the switch between growth and defence lifestyles in plants

Ballare et al. (2012) suggest the hypothesis that UV-B acts in tandem with background light (red : Far red) to signal if the plant should partition resources to vertical growth or defence (Figure 1.13). In a shaded environment, higher plant canopies absorb UV-B light as well as red (R) light, but not far red (FR) light (Ballare et al., 2012). Plants below the canopy therefore receive a low UV-B level and R:FR ratio. The combination of low UV-B and R:FR acts as a signal to the plant to initiate vertical growth to escape the shaded environment (Mazza and Ballare, 2015). To increase growth, resources are partitioned from other responses; such as defence against pathogens to growth pathways; such as up-regulation of the growth hormone auxin (Figure 1.13)(Mazza and Ballare, 2015). However, when the plant is no longer shaded, UV-B and R:FR levels are high. High UV-B and R:FR acts as a signal that enhanced upward growth is no longer required (Mazza and Ballare, 2015). The plant then redirects these resources to protection by switching on defence pathways (Figure 1.13) (Mazza and Ballare, 2015).

Application of pretreatment with UV-B light may use this switch between pathways to enhance resistance against pathogens. An increase of UV-B light signals no competition, resulting in down-regulation of growth pathways and up-regulation of defence pathways. This would explain the commonly witnessed observation of decreased size of UV-B-treated plants upon treatment but rapid growth in the field, as well as provide a possible explanation for increased resistance to disease. If UV-B acts as a signal to switch to defensive growth, this would provide an initial protection, resulting in lettuce plants in a seedling stage in the nursery would benefit from protection. However, when placed into a field environment, similar light levels would be received, and its protection levels would be similar to non-treated plants.

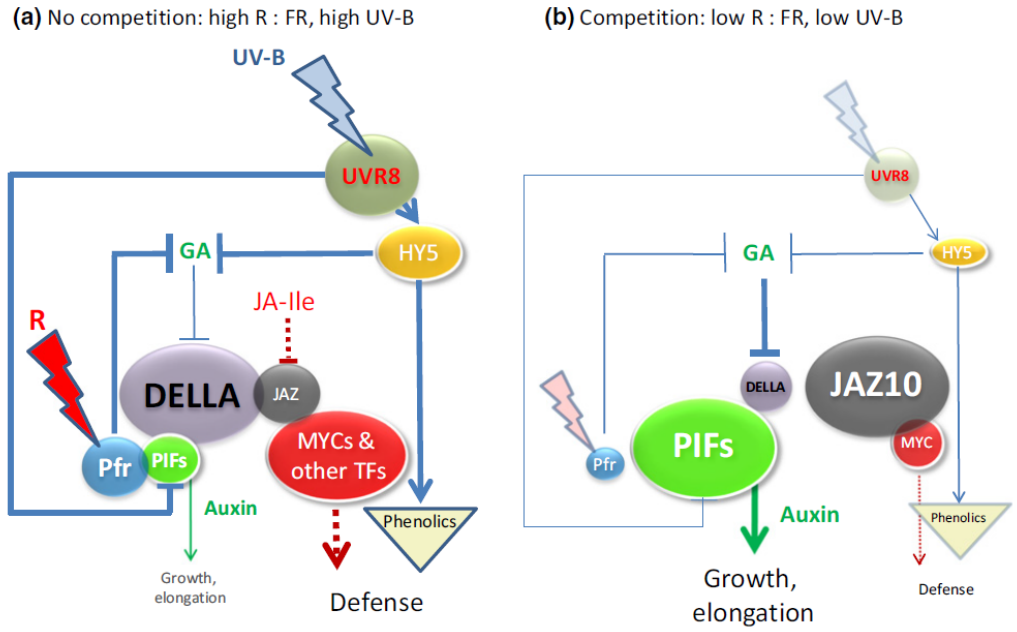


Figure 1.13.: When a plant is not shaded (a) UV-B and Red:Far red (R : FR) levels are high signalling a reduced need for growth and so plant resources are instead used for defence. When a plant is shaded (b), UV-B and R:FR levels reduce, signalling a need for growth to move from the shade and so resources are switched from defensive to growth and elongation. Application of UV-B-pretreatment therefore may act as a signal to switch the plant into defensive growth resulting in an increased disease resistance. Adapted from Mazza and Ballare (2015). GA = gibberellin, HY5 = ELONGATED HYPOCOTYL5, JA-Ile = jasmonyl-L-isoleucine, JAZ = JASMONATE ZIM domain protein, PIF = PHYTOCHROMEINTERACTINGFACTORS, UVR8 = UV-B resistance 8 .

1.3.7.3. UV-B amplifies plant defensive barriers to pathogen entry

Outer barriers play a major role in prevention of entry of biotrophic pathogens into the plant. Trichomes aid in protection by trapping spores thus preventing germination on the leaf surface (Lebeda et al., 2008). This defensive activity may be enhanced by UV-B-pretreatment as UV-B exposure causes increases in both trichome size, (Pandey and Pandey-Rai, 2014) and number (Yamasaki and Murakami, 2014; Yan et al., 2012). Increases in trichome density would increase the spore trapping ability of the leaf and may aid in prevention of infection resulting in increased resistance. Once past the trichomes, most downy mildew infections occur through penetration of cuticle (Lebeda et al., 2008). Enhanced UV-B has been shown to alter the compositions of epicuticular waxes and the cuticle on a range of plants species (Barnes et al., 1996; Fukuda et al., 2008; Ni et al., 2015, 2014; Pilon et al., 1999; Steinmuller and Tevini, 1985). These alterations may further aid in prevention of entry of *B. lactucae* by strengthening the cuticular barrier. UV-B tends to

increase the overall amount of wax (Fukuda et al., 2008; Kakani et al., 2003; Qaderi and Reid, 2005); however, at higher doses either no change or a decrease in wax was observed (Fukuda et al., 2008; Ni et al., 2014). In many cases, the structure of the alkanes and alcohol which make up the waxes changed after exposure to UV-B light (Fukuda et al., 2008; Grammatikopoulos et al., 1998; Ni et al., 2014; Pilon et al., 1999; Qaderi and Reid, 2005). Most commonly the length of alkyl chains was decreased in UV-B-treated plants (Fukuda et al., 2008; Steinmuller and Tevini, 1985).

Pathogens which reach the cell surface are confronted with the cell wall. An increase in sinapate production as a result of UV-B-induced up-regulation of FAH1 (ferulic acid 5-hydroxylase 1) transcription, was revealed to have a role in the increased defence of *Arabidopsis* plants against *B. cinerea* (Demkura and Ballare, 2012). Sinapate acts as a precursor to a defensive syringyl type lignin which strengthens cell walls (Demkura and Ballare, 2012). As described in Section 1.1.3 and 1.3.7.6, the cell wall, and these other external barriers are of high importance to disease defence.

1.3.7.4. UV-B manipulates defence phytohormones

The biotrophic associated defence hormone, SA, is known to be induced by UV-C light (Yalpani et al., 1994). This is thought to occur through a UV-C induced up-regulation of the phenylpropanoid pathway which produces SA (Yalpani et al., 1994). UV-B is well known to also up-regulate the phenylpropanoid pathway through induction of CHS (Tilbrook et al., 2013). This suggests the possibility that UV-B may also influence SA production. Some evidence has been provided for UV-B-induced SA production, but not in conjunction with the effect this has on disease resistance. A daily dose of 24 kJ m^{-2} d^{-1} applied to barley resulted in up to a 150% increase in SA in the roots and 100% in the leaves compared to control plants (Bandurska and Cieslak, 2013). At a much higher dose of 248 KJ m^{-2} d^{-1} , increases to SA in wheat roots and leaves was also witnessed (Kovacs et al., 2014). ROS have been noted to accompany the increase in SA as a response to higher doses of UV-B (51.84kJm^{-2} d^{-1})(Surplus et al., 1998). Increases to ROS levels are a good indication of plant stress, so induction of SA under high UV-B doses is likely a general stress response rather than a specific UV-B response. Although a range of doses have been used, these experiments provide some support towards the hypothesis that increased disease resistance could be caused by induction of the defence hormone SA.

Demkura and Ballare (2012) also showed that although JA has a major role in defence

against the necrotrophic pathogen *B. cinerea*, UV-B does not alter the JA defence response in this system. However, in previous studies, UV-B exposure amplified a plant's sensitivity to JA resulting in increased production of JA-inducible genes such as methyl jasmonate, and phenolic compounds (Demkura et al., 2010). Increased sensitivity to JA was observed after herbivory rather than a necrotrophic system, indicating that UV-B-induced defence is complex and may depend on the plant-pathogen system involved.

1.3.7.5. UV-B can up-regulate PR protein expression

In support of UV-B induction of SA, UV-B exposure increases expression of PR proteins. PR proteins are those associated with increased resistance to pathogens (Section 1.1.10.1). In many cases this occurs following a higher, stress causing dosage of UV-B. For example, at a daily dose of 51.84KJm^{-2} (3.0 µmol m^{-2}s^{-1} for 12 h), the transcripts of PR2, PR2 and PR5 have been shown to increase (Surplus et al., 1998). Although this suggests PR protein expression may be a general stress response, increases in PR protein are also noted in some lower dosage experiments. Green and Fluhr (1995) showed PR1 accumulation increased at a UV-B fluence rate as low as 0.08-0.2 µmol m^{-2}s^{-1} after a period of 10 h with a maximum increase at 1.0µmol m^{-2}s^{-1} (22 h). To further support that the accumulation of PR1 is UV-B-specific, at a higher, stress dose of 10 µmol m^{-2}s^{-1}, accumulation of PR1 decreased (Green and Fluhr, 1995). It was noted that increased accumulation of PR1 was localised to areas of the leaf which were exposed to the UV-B irradiation (Green and Fluhr, 1995). UV-B-specific accumulation of PR proteins is also supported by Fujibe et al. (2000). PR5, PR1 and PR1 were induced by UV-B irradiance as well as increases to SA (Fujibe et al., 2000). It is clear that UV-B can induce SA accumulation; however, whether this is a UV-B-specific response or general stress response remains uncertain.

1.3.7.6. UV-B increases the accumulation of many defensive secondary metabolites

Up-regulation of the phenylpropanoid pathway is one of the most supported UV-B responses (Dolzhenko et al., 2010; Jansen et al., 1998; Park et al., 2013; Strid et al., 1994; Tattini et al., 2000). Many phenolic compounds from this pathway are noted to have antimicrobial properties as described in Section 1.1.10.2. It therefore follows that in several disease systems, up-regulation of the phenylpropanoid pathway has been correlated with an increased disease resistance, such as reduction in powdery mildew infection in plant foliage (Foster-Hartnett et al., 2007; Iqbal et al., 2005; Torregrosa et al., 2004; Maher et al., 1994).

The most likely role of UV-B-induced phenolics in disease defence is increased phytoanticipins. UV-B-induced flavonoids usually have a primary role in protection against high energy UV-B light. This includes quercetin, rutin and kaempferol (Reifenrath and Muller, 2007; Winter and Rostas, 2008; Hectors et al., 2012; Cvetkovic et al., 2011; Kuhlmann and Muller, 2010; Fraser et al., 2017). Many of these have been shown to have antibacterial and antifungal activity (Zhu et al., 2004; Gatto et al., 2011; Rauha et al., 2000). Several flavonoids have been observed to induce innate immunity. Rutin has been shown to activate defences against *Xanthomonas oryzae pv. oryzae, Ralstonia solanacearum,* and *Pseudomonas syringae* pv. tomato strain DC3000 in rice, tobacco and *A. thaliana* respectively (Yang et al., 2016). Flavonoids may also aid in prevention of fungal attack through hindering initial spore germination and growth on the plant such as quercetin-3-galactoside inhibition of germtube elongation of *B. cinerea* (Tao et al., 2010). UV-B-induced increases to lignin levels as described in Section 1.3.7.3, which also increases the barriers to pathogen penetration. Therefore, a UV-B-induced increase to phenolic compound levels is likely to increase the preformed phenolic defences thus priming the plant for attack resulting in an increased pathogen resistance.

UV-B light may also influence levels of phytoalexins. UV-C light is commonly noted to increase levels of phytoalexins (Douillet-Breuil et al., 1999; Horie et al., 2015; Marti et al., 2014; Mercier et al., 1993; Park et al., 2013). As a UV-C response, phytoalexin induction may be related to more of a general stress/wounding signalling pathway induced by a high UV dose. There have been a few cases in which UV-B has increased phytoalexin levels in Brassicas (Mewis et al., 2012; Mert-Turk et al., 2003). Mert-Turk et al. (2003) treated plants with UV-B light (280 nm) for two days, 12 h per day and found that the phytoalexin; camalexin, was induced in some wild type accessions (Ws-3, Oy-0, and Ler) but not others (Col-0, and Nd-1). As 280nm is near UV-C range, these results favour a general response explanation rather than specific UV-B response; however, as the dose received by the plants is ill described in this paper, it presents little insight on the dose required to induce phytoalexins. Mewis et al. (2012) provides stronger evidence that UV-B can induce phytoalexins; 12 day old brocolli sprouts exposed to UV-B irradiation for 240 min (0.6 kJ m–2 d–1) had an almost 2 fold increase in two genes which were putatively indicated to be involved in phytoalexin synthesis (JCVI_19911: 2.12 fold increase, DY019565: 1.86 fold increase). Glucosinolates (Section 1.1.10) are also shown to increase following UV-B

exposure (Mewis et al., 2012; Reifenrath and Muller, 2007; Tohidi-Moghadam et al., 2012; Wang et al., 2011) as well as their antimicrobial side products (Mewis et al., 2012; Topcu et al., 2015). Evidence of induction of phytoalexins in other plant groups is not well investigated but provide an promising opportunity for further investigation.

Although many UV-B-induced phenolic compounds appear to have a role in plant defence, flavonoids are often down-regulated following pathogen recognition (Hahlbrock et al., 1981; Gleitz et al., 1991; Lo and Nicholson, 1998; McLusky et al., 1999; Schenke et al., 2011, 2018). It is argued by Schenke et al. (2018) that there is reciprocal crosstalk between the UV-B flavonoid pathway and biotic defence pathways in which flavonoid biosynthesis is down-regulated to prioritise resources towards other defensive phenolics, such as lignin production, of the phenylpropainoid pathway. However, a UV-B-pretreatment could still provide a priming for resistance with an increase in flavonoids prior to infection. This would help prevent or slow entry of pathogens. Once the plant is inoculated and the pathogen is recognised, the flavonoid pathway is down-regulated; however, the flavonoids which were already induced by UV-B may still have a role in increasing resistance at the early stage of infection.

1.3.7.7. UV-B can induce an oxidative burst

As described in Section 1.1.10.3, the oxidative burst is an important part of disease defence, not only as a antimicrobial agent but also as a signal. UV-B has been shown to increase the level of ROS, usually following a high (stress) dose of UV-B (Hideg et al., 2013).The MAPK-induced signal cascade induced by high UV-B exposure is similar to that induced by pathogens resulting in many similar genes being expressed (Kalbina and Strid, 2006; Hideg et al., 2013), indicating that it would likely contribute to the disease defence response pathway also (see 1.3.6).

1.3.7.8. UV-B likely causes enhanced disease protection through a combination of responses

A UV-B-induced increase to disease defence is likely through a combination of protective responses (Figure 1.14). Plants respond to UV-B through a photomorphogenic UVR8-dependent pathway or general UVR8 independent pathways. These pathways may contribute differently to the susceptibility of the plant to disease. High doses of UV-B tends to lead to induction of the defence hormone SA, and subsequent PR proteins as

well as induction of phytoalexins and a more pronounced oxidative burst. Accumulation of some defensive secondary metabolites can be assumed if the UV-B stress pathway is thought to be similar to the UV-C response pathway. Hideg et al. (2013) noted a high dose of UV-B which activates genes through the MAPK cascade has a similar gene response to that of pathogen attack indicating there may be an overlap between these pathways. Low doses of UV-B may result in SA and PR protein production; however, evidence is weaker for this response in low dose than high dose experiments. The up-regulation of the phenylpropanoid pathway, resulting in increases to phytoanticipins such as cell wall strengthening with lignin, and other secondary metabolites is more effective at a lower dose. Increases to phenolics which prevent entry of the pathogen and restrict growth are therefore more likely to contribute to lower UV-B dose response. The type of protective mechanisms induced by UV-B are therefore dose dependent, with perhaps a high, stressful dose of UV-B generating a wound like response whereas a low UV-B dose induces a fortifying of the plant cell to prevent entry and a bolster to antimicrobial phenolics. Overall UV-B light exposure results in a tougher plant which is generally more resistant to stress, including biotic stress. Possible mechanisms need to be investigated further to understand the key parts of the UV-B response which contribute to increased disease resistance and the ideal dose to maximise this defence.

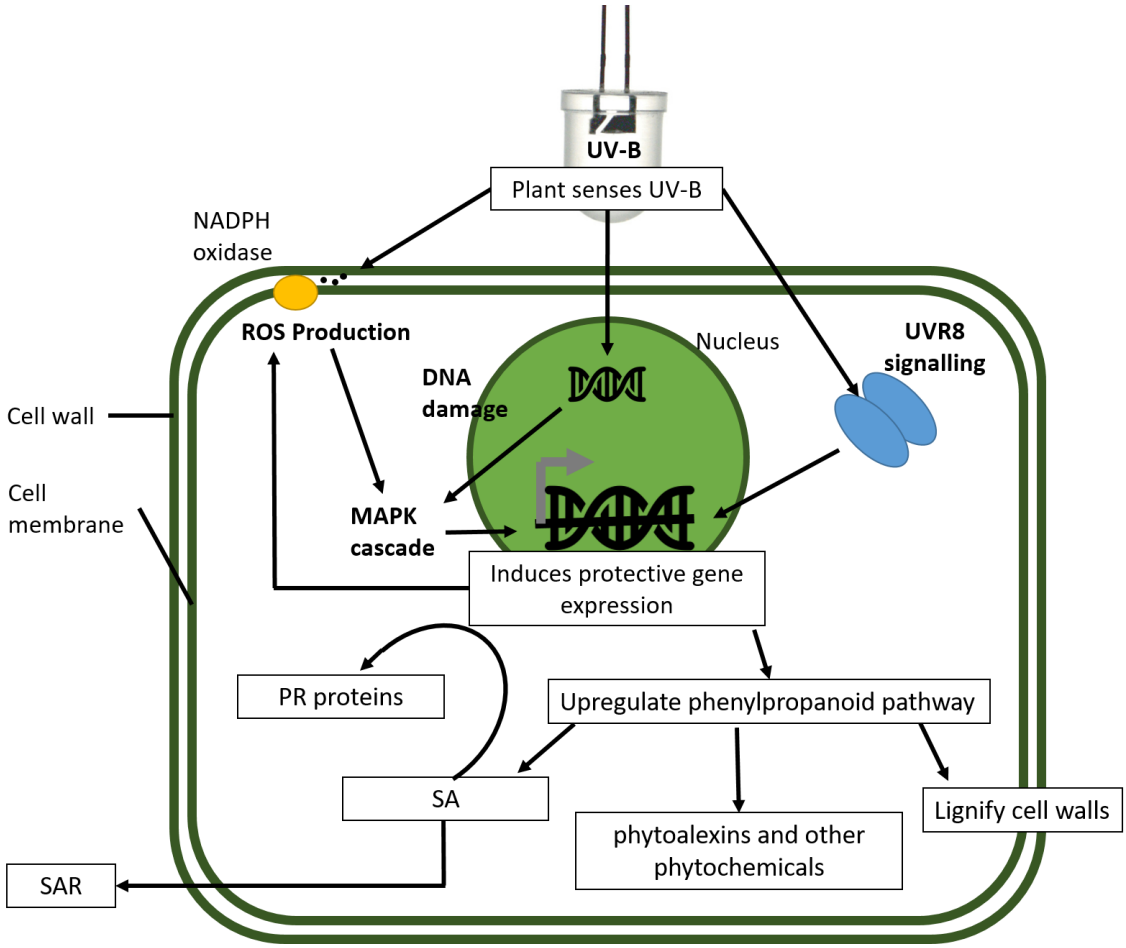


Figure 1.14.: Hypothetical model of UV-B/pathogen response [suggested by the author]. Upon reception of UV-B light, the plant induces a range of protective responses to reduce damage caused by the high energy of UV-B light. Perception and signalling of UV-B light can be achieved through stress-like responses such as DNA damage or induction of reactive oxygen species (ROS) or specifically through a UV-B photoreceptor; UVR8 (UV-B resistance 8). Although these pathways can induce different UV-B response genes, they tend to both increase up-regulation of the phenylpropanoid pathway. The phenylpropanoid pathway includes many defensive compounds, such as flavonoids, and lignin which protect the plant from UV-B light as well as pathogens. Other phenolic compounds which contribute to pathogen defences, such as phytoalexins or the phytohormone salicylic acid, have been observed to up-regulated by UVR8-independent pathways only. Salicylic acid controls the biotrophic defence response through pathogenesis-related (PR) proteins and a systemic acquired resistance (SAR) response. The combination of these UV-B-induced protective responsive can result in an increase resistance of the plant to both abiotic and biotic stress.

1.4. UV-B-pretreatments could be used as a disease control tool

Previous studies reveal many overlaps between the response of a plant to UV-B light and pathogen attack. However, the effect of UV-B exposure on the resistance of a plant to disease is a relatively unexplored area. Two studies have observed that UV-B-pretreatments can increase resistance of lettuce to a biotrophic pathogen (*B. lactucae*) (Wargent et al., 2006) and *Arabidopsis* to a necrotrophic pathogen (*B. cinerea*) (Demkura and Ballare, 2012). The mechanism in which UV-B causes this increased resistance is also largely unknown. My project aims to address the lack of research into a UV-B-induced disease resistance with a side focus in commercial adaptation to a disease control tool.

The case study for this project is lettuce downy mildew caused by *B. lactucae*. This case study was chosen due to past evidence towards a UV-B-induced resistance and the need for improved disease control methods in lettuce downy mildew. Many phenolics induced by UV-B have also been observed to have a role in disease resistance. Therefore I hypothesise:

1. UV-B-pretreatments can reduce disease severity in lettuce against *B. lactucae*
2. UV-B-induced phenolics in part mediate this reduced disease severity phenotype

Hypothesis 1 was tested through assessment of disease following a range of UV-B-treatments in different cultivars. The objectives used to test this hypothesis were:

1. Determine the optimal UV-B (300nm) treatment required for disease incidence and severity reduction in lettuce cv. Casino
2. Determine if UV-B-induced disease defence is conserved across a range of cultivars
3. Determine if a UV-B-reduced disease severity alters the ability of disease to spread between plants
4. Determine if *B. lactucae* growth is reduced within UV-B pre-treated plants

Hypothesis 2 was tested through examination of gene expression changes in *Arabidopsis* following UV-B exposure and an incompatible biotrophic interaction to confirm overlap between phenolic and disease resistance pathways. The role of UV-B-induced phenolic compounds were further investigated through measurement of flavonoids, LC-MS and compound infiltration in UV-B-treated plants and correlated with subsequent disease severity.The objectives used to test this hypothesis were:

1. Determine similarities between gene expression in a plant exposed to UV-B and an incompatible pathogen

2. Determine if flavonoid levels correlate with disease changes

3. Determine which phenolic compounds are up-regulated by UV-B light in plants

4. Determine if any UV-B-induced phenolic compounds correlate with disease reduction\

5. Determine if UV-B induced phenolics can reduce disease severity in isolation

Chapter 2

Building a UV-B dose response for disease reduction

2.1. Introduction

Plants experience a reduction in disease severity following UV-B exposure (Demkura and Ballare, 2012; Wargent et al., 2006). However, there is still limited information known regarding how disease protection can be triggered by different UV-B-treatments. Here, I used a model system of lettuce (*Lactuca sativa*) and the obligate biotrophic pathogen, *Bremia lactucae*, the causative oomycete of lettuce downy mildew, to characterise the optimum dose for disease reduction efficacy. Dose response tests were completed in a controlled environment as well as using commercial light treatment systems. A range of UV-B fluence rates at 300nm were tested, with a dose of 0.5 µmol $m^{-2}s^{-1}$ identified as most successful at reducing downy mildew disease severity on a lettuce cultivar Casino. This optimised dose was applied to a range of lettuce cultivars resulting in reduced disease severity in a cultivar dependent manner. UV-B-pretreated plants had significant reductions of disease incidence, sporulation severity, disease damage and conidia count compared to control plants (photosynthetically active radiation (PAR) only). The reduction in conidia

count was significant enough to reduce severity of secondary infections.

2.2. Methods

2.2.1. Experimental treatments

2.2.1.1. Plant growth

Lettuce (*L. sativa*) seeds were sown 0.5cm deep into black plastic trays, with a cell size of 3cm^2, containing 'Daltons Seedling Raising Mix'. The tray was spread with a single layer of grade 3 medium vermiculite [Auspari pty LTD, NSW]. Sown trays were misted with water then placed in darkness at 14°C for 48 h for vernalisation. Following vernalisation, plants were grown for 14 days in a controlled temperature room (CTR). The CTR had growth conditions of 17°C, 215µmol m^{-2}s^{-1} with a photo-period of 10 h supplied by FL58W/965 super daylight deluxe fluorescent tubes [Slyvania Premium Extra, China]. Capillary matting beneath the trays was watered daily. In the majority of experiments, cultivar Casino [Terranova Seed, NZ] was used.

2.2.1.2. Light treatments

Light treatments were applied with a stationary light-emitting diode (LED) array designed by Biolumic Ltd. Treatments were either control (PAR only) or UV-B (PAR +300nm UV-B). PAR light consisted of red and blue LEDs. Dose response included UV-B doses of 0.1, 0.3 and 0.5µmol m^{-2}s^{-1}UV-B light with a background of 215µmol m^{-2}s^{-1}PAR (Table 2.1). Light quality and quantity were confirmed with a radiometer [Optronic Laboratories OL756] or spectroradiometer [Spectrilight ILT950] prior to each treatment. Treatments were applied to two-week old-plants (post sowing) for a photo-period of 10 hs over three days. Following light treatments, plants received a darkness period of 14 hs prior to infection with disease. Treatments were conducted in a 17°C CTR with the LED array acting as a sole light source. Set A (Table 2.1) treatment was used for method development prior to the availability of a radiometer to measure PAR and UV-B levels. Measurement of light levels in set A was taken after experiments had begun and were lower than expected; however, the experimental set was run to completion regardless.

Table 2.1.: List of UV-B-treatments used in dose screening.
PAR; photosynthetically active radiation

Experiment Set	UV-B (μmol m^{-2} s^{-1})	PAR (μmol m^{-2} s^{-1})	Length (hours)	Reps
A	0.1	90	72	4
B	0.3	215	72	4
C	0.1	215	72	4
D	0.5	215	72	3

2.2.2. Application of commercial treatments

2.2.2.1. Plant growth

Lettuce (*L. sativa*), were sown as for experimental treatments. Growth chamber experiments used cultivars; El Dorado, Iceberg and Salinas [Richard Michlemore, UC Davis, CA; USA] and were raised as for experimental treatments. Glasshouse lettuce plants (cultivar Casino) were grown on a flood and drain table for five days then moved to matting with drip irrigation under standard glasshouse conditions in Palmerston North, New Zealand. A cooling fan maintained temperature under 20^{o}C.

2.2.2.2. Light treatments

Two-week-old plants were treated with a moving (52.8 mm/s) UV-B (280nm peak) LED array designed by Biolumic Ltd in either a growth chamber or glasshouse.

Growth chamber treatments were performed on plants using recipe BL3 for a 10 h photoperiod over three days within a growth chamber with a temperature of 17^{o}C. Background lighting (PAR) was supplied by overhead stationary red and blue LEDs (100 µmol m^{-2}s^{-1}). Control plants received PAR from overhead stationary red and blue LEDs (100 µmol m^{-2}s^{-1}) only.

Glasshouse treatments were performed using recipes BL1 and BL2 for a photoperiod of 16 h over three or 7 days under stadnard glasshouse conditions. Two sets of control plants were used in glasshouse experiments; PAR only or no supplementary light (i.e. standard glasshouse conditions). Due to chronic pest pressure from fungus gnats, combined with lighting equipment failure, these glasshouse trials could not be successfully completed [data not shown].

Treatment BL1, BL2 and BL3 are intellectual property of Biolumic Ltd, therefore, were subject to commercial confidentiality, thus further recipe treatment information can not be

provided. Light quality and quantity was confirmed to meet the specifications of BioLumic treatment recipes using a radiometer or spectroradiometer prior to each treatment.

2.2.3. Pathogen inoculation and disease assessment

Plants were misted with 10^5 conidia mL^{-1} of *B. lactucae* (sextext code IBEB-C 36-01-00 or EU-B 16-63-40-00) using a pressure sprayer until plants were saturated. Inoculated plants were kept in a misting tent at a temperature of 17^{o}C and misted twice daily with water to encourage a high humidity. Disease was visually assessed using a disease scale (Table 2.2) or a sporulation scale (Table 2.3) daily from 6 days post-inoculation (DPI) till 12 DPI. Rating scales were created based on observations of growth patterns of *B. lactucae* on lettuce cultivar Casino seedlings (2-4 weeks old) between 6 and 12 DPI.

Conidia counts were taken 12 DPI. Count samples consisted of a number of randomly chosen plants washed in distilled water at a ratio of 1 plant per 20mL depending on the number of plants available and the accuracy of equipment available. An aliquot (5µL) ofthe resulting suspension was pipetted into a haemocytometer (0.1 mL depth) [BRAND® counting chamber BLAUBRAND® Neubauer improved, Sigma - Aldrich] and conidia were counted in the four corner squares. The average of these four squares was multiplied by 10,000 to give a measurement in conidia mL^{-1}.

Table 2.2.: Disease rating scale used to assess the total downy mildew disease (sporulation and damage) severity of iceberg type lettuce seedlings infected with *B. lactucae.*

Rating	Description
0	No visible disease
1	Cotyledons infected : yellowing or sporangia
2	Singular patch of sporangia on a singular leaf
3	Sporangia covering entire leaf
4	Sporangia on more then one leaf. Signs of yellowing
5	Majority of plant covered , brown lesions and yellowing evident, very severe infection/dead

Table 2.3.: Sporulation rating scale used to assess *B. lactucae* sporulation level on infected iceberg type lettuce seedlings.

Rating	Description
0	No signs of spores
1	Cotyledon infected
2	Singular patch of spores 1-10% covered on 1 leaf only
3	Spores 10-25% covered on 1 leaf only
4	Spores 25-75% covered on 1 leaf only
5	Spores on multiple leaves <25% covered
6	Spores on multiple leaves AND 25-75% covered on 1 leaf
7	Spores on multiple leaves AND 50-75% covered 2 leaf
8	Spores on multiple leaves AND 50-75% covered on 3 leaf
9	Spores on multiple leaves AND 75-100% covered on >3 leaf

2.2.4. Dose response screening

Light treatments B-D (Table 2.1) were carried out in separate experiments using lettuce cultivar Casino followed by disease inoculation and assessment. Differences in disease severity between control and UV-B-pretreated plants were determined. Measurements based on rating scales (incidence, damage, disease rating and sporulation rating) used one plant per sample. The total number of rating samples per experiment are; B = 108-205, C = 279-281, D = 173 per treatment. Conidia counts used four plants per sample. The total number of conidia count samples per experiment are: B = 36, C = 42, D = 32 per treatment. The difference in sample numbers between expreiments was due to size of available growth trays, the sensitivity of measurement equipment available, and treatment area of the UV-B array.

2.2.5. Multiple cultivar screening

Experiments were run as for dose response light treatment D (Table 2.1); however, used a range of lettuce cultivars [Casino], El Dorado, Iceberg, Pavane, Pedrola, Salinas [Richard Michlemore, UC Davis, CA;USA] sown in a Latin square design. At 72 h, a non-destructive hand-held flavonoid meter; Dualex [Force A, Orsay; France] (Goulas et al., 2004) was used to take flavonoid measurements. Plants were inoculated with *B. lactucae* conidia at 72 h and assessed as described in Section 2.2.3. Four repeats were completed with a sample size of 8 plants per cultivar per treatment. Total sample number for ratings was 18-24, flavonoids; 9-20, conidia count; 13-16 per cultivar per treatment. Ratings and flavonoid

measures used one plant per samples. Conidia counts were calculated using four plants per sample.

2.2.6. Commercial treatment screening

Lettuce plants underwent growth and treatment conditions as described in Section 2.2.2. In growth chamber experiments, plant flavonoid index was taken using a Dualex (Goulas et al., 2004) at 0, 24, 48, and 72 h after treatment as well as 1, 7, 12 DPI. The total number of samples measured for flavonoid levels was 38-45 per treatment. Plants were inoculated with *B. lactucae* conidia and disease was assessed as described in Section 2.2.3. The total number of rating samples was 57-60 per treatment. The total number of conidia count samples was 16 (three plants per sample). In glasshouse experiments, a high fungus gnat population caused heavy yellowing, wilting and some cases death of the plant. In these cases, the experiment was discarded.

2.2.7. Secondary inoculation experiments

Light treatment D was used for secondary inoculation experiments (Table 2.1). The primary lettuce plants (A) were light-treated then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. At 7 DPI, secondary light-treated plants (B) were placed in the tent (Figure 2.1 & 2.2). Inoculation of secondary plants by primary was encouraged by lightly pumping air over the plants thrice a day for seven days. Plants were misted with water twice daily. Disease was assessed on B plants as described in Section 2.2.3; however, daily visual disease assessment ceased at 11 DPI, with conidia counts being taken at 11 DPI also. The total number of samples used in rating scales was 58-62 per treatment. The total number of samples measured in conidia counts was 11-12 (four plants per sample) per treatment.

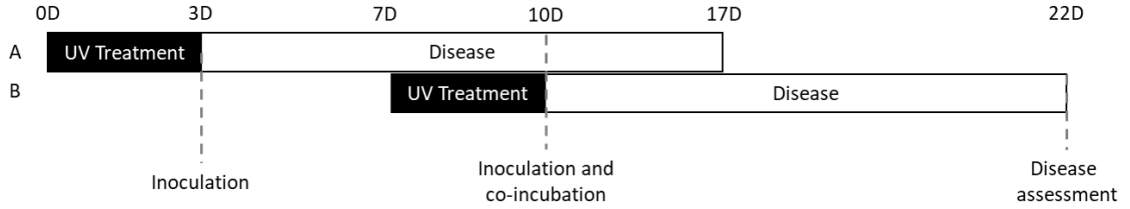


Figure 2.1.: Time line of secondary inoculation experiments. "A" indicates the primary plants which act as the source of inoculum. "B" indicates the secondary plants. The time line progresses in days (D). At 0D, plant set A are treated with UV-B light for 3 days. Plant set A are then inoculated with 10^5conidia mL^{-1} *B. lactucae* and placed in a misting tent. At 7D, plant set B are treated with UV-B for three days then placed in the same misting tent as plant set A (10D) for inoculation. 10D is 7 DPI for plant set A. At 22D (11 DPI for plants set B), plant set B are removed from the misting tent for conidia counts.

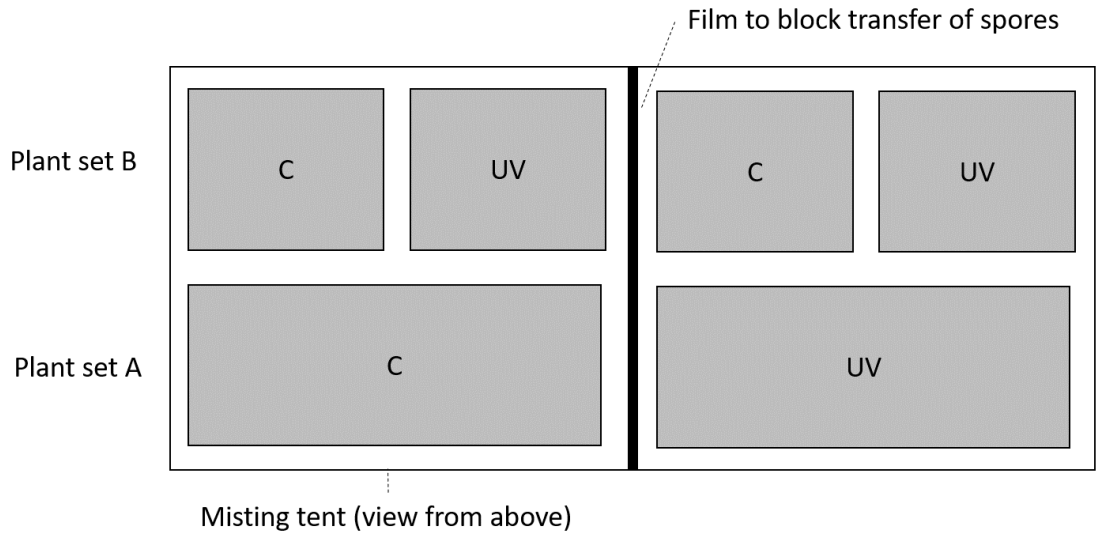


Figure 2.2.: Layout of trays of lettuce cv. Casino plants [grey box; C=control, UV=UV-B-pretreated] within a misting tent. Plant set A were inoculated with 10^5 conidia mL^{-1} of *B. lactucae.* Plant set B were naturally infected from plant set A and resulting disease symptoms of plant set B were measured.

2.2.8. Data analysis

IBM SPSS Statistics for Windows, Version 23.0 was used for quantitative summaries and statistical analysis of results.

Graphs were produced using Microsoft Excel 2016, IBM SPSS Statistics for Windows, Version 23.0, or Minitab 17. To determine significance between means of normally distributed data; t-tests or ANOVAs were used. To determine significant differences between non-normally distributed data, such as rating scales, non-parametric tests including Mann-Whitney U and Kruskall-Wallis were used. Following Kruskall-Wallis tests, multiple comparisons were made using Mann-Whitney U. To avoid the occurrence of a type I error, Bonferroni correction was applied (Armstrong, 2014). To determine differences between counts, a two-tailed Fisher's Exact test was used.

The % decrease compared to a control was calculated by:$(1 - Treatment/Control) \times 100$

Degree of Infection (DoI) was used to summarise the final value of sporulation intensity, as a percentage of the maximum score for rating scales based on Lebeda and Petrzelova (2010); Townsend and Heuberger (1943).

$DI = \Sigma(n \times v) \times 100/(x \times N)$

n Number in each rating category

v Rating x scale range

x Scale range

N Total number assessed

Rating scales were normalised to improve comparability between repeats and treatments. The normalised scale was presented as a percentage of the mean rating of control plants at 12 DPI.

Normalised rating = rating/(mean control rating at 12 DPI) x 100

This attempted to account for the differences in disease pressure, by expressing the value as a percentage of the maximum rating gained in each repeat.

Results and Discussion

2.3. UV-B-pretreatment can reduce severity of downy mildew disease in lettuce

Three doses of UV-B (0.1, 0.3 and 0.5 µmol $m^{-2}s^{-1}$) were screened for their effectiveness of decreasing disease severity in lettuce cultivar Casino. This included assessment of disease incidence, severity throughout the disease period as well as a final conidia count at 12 DPI. The highest dose, 0.5 µmol $m^{-2}s^{-1}$, was the best dose tested based on UV-B-induced decreases to all of the assessment measures.

2.3.1. UV-B-pretreatment (0.5 µmol $m^{-2}s^{-1}$) delays disease incidence

Disease incidence (% of plants infected) increased over time, with a steeper rate of infection at early stage disease (6-8 DPI), followed by a plateau of incidence at later disease stages (9-12 DPI). Disease incidence did not significantly differ between UV-B and control plants of the 0.1 or 0.3 µmol $m^{-2}s^{-1}$dose experiments at any time. Plants pretreated with 0.5 µmol $m^{-2}s^{-1}$UV-B had significantly reduced incidence at 7 DPI compared to control plants (Fisher's Exact test, two-tailed, $p = 0.003$) (Figure 2.3). As this difference in incidence was lost as the disease stage progressed (Figure 2.3), the UV-B-pretreatment causes a delay in infection rather than a reduction in overall incidence. A delay in infection suggests that the pathogen takes longer to produce sporangiophores within the UV-B (0.5 µmol $m^{-2}s^{-1}$)-pretreated plants compared to control plants.

A delay in infection suggests UV-B-induced defence is important at the establishment stage of disease; i.e. penetration and spread of mycelium throughout the plant resulting in a longer incubation/latent period. Plant cell wall-related defences, such as the level of pre-formed secondary metabolites (phytoanticipins), papillae response and lignification, can prevent or delay the establishment of disease (Underwood, 2012). As outlined in Sections 1.3.7.3 and 1.3.7.6, a typical UV-B response involves formation of protective secondary metabolites and strengthening of barriers. These UV-B-protective responses may cause prevention of entry or germination of some *B. lactucae* conidia. The implications for a delay in disease in agriculture are discussed in Section 5.3. If entry is not prevented, UV-B bolstered defence may increase the time and resources required for successful germtubes to penetrate the cuticle and cell wall of an epidermal cell thus increasing the incubation

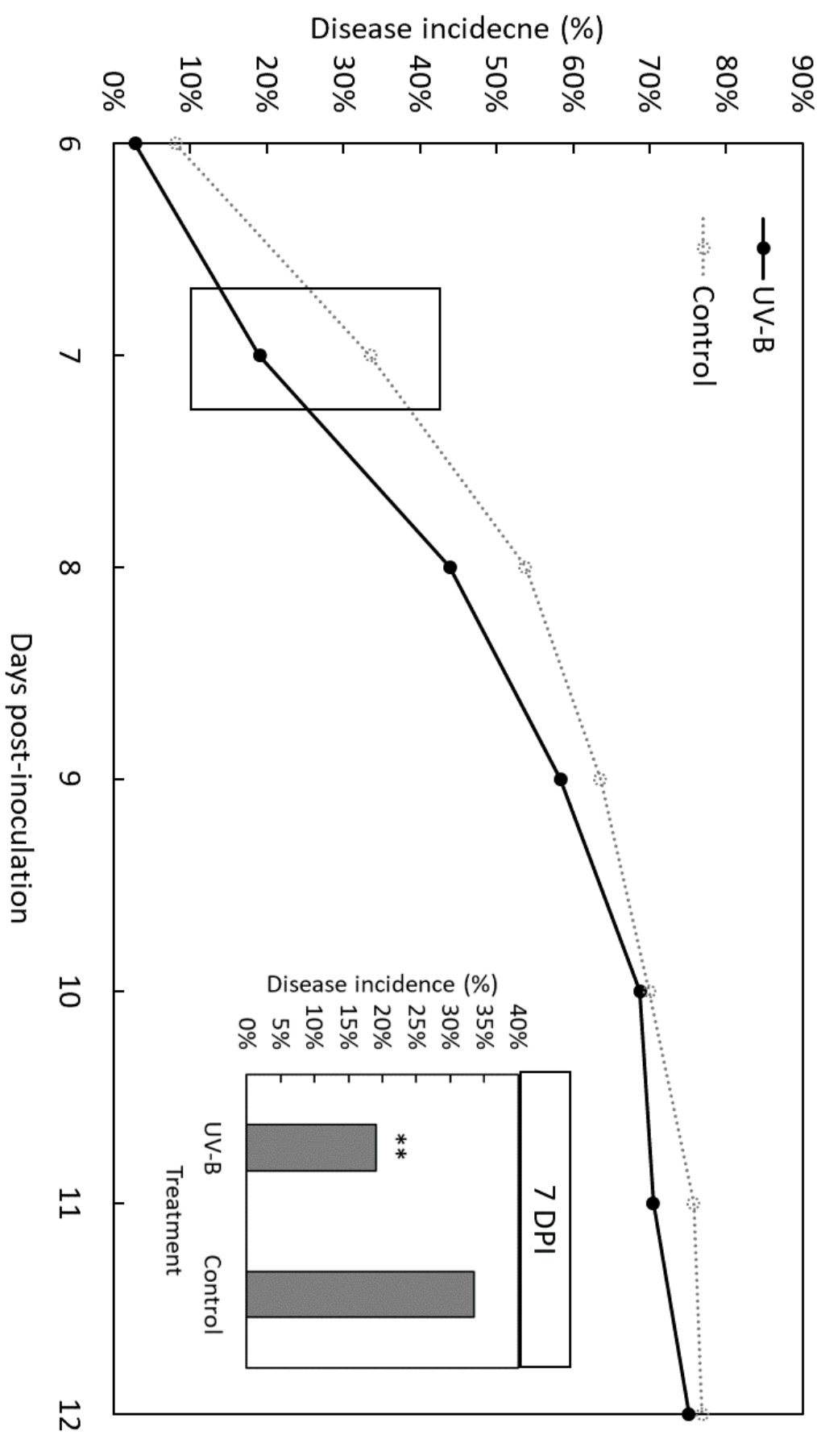


Figure 2.3.: UV-B (0.5µmol $m^{-2}s^{-1}$)-pretreated lettuce (*L. sativa*) cv. Casino [black line] plants had a lower percentage of plants infected (incidence) with *B. lactucae* then control plants [grey line] over time (days post-inoculation). At 7 DPI [bar chart] the number of infected plants was significantly lower in UV-B-treated than control plants (Fisher's Exact test, two-tailed, p = 0.003). Lettuce plants were treated with photosynthetically active radiation (PAR) + 0.5µmol $m^{-2}s^{-1}$UV-B or PAR only (control) for three days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. From 6 DPI onwards, the disease incidence was measured as the number of plants displaying disease symptoms divided by the total number of plants.

time.

2.3.2. UV-B-pretreatment (0.3 and 0.5 µmol $m^{-2}s^{-1}$) reduces distribution of disease ratings

The disease rating scale (Table 2.2) assesses total disease severity including both sporulation intensity and the presence of disease damage such as yellowing. The severity of disease ratings is summarised by degree of infection (DoI), with significant differences in distribution given by daily Mann Whitney U analysis of normalised ratings. Disease severity increased over time with many plants reaching a maximum disease rating by 12 DPI. In no cases did the DoI from pooled ratings reach 100% by 12 DPI.

DoI of control and UV-B-pretreated plants of the 0.1 µmol $m^{-2}s^{-1}$experimental set were similar throughout the disease period (Figure 2.4). The distribution of normalised ratings was significantly higher in UV-B-pretreated plants (0.1 µmol $m^{-2}s^{-1}$) at 8 and 9 DPI; however, was not significantly different at any other time point (Table 2.4). The disease pressure in the 0.1 µmol $m^{-2}s^{-1}$experimental set was lower than other experiments. This low disease pressure meant only lower ratings were observed, thus reducing the sample size and potential rating difference. The lower disease pressure meant when considering normalised ratings (excluded non-infected plants) a significant difference occurred even though the overall DoIs of the UV-B and control plants of the 0.1 µmol $m^{-2}s^{-1}$experimental set did not appear to be very different.

At early stage disease (6-9 DPI), the DoI of UV-B and control plants of the 0.3 µmol $m^{-2}s^{-1}$experimental set were similar. At later-stage disease (10-12 DPI), UV-B-pretreated plants had a lower DoI than control (Figure 2.4). This is supported by Mann Whitney U analysis of normalised ratings, in which distribution of ratings did not significantly differ from 7 to 9 DPI; however, were significantly lower in UV-B plants from 10 DPI onwards (Table 2.4).

Throughout the disease period, UV-B plants of the 0.5 µmol $m^{-2}s^{-1}$experimental set had a lower DoI than control plants (Figure 2.4). The distribution of normalised ratings was significantly lower at all times except 9 and 10 DPI in which the decrease was not significant (Table 2.4).

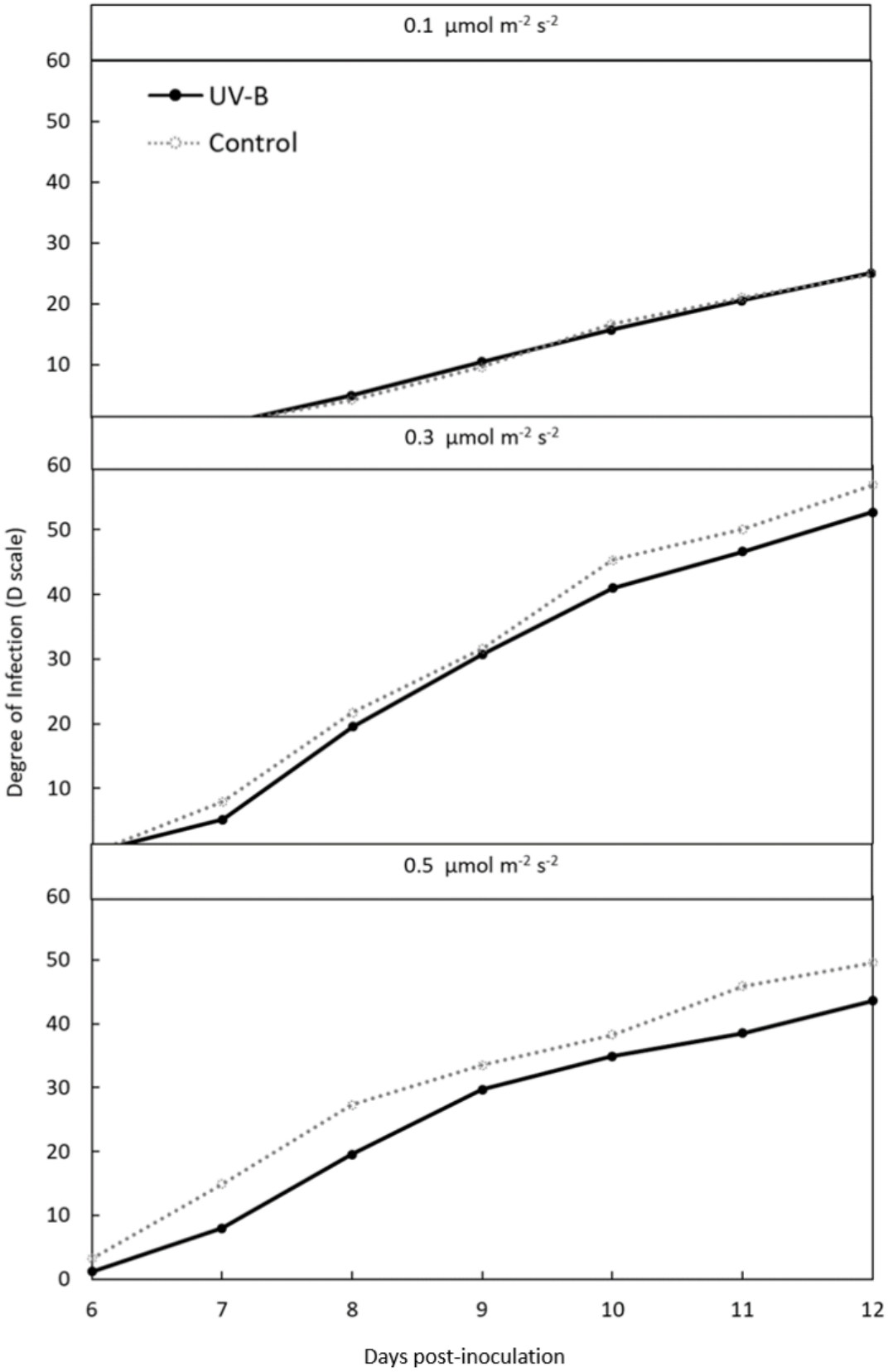


Figure 2.4.: Degree of infection (DoI) by downy mildew in lettuce (*L. sativa*) cv. Casino based on the disease (D) scale was similar in UV-B [black line] and control [grey dotted line] in 0.1 and early stage 0.3 µmol $m^{-2}s^{-1}$experiments. UV-B plants had a lower DoI than control plants in 0.5 and late stage 0.3 µmol $m^{-2}s^{-1}$experiments. Lettuce plants were treated with PAR + 0.1, 0.3 or 0.5µmol $m^{-2}s^{-1}$UV-B or PAR only (control) for three days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. From 6 DPI til 12 DPI, plants were rated on a disease scale.

Table 2.4.: Change in distribution of UV-B pooled normalised downy mildew disease ratings compared to control lettuce (*L. sativa*) cv. Casino plants. Lettuce plants were treated with photosynthetically active radiation (PAR) + 0.1, 0.3 or 0.5µmol $m^{-2}s^{-1}$UV-B or PAR only (control) for three days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae* . From 6 days post-inoculation (DPI) til 12 DPI, plants were rated on a disease scale. The disease ratings were normalised based on the maximum rating for each repeat. Normalised ratings of UV-B and control plants were compared using Mann Whitney U tests. Asterisks indicate significant differences where *=p<0.05, ***=p<0.0005 and ns is no significant difference.

Treatment (µmol m^{-2} s^{-2})	7 DPI		8 DPI		9 DPI		10 DPI		11 DPI		12 DPI	
0.1	Nil	ns	Increase	*	Increase	*	Nil	ns	Nil	ns	Nil	ns
0.3	Nil	ns	Nil	ns	Nil	ns	Decrease	*	Decrease	*	Decrease	*
0.5	Decrease	*	Decrease	***	Nil	ns	Nil	ns	Decrease	***	Decrease	***

As the UV-B dose increases, the disease rating severity decreases. The lowest dose; 0.1µmol $m^{-2}s^{-1}$ UV-B, resulted in no change or a (slight) increase in disease severity. The medium dose; 0.3 µmol $m^{-2}s^{-1}$ UV-B, resulted in a decrease in disease severity at late stage only. The highest dose; 0.5 µmol $m^{-2}s^{-1}$ UV-B, resulted in decreases to disease severity throughout the disease period (except 9 and 10 DPI).

UV-B-pretreatment may cause a more noticeable reduction to disease at higher severity levels. When the overall disease pressure was low (entire 0.1 µmol $m^{-2}s^{-1}$experimental set, or early stage disease in 0.3 and 0.5 µmol $m^{-2}s^{-1}$experimental set), UV-B mediated decreases to disease severity did not consistently occur. However, at higher disease pressure (late stage 0.3 and 0.5 µmol $m^{-2}s^{-1}$experimental sets), UV-B-induced decreases to disease severity were observed. The observation of increasing UV-B effect with disease severity is likely a statistical artefact. A higher disease incidence increases sample size of normalised ratings, thus increasing the statistical power of the experiment. To add, at a high disease severity, ratings of infected plants will be higher. As ratings are a fixed scale, as the median rating increases, the range of ratings which are considered 'lower' is greater. Therefore, in order for a UV-B-induced disease reduction to be observed, plants must be past a severity threshold. UV-B likely results in reduced disease severity throughout the disease period; however, at early stage disease (or low severity), the lack of samples and lower achievable rating range masks differences.

2.3.3. UV-B-pretreatment (0.5 µmol $m^{-2}s^{-1}$) reduces sporulation severity

The sensitivity of the disease scale can detect major differences in disease; however, after

several screens, it became obvious a more sensitive scale to judge the level of *B. lactucae* growth only (not damage) was required. The sporulation scale (Table 2.3) was used in all replicates for experiment sets C (0.1 µmol $m^{-2}s^{-1}$ UV-B) and D (0.5 µmol $m^{-2}s^{-1}$ UV-B).

At a dose of 0.1 µmol $m^{-2}s^{-1}$ UV-B, sporulation level did not significantly differ from control plants at any time point. This may be due to the dose of UV-B being too low to elicit a response in the plant or could be due to a lower disease pressure in this set of experiments. Following 0.5 µmol $m^{-2}s^{-1}$ UV-B-pretreatment, plants had a lower DoI than control plants throughout the disease period, with a significant reduction in normalised sporulation rating compared to control at all days except 11 DPI (Figure 2.5). Data from sporulation rating confirmed findings from disease rating analysis that 0.5 µmol $m^{-2}s^{-1}$ UV-B can reduce downy mildew disease severity in lettuce.

2.3.4. UV-B-pretreatment resulted in a reduced number of plants displaying damage caused by disease

The disease scale used incorporated both *B. lactucae* reproductive growth and disease damage on the plant. At a rating of four or above, the plant exhibited damage due to disease such as yellowing or the formation of lesions. In all doses, UV-B-pretreated plants exhibited less damage due to disease 12 DPI (Figure 2.6); however, was only significantly reduced following 0.5 µmol $m^{-2}s^{-1}$ UV-B-pretreatment (Fisher's Exact test, two-tailed, $p = 0.021$).

Control plants did not have similar number of damaged plants across experimental sets (Figure 2.6). Control plants of the 0.3 and especially 0.1 µmol $m^{-2}s^{-1}$ UV-B sets had a much lower number of damaged plants than control plants of the 0.5 µmol $m^{-2}s^{-1}$ UV-B set. Although damage levels of UV-B-pretreated plants from the 0.3 and 0.5 µmol $m^{-2}s^{-1}$ UV-B sets were similar (and 0.1 much lower), the damage levels of control plants were also lower resulting in no significant difference. Therefore a lack of difference between disease tolerance of treatments in 0.1 and 0.3 µmol $m^{-2}s^{-1}$ UV-B experimental sets may be due to a lower disease pressure rather than a lack of UV-B response.

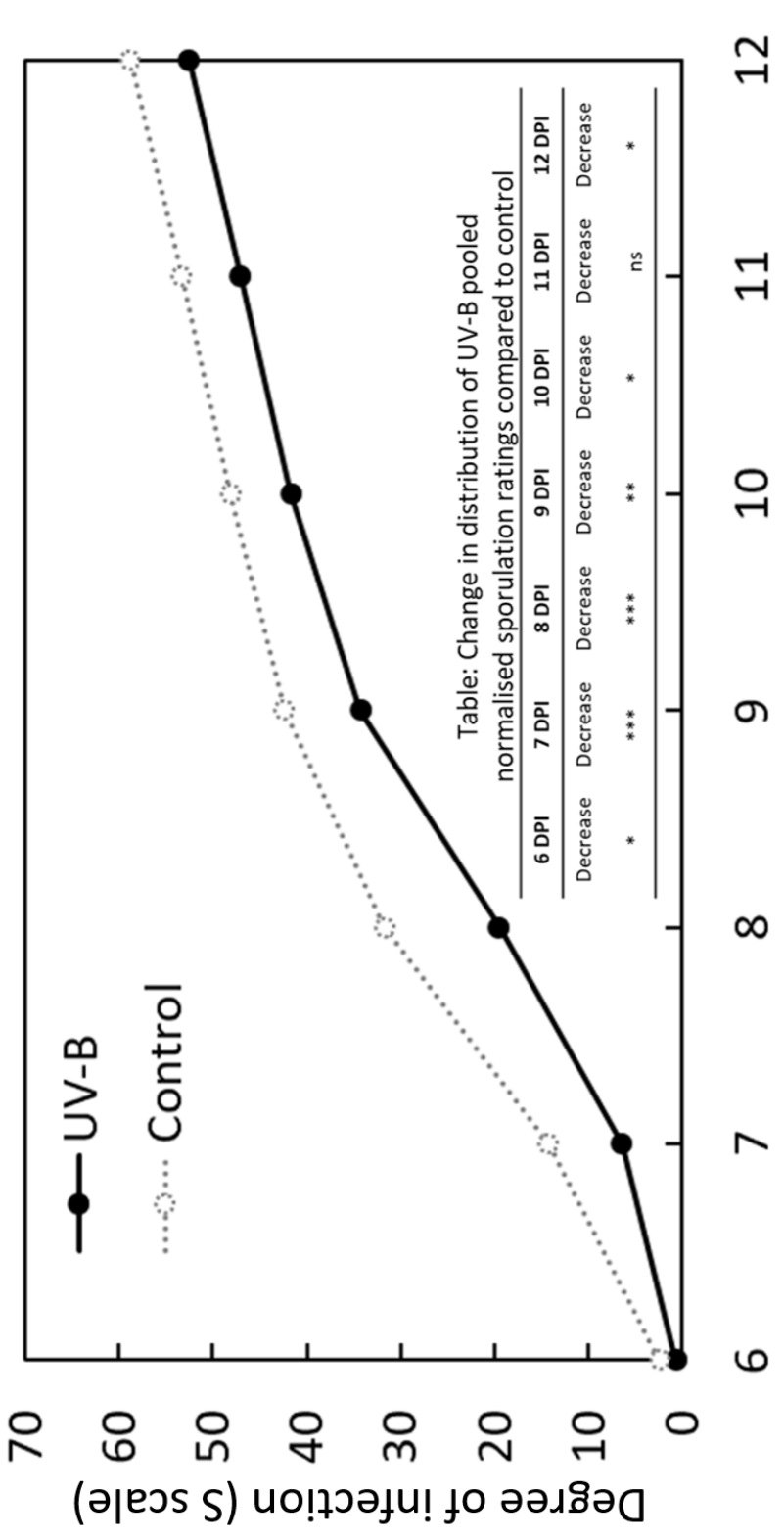


Figure 2.5.: Degree of infection (DoI) based on pooled downy mildew sporulation ratings were lower in UV-B (0.5 µmol $m^{-2}s^{-1}$) treated lettuce (*L. sativa*) cv. Casino plants [black line] compared to control plants [grey dotted line] at all time points. Lettuce plants were treated with photosynthetically active radiation (PAR) + 0.5µmol $m^{-2}s^{-1}$UV-B or PAR only (control) for three days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. From 6 days post-inoculation (DPI) until 12 DPI, plants were rated on a sporulation scale. Overall sporulation severity per day was summarised as degree of infection. Table shows significant changes of UV-B normalised sporulation ratings compared to control according to daily Mann Whiteny U tests [* = p <0.05, ** = p <0.005, *** = p <0.0005, ns = non-significant].

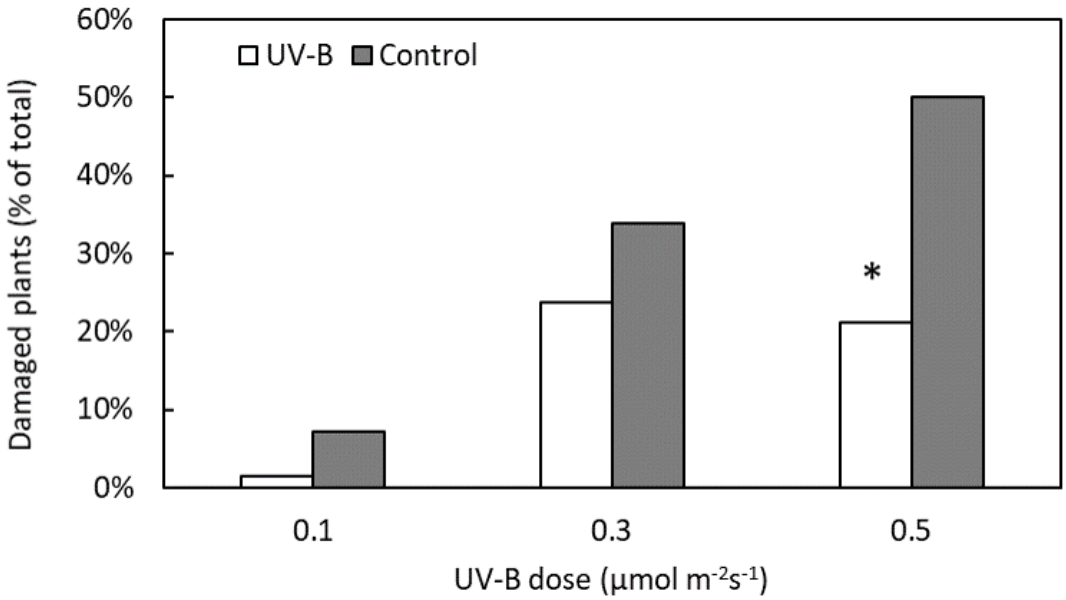


Figure 2.6.: The highest UV-B [white bar] dose tested, 0.5 µmol $m^{-2}s^{-1}$, displayed a significantly reduced % of lettuce (*L. sativa*) cv. Casino plants displaying damage caused by downy mildew disease compared control [grey bar] plants (Fisher's Exact test, two-tailed, $p = 0.021$) at 12 days post-inouclation (DPI). Lower doses also displayed a reduction in disease damage in UV-B-pretreated plants; however, this difference was not significant (Fisher's Exact test, two-tailed, $p < 0.05$). Lettuce plants were treated with photosynthetically active radiation (PAR) + 0.1, 0.3 or 0.5µmol $m^{-2}s^{-1}$UV-B or PAR only (control) for three days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae.* From 6 DPI til 12 DPI, plants were rated on a disease scale. At a rating of 4 or 5, plants displayed yellowing or necrotic lesions (disease damage). The number of damaged plants as a percent of the total number of plants are shown.

A reduction in disease damage in 0.5 µmol $m^{-2}s^{-1}$ UV-B-pretreated plants indicates UV-B light may affect the tolerance of a plant to disease, i.e. its ability to continue growing normally despite infection. An increased tolerance caused by UV-B light may be caused by an increase to antioxidants. Reactive oxygen species (ROS) are produced upon pathogen attack, and although they have a major role in defence against the pathogen, they can also damage the host plant (Heller and Tudzynski, 2011). In most cases, including lettuce defence against *B. lactucae,* antioxidants are produced alongside ROS in order to reduce the self damage caused by ROS (Sedlarova et al., 2007). UV-B is well known to up-regulate antioxidants (Rao et al., 1996; Carletti et al., 2003; Agarwal, 2007; Jordan, 2002; García-Macías et al., 2007). Increases to antioxidants will increase the ROS scavenging ability of the lettuce plant resulting in a reduction in ROS damage that may contribute to visible disease damage.

2.3.5. UV-B-pretreatment reduced the number of conidia harvested per plant

The conidia count measured the number of spores per plant washed from UV-B-pretreated or control plants. The conidia count was significantly (t-test, $p < 0.05$) reduced in UV-B plants of every dose compared to control plants (Figure 2.7). A dose of 0.1 µmol $m^{-2}s^{-1}$ UV-B resulted in the lowest mean decrease in conidia count of 29% (t-test, $p = 0.012$). Doses 0.3 and 0.5 µmol $m^{-2}s^{-1}$ UV-B resulted in similar mean decreases of 57% (t-test, $p < 0.0001$) and 58% (t-test, $p < 0.0001$) in conidia count respectively. Consistency in % decrease was high across repeats of each dose.

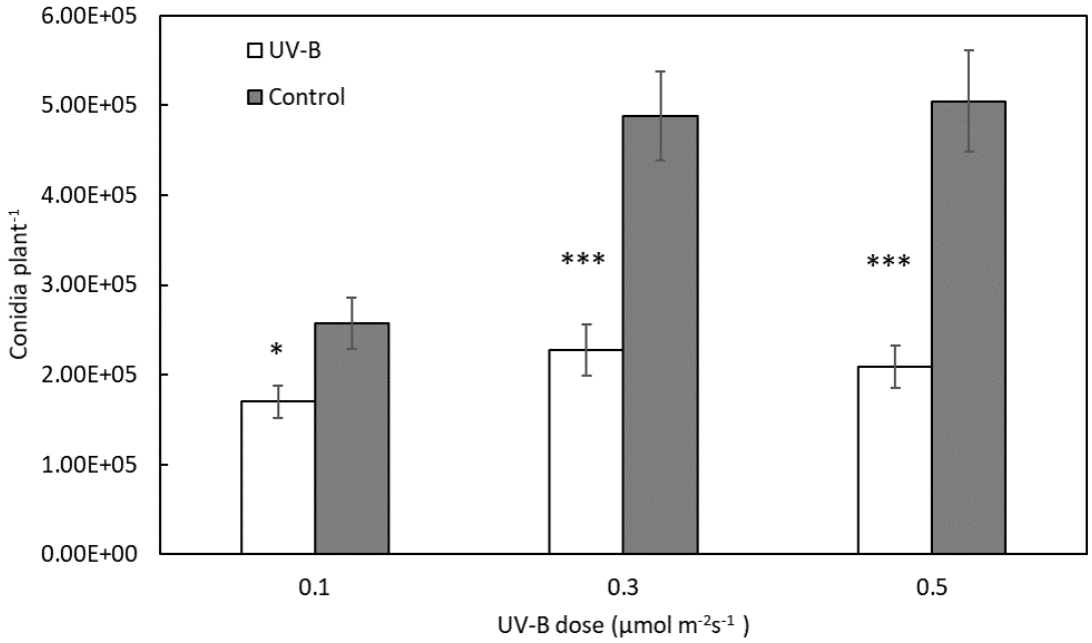


Figure 2.7.: The number of *B. lactucae* conidia harvested per plant in lettuce (*L. sativa*) cv. Casino was significantly lower in UV-B [white]-pretreated plants than control [grey] plants over all three UV-B doses tested. Lettuce plants were treated with photosynthetically active radiation (PAR) + 0.1, 0.3 or 0.5µmol $m^{-2}s^{-1}$UV-B or PAR only (control) for three days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae.* At 12 days post-inoculation, plants were washed and the resulting conidia suspension counted . Significant differences between control and UV-B plants are shown by an asterisk where *=p<0.05 and ***=p<0.0005 according to t-tests. Error bars indicate 1 S.E.

Decreases to *B. lactucae* conidia count (0.3 and 0.5 µmol $m^{-2}s^{-1}$ UV-B-pretreatment) were comparable to treatment of lettuce with several carboxylic acid amide (CAA) fungicides. Leaf disks treated with the fungicides mandipropamid, dimethomorph and iprovalcarb had a 48-55% decrease in conidia count (Cohen et al., 2008). These fungicide reductions are slightly lower than the greatest reductions achieved by a UV-B-pretreatment in this

work (58% reduction in conidia count). Conidia count reductions were greater in leaf disks treatment with benthiavalicarb (85% reduction) than following my UV-B-treatment. Reductions in disease caused by these UV-B-treatments are commercially comparable to several CAA fungicides currently used in downy mildew disease control. Therefore, UV-B can be as successful at reducing downy mildew disease (as conidia count) as commercial standards.

These results strongly support a UV-B-induced reduction in conidia harvested per plant. The level of airborne conidia directly relates to the risk of downy mildew disease development and resulting yield loss (Fall et al., 2015). Airborne conidia level is not only dependent on the sporulation intensity of conidiophores (measured by sporulation rating) but also the proportion of conidia released, can escape the canopy layer, become airborne then become deposited on a susceptible host whilst still viable (Fall et al., 2016). A lower conidia count suggests several possibilities of UV-B-pretreated plants infected with downy mildew disease:

1. Fewer conidiophores are produced in UV-B-pretreated plants
2. Fewer conidia are produced in UV-B-pretreated plants
3. Attachment of conidia to conidiophores is increased in UV-B-pretreated plants
4. Conidia are less stable and more likely to burst when produced on UV-B-pretreated plants

Regardless of mechanism, this suggests:

1. UV-B-pretreated plants interfere with the development of conidiophores/conidia
2. UV-B-pretreated plants are less likely to cause secondary infections of downy mildew due to reduced release of conidia

Disease assessment in dose response indicates that the 0.5 µmol $m^{-2}s^{-1}$ UV-B-pretreatment reduced sporulation intensity (measured by sporulation rating) as well as the proportion of

conidia released (conidia count). Therefore UV-B-pretreated plants produce a lower level of airborne conidia and reduce the risk of potential epidemics.

2.3.6. The most effective disease-reducing dose characterised was 0.5 µmol $m^{-2}s^{-1}$ UV-B

The dose response screening suggests that treatment D: 0.5 µmol $m^{-2}s^{-1}$ UV-B, 215 µmol $m^{-2}s^{-1}$ PAR is the most effective at decreasing downy mildew disease susceptibility in lettuce cv. Casino of the treatments tested. A similar decrease in conidia count compared to control was achieved in the 0.3 and 0.5 µmol $m^{-2}s^{-1}$ UV-B experiment sets. The 0.5µmol $m^{-2}s^{-1}$ dose was observed to decrease the additional disease measures of incidence, number of damaged plants and disease rating throughout the entire disease period also. The 0.3 µmol $m^{-2}s^{-1}$ treated plants only displayed disease severity decreases at late stage disease. To add, consistency in UV-B reductions was higher in the 0.5 µmol $m^{-2}s^{-1}$ experimental set than other treatments. As disease severity decreased with increasing UV-B dose, it may be further increased by higher UV-B doses; however, current light modules at this wavelength cannot provide a higher dose.

2.3.7. Variation in disease pressure masked the effect of UV-B on disease severity

The disease pressure varied greatly, not only between experimental sets but also between repeats. This had a major impact on the potential occurrence of a UV-B effect on disease levels in plants. Variations in disease complicated the analysis of results, pooling data, and summaries, as it may cause skewing by high/low ratings caused by disease pressure rather than UV-B-induced resistance. Control plants in all these sets were under the same conditions and it would be expected that infection rates would be similar; however, they were not.

Observation of a significant difference was largely dependent on disease pressure in rating scales but not conidia count measurements. Therefore, disease pressure changes have a greater effect on UV-B reductions to disease rating scales than conidia count measures. Lack of consistency in rating data may be due to limitations in the design of the disease scale (Table 2.2). As the disease scale attempts to account for both damage and sporulation caused by *B. lactucae* with descriptive qualifiers, ratings are very subjective and may not

always fit perfectly within one of the five categories. A more sensitive scale, which described sporulation only on a numeric basis (Table 2.3) was introduced in later experiments (0.1 and 0.5 µmol $m^{-2}s^{-1}$UV-B experimental sets) as well as future experiments. Results from the sporulation scale had lower variation than the disease scale. Experiments in following chapters used the sporulation scale to describe sporulation severity with the disease (or a damage) scale to describe damage only.

Attempts to improve consistency of downy mildew infections were implemented; however, these had little success. As the age of the inoculum and environmental factors, such as humidity, can alter germination and subsequent success of disease (Fletcher, 1976; Wu et al., 2000), steps were taken to maintain consistency of inoculation method. The Casino-*B. lactucae* strain combination was observed to take about seven days to begin sporulation. *B. lactucae* releases the largest amount of inoculum in the first four hours of light following at least six hours of darkness (Raffray and Sequeira, 1971; Scherm and Bruggen, 1995; Su et al., 2000). Inoculum was harvested two hours after the start of the photoperiod (11 AM), seven days after inoculation, from 21-day old Casino lettuce plants. The inoculum was reduced to 10^5 conidia mL^{-1} and sprayed at 20psi onto each plant for two seconds resulting in saturation of the plant. Following inoculation, plants were misted twice daily; at the start (9 AM) and halfway through (2 PM) a day with water to attempt to maintain a similar humidity. Although experiments were run with these consistency measures applied, there were still changes in disease pressure between experiments.

2.4. The optimal UV-B dose reduces disease susceptibility in a range of lettuce cultivars

The dose 0.5 µmol $m^{-2}s^{-1}$had been shown to decrease disease in lettuce cv. Casino; however, in order to test if a UV-B disease defence response is conserved across lettuce, it was then tested across a range of cultivars with varying levels of disease susceptibility.

2.4.1. UV-B-pretreatment delayed downy mildew disease incidence in El Dorado plants only

Lettuce plants of cultivar El Dorado had an extremely high susceptibility to disease infection, with 100% of control plants displaying sporulation symptoms on the first day of measurement (6 DPI) (Figure 2.8). Pedrola plants were completely resistant to the

B. lactucae strain used (0% incidence). All other cultivar (except Casino) control plants reached near 100% incidence by 10 DPI (Casino: 11 DPI). UV-B-pretreated El Dorado plants had a 32% lower disease incidence (% of plants infected) at 6 DPI (Fisher's Exact test, two-tailed, $p = 0.009$) than control plants with no significant difference in incidence at later time points. Disease incidence was therefore delayed by one day in UV-B-pretreated cultivar El Dorado plants. All other cultivars did not significantly differ in disease incidence at any time point.

The implications of a delay in disease are discussed in Section 5.1. Of the five susceptible cultivars tested, only one (El Dorado) experienced a delay in infection. Therefore there may be a cultivar effect (susceptibility and UV-B responsiveness) on the ability of UV-B-pretreatments to delay disease incidence. As El Dorado control plants reached 100% infection by 6 DPI, El Dorado plants likely have minimal defensive barriers to downy mildew disease. UV-B-pretreatment may bolster these defences enough to cause a delay in incidence.

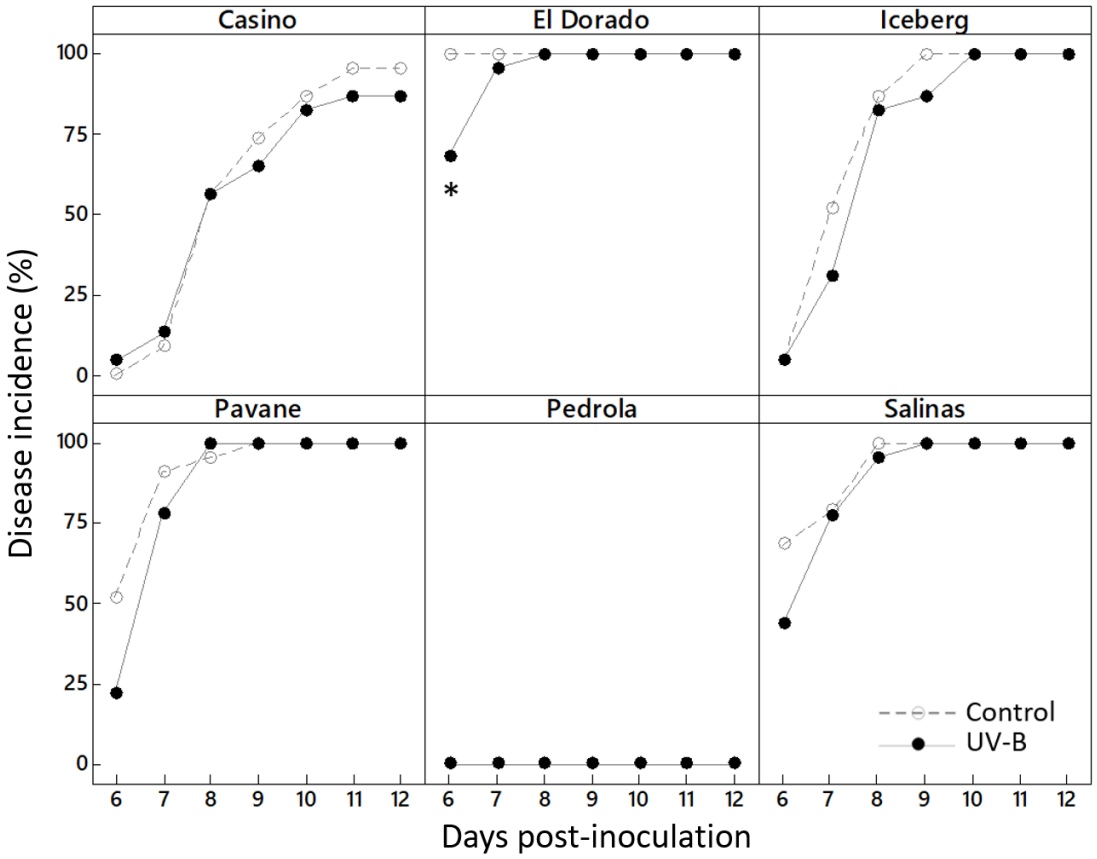


Figure 2.8.: Incidence of sporulation (% of plants infected) over time (days post-inoculation; DPI) in UV-B-pretreated [full black line] and control [dashed grey line] lettuce (*L. sativa*) plants of multiple cultivars [panel titles]. Lettuce plants were treated with photosynthetically active radiation (PAR) + 0.5µmol $m^{-2}s^{-1}$UV-B or PAR only (control) for three days then inoculated with 10^5conidia mL^1 of *B. lactucae*. From 6 DPI onwards, the disease incidence was measured as the number of plants displaying disease symptoms divided by the total number of plants. At 6 DPI, UV-B-treated plants of cultivar El Dorado had significantly lower incidence than control Fisher's Exact test, two-tailed, p = 0.009) as indicated by *.

2.4.2. UV-B-pretreatment reduced downy mildew sporulation rating severity in a range of lettuce cultivars

Sporulation severity over time, as shown by degree of infection (DoI), varied by lettuce cultivar (Figure 2.9). DoI of control plants confirmed cultivar susceptibility levels described by incidence. Pedrola was the least susceptible cultivar (complete resistant) followed by Pavane and Salinas, Iceberg, Casino and El Dorado (highly susceptible).

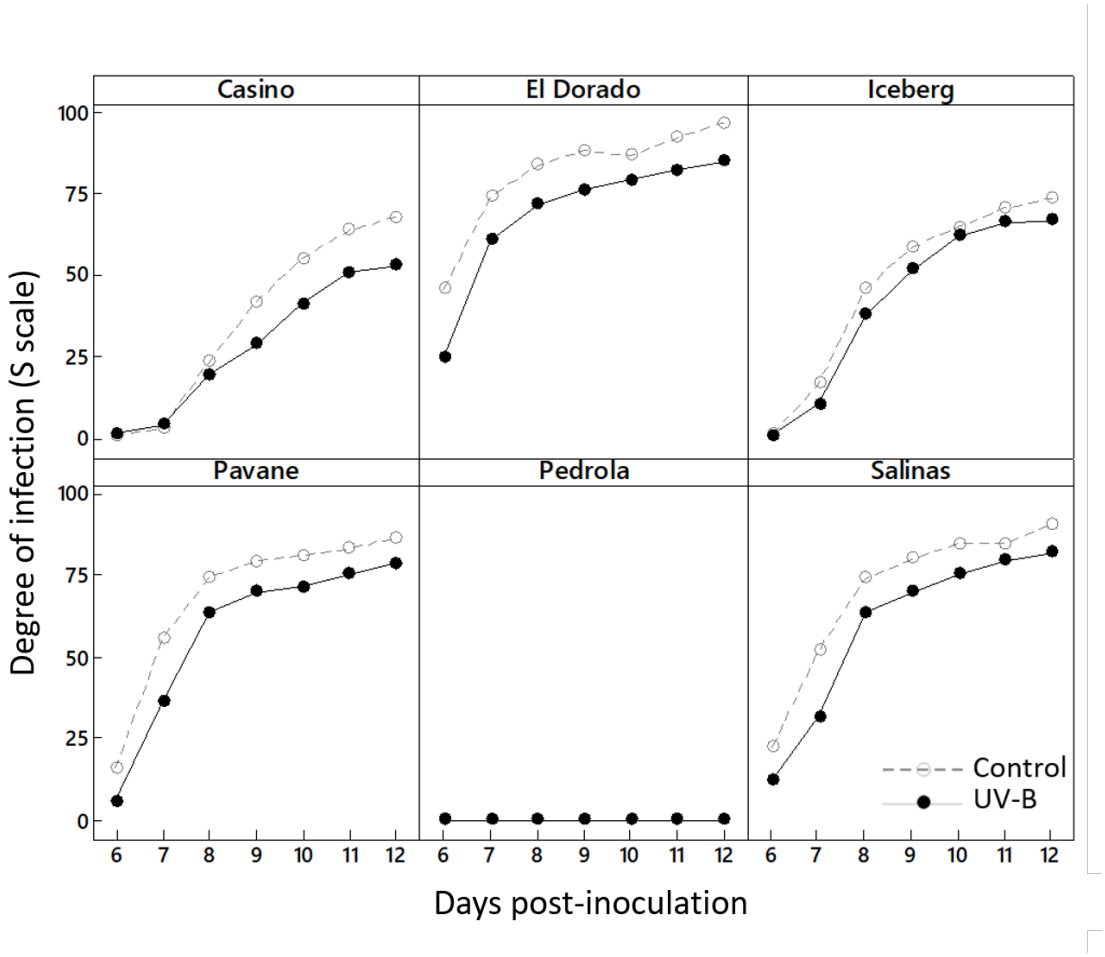


Figure 2.9.: DoI was lower in UV-B-treated plants [black line] of all lettuce (*L. sativia*) cultivars [panel title] compared to control plants [grey dotted line] at all time points except for cv. Pedrola which was completely resistant. Lettuce plants were treated with photosynthetically active radiation (PAR) + 0.5µmol $m^{-2}s^{-1}$UV-B or PAR only (control) for three days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. From 6 days post-inoculation (DPI) til 12 DPI, plants were rated on a sporulation scale. Overall sporulation severity per day was summarised as degree of infection.

For all cultivars, UV-B-pretreated plants had lower DoIs than control plants over time (from 8 DPI only in Casino). UV-B-pretreated plants of cultivars El Dorado and Pavane had a significantly lower distribution of normalised disease ratings than control plants at all time points (Table 2.5). At late-stage disease, UV-B-pretreated plants had significantly lower rating distributions in cultivar Salinas (10 to 12 DPI) and Iceberg (12 DPI only). At no time point did Casino significantly differ in rating distribution between control and UV-B plants.

Table 2.5.: Change in distribution of pooled normalised downy mildew sporulation ratings in UV-B-pretreated compared to control lettuce (*L. sativa*) plants in a range of cultivars. Lettuce plants were treated with photosynthetically active radiation (PAR) + 0.5µmol $m^{-2}s^{-1}$UV-B or PAR only (control) for three days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. From 6 days post-inoculation (DPI) till 12 DPI, plants were rated on a sporulation scale. The sporulation ratings were normalised based on the maximum rating for each repeat. Normalised ratings of UV-B and control plants were compared using Mann Whitney U tests. Asterisks indicate significant differences according where *=p<0.05, ** = p<0.005 ***=p<0.0005, ns indicates a non-significant change.

Cultivar	6 DPI		7 DPI		8 DPI		9 DPI		10 DPI		11 DPI		12 DPI	
Casino	Nil	ns	Nil	ns	Nil	ns	Nil	ns	Nil	ns	Nil	ns	Nil	ns
El Dorado	Decrease	*	Decrease	*	Decrease	***	Decrease	***	Decrease	**	Decrease	***	Decrease	***
Iceberg	Nil	ns	Nil	ns	Nil	ns	Nil	ns	Nil	ns	Nil	ns	Decrease	*
Pavane	Decrease	*	Decrease	*	Decrease	**	Decrease	*	Decrease	*	Decrease	*	Decrease	*
Salinas	Nil	ns	Nil	ns	Nil	ns	Nil	ns	Decrease	*	Decrease	*	Decrease	*

2.4.3. UV-B-pretreatment increased damage tolerance to downy mildew in a range of lettuce cultivars

Tolerance to damage caused by downy mildew disease was cultivar-dependent. At 12 DPI, control lettuce plants of cultivar Casino had the greatest tolerance, followed by Iceberg (Figure 2.10). Both El Dorado and Pavane controls reached 100% of plants damaged, with high levels of damage in Salinas also (89% damaged) indicating low tolerance levels for these three cultivars. At 12 DPI, Casino (Fisher's Exact test, two-tailed, p = 0.029), El Dorado (Fisher's Exact test, two-tailed, p = 0.004), Pavane (Fisher's Exact test, two-tailed, p = 0.049) and Salinas (Fisher's Exact test, two-tailed, p = 0.017) UV-B-pretreated plants had significantly lower number of plants damaged by disease compared to control plants of each cultivar (Figure 2.10). As the experiment ended at 12 DPI, it is uncertain if the decrease in damage is a delay, and if given time damage levels would reach similar levels or if the reduction is a more permanent effect. El Dorado (Fisher's Exact test, two-tailed, p = 0.034, P <0.000) and Salinas (Fisher's Exact test, two-tailed, p = 0.043, p = 0.011) UV-B-pretreated plants had a lower number of damaged plants than control at 10 and 11 DPI also.

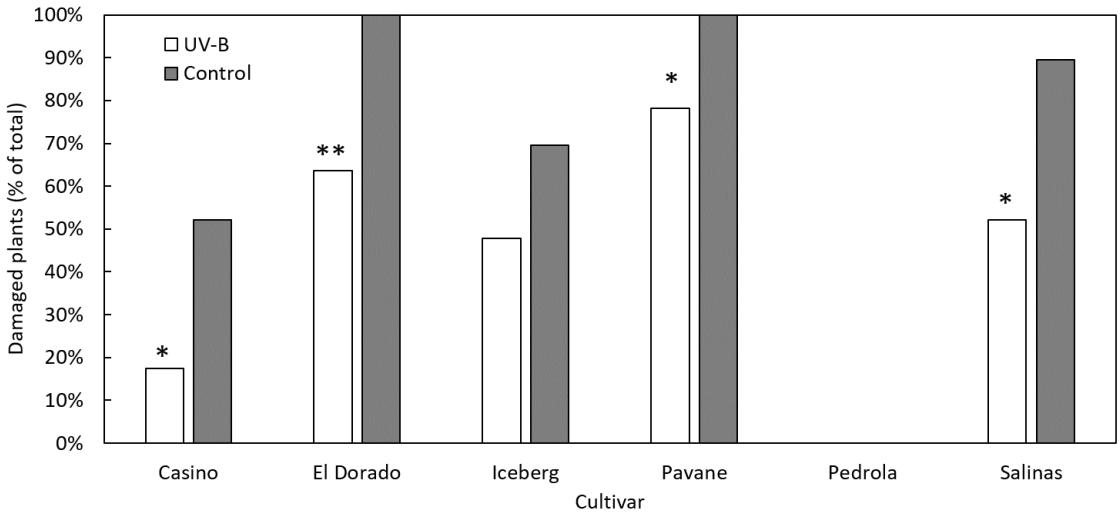


Figure 2.10.: Percent (%) of plants displaying yellowing or lesions (disease damage) at 12 DPI in multiple lettuce (*L. sativa*) cultivars. Colour indicates light pretreatment [White = UV-B, Grey = control]. Lettuce plants were treated with photosynthetically active radiation (PAR) + 0.5µmol $m^{-2}s^{-1}$UV-B or PAR only (control) for three days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae.* From 6 days post-inoculation (DPI) till 12 DPI, plants were rated on a disease scale. At a rating of 4 or 5, plants displayed yellowing or necrotic lesions (disease damage). The number of damaged plants as a percent of the total number of plants are shown. Asterisks indicate significant differences in the number of damaged plants between control and UV-B-pretreated plants of each cultivar according to a Fisher's Exact test, two-tailed, where * = $p<0.05$ and **=$p<0.005$.

2.4.4. UV-B-pretreatment reduced the number of conidia harvested per plant in a range of cultivars

Cultivar susceptibility order observed in incidence and sporulation rating is confirmed by conidia counts. UV-B-pretreatment reduced conidia count at a similar level in all cultivars compared to control (Figure 2.11). The conidia count of UV-B-pretreated plants was lower by 58% in cv. Salinas (t-test, $p < 0.0005$), 54% in cv. Casino (t-test, $p = 0.031$), 60% in cv. El Dorado (t-test, $p < 0.0005$), 62% in cv. Iceberg (t-test, $p < 0.0005$) and 61% in cv. Pavane (t-test, $p < 0.0005$). Variation within repeats was very small for conidia count measures which likely contributes to a very low p value in many cultivars.

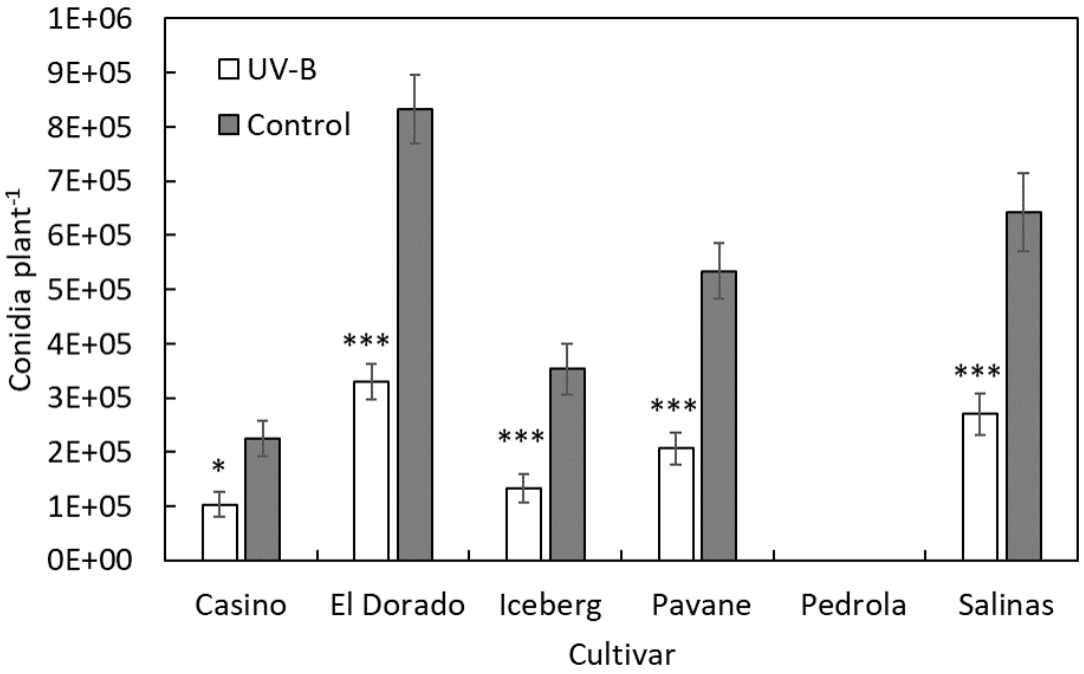


Figure 2.11.: *B. lactucae* conidia per plant were lower in UV-B [white bar]-pretreated plants than control [grey bar] plants of multiple lettuce (*L. sativa*) cultivars. Lettuce plants were treated with photosynthetically active radiation (PAR) + 0.5µmol $m^{-2}s^{-1}$UV-B or PAR only (control) for three days then inoculated with 10^5conidia mL^{-1} of *B. lactucae.* At 12 days post-inoculation, plants were washed and the resulting conidia suspension counted. Error bars indicate 1 S.E. Asterisks indicate significant differences between control and UV-B plants according to a t-test within each cultivar where *=$p<0.05$, ***=$p<0.0005$.

2.4.5. The optimal dose of 0.5 µmol $m^{-2}s^{-1}$UV-B causes downy mildew disease reduction across a range of lettuce cultivars

The optimal UV-B dose from dose response experiments (0.5 µmol $m^{-2}s^{-1}$) was assessed on multiple lettuce cultivars with a range of downy mildew susceptibility. UV-B-pretreatment had the greatest effect on cultivar El Dorado. El Dorado plants pretreated with UV-B had a reduced conidia count, incidence (6 DPI), sporulation rating and number of plants damaged. Other cultivars (Pavane and Salinas) had a moderate response to UV-B-treatment [decreased sporulation rating, damage and conidia count]. Cultivars Iceberg and Casino experienced reduced UV-B-induced effect, with a decrease in conidia count and either sporulation rating (Iceberg, 12 DPI) or damage (Casino, 12 DPI). Overall, my best UV-B-pretreatment can reduce disease in many cultivars; however, some cultivars have a greater response to 0.5 µmol $m^{-2}s^{-1}$ UV-B than others.

2.4.6. Casino displayed an inconsistent response to UV-B light across experimental sets

Lettuce cultivar Casino was used in both the dose response (DR) and multiple cultivar experiments; however, yielded different disease severity following the 0.5 µmol $m^{-2}s^{-1}$treatment used in both experimental sets. The delay in incidence and reduction of sporulation rating described in DR was not observed in the multiple cultivars experiments. This may be explained by the lower sample size (n = 24) in the multiple cultivar experiments compared to DR (n = 173). As rating scales were noted in DR experiments as a highly variable measure, the reduced sample size may have been too small for significance to occur within the level of variation. The more reliable measures; conidia count as well as tolerance, yielded a significant decrease in UV-B plants in both DR and multi cultivar experiments. UV-B-pretreatments can reduce disease severity in cultivar Casino; however, this is only observed as a reduction in conidia count and disease damage in a lower sample size.

2.4.7. El Dorado, Iceberg and Salinas were chosen as future working lettuce cultivars

Experiments which used multiple cultivars (semi-commercial response, LC-MS-1, and phenolic infiltrations) used cultivars El Dorado, Iceberg and Salinas based off results from this multiple cultivar experimental set. As the cultivar Casino was discontinued by Terranova seeds, and limited stock remained, this cultivar could not be logistically included in future experiments. El Dorado, Iceberg and Salinas showed both a range of disease susceptibility and UV-B response. These cultivars were readily available, had good germination qualities as well as uniform growth. To add, Iceberg is noted to display field resistance to downy mildew, which is described as a reduction of lesion size and slower disease progress of adult plants (Grube and Ochoa, 2005), which may add an interesting element to analysis.

2.5. UV-B-pretreatment using commercial UV devices can reduce disease susceptibility

The UV-B-pretreatment used to decrease downy mildew disease severity in dose response experiments (Section 2.3.1) varies in dose, wavelength and plant growth method from BioLumic commercially applied UV-B-treatments. In order to test whether current commercial treatments can produce a similar reduction of disease susceptibility, commercial treatments using BioLumic arrays, growth conditions and treatment recipes were carried out on lettuce plants followed by disease inoculation and assessment. Unfortunately, these commercial experiments ran into several unexpected complications resulting in unreliable data. Although measures were taken to attempt to fix these issues, due to time constraints, these experiments were abandoned. To give a projection of application, semi-commercial experiments, which used BioLumic arrays and recipes combined with controlled growth conditions were carried out. This removed the majority of the limitations of the fully commercial glasshouse experiments and the resulting more reliable data is analysed in this section.

2.5.1. Semi-commercial UV-B-pretreatment decreased downy mildew disease severity in lettuce

The semi-commercial treatment did not affect disease incidence; however, the severity of sporulation as shown by DoI was lower in UV-B-pretreated plants of each cultivar (Figure 2.12). The distribution of pooled normalised sporulation rating was significantly lower in UV-B-pretreated plants of lettuce cultivar El Dorado (6-12 DPI), and Salinas (6 DPI, 8-12 DPI). Sporulation severity (distribution of normalised sporulation rating) was only significantly reduced at 12 DPI only in Iceberg.

Conidia count was significantly reduced in UV-B-pretreated plants of all cultivars (Figure 2.13). Salinas had the largest decrease in conidia count (54%) followed by Iceberg (41%) and El Dorado (40%). Reductions to conidia count were greater in the stationary 300nm treatment (Section 2.4.4) in El Dorado (60%) and Iceberg (62%) than the semi-commercial treatment but were similar in Salinas (58%).

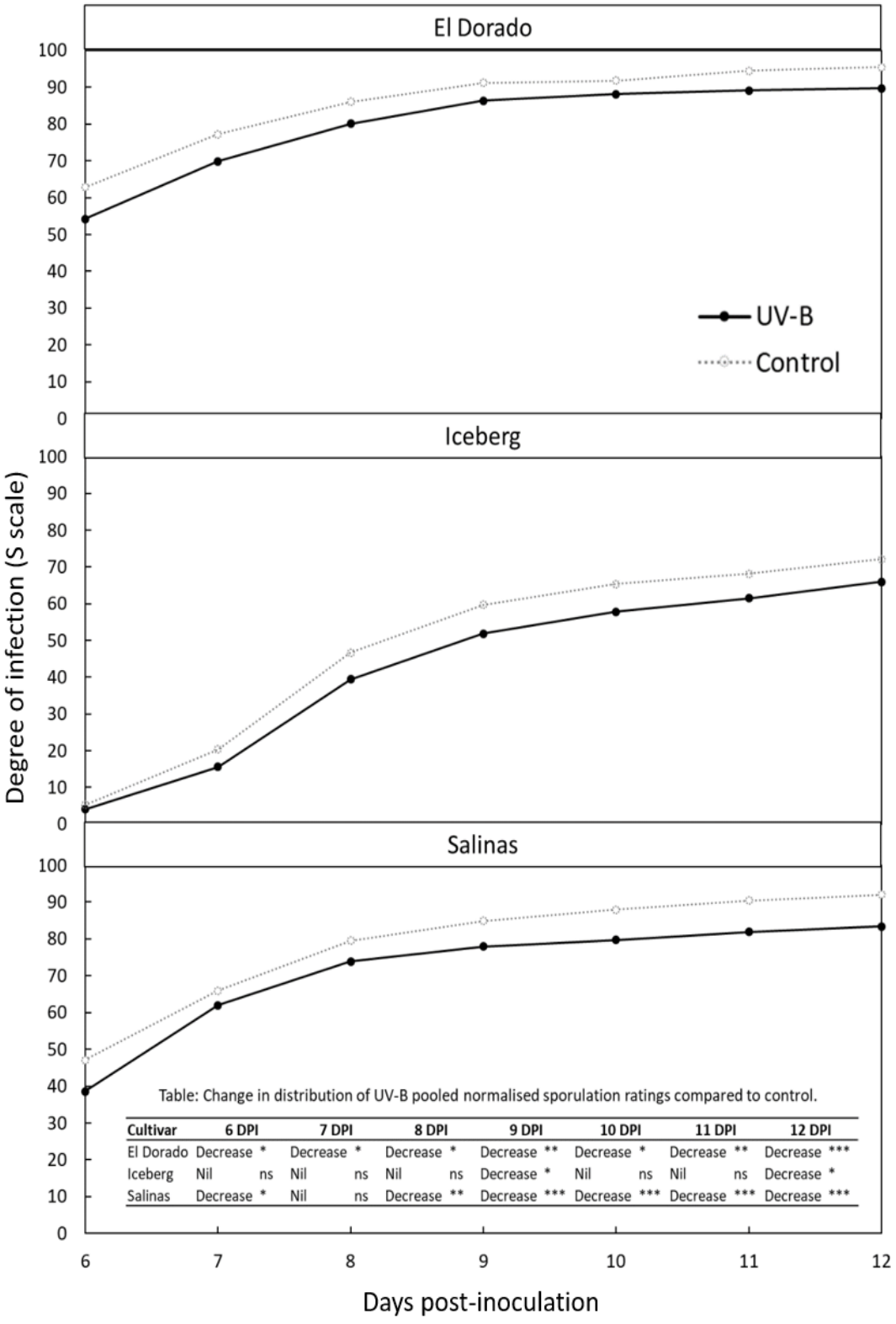


Table: Change in distribution of UV-B pooled normalised sporulation ratings compared to control.

Cultivar	6 DPI		7 DPI		8 DPI		9 DPI		10 DPI		11 DPI		12 DPI	
El Dorado	Decrease	*	Decrease	*	Decrease	*	Decrease	**	Decrease	*	Decrease	**	Decrease	***
Iceberg	Nil	ns	Nil	ns	Nil	ns	Decrease	*	Nil	ns	Nil	ns	Decrease	*
Salinas	Decrease	*	Nil	ns	Decrease	**	Decrease	***	Decrease	***	Decrease	***	Decrease	***

Figure 2.12.: Degree of infection (sporulation scale) of UV-B-pretreated lettuce (*L. sativa*) plants [black line] was lower than control plants [grey dotted line] of all cultivars [panel title] at all time points. Lettuce plants were treated with photosynthetically active radiation (PAR) + UV-B (280nm) or PAR only (control) for three days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. From 6 days post-inoculation (DPI) till 12 DPI, plants were rated on a sporulation (S) scale. Normalised ratings of UV-B and control plants were compared using Mann Whitney U tests [Table]. Asterisks indicate significant differences according where *=$p<0.05$, ** = $p<0.005$ ***=$p<0.0005$, ns indicates a non significant change.

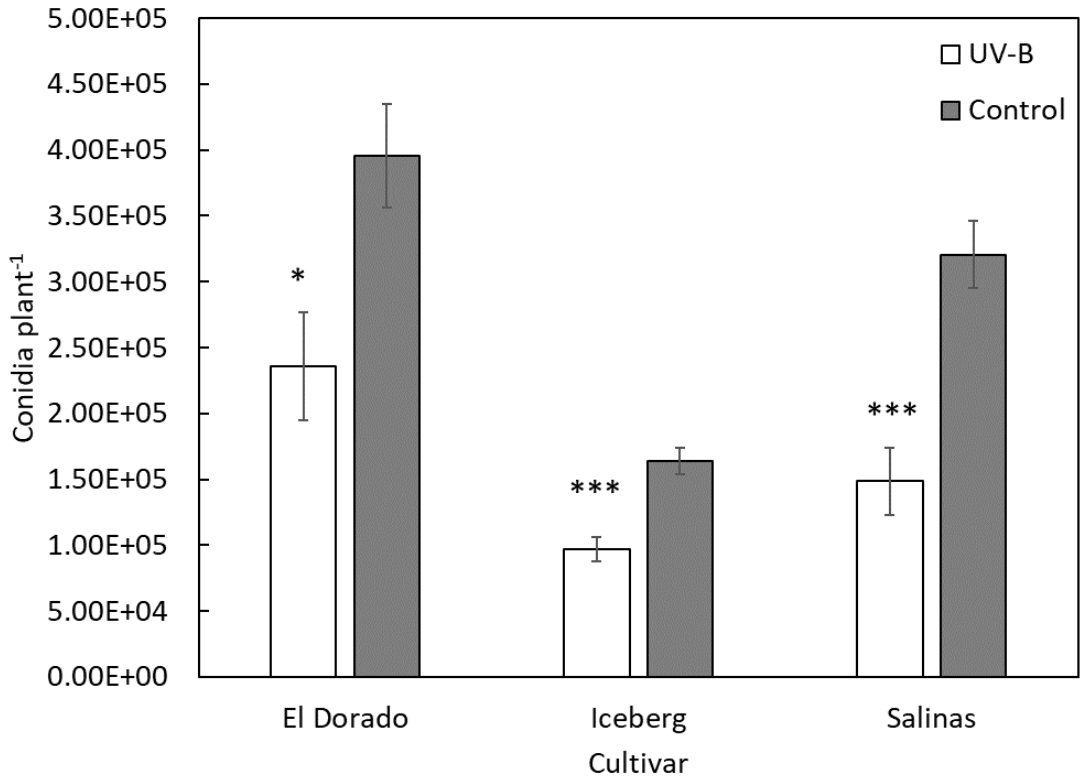


Figure 2.13.: *B. lactucae* conidia count of UV-B-pretreated (*L. sativa*) plants were significantly lower then control plants of lettuce cv. El Dorado, Iceberg and Salinas. Lettuce plants were treated with photosynthetically active radiation (PAR) + UV-B (280nm) or PAR only (control) for three days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae.* At 12 days post-inoculation, plants were washed and the resulting conidia suspension counted . Error bars indicate 1 S.E. Asterisks indicate significant difference between control and UV-B of each cultivar according to a t-test where * = $p<0.05$, *** = $p<0.0005$.

A commercial UV-B-pretreatment can reduce disease susceptibility as sporulation severity and conidia count. As the main goal of a commercial treatment is increased yield and uniformity, this experiment adds the claim that the commercial UV-B-treatment can also decrease disease susceptibility increasing the value of the treatment.

2.5.2. Adaptation of lettuce growth conditions and disease assessment protocol is required to test a glasshouse comercial treatment

Application of a BioLumic developmental treatment to glasshouse-grown and UV-B-treated lettuce plants was attempted; however, due to issues in growth and treatment systems, these experiments were unable to be completed. The glasshouse set-up used to grow plants had an irregular irrigation system and chronic fungus gnat infestation [families Mycetophilidae or Sciaridaeinfection]. Although steps (tray rotation, insecticide spray) were taken to improve growing conditions, plants were often not uniform or healthy

enough for further experimentation. The light treatment system was at an early stage of development at the time of this experimental set. The system had a number of software bugs that resulted in uncertainty on whether the treatment had correctly been applied for the appropriate time and dose. As time limited further correction of the protocol, and there were insufficient successful consistent repeats to draw conclusions, this experimental set was discontinued. Several changes to the protocol used to test a commercial UV-B disease treatment could improve the success of the experiments.

- Plants must have growth conditions which produce a uniform tray. An even growth bench, with adjustments to the irrigation system to prevent pooling of water may aid in providing a set of uniform plants.

- A complete clean out of growth and disease areas to remove present gnats and potential breeding grounds is required. Plants should be sown in predator treated soil [nematodes (*Steinernema feltiae*)] (Bethke, 2010). On the final day of light treatment, they should be sprayed with Orthene T (Bethke, 2010) as a precautionary measure. Once in the misting tent, fungus gnat presence should be carefully monitored by sticky traps and visible damage. Plants will forgo misting at 7 DPI, and another precautionary insecticide spray applied.

- Glasshouse grown plants have a different phenotype and disease progression than plants grown in a controlled environment. Disease on several sets of glasshouse grown lettuce needs to be observed and characterised to develop a glasshouse disease rating scale. Conidia counts should be carried out with a reduced ratio of 1 plant per 15mL rather then the standard 1 plant per 20mL used in growth chamber experiments.

2.6. Secondary downy mildew infections have reduced severity when spread between UV-B-pretreated plants

I have shown UV-B-pretreatment (0.5 µmol $m^{-2}s^{-1}$) can decrease *B. lactucae* conidia count in lettuce plants (Section 2.3). As conidia count indicates the level of inoculum produced on a plant; UV-B-pretreated plants which have downy mildew disease have a lower potential to start secondary infections. To test this hypothesis, UV-B-pretreated, or control plants were used as a source of inoculum (A) to infect a new set of UV-B-pretreated or control plants (B). Treatments are described in the format of A→B. E.g. UV→C indicates the inoculum came from UV-B-pretreated plants (A), and infected control (C) plants (B). The disease symptoms of the secondary plant (B) were assessed.

2.6.1. Downy mildew disease incidence was delayed in UV→UV lettuce plants

Disease incidence rate was rapid in this experimental set. As all treatments were almost saturated with disease incidence by 8 DPI, from this time point onwards there were no differences in incidence. The most effective treatment combination; UV→UV, resulted in plants with a significantly lower incidence than C→C at both 6 and 7 DPI (Fisher's Exact test, two-tailed, p = 0.04, 0.046 respectively) (Figure 2.14). At 7 DPI, UV→UV plants had a significantly lower incidence than C→UV plants also (Fisher's Exact test, two-tailed, p = 0.031). In order for a reduction/delay in disease incidence to occur, both the primary and secondary plant must be UV-B-pretreated.

2.6.2. Plants infected with inoculum from a UV-B-pretreated plant have reduced sporulation severity

The DoI (based on sporulation scale) of UV→UV plants is lower than C→C plants throughout the disease period (Figure 2.15). A Kruskall Wallis test with Bonferroni correction shows the distribution of normalised ratings were significantly lower in UV→UV and UV→C plants than C→C plants from 8 DPI onwards. At two time points (9 and 11 DPI), UV→UV plants had a significantly lower sporulation rating distribution than UV→C plants also. Both UV→UV and UV→C received inoculum from a UV-B-pretreated plant. Therefore, the level of inoculum produced by an infected UV-B-pretreated plant is sufficiently reduced for a resulting secondary infection to have reduced sporulation severity also. The disease reduction effect is enhanced when the secondary plant is also

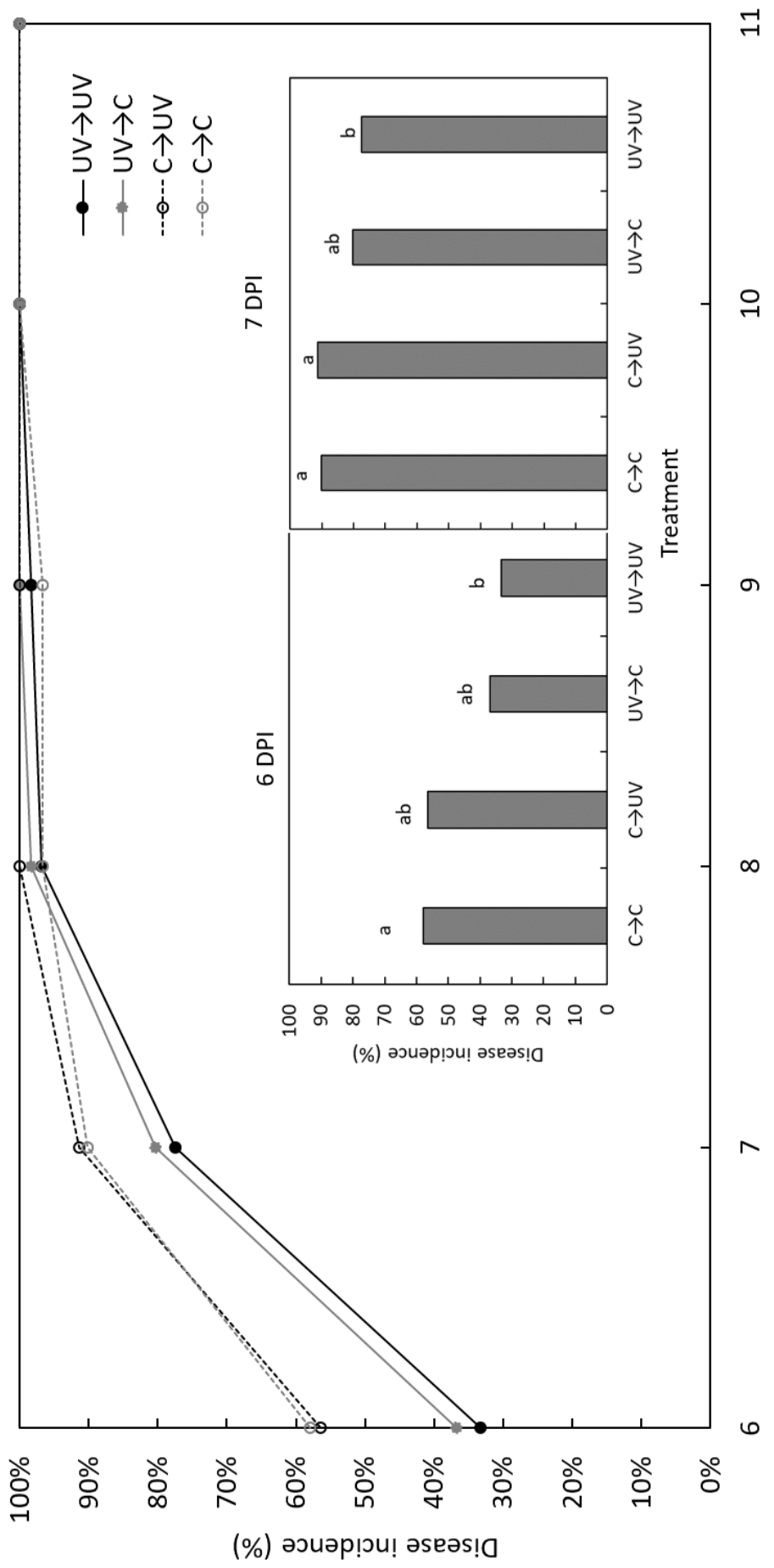


Figure 2.14.: Incidence (% of plants infected) of secondary lettuce (*L. sativa*) cv. Casino plants was significantly lower in UV→UV plants than C→C plants at 6 and 7 days post-inoculation (DPI) [insets]. Marker colour indicates treatment of primary plant [black = UV-B, grey = control] and fill indicates treatment of secondary plant [full = UV, empty = control]. Primary plants were treated with photosynthetically active radiation (PAR) + UV-B or PAR only (control) then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. At 7 DPI, a secondary set of UV-B or control-treated plants were placed in a misting tent with primary plants. Disease of secondary plants was assessed. The number of plants as a percent of the total displaying downy mildew symptoms are shown between 6 and 11 DPI. Treatments are coded in the format of primarysecondary plant treatment where C = control and UV = UV-B-pretreated. Lower case letters indicate significance groups (Fisher's Exact test, two-tailed, $p<0.05$).

UV-B-pretreated.

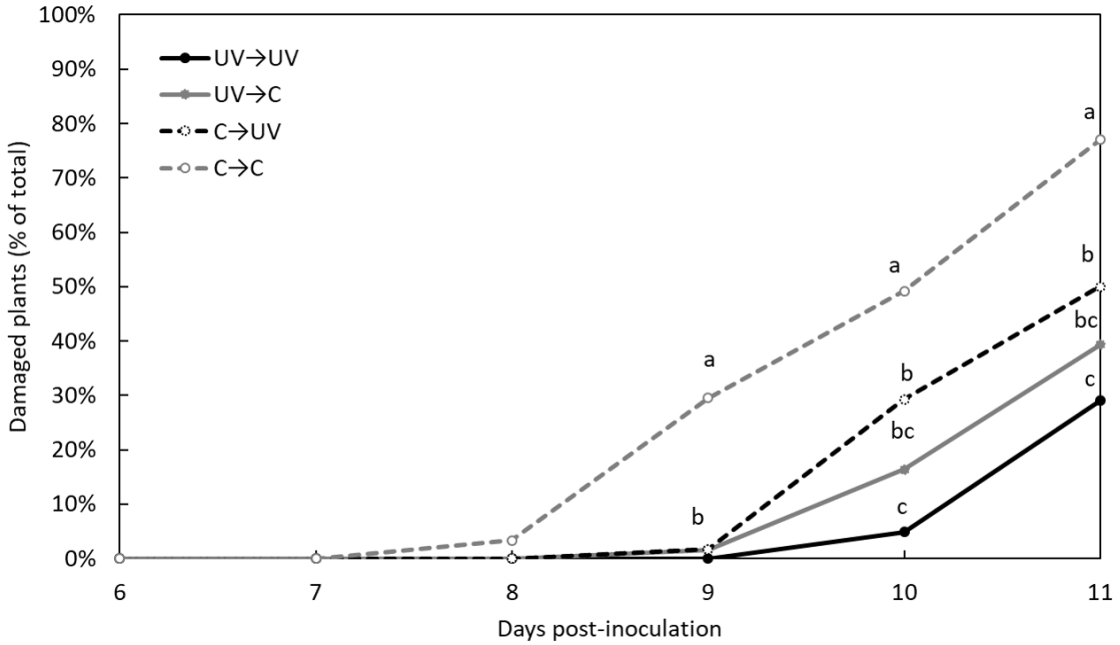


Figure 2.15.: Degree of infection (DoI) of UV→UV lettuce (*L. sativa)* cv. Casino plants was lower than that of C→C plants over time. Marker colour indicates treatment of primary plant [black = UV-B, grey = control] and fill indicates treatment of secondary plant [full = UV, empty = control]. Primary plants were treated with photosynthetically active radiation (PAR) + UV-B or PAR only (control) then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. At 7 days post-inoculation (DPI), a secondary set of UV-B or control treated plants were placed in a misting tent with primary plants. Treatments are coded in the format of primary→secondary plant treatment where C = control and UV = UV-B-pretreated. The Table shows significant differences between groups where different letters indicate $p<0.05$ according to Kruskal Wallis test with bonferroni correction.

2.6.3. Downy mildew disease damage was reduced in lettuce plants in which either primary or secondary plant was UV-B-pretreated

Ratings of 4 or 5 on the disease scale indicated a plant displayed damage symptomatic of downy mildew disease. All treatments containing at least one set of UV-B-treated plants (C→UV, UV→C, and UV→UV) had significantly fewer disease-damaged plants than C→C on 9, 10 and 11 DPI (Figure 2.16). UV→UV treatment had significantly fewer damaged plants than C→UV at 10 and 11 DPI also. UV-B-pretreatment of either the secondary or primary plant alone is enough to reduce the number of plants displaying disease damage. When both sets of plants are treated, the effect is amplified, with an even greater reduction in damaged plants.

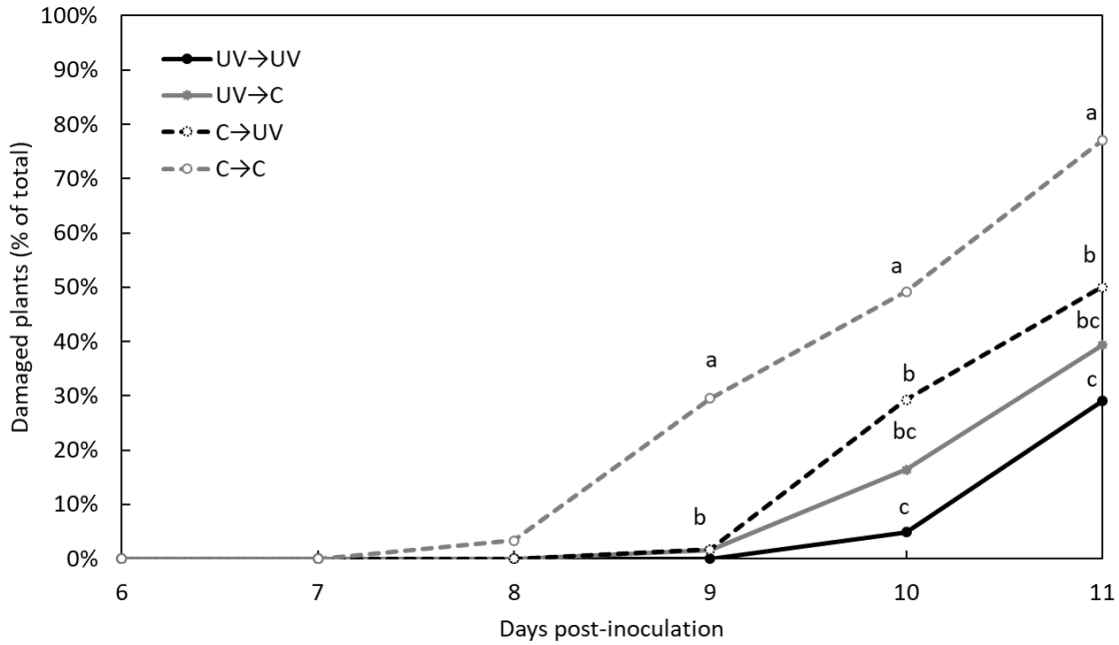


Figure 2.16.: Pooled counts of lettuce (*L. sativa*) cv. Casino plants displaying disease damage (rating 4 or 5 on disease scale) as a % of total number of plants over time. Marker colour indicates treatment of primary plant [black = UV-B, grey = control] and fill indicates treatment of secondary plant [full = UV, empty = control]. Primary plants were treated with photosynthetically active radiation (PAR) + UV-B or PAR only (control) then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. At 7 days post-inoculation, a secondary set of UV-B or control treated plants were placed in a misting tent with primary plants. The number of secondary plants displaying damage due to disease as a percent of total plants are shown. Treatments are coded in the format of infecting→infected treatment where C = control and UV = UV-B-pretreated. Lower case letters indicate significance groups (Fisher's Exact test, two-tailed, $p<0.05$).

Control plants which received inoculum from a UV-B-pretreated source (UV→C) had a greater tolerance than C→C plants. In Section 2.3.4, I hypothesised that UV-B-pretreatment might increase tolerance through increased antioxidants. However, results in this Section, indicate that receiving a reduced inoculum alone (UV→C) can result in increased tolerance. Therefore, reduced disease damage may be caused simply by a reduction of *B. lactucae* growth within the plant as a result of reduced inoculum or UV-B defences. Reduced pathogen biomass would result in fewer nutrients syphoned from the host plant by the pathogen, resulting in the ability of the plant to continue to grow normally despite the disease. However, this hypothesis is not supported by later microscopy experiment (Section 3.3), in which *B. lactucae* hyphal density did not significantly differ between UV-B-pretreated and control plants at 7 DPI indicating that UV-B-pretreatment may not cause a significant difference in biomass at this timepoint. The cause of increased tolerance

by either UV-B-pretreatment or inoculum from a UV-B-pretreated source requires further investigation for strong conclusions.

2.6.4. Treatments with at least one phase of UV-B had a reduced conidia count

All treatments where the primary, secondary or both plants received a UV-B-pretreatment had a significantly lower conidia count than control plants (C→C) (Figure 2.17). Intermediate treatments reduced conidia count by 35% (C→UV, ANOVA LSD; p <0.0005) and 42% (UV→C, ANOVA LSD; p <0.0005). When both primary and secondary plants received a UV-B-pretreatment (UV→UV); disease was further reduced (67%, ANOVA LSD; p <0.0005).

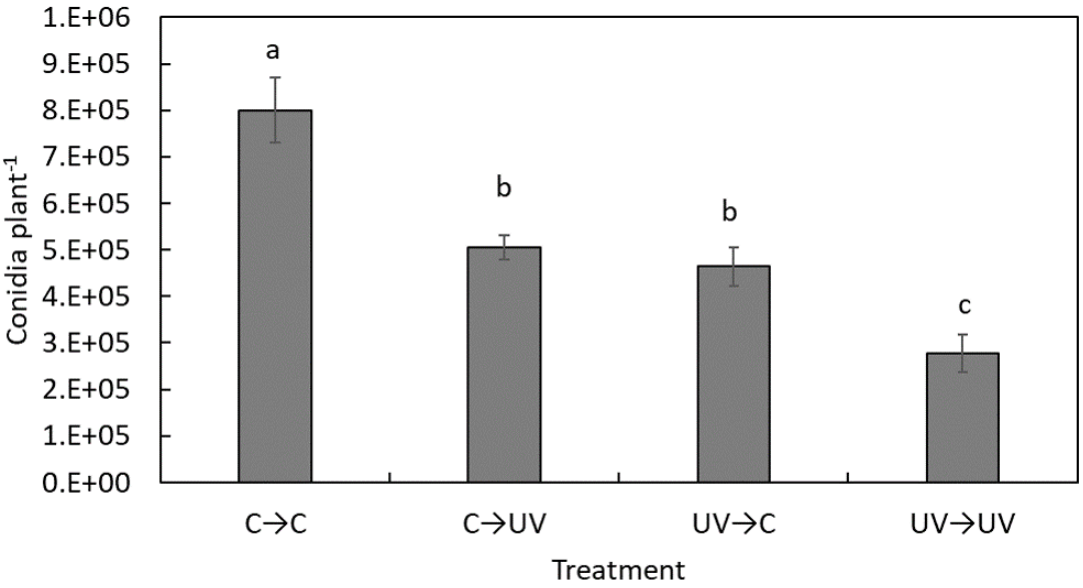


Figure 2.17.: Pooled number of conidia harvested per lettuce (*L. sativa*) cv. Casino plant are lower in UV→UV plants than any other treatment combination. Primary plants were treated with photosynthetically active radiation (PAR) + UV-B or PAR only (control) then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. At 7 days post-inoculation, a secondary set of UV-B or control treated plants were placed in a misting tent with primary plants.At 11 days after being placed in the misting tent, secondary plants were washed and the resulting conidia suspension counted . Treatments are coded in the format of primary→secondary treatment where C = control and UV = UV-B-pretreated. Lower case letters indicate significance groups (ANOVA, p<0.05). Error bars indicate 1 S.E.

Significantly fewer conidia were harvested from UV→UV plants than any other treatment. Plants of the intermediate treatments (C→UV and UV→C), had a significantly higher conidia count than plants of the UV→UV treatment but significantly lower conidia count than plants of the C→C treatment. Conidia count data clearly shows a progressive effect,

where one set of UV-B plants (primary or secondary) causes an intermediate decrease; however, when both sets of plants are UV treated (UV→UV) an amplified decrease occurs. The amplified effect is caused by the combination of a decreased inoculum from a UV-B-pretreated plant source and the additional UV-B-protective response in the secondary plant.

2.6.5. UV-B-pretreatment reduces the spread of disease between treated plants

Disease assessment clearly indicates that disease susceptibility is lower in plants which have had two generations of UV-B-treatments. This is shown by a lower sporulation rating, decreased damage resulting from disease and fewer conidia harvested from in UV→UV plants compared to C→C plants. Intermediate stages, in which either the inoculum (A) or the secondary infection (B) plants have experienced UV-B (C→UV or UV→C), had some decreases in disease symptoms; however, was rarely as strong as the effect of UV-B-treatment of both A & B plants. Disease in plants with no UV-B at either stage (C→C) had the greatest disease severity, damage and conidia count. UV-B defence appears to be accumulative, with the more generations of UV-B-treated plant the disease is spread through, the weaker the disease severity becomes.

Where inoculum from a UV-B-pretreated plant is used to infect a control plant (UV→C) a significantly lower disease severity than C→C was commonly observed. As dose response experiments (Section 2.3) indicate a reduced conidia count from UV-B plants, the UV→C treatment shows this reduction in conidia count (inoculum) is sufficient to reduce disease symptoms in the secondary generation. However, as the UV→UV plants had an even greater resistance, as well as this reduced inoculum, further defence against infection must be induced to further decrease disease.

The secondary inoculation experiments have some implications on situations in an applied setting. Although only two generations of UV-B-treatment were tested, it presents the possibility that virulence of disease will decrease over generations if infection cycles occur on UV-B-pretreated plants (Figure 2.18). Downy mildew disease is polycyclic in lettuce. Commonly a small number of plants will become infected, develop disease and become an inoculum source resulting in the infection of a secondary plant. This cycle continues until all nearby plants are infected unless disease control measures are taken. If infected plants were UV-B-pretreated, disease is limited, resulting in a reduced amount of inoculum

on maturation of disease. This inoculum may spread to nearby UV-B-pretreated plants; however, again, disease will be restricted by UV-B-induced defences resulting in a further reduced disease level in the secondary plant. The UV-B-pretreated secondary plant will then produce a smaller level of inoculum, and the cycle continues with reduced inoculum levels as disease passes through UV-B-pretreated plants. Therefore, over generations of disease produced on UV-B-pretreated plants, the quality of disease may be reduced, resulting in a limited spread of disease.

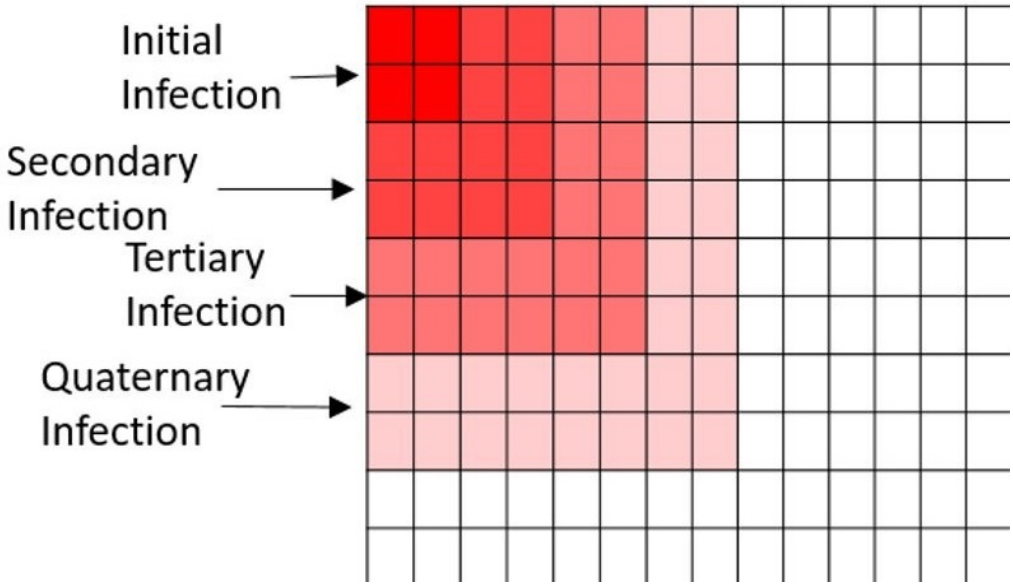


Figure 2.18.: Possible spread of downy mildew disease across a UV-B-pretreated lettuce nursery. Each box indicates a tray of lettuce plants in a nursery with red intensity indicates the disease severity [more red = higher disease severity]. Following initial infection, each generation of conidia produced from UV-B plants results in reduced disease severity. Over several generations, this may prevent disease from spreading across an entire nursery.

Chapter 3

Investigation into putative UV-B-induced disease defence mechanisms

3.1. Introduction

UV-B light can reduce the susceptibility of a plant to disease. Evidence was provided towards a UV-B-induced defence response in lettuce (*Lactuca sativa*) against downy mildew disease caused by *Bremia lactucae* in Chapter 2. However, little is known about the mechanism in which this defence is induced. Previous studies suggest several possibilities such as UV-B-induced increases to phenylpropanoid pathway, changes to hormones, induction of reactive oxygen species or thickening of the cell wall (Section 1.3.7.3). With such a range of overlap between UV-B and disease defence pathways, a screen of possible mechanisms was required to highlight the more promising pathways for further work. In this Chapter, several investigative experiments were carried out to probe into the mechanism which may cause reduced susceptibility in UV-B-pretreated plants. Microscopic investigation of *B. lactucae* growth indicated that UV-B-induced changes to the plant affect hyphal morphology and density. UV-B-pretreatment increased flavonoid levels and decreased disease levels presenting the possibility of a correlation. A role of UV-B-induced flavonoids and other phenolic secondary metabolites was confirmed through re-examination of previously published transcriptomics data on UV-B and biotrophic disease

(*Hyaloperonospora arabidopsidis*) in *Arabidopsis*. Transcriptomics data also highlighted several genes which were important to disease resistance as well induced by UV-B light. Due to the lack of availability of lettuce mutants, and a time limitation on creating them, the role of these genes in a UV-B-induced disease defence could not be further investigated in lettuce. Transcriptomics data indicated several pathways were unlikely to have a major role in a UV-B-induced defence due to little overlap in gene expression changes of UV-B and resistance to biotrophic disease. Unlikely candidates included defensive hormones (jasmonic (JA) and salicylic acid (SA)). A UV-B incubation of flavonoids and other phenolics showed the most promising links to a reduced disease severity. The role of flavonoids was investigated further in Chapter 4.

3.2. Methods

3.2.1. Plant growth

Lettuce (*Lactuca sativa*), seeds were sown into black plastic trays, with a cell size of 3cm^2, containing 'Daltons Seedling Raising Mix'. A single layer of grade 3 medium vermiculite [Auspari pty LTD, NSW] was spread over the tray. Sown trays were misted with water then placed in darkness at 14^{o}C for 48 h for vernalisation. Following vernalisation, plants were moved to a controlled temperature room (CTR) and grown for 14 days. The CTR had growth conditions of 17^{o}C, with a photo-period of 10 h supplied by 215µmol m^{-2}s^{-1} white light from FL58W/965 super daylight deluxe fluorescent tubes [Slyvania Premium Extra, China]. Water was applied daily to capillary matting underneath the trays.

3.2.2. Microscopy experiments

Light treatment D, lettuce cv. Casino was used for microscopy experiments (Table 2.1). Following pathogen inoculation (as for Section 2.2.3 without assessment), plants were misted daily. Ten randomly selected plants were harvested for microscopy at four, seven and 12 days post-inoculation (DPI). The second true leaf from each harvested plant was cleared with 1m KOH and stained with aniline blue using the method described by Diez-Navajas et al. (2007). Slides were observed with an Olympus BX51 Microscope with Micropublisher 5 colour CCD camera, excitation filter of 330-385nm within the Manawatu Microscopy Imaging Centre

(http://www.massey.ac.nz /massey/learning/departments/centres-research/ manawatu-microscopy-imaging-centre/ mmic_home.cfm). Images were taken at three to five locations on the leaf. If hyphae were present in an area, ImageJ (https://imagej.nih.gov/ij/) was used to analyse the area of fluorescence, as well as the width of fungal structures. Area of fluorescence or hyphal density, was measured as the area of hyphae dived by the total image area and averaged over positions for each plant leaf. Hyphal width was measured as the average perpendicular width of four distinct hyphae within each area. The average hyphal width of each area was then averaged between positions for each plant leaf. The total number of samples per treatment was 9-18 leaves in hyphal width measurements and 24-26 leaves in density measurements.

3.2.3. Flavonoid measurements

The Dualex [Force A, Orsay; France] is a hand held, non-destructive tool which uses light fluorescence and transmission through a leaf clip to estimate leaf chlorophyll content (Chl), epidermal UV-absorbance (Flv), anthocyanins and a Nitrogen Balance Index (NBI, patented) (Goulas et al., 2004). It was used to take flavonoid measures at 72 h during multiple cultivar experiments and at 0, 24, 48, and 72 h after treatment as well as 1, 7, 12 DPI in semi-commercial experiments in Chapter 2.

3.2.4. Micro-array methods

Gene expression data sets for *Arabidopsis thaliana* cv. Col-0 treated with high and low UV-B-treatments as well as infection with biotrophic oomycete *H. arabidopsidis,* previously *Peronospora parasitica*, were obtained from Asai et al. (2014); Ulm et al. (2004) and https://www.ebi.ac.uk (E-GEOD-5731) (Table 3.1). The Log_2fold change (FC) against the control from each experiment was analysed with Mapman and Pageman (https://mapman.gabipd.org/). Log_2FC is the standard expression change format used by this program. A threshold of +/- 1 Log_2FC was used to identify strong changes. Mapman / Pageman identified putative gene IDs based on the Affy matrix tag and placed the gene into a bin related to its function. Bins for secondary metabolism, biotic stress and hormone metabolism were investigated further.

Table 3.1.: Microarray data sets used for *Aarabidopsis* plants treated with UV-B light (a) or infected with *Hyaloperonospora arabidopsidis* (b).

Label	UV-B irradience	UV-B Source	Photoperiod	Timepoint	Interaction	Plant	Source
Low UV-B	1.5 µmol m^{-2} s^{-1}	Philips TL 40W/12 UV fluorescent tubes; transmission cutoff filters of WG295, WG305, and WG327	6 hours	6 hours	UV-B + PAR vs PAR	Col-0	Ulm, et. al. (2004). PNAS: 101(5), 1397-1402.
High UV-B	4.5 µmol m^{-2} s^{-1}	Philips TL12 40W tubes, "Diacel" cellulose diacetate filter: 280nm-320nm	18 hours	30 hours	UV-B + PAR vs PAR	Col-0	E-GEOD-5731; https://www.ebi.ac.uk

(a) UV-B data sources. UV-B = Ultraviolet-B, PAR = photosynthetically active radiation

Label	Pathogen	Disease	Time point	Interaction	Plant	Source
Compatible	Hyaloperonospora arabidopsidis; Waco9	Biotrophic	1 DPI	Compatible vs mock	Col-0	Asai et. al. (2014). PLoS Pathogens: 10, 1-14.
Incompatible	Hyaloperonospora arabidopsidis; Emoy2	Biotrophic	1 DPI	Compatible vs incompatible	Col-1	Asai et. al. (2014). PLoS Pathogens: 10, 1-14.

(b) Disease data sources. DPI = Days post-inoculation

Results and Discussion

3.3. *B. lactucae* hyphal density and width within UV-B-pretreated plants was reduced

The growth of *B. lactucae* within UV-B-pretreated and control (Photosynthetically active radiation (PAR) only) plants was examined using aniline blue fluorescent microscopy (Figure. 3.1). Differences in growth patterns of *B. lactucae* provides further evidence towards the presence of a UV-B-induced disease defence and may provide insight on if any localisation effects occur. The number of leaves containing *B. lactucae* hyphae did not significantly differ between UV-B or control lettuce cultivar Casino plants at 4 or 7 DPI (Fisher's Exact test, two-tailed, $p > 0.05$). *B. lactucae* hyphal width was significantly reduced in UV-B-pretreated plants at 7 DPI compared to control plants (t-test, $p = 0.019$) but not 4 DPI (Figure 3.2).

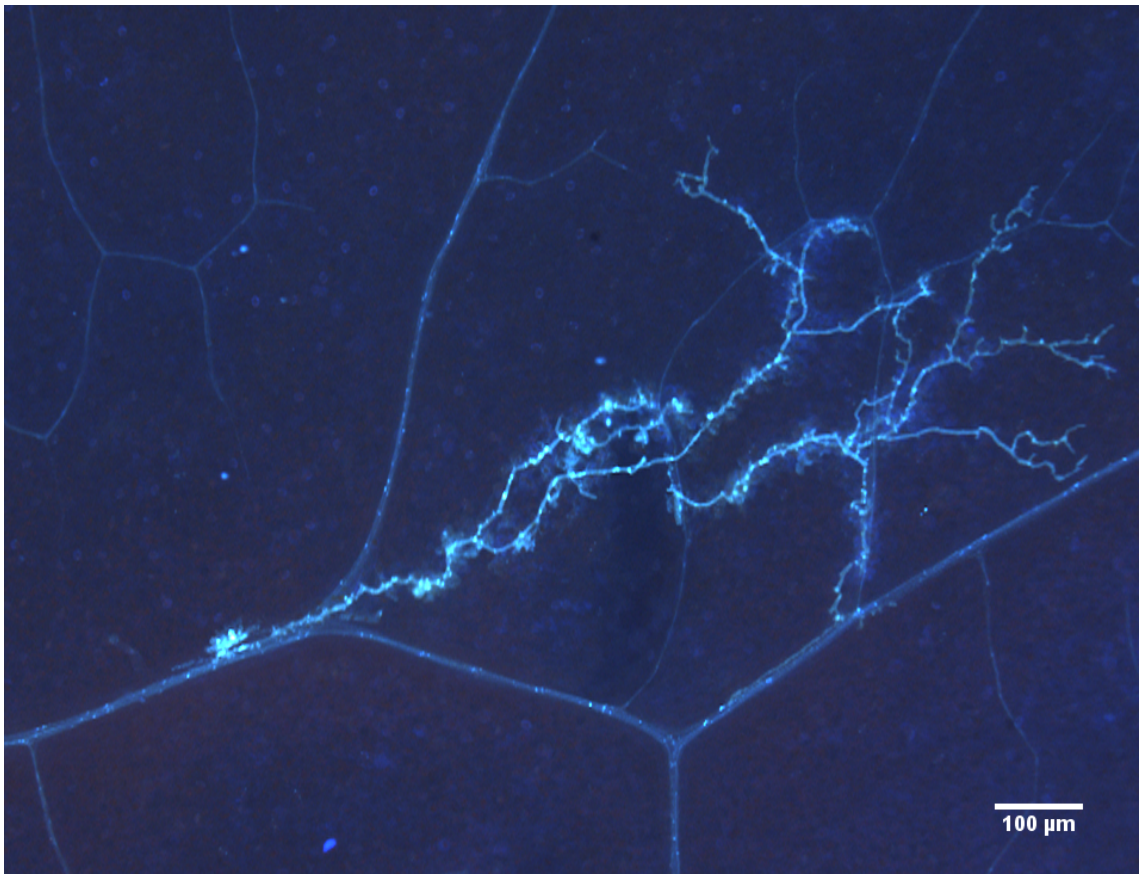


Figure 3.1.: *B. lactucae* hyphae (light blue) stained by aniline blue within *L. sativa* cv. Casino tissue. Image analysis of width and area was achieved by selecting *B. lactucae* tissue over a threshold brightness level. Scale bar indicates 100µm.

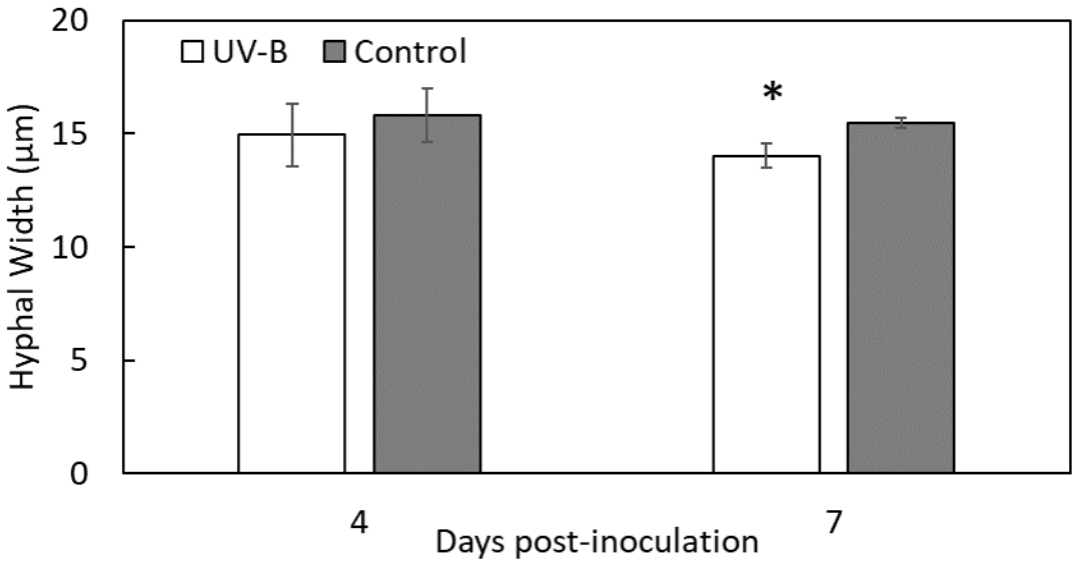


Figure 3.2.: UV-B-pretreated lettuce (*L. sativa*) cv. Casino plants [white bar] contained *B. lactucae* with a significantly reduced hyphal width at 7 days post-inoculation (DPI) but not 4 DPI compared to control plants (t-test). Lettuce plants were treated with photosynthetically active radiation (PAR) + UV-B light or PAR only (control) for 3 days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. At 4 and 7 DPI, leaves were cleared with 1m KOH and stained with aniline blue. Once the stain set, images were taken using a fluorescence microscope and hyphal width measured. Error bars indicate 1 S.E. Asterisks indicate significant differences where * = $p < 0.05$ (T-Test).

Hyphal density was sampled at three/five locations on a lettuce leaf. To calculate hyphae density for the leaf, the density of the three/five locations was averaged irrespective of the presence of hyphae. Hyphal density was significantly reduced at 4 DPI in UV-B-pretreated plants (t-test, $p = 0.038$) but not 7 DPI (Figure 3.3). When pre-determined sample locations that lacked *B. lactucae* growth were excluded, this significant difference was lost. Hyphal density at 12 DPI was too high to distinguish individual hyphae and resulted in fluorescence saturation. Data at 12 DPI could not be accurately extracted from the resulting images. Reduction of both hyphal density and hyphal width provides evidence that UV-B-pretreatment of a lettuce plant can alter the growth of *B. lactucae* within it.

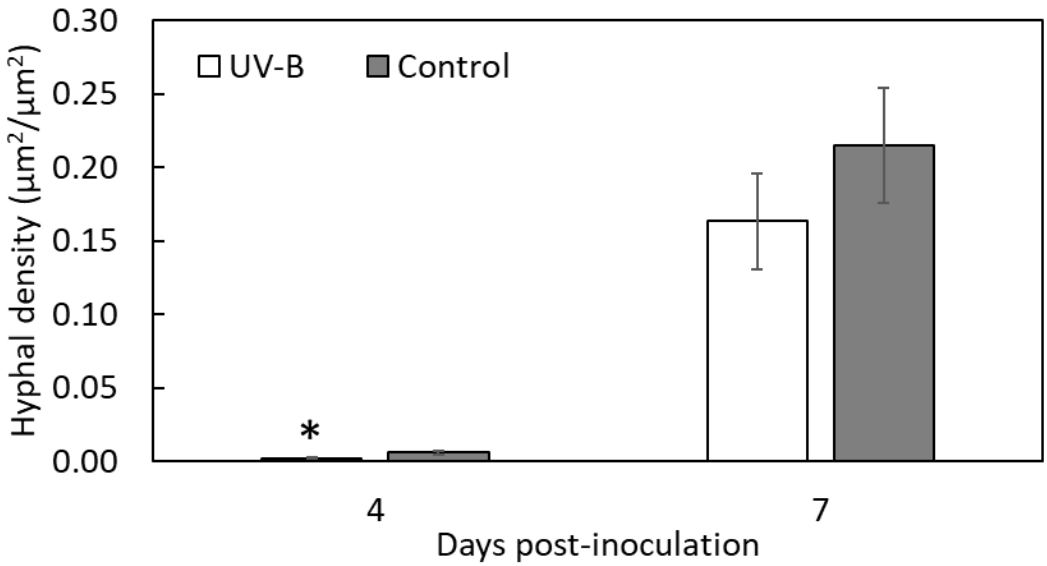


Figure 3.3.: UV-B-pretreated lettuce (*L. sativa*) cv. Casino plants [white bar] contained *B. lactucae* with a significantly reduced hyphal density at 4 days post-inoculayion (DPI) but not 7 DPI compared to control plants (t-test). Lettuce plants were treated with photosynthetically active radiation (PAR) + UV-B light or PAR only (control) for 3 days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. At 4 and 7 DPI, leaves were cleared with 1m KOH and stained with aniline blue. Once the stain set, images were taken using a fluorescence microscope and hyphal density measured. Error bars indicate 1 S.E. Asterisks indicate significant differences where * = $p<0.05$ (T-Test).

A reduction of hyphal density at 4 DPI, but not 7 DPI also provides evidence that UV-B causes a delay in the establishment of disease. A reduction in hyphal density was only observed when the density of all sampled locations were averaged per leaf irrespective of hyphal presence. The difference in hyphal density was caused by the lack of hyphal mass in a higher number of sample locations in UV-B-treated than control plants. For example, hyphae may have been present at sample points A and B in a control leaf but only sample point A in a UV-B-pretreated leaf resulting in a lower average density in UV-B compared to control. A Fisher's Exact test confirms that a significantly lower number of sample points (two-tailed, $p = 0.001$) contained hyphae in UV-B plants compared to control at 4 DPI (Figure 3.4). The hyphal density measure is therefore a better indicator of hyphal spread than hyphal mass.

At 4 DPI, hyphal density was lower in UV-B-pretreated plants indicating a reduced percentage of the plant area contains hyphae, but by at 7 DPI, hyphae had spread throughout the plant resulting in loss of a significant difference. Hyphal establishment and spread throughout the plant is therefore delayed in UV-B-pretreated plants. The delay may be caused by fewer successful infection points, i.e. fewer conidia successfully

forming germ tubes and penetrating the leaf at fewer points, or a reduced growth rate of established hyphae through-out the plant. Based on images collected, the entry point was not distinguishable so no evidence for either reason was available.

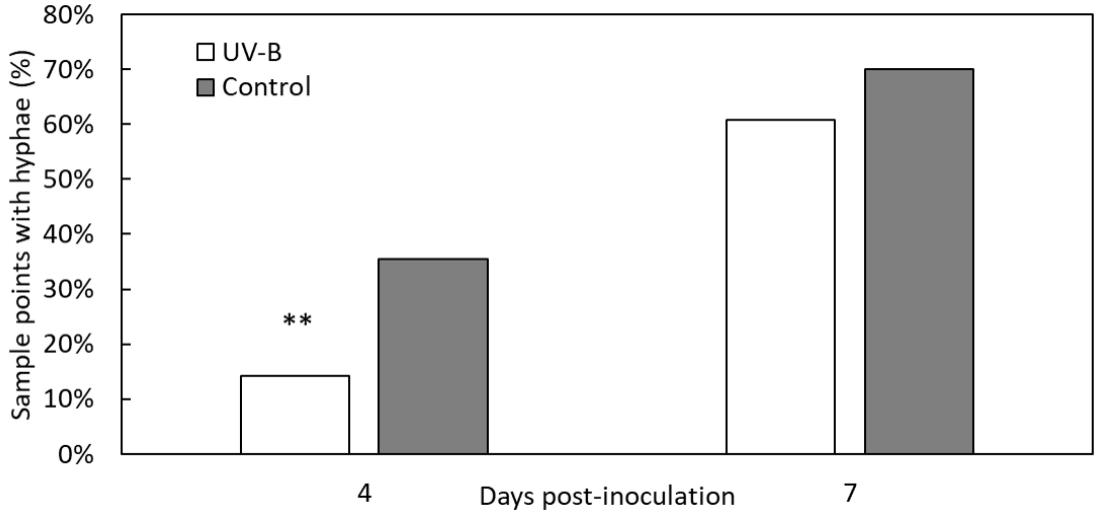


Figure 3.4.: Fewer sample locations contained hyphae in UV-B-pretreated lettuce (*L. sativa*) cv. Casino plants [white bar] than control plants [grey bar] at 4 days post-inoculation (DPI) but not 7 DPI. Lettuce plants were treated with photosynthetically active radiation (PAR) + UV-B light or PAR only (control) for 3 days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. At 4 and 7 DPI, leaves were cleared with 1m KOH and stained with aniline blue. Once the stain set, five images per leaf were taken using a fluorescence microscope. The number of images containing *B. lactucae* as a percent of total images was calculated to determine sample points with hyphae (%). Asterisks indicate significance according to a fishers exact test (two-tailed) where **=$p<0.005$.

Hyphal width was reduced at 7 DPI in UV-B-pretreated lettuce plants; however, hyphal density (of sample points with hyphae present) was not. The reduction in width was therefore not high enough to cause an effect on density or other aspects of hyphal morphology (e.g number, branching or length of hyphae) were increased.

Due to the 2D nature of fluorescence microscopy, it was difficult to assess other hyphal or sporangiophore traits. Hyphae tend to grow through multiple cell layers in the plant; however, fluorescence microscopy views these all as one plain. In higher density samples, overlapping layers of hyphae, or vertically growing hyphae can complicate the ability to accurately measure total fungal mass in an area as well as the ability to distinguish edges of hyphae for width measurements. Although 12 DPI samples were taken in all cases, when hyphae were present, it was so dense that the limitations of the microscopy technique chosen meant samples could not be accurately used. Fluorescence microscopy work provided some insight into the UV-B disease phenotype; however, other microscopy

techniques, such as confocal microscopy which works in 3D may provide more data on *B. lactucae* vertical growth and infection structures throughout a UV-B-pretreated plant.

3.4. UV-B increases total flavonoid level in a range of lettuce cultivars

The phenylpropanoid pathway is generally regarded to both be heavily up-regulated by UV-B light (Section 1.3.3) and influence the resistance of plants to disease (Section 1.1.10.2). Flavonoid level, as measured non-destructively with a Dualex were used as an indicator of total flavonoid level to probe into the role of these compounds in UV-B-induced disease defence.

Flavonoid levels were measured at 72 h during the multiple cultivar experimental set (Section 2.2.5). Lettuce cultivars had a similar basal flavonoid level (72 h) across control plants (Figure 3.5). Flavonoid level was significantly increased in UV-B plants of all cultivars (t-test, $p < 0.05$). Lettuce cultivar Iceberg displayed the largest increase (54%) followed by El Dorado (44%) in flavonoids. UV-B light induced flavonoids to a lesser degree in lettuce cultivars; Casino (26%), Pavane (27%), Pedrola (30%), and Salinas (25%).

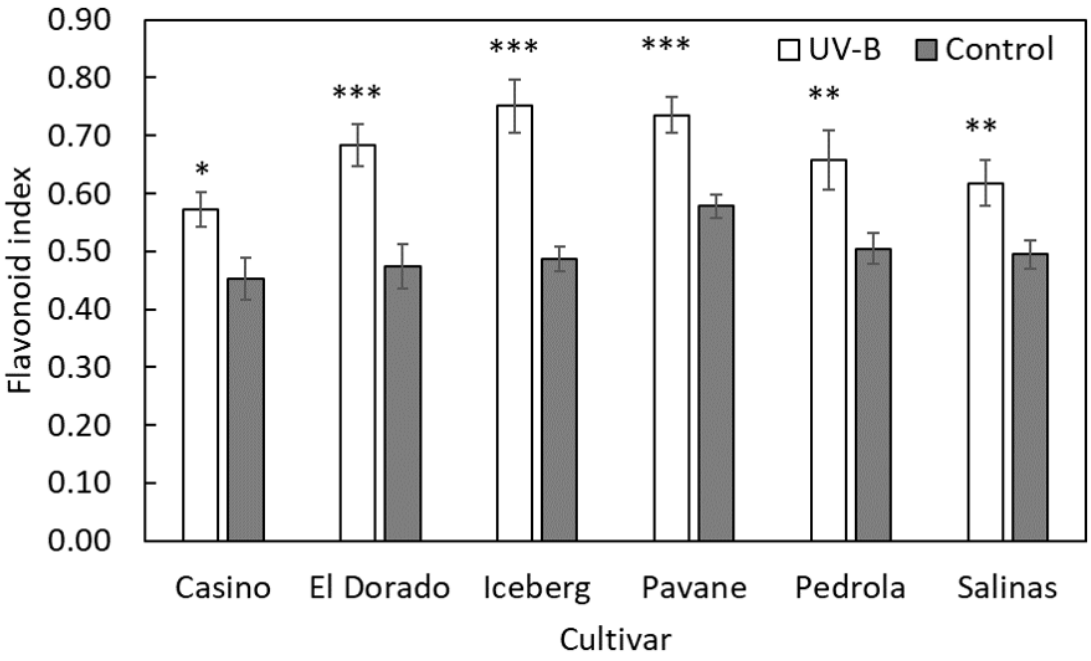


Figure 3.5.: Flavonoid index at 72 h was significantly higher in UV-B [white bars] plants compared to control plants [grey bars] for every lettuce (*L. sativa*) cultivar tested. Lettuce plants were treated with photosynthetically active radiation (PAR) + UV-B light (300nm) or PAR only (control) for three days followed by measurement of flavonoids using a Dualex. Error bars are 1 S.E. Asterisks indicate significant differences between control and UV-B according to t-tests within cultivar where *=p<0.05, **=p<0.005, ***=p<0.0005.

Flavonoids were taken throughout the light and disease period in the semi-commercial experimental set. Lettuce plants received UV-B light treatment between 0 and 72 h followed by a disease period of 72 to 360 h. Dualex measurements for flavonoids were taken every 24 h during the light treatment period (0, 24, 48, 72 h) and at 96, 168, and 360 h during the disease stage. UV-B-treated lettuce plants had higher flavonoid levels at some time points but not continuously (Figure 3.6). UV-B-treated cultivar El Dorado plants had a significantly higher flavonoid content at 48, 96 and 168 h compared to El Dorado control plants (daily t-test, $p < 0.05$). UV-B-treated cultivar Iceberg plants had significantly higher flavonoids at 96 and 168 h, whilst UV-B-treated cultivar Salinas plants had significantly higher flavonoids at 168 h only (daily t-test, $p < 0.05$).

Flavonoids would be expected to be induced by UV-B resulting in elevated levels shortly after the beginning of a treatment. Although overall flavonoid levels were higher in the treatment phase than the disease phase, control lettuce plants, as well as UV-B plants, experienced a flavonoid increase. Total flavonoid increase could have occurred due to the change in light environment between growth (white fluorescent tubes) treatment (red and

blue LEDs) rather than specifically as a response to UV-B light (Discussed in Section 4.3.2). Following an initial increase at 24 h, a decrease in flavonoids was observed from 48 h onwards. This suggests that following an initial increase in flavonoids, additional time receiving a UV-B dose did not cause further increases. In the disease stage, the effects of the UV-B-treatment on flavonoid levels is more pronounced. Overall flavonoid levels decrease over time throughout the disease period however, UV-B-pretreated plants can maintain flavonoid levels more effectively than control plants. This improved flavonoid maintenance results in UV-B-pretreated plants with significantly higher flavonoids (at certain time periods) during the disease period.

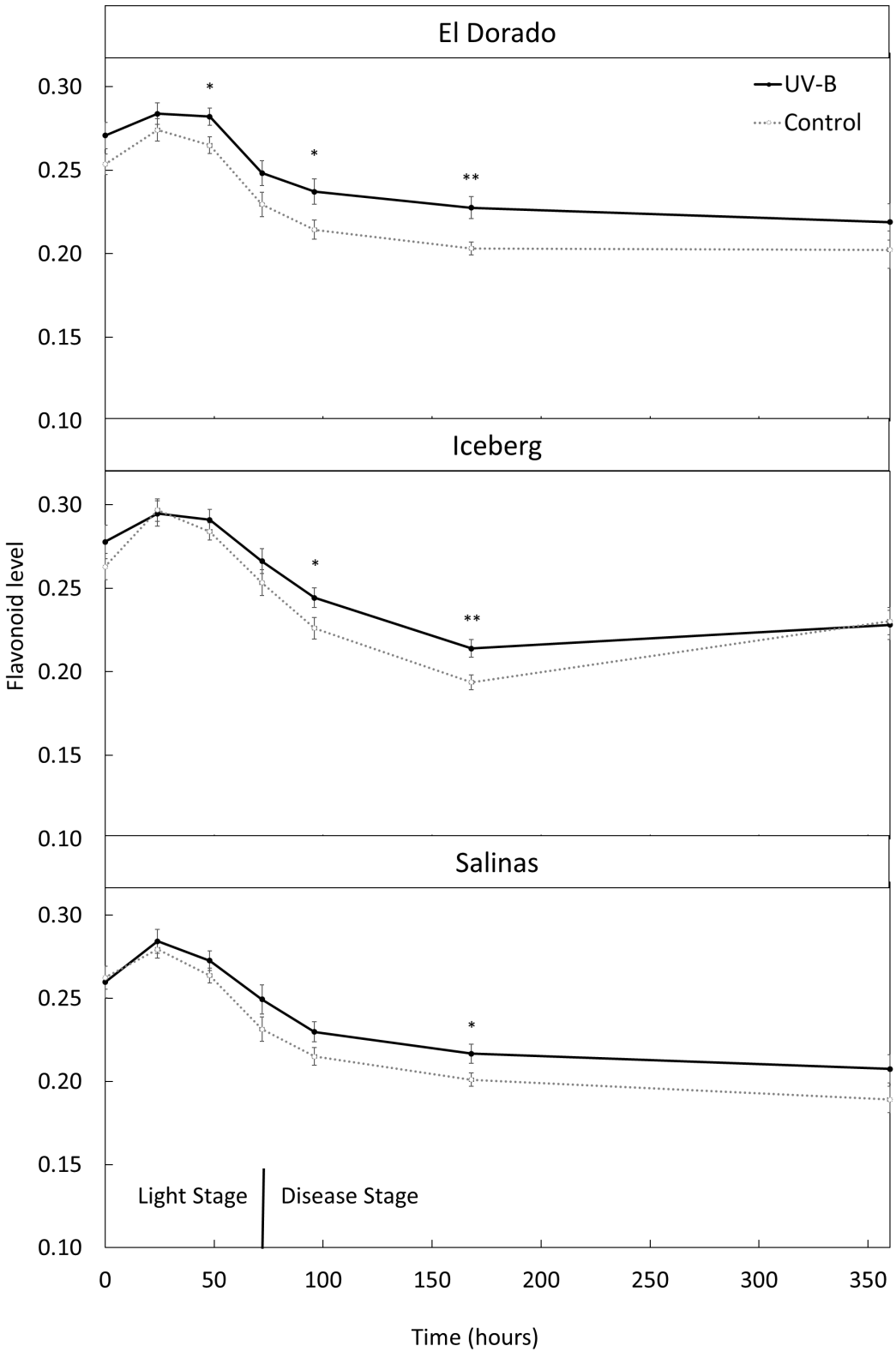


Figure 3.6.: Flavonoid levels of UV-B exposed lettuce (*L. sativa*) plants [black full line] are generally similar to control plants [grey dashed line] during the light phase and higher during the disease phase. Lettuce cv. El Dorado, Iceberg or Salinas plants were treated with photosynthetically active radiation (PAR) +UV-B light (280nm) or PAR only (control) for three days followed by inoculation of 10^5 conidia mL^{-1} of *B. lactucae*. Flavonoids were measured throughout the treatment and disease phase using a Dualex . Error bars indicate 1 S.E. Asterisks indicate significant differences between control and UV-B plants according to time point t-tests where *=$p<0.05$, **=$p<0.005$.

3.4.1. UV-B dose and wavelength can influence flavonoid response

Flavonoid levels were measured in two different treatment set-ups. A stationary array with 300nm LEDs was used for the majority of experiments (Section 2.2.1.2); however, the growth chamber commercial experiment used a moving 280nm array (Section 2.2.2.2). Although wavelengths were a major difference between the experiments, they also varied in dose and duration (due to motion). Therefore, although it can be suggested that the different set-ups induced different response pathways, it is not appropriate to attribute this purely to wavelength, dose or duration. The set-ups are identified by the wavelengths used in this Section. The 300nm set-up treatment resulted in a significantly higher increase in flavonoids in lettuce cultivars El Dorado and Iceberg (t-test, $p<0.05$) than the 280nm set-up at 72 h (Figure 3.7).

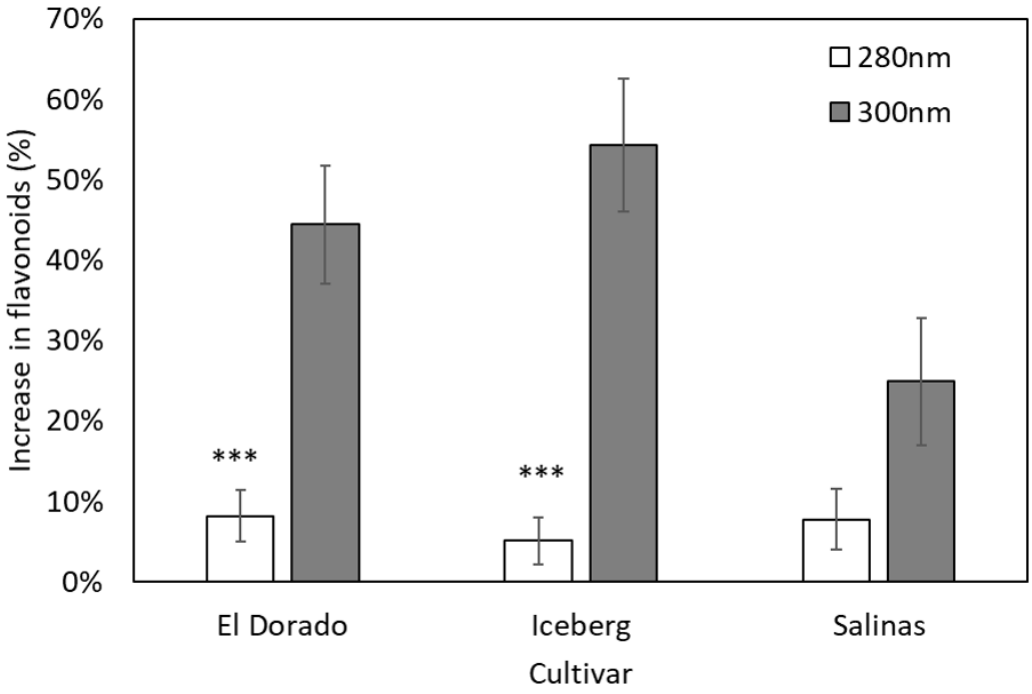


Figure 3.7.: Lettuce (*L. sativa*) cv. El Dorado and Iceberg had a significantly higher UV-B increase in flavonoids at 72 h following a 300nm [grey bar] treatment than a 280nm treatment [white bar]. Plants underwent a three day treatment of photosynthetically active radiation (PAR) + UV-B or PAR only (control). Following treatment, flavonoid levels were measured using a Duaelx. Flavonoid level is shown as % increase of UV-B plants compared to control. Error bars indicate 1 S.E. Asterisks indicate significant difference in UV-B increase in flavonoids according to a t-test where *** = $p<0.0005$.

UV-B light treatment is more effective at increasing flavonoids at 72 h using the 300nm experimental set-up than the 280 semi-commercial set-up. It was noted that significantly

higher flavonoids were present in UV-B-treated than control plants of the 280nm set-up treatment in the disease phase; however, these time points were not used in the multiple cultivar experimental set. Compared to a later experiment used in metabolomics (Section 4.3.2), a similar 300nm treatment was observed to increase the level of flavonoids at certain time points in lettuce cultivar Iceberg and Salinas, throughout the disease period also.

The difference in flavonoid levels between set-ups could be due to different primary UV-B response pathways induced by the treatment. The 300nm treatment can be considered a low dose and is therefore, more likely to use the photomorphogenic signalling pathway perceived by UVR8 as the primary UV-B response pathway (Brown and Jenkins, 2008). The UVR8-dependent pathway is well documented to result in increases to flavonoids and other photo-protective pigments (Coffey et al., 2017). Although the fluency rate was still relatively low in the 280nm set-up, and duration is reduced by the sweeping motion of the array, the wavelength is also much lower, resulting in a high energy light not present in ambient sunlight. This higher energy light may induce a more general UV-B pathway that results in a lower induction of flavonoids or perhaps a more prolonged/delayed production of flavonoids resulting in the significant difference found later in the disease period.

3.4.2. UV-B-induced increases to lettuce flavonoids may be involved in the UV-B-induced disease defence

There was no significant correlation between any measure of disease severity and flavonoid level at 72 h in the multiple cultivar 300nm experimental set. As the 300nm set-up resulted in both a higher induction of flavonoids and decrease in conidia count than the 280nm set-up (at 72 h), it would be expected that a significant correlation would have been observed in the multi-cultivar experimental set. In the later metabolomics experiment (Section 4.3.2), however, a highly significant negative correlation between conidia count and flavonoid level was observed. This experimental set followed the same method; however, included a higher number of samples, which may have contributed to the statistical power of the experiment.

Within the 280nm semi-commercial experiment, flavonoid level at 360 h (12 DPI) correlated negatively ($r = -0.666$, $p = 0.003$) with conidia count (Figure 3.8). Significant correlations between disease severity and flavonoid level were not present at any other time point. As 360 h, was the last recorded time point, high flavonoids at late-stage disease may influence conidia count following a 280nm treatment. This flavonoid probe indicates

that UV-B can both increase flavonoids and decrease disease, which at late-stage disease forms a negative correlation.

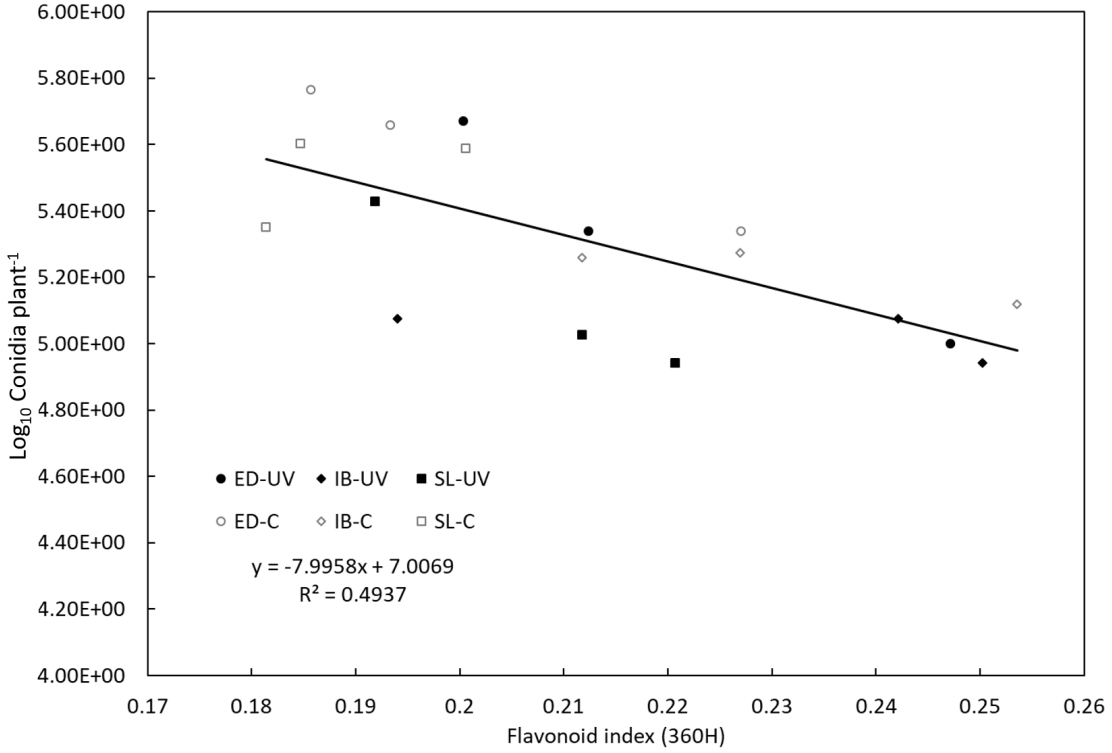


Figure 3.8.: Log10 Conidiaplant^{-1} negatively correlate with flavonoid level at 360 h (12 DPI) across UV-B [black marker] and control [grey marker] lettuce (*L. sativa*) plants of cv. El Dorado [ED = circle], Iceberg [IB = diamond] and Salinas [SL = square]. Lettuce plants were treated with photosynthetically active radiation (PAR) +UV-B light (280nm) [UV = black] or PAR only [C = gray] for three days followed by inoculation of 10^5conidia mL^{-1} of *B. lactucae*. At 360 h, plants were measured for flavonoid levels using a Dualex. Plants were then washed and the resulting conidia suspension counted.

3.5. Gene expression analysis highlights similarities between gene expression responses in plants to biotrophic disease and UV-B light

Transcriptomics studies in *A. thaliana* examining differential expression to UV-B light as well as response to a biotrophic (*H. arabidopsis* (*Hpa*)) filamentous oomycete pathogen have been completed in the past. Re-examination of these published data sets indicates some overlaps between the expression changes in a plant treated with UV-B or infected with a biotrophic disease. The overlap in these pathways may provide some insight into possible mechanisms in which UV-B light can influence the susceptibility to disease. Two doses of

UV-B light were assessed; low or high depending on dose and duration, as an example of UV-B gene expression response (Table 3.1a). The "low UV-B" light treatment has a higher dose similarity to the treatments used in this project than "high UV-B". An incompatible (avirulent strain) and a compatible (virulent strain) interaction of *Hpa* with *Arabidopsis* at 1 DPI were included in determining gene expression changes important to biotrophic disease resistance (Table 3.1b). At 1 DPI, successful penetration and the formation of haustoria were formed in both interactions (Asai et al., 2014). At 3 DPI, a hypersensitivite response (HR) and a halt in disease progression occurred in the incompatible interaction; however, hyphal growth and eventual sporulation (5 DPI) was observed in the compatible interaction (Asai et al., 2014). Therefore gene changes at 1 DPI likely highly influence the induction of a successful defence against the pathogen. Asai et al. (2014) suggest that up-regulation at 1 DPI in the incompatible interaction represent disease resistance genes, whilst down-regulation in the compatible interaction suggests pathogen induced-susceptibility.

Review of the current literature (Section 1.3.7) suggests UV-B induces many protective responses which may contribute to reduced disease susceptibility. These include increases to secondary metabolites, defensive hormone activity and pathogenesis-related (PR) proteins. Expression of genes grouped into these bins in *Arabidopsis* plants treated with either UV-B or infected with *Hpa* supports these claim with similarities in overall expression change (Figure 3.9).

Secondary metabolite-related genes tend to be up-regulated by both low and high UV-B light. Secondary metabolite genes are also up-regulated by *Arabidopsis* when confronted with an avirulent *Hpa* strain but down-regulated by a virulent *Hpa* strain. Therefore, secondary metabolite genes up-regulation marks successful resistance whilst down-regulation marks disease susceptibility. As both UV-B doses up-regulated overall secondary metabolite expression, UV-B-treatment would likely contribute to a secondary metabolite mediated disease defence.

Induction of the PR proteins occurred in all UV-B-treatments and biotrophic interactions. The highest up-regulation occurred in the high UV-B-treatment and incompatible interaction. High UV-B would likely have a major (and low UV-B a minor) effect on PR protein-mediated defence against a biotrophic pathogen.

Defensive hormones jasmonic acid (JA) and salicylic acid (SA)-related genes are both down-regulated by low UV-B light but up-regulated (JA only) by high UV-B light (Figure

3.9). Defence hormone induction by UV-B light is likely a more general stress/wounding response caused by the high energy light rather than a UV-B-specific response. Both hormone-related gene sets are down-regulated in a compatible interaction. A very slight up-regulation of JA-related genes and slight down-regulation of SA-related genes occurs during the incompatible interaction. Genes in these defence hormone bins are likely not as crucial to resistance as those in the secondary metabolism or PR proteins. An up-regulation of JA-related genes by high UV-B may affect biotrophic defence but is unlikely to be a major contributor.

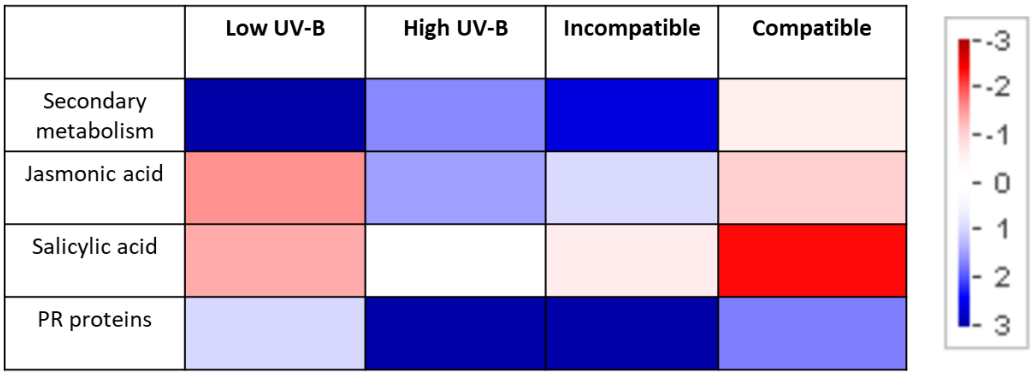


Figure 3.9.: Gene expression levels of *Arabidopsis thalina* treated with a low/high UV-B dose or incompatible/compatible infection with the biotrophic pathogen *Hyaloperonospora arabidopsidis*. Expression level is indicated by colour intensity [blue = up-regulated, red = down-regulated] as coded by the scale.

3.5.1. UV-B and incompatible biotrophic infection up-regulate secondary metabolites

Both UV-B and biotrophic disease can up-regulate the overall phenylpropanoid pathway. 4-coumarate-CoA ligase (4CL) proteins form the branch point between the primary metabolite pathway and phenylpropanoid pathway (Schneider et al., 2003). 4CLs convert cinnamic acid derivatives 4-coumaric acid (4-hydroxycinnamic acid), caffeic acid (3,4-dihydroxycinnamic acid), and ferulic acid (3-methoxy-4-hydroxycinnamic acid) into their activated ester form (Schneider et al., 2003). These activated esters serve as precursors for several phenolic compounds including flavonoids and lignin (Schneider et al., 2003). A number of 4CL isoforms are present in *Arabidopsis* plants with different substrate affinities. 4CL12 was identified in transcriptomics data as up-regulated by an incompatible *Hpa* inter-

action indicating a role of 4CL12 in biotrophic defence; however, as it is also up-regulated by an compatible interaction, it is not a marker for resistance. Both UV-B doses also up-regulate 4CL12. Although the substrate affinity of 4CL12 is uncertain, it can be said to contribute to the overall pool of phenolic precursors. UV-B can up-regulate 4CL12, resulting in increased channelling of the primary metabolism into the phenylpropanoid pathway in a manner relevant to *Hpa* defence in *Arabidopsis*. To determine which specific phenolics may be involved in a UV-B-induced *Hpa* defence, I investigated differential expression of phenolic groups.

3.5.1.1. UV-B up-regulates flavonoid-related genes which are important to biotrophic pathogen resistance

A major phenolic group commonly reported to be induced by both UV-B and disease resistance are the flavonoids. One of the major specialisation steps in flavonoid synthesis is further differentiation of dihydroflavonols (Dubos et al., 2010). Both doses of UV-B light heavily up-regulated the formation of a dihydroflavonol though up-regulation of flavanone 3-hydroxylase (F3H) (Figure 3.10). F3H is up-regulated in an incompatible *Hpa* interaction but heavily down-regulated in a compatible interaction. Therefore formation of a dihydroflavonol through regulation of F3H is important for resistance to *Hpa* and is also induced by UV-B light.

Dihydroflavonol is channelled into a flavonoid pathway (kaempferol or quercetin) through flavonoid 3´-hydroxylase (F3´H) and flavonol synthase (FLS) enzymes (Peer et al., 2001) or to the anthocyanin pathway through dihydroflavonol reductase (Tanaka et al., 2008). UV-B or biotrophic pathogen exposure did not alter *FLS* gene regulation by over $1\log_2$ FC. *Hpa* pathogen attack (incompatible and compatible) up-regulated the gene for psuedoenzyme FLS2; however, the activity of this enzyme is unknown. FLS2 has no FLS function due to a premature stop codon (Owens et al., 2008) so may have no relevance to flavonoid synthesis.

F3'H controls the ratio between quercetin or kaempferol flavonoids through facilitating the conversion of kaempferol into quercetin (Peer et al., 2001). Low UV-B and incompatible *Hpa* interaction both up-regulate a *F3'H* gene, resulting in a higher quercetin:kaempferol ratio. Up-regulation of quercetin is therefore key for *Hpa* resistance and is also heavily up-regulated by UV-B light. Quercetin flavonoids therefore may have a role in a UV-B-induced disease defence.

Table 3.2.: Log_2 fold change of genes involved in secondary metabolism in *Arabidopsis* when exposed to high or low UV-B light or when involved in an incompatible or compatible biotrophic interaction with *Hpa*. Expression level is indicated by colour intensity [blue = up-regulated, red = down-regulated].

Locus	Type	Name	Low UV-B	High UV-B	Incompatible	Compatible
at5g61160	anthocyanins	AACT1	-0.4	3.2	-1.7	-0.9
at1g56650	anthocyanins	PAP1, MYB75	0.3	1.1	1.8	-0.3
at3g55970	anthocyanins	oxidoreductase, 2OG-Fe(II) oxygenase family protein	0.1	3.4	-1.5	-1.4
at4g22870	anthocyanins	leucoanthocyanidin dioxygenase, putative / anthocyanidin synthase, putative	2.3	0.4	2.0	1.2
at5g65550	anthocyanins	UDP-glucoronosyl/UDP-glucosyl transferase family protein	-1.1	-0.8	1.1	1.2
at1g03940	anthocyanins	transferase family protein	1.7	1.6	1.5	-0.2
at5g54060	anthocyanins	UF3GT	-0.2	1.5	2.4	0.5
at2g22590	flavonoids	transferase, transferring glycosyl groups	5.4	1.0	-1.4	0.2
at1g25460	flavonoids	oxidoreductase family protein	1.0	0.4	-0.2	-1.0
at3g51240	flavonoids	F3H, TT6, F3'H	4.4	4.9	1.9	-2.9
at5g07990	flavonoids	TT7, F3H	3.0	-0.1	1.6	0.0
at5g63580	flavonoids	FLS2	-2.8	-0.3	1.8	1.4
at2g36800	flavonoids	DOGT1, UGT73C5	1.6	4.4	0.2	1.6
at1g75290	flavonoids	oxidoreductase, acting on NADH or NADPH	0.8	-1.2	1.4	1.4
at1g52040	glucosinolates degradation (myrosinase)	MBP1	1.3	-1.5	-1.1	-0.8
at1g54020	glucosinolates degradation (myrosinase)	myrosinase-associated protein, putative	-2.4	-1.0	-1.9	-2.8
at5g07700	glucosinolates regulation	MYB76	-0.6	-1.1	1.5	1.2
at5g57220	glucosinolates synthesis	CYP81F2	-1.2	3.1	1.0	1.0
at4g37990	lignin biosynthesis	ELI3-2, ELI3, ATCAD8, CAD-B2	-0.1	5.0	0.4	-1.4
at1g67980	lignin biosynthesis	CCOAMT	-0.3	2.4	1.0	1.3
at2g29130	lignin biosynthesis	LAC2	-1.2	-0.3	1.0	1.7
at5g05390	lignin biosynthesis	LAC12	1.6	-1.1	1.0	-1.4
at1g20500	phenylpropanoids	4-coumarate-CoA ligase 12 (4CL12)	1.1	0.8	1.4	1.4
at5g38130	phenylpropanoids	transferase family protein	0.0	-1.0	-3.4	-2.0
at5g42830	phenylpropanoids	transferase family protein	0.3	1.9	3.1	2.0
at2g37140	terpenoids	terpene synthase/cyclase-related	0.0	1.7	1.0	1.0
at1g61120	terpenoids	TPS04, GES	-0.8	3.7	1.3	-0.7
at3g45130	terpenoids	LAS1	-1.9	0.0	-1.9	-3.0
at5g51420	wax	long-chain-alcohol O-fatty-acyltransferase family protein / wax synthase family protein	1.4	-1.8	0.7	-1.2

Quercetin (and other secondary metabolites) can be glycosylated by glycosyltransferases (UGTs) to alter their biological activity (Rehman et al., 2018). Glycosylation is often associated with defensive compounds and increased tolerance to stress (Rehman et al., 2018). The UGTs; *UGT73C5* and locus *at2g22590*, were up-regulated by UV-B light but were associated with susceptibility (*UGT73C5*) or were unimportant (*at2g22590*) to biotrophic disease (Table 3.2). Glycosylation in these cases is likely to aid in protection against high light stress rather than disease defence.

Dihydroflavonols are also precursors for anthocyanins. The transcription factor PAP1 activates dihydroflavol reductase and Leucoanthocyanidin dioxygenase (*LDOX*) genes which catalyse the synthesis of anthocyanins (Dubos et al., 2010). PAP1 is important to *Hpa* resistance as indicated by *Hpa* incompatible induced up-regulation but (weak) *Hpa* compatible induced down-regulation (Table 3.2). UV-B also up-regulates PAP1; however, induction is much stronger by higher UV-B light. PAP1 responsive gene; a putative *LDOX* (*at4g22870*) is heavily up-regulated by low UV-B light as well biotrophic pathogen attack. Glycosylation may be involved in UV-B-induced biotrophic defence due to up-regulation of several UGTs specific to anthocyanins by UV-B light and *Hpa*. Anthocyanins may have a role in a UV-B-induced disease defence; however, is less likely a UV-B-specific effect due to a dependence on high UV-B for the key anthocyanin synthesis transcription factor PAP1.

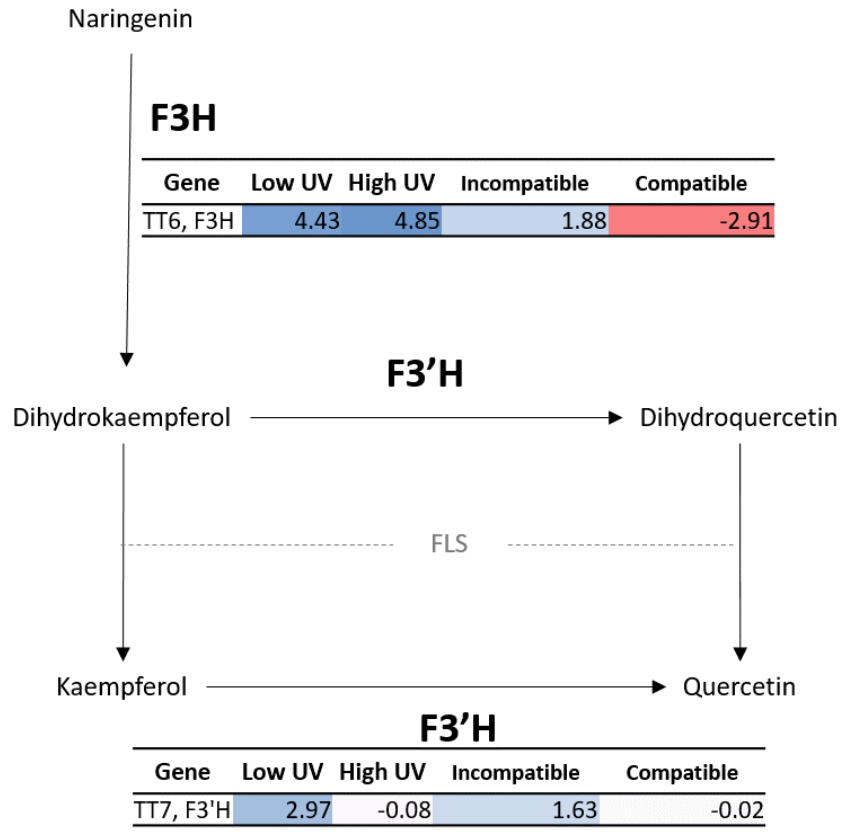


Figure 3.10.: Specialisation of naringenin into kaempferol or quercetin (Peer et al., 2001). The $\log_2$ fold change of key genes in this pathway following UV-B-treatment and pathogen infection are shown. Intensity of colour indicates intensity of change.

3.5.1.2. UV-B may increase phenolic barriers to pathogen attack

Lignin protects plants against pathogen attack through increased resistance of pathogen penetration through the strengthening of cell walls (Bhuiyan et al., 2009) and formation of papillae (Zeyen et al., 2002). High UV-B up-regulates two lignin synthesis genes; Caffeoyl-CoA 3-O-methyltransferase (*CCOAMT*) and Cinnamyl alcohol dehydrogenase 8 (*CAD8*) (Table 3.2). *Hpa* (incompatible and compatible) up-regulates *CCOAMT*. Only an incompatible *Hpa* infection up-regulated *CAD8* expression. High UV-B may enhance disease defence through lignin synthesis.

Lignin is a polymer formed from monolignin units (Vanholme et al., 2010). Laccases are multi-copper containing glycoproteins (Mayer, 2002) which are thought to be associated with polymerisation of lignin in plants (Bao et al., 1993). Incompatible *Hpa* infection up-regulated two laccases (*LAC2* and *LAC12*) indicating lignin polymer formation may

be important to resistance. *LAC2* was down-regulated by both UV-B doses; however, *LAC12* was only up-regulated by low UV-B. The role of different laccase is unknown, so it is uncertain the impact of down-regulation of *LAC2* but up-regulation of *LAC12* (low UV-B) would have on lignin polymerisation and resulting biotrophic defence.

A single wax synthesis-related gene (*at5g51420*) was also up-regulated by low UV-B and resistance to *Hpa*. UV-B increased wax levels may contribute to increased barriers to pathogen entry.

3.5.1.3. High UV-B induces several wounding-related secondary metabolites which may contribute to an increased biotrophic pathogen resistance

High UV-B light up-regulates two terpene synthase genes (*TPS04* and *at2g37140*) which are important to resistance against *Hpa* (Table 3.2). TPS04 catalyses the formation of (E,E)-geranyllinalool which can then be degraded to several terpenes including the C16-homoterpene TMTT (4,8,12-trimethyltrideca-1,3,7,11-tetraene) (Herde et al., 2008). The terpenes formed by *at2g37140* are not yet characterised. TMTT appears to be a result of induction JA signalling (Attaran et al., 2008). As there is some overlap between resistance and UV light expression, terpenes may be worth further investigation.

High UV-B also induces the glucosinolate synthesis gene *CYP81F2*. *CYP81F2* encodes the glucosinolate 4-methoxyindol-3-ylmethylglucosinolate (4M3M), which can be hydrolysed into active end products capable of halting non-adapted powdery mildew attack (Bednarek et al., 2009). *CYP81F2* is up-regulated at similar levels in both compatible and incompatible *Hpa* infections. As downy mildew disease and powdery mildew are both caused by biotrophic pathogens, increased 4M3M may contribute to a UV-B-induced defence in downy mildew disease also. However, other genes, such as positive regulator of glucosinolate synthesis *MYB76* (Li et al., 2013b) were up-regulated by *Hpa* but down-regulated by UV-B light providing evidence against a role of glucosinolates in defence. Myrosinase glucosinolate degradation enzymes hydrolyse glucosinolate to produce active end products (Wittstock and Burow, 2010). Expression of specific myrosinase enzymes can determine which glucosinolate end products are produced. Both myrosinase genes altered by *Hpa* in this data set were down-regulated and thus are unlikely to produce end products which contribute to *Hpa* resistance.

3.5.2. UV-B-induced defence hormone synthesis gene changes are unlikely to contribute to biotrophic disease defence

JA is generally accepted as a wounding-induced hormone which recruits protection against necrotrophic and herbivore attack (Vasyukova and Ozeretskovskaya, 2009). It is an oxylipin synthesised through serial modification (Shah, 2005). Many of the enzymes involved in this synthesis pathway such as lipoxygenases (LOX), and lipases, can result in a range of products, mostly antimicrobial, anti-insect or defence regulator signals (Farmer et al., 2003). No gene involved in the biosynthesis of JA was differently expressed by UV-B and biotrophic infection (incompatible or compatible) by +/- 1 $\log_2$FC (Table 3.3). High UV-B up-regulated some JA synthesis genes including *LOX3*, putative lipoxygenase (*at1g72520*), *AOC1*, *OPR1* and *OPR3*. Low UV-B resulted in down-regulation of many JA synthesis genes with only *LOX1* and *LOX3* up-regulated. High UV-B may contribute to defence against a necrotrophic pathogen through up-regulation of JA biosynthesis; however, low UV-B is unlikely to affect JA-mediated defence against a necrotrophic pathogen.

SA is generally associated with biotrophic disease defence (Vasyukova and Ozeretskovskaya, 2007). The binning system in MapMan only captured highly differentiated expressed methyl transferase genes (Table A.1). The majority of these were S-adenosyl-L-methionine:carboxyl methyltransferases, as well as a farnesonate methyl transferase (FAMT) and nicotine methyl transferase (NAMT), which are predicted to act as volatile signals in pest/pathogen defence but functions are largely unknown (Wu et al., 2018; Yang et al., 2006). Another methyl transferase, *BSMT1*, had high differential expression in all interactions. BSMT1 methylates SA to create a volatile signal molecule; MeSA (Chen et al., 2003). Increases to BSMT1 and therefore MeSA resulted in increased susceptibility to pathogens and reduced SA and PR1 production in the cell it was created but increased levels in neighbouring plants (Koo et al., 2007). Up-regulation of BSMT1 therefore, would provide decreased disease protection of the producing plant but increased protection in neighbouring plants. High UV-B up-regulated *BSMT1* (2.8, $\log_2$FC). Low UV-B and biotrophic interactions resulted in down-regulation of *BSMT* (-1.6, -0.41, -1.08 $\log_2$FC respectively). High UV-B may therefore aid in protection of neighbouring plants through increases to MeSA, whereas low

Table 3.3.: Log_2 fold change of Jasmonic acid-related genes in *Arabidopsis* plants treated with UV-B or infected with the biotrophic pathogen *Hpa* in a compatible or incompatible interaction.

Locus	Type	Name	Low UV-B	High UV-B	Incompatible	Compatible
at1g18020	12-Oxo-PDA-reductase	12-oxophytodienoate reductase, putative	-1.14	0.34	0.45	0.22
at1g09400	12-Oxo-PDA-reductase	12-oxophytodienoate reductase, putative	-1.62	0.96	-0.87	0.11
at1g76680	12-Oxo-PDA-reductase	OPR1	0.24	1.27	0.11	0.94
at2g06050	12-Oxo-PDA-reductase	OPR3	-0.62	1.78	0.28	-0.18
at1g13280	allene oxidase synthase	AOC4	-1.29	-1.81	0.13	0.06
at3g25780	allene oxidase synthase	AOC3	-0.77	0.90	0.77	0.02
at3g25760	allene oxidase synthase	AOC1, ERD12	0.81	1.04	-0.21	0.21
at1g67560	lipoxygenase	lipoxygenase family protein	-0.37	-1.13	0.09	0.23
at1g55020	lipoxygenase	LOX1	1.38	-0.21	0.38	0.34
at3g22400	lipoxygenase	LOX5	-0.66	-1.14	-0.16	-0.19
at1g17420	lipoxygenase	LOX3	1.11	1.79	0.12	-0.28
at1g72520	lipoxygenase	lipoxygenase, putative	-0.37	3.80	0.44	-0.25

UV-B contributes to the protection of a single plant.

3.5.3. UV-B induces several biotic stress-related genes which may contribute to increased resistance

3.5.3.1. High UV-B up-regulates similar PR genes to an incompatible infection with *Hpa*

Several PR proteins (at2g43570, at1g78780, PR5 and PR1) are important to the resistance of *Arabidopsis* to *Hpa* as indicated by an increased up-regulation in an incompatible but not a compatible *Hpa* infection (Table 3.4). A high UV-B-treatment also up-regulated all of these genes. A low UV-B-treatment only resulted in up-regulation of PR5 and a putative chitinase which were not required for resistance. High UV-B may contribute to a PR-mediated defence, but low UV-B would have little effect. All UV-B altered defensins attributed to susceptibility rather than resistance to *Hpa.* High UV-B up-regulated defensin genes *PDF1.2A*, *PDF1.2b* and a defensin like family protein gene. Defensins are generally associated with defence against fungal pathogens (Thomma et al., 2002), as *Hpa* is an oomycete, it may lack the target site for the UV-B-induced defensins. There is still potential that UV-B-induced defensins may contribute to a different pathogen; however, this data indicates UV-B-induced defensins do not contribute to resistance against *Hpa.*

3.5.3.2. UV-B up-regulates several salicylic response genes which are important to *Hpa* resistance

Although SA synthesis genes were not altered by UV-B or disease, several SA response genes were increased by UV-B light treatment (Table. 3.4). Several SA response genes had an increased up-regulation in incompatible compared to compatible *Hpa* infection indicating an importance to resistance. These genes were also all up-regulated by high UV-B; however, only two genes (*AIG1* and *PAD4*) were up-regulated by low UV-B. Previous studies confirm the role of these genes in successful defence.

UV-B up-regulated *AIG* expression. AIG is associated with increased resistance, through a RPS2-dependent pathway (Reuber, 1996); however, a role in defence against *Hpa* has not yet been explored.

SARD4 is involved in the synthesis of pipecolic acid which is then further reduced to form NHP; the activator of systemic acquired resistance (SAR) (Hartmann and Zeier,

Table 3.4.: Log_2 fold change in biotic stressrelated genes for high and low UV treatments in *Arabidopsis* as well as incompatible and compatible interactions with a biotrophic pathogen *Hpa.*

Locus	Type	Name	Low UV-B	High UV-B	Incompatible	Compatible
at3g59930	plant defensins	defensin-like (DEFL) family protein.	-0.28	2.42	-4.26	-0.44
at2g26020	plant defensins	PDF1.2b	1.11	4.47	-3.08	-2.42
at5g44420	plant defensins	PDF1.2, PDF1.2A, LCR77	-0.75	3.77	-3.06	-2.71
at5g40155	plant defensins	defensin-like (DEFL) family protein.	0.02	-1.32	0.74	1.30
at1g75830	plant defensins	LCR67, PDF1.1	-1.13	-1.49	-1.98	1.56
at1g75040	PR-proteins	PR5	1.86	1.95	2.01	0.73
at2g43570	PR-proteins	chitinase, putative	-0.32	2.85	2.13	0.97
at1g78780	PR-proteins	pathogenesis-related family protein	-0.91	1.64	2.78	0.30
at2g43580	PR-proteins	chitinase, putative	1.91	-0.36	-1.99	-2.80
at2g14610	PR-proteins	PR1	-0.17	1.55	5.18	2.57
at1g33960	SA response	AIG1, IAN8	1.76	2.74	3.95	1.26
at5g52810	SA response	SARD4	-0.24	1.48	1.90	0.24
at3g50480	SA response	HR4	-0.04	1.60	2.63	1.52
at3g48080	SA response	EDS1B	-0.36	1.41	2.70	1.56
at3g52430	SA response	PAD4	1.05	1.50	1.69	0.43
at1g73260	SA response	KTI1, KTI4	-0.15	3.03	1.31	0.61
at3g09590	undefined biotic stress	CAP superfamily protein	-0.94	1.37	-1.43	0.00
at1g52660	undefined biotic stress	P-loop containing nucleoside triphosphate hydrolases superfamily protein	-0.55	-4.32	1.28	0.81
at2g34315	undefined biotic stress	avirulence induced family protein	0.21	-1.40	-2.24	-1.15
at3g22231	undefined biotic stress	PCC1	2.57	-0.26	3.38	1.57
at3g23190	undefined biotic stress	HR lesion inducing protein-related	-1.05	-2.65	3.30	2.21
at3g02840	undefined biotic stress	immediate-early fungal elicitor family protein	1.32	1.05	0.65	1.17
at1g11770	undefined biotic stress	OGOX3	2.59	-1.83	2.09	2.2

2018). Induction of SARD4 by high UV-B may result in increased resistance in non-light treated plant parts also due to induction of a SAR response.

HR4 is a homolog to RPW8 (Sáenz-Mata and Jiménez-Bremont, 2012). RPW8 is associated with broad-spectrum resistance to powdery mildew (Orgil et al., 2007). As downy mildew and powdery mildew are both caused by biotrophic pathogens, there may be some similarities in plant defence mechanism, and so a high UV-B up-regulation of HR4 would contribute to this defence.

PAD4 is a lipase-like protein that associates with EDS1 (enhanced disease susceptibility) for accumulation of SA in an R-mediated defence (Feys et al., 2001). EDS1 is required for R gene-immunity dependent programmed cell death (Feys et al., 2001). Both PAD4 and EDS1 are required for resistance against *Hpa* (Glazebrook et al., 1997; Parker, 1996). Therefore up-regulation of *PAD4* by both high and low UV-B, and *EDS1B* by high UV-B could increase SA dependent defence against *Hpa* and promote R gene-related defences.

Kunitz typsin inhibitor 1 (KTI1) is induced by both fungal and bacterial elictors as well as SA. KTI1 is associated with decreased lesion size (Li et al., 2008). Park et al. 2001 suggest that KTI1 may be involved in HR through regulation of programmed cell death. High UV-B may contribute to defence through *KT1* up-regulation.

UV-B-treatment (particularity high UV-B) may not increase SA levels; however, the common theme of up-regulation of SA response genes indicates that UV-B may influence the SA pathway. Further research into the interactions between SA and UV-B could provide insight on a possible role of SA response in a UV-B-induced disease defence.

3.5.3.3. High UV-B up-regulates many of the same receptors as an incompatible *Hpa* infection

An incompatible infection with *Hpa* induced the up-regulation of 14 receptor genes over 1$\log_2$FC (Figure 3.11, Table A.2). Most of these receptors had an undefined function; however, resembled receptor-like proteins, or contained a toll-interleukin resistance domain indicating they were likely involved in defence against pathogens through either recognition of common pathogen epitopes (PAMP mediated immunity) or specific pathogens through elicitors (Effector triggered immunity) (Morris and Walker, 2003). These receptors may be involved in recognition of other cues, such as release of defence hormones, DAMPS, or environmental cues (Morris and Walker, 2003). Of the 14 genes which differentiate *Hpa*

resistance, 50% of these (7 genes) were also up-regulated by high UV-B light. A plant treated with high UV-B light has higher potential for perception of a pathogen through increased receptor capacity. High UV-B may prime a plant resulting in a quicker response to the pathogen allowing less time for the pathogen to become established and heightened defences.

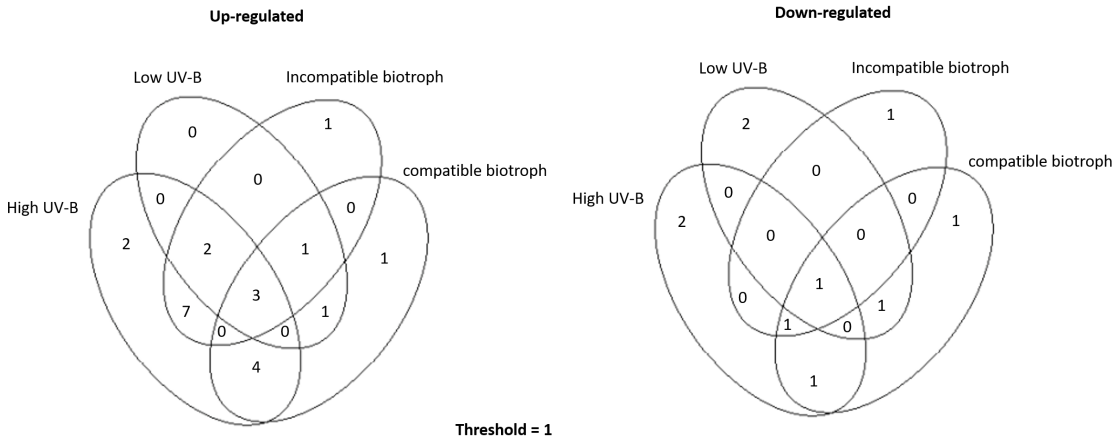


Figure 3.11.: Several biotic stress-related receptors had the same differential pattern between treatment with UV-B light and infection with *Hpa* in *A. thalina*. A threshold of 1 $\log_2$ fold change was used to determine large changes in gene expression.

3.5.4. High UV-B may result in gene expression more suited to necrotrophic fungal defence

Many of the gene expression changes caused by a high UV-B-treatment are similar to what would be expected to be important to necrotrophic fungal disease defence. High UV-B induces JA synthesis genes, as well as PR proteins, defensins, and terpenes. These defensive genes are all associated with herbivore or necrotrophic responses as well as a general wounding response (Glazebrook, 2005; Oliver and Ipcho, 2004). High UV-B may therefore contribute more to a necrotrophic defence then to a biotrophic defence. Although qualitative resistance data is readily available for biotrophic interactions, necrotrophic defence is largely quantitative and published expression data on incompatible interactions are not readily available (Zhang et al., 2013a). Distinction between genes up-regulated which contrite to resistance or susceptibility is more of a challenge when examining qualitative resistance, so comparison of UV-B gene expression and necrotrophic pathogens is not explored in this project.

3.6. UV-B-induced flavonoids is the most promising mechanism to explore further

The UV-B-treatments used in this project more closely resembles the "low UV-B" over the "High UV-B" dose in gene expression data. Gene expression analysis indicates that secondary metabolites, in particular flavonoid gene expression, provides the most persuasive evidence for a role of low UV-B light in an increased disease defence against *Hpa.* Flavonoids, in particular, specialization into quercetin compounds, are up-regulated by low UV-B and an incompatible *Hpa* attack but down-regulated by compatible *Hpa* attack. Flavonoid synthesis is therfore up-regulated by UV-B and is important to resistance of *Arabidopsis* to *Hpa.* Within this Chapter, lettuce treatments with UV-B have been shown to increase overall flavonoid level as well as increase disease resistance to oomycete *B. lactucae.* Therefore flavonoid genes important to *Arabidopsis* UV-B disease defence may also have a role in lettuce UV-B-induced disease defence. Of the investigative probes used in this chapter, the role of UV-B-induced flavonoids is most promising for further investigation into a UV-B-induced biotrophic disease defence.

Chapter 4

Role of phenolics in the UV-B induced disease defence

4.1. Introduction

The phenylpropanoid pathway is key for both UV-B light protection and defence against disease in plants (Chapter 1). This overlap between pathways is greater than simply a stress response. UV-B and pathogens increase phenolic defences (Demkura and Ballare, 2012), such as glucosinolates, flavonoids and terpenes with protective properties (Mewis et al., 2012). Flavonoids are a phenolic group which are most commonly associated with a UV-B response (Caldwell et al., 1983; Choudhary and Agrawal, 2014; Dolzhenko et al., 2010; Lancaster et al., 2000; Lavola, 1998) as well as an association with increased disease resistance (Yang et al., 2016; Tao et al., 2010; Jia et al., 2010; Sanzani et al., 2010, 2008).

In Chapter 3 I confirmed that UV-B light can up-regulate flavonoids in lettuce (*Lactuca sativa*) as well as cause the up-regulation of flavonoid genes that are differential expressed between a incompatible and compatible biotrophic interaction in *Arabidopsis* such as flavonoid 3´-hydroxylase (*F3´H*) and flavonol synthase (*FLS*). In this Chapter, a range of lettuce cultivars with varying flavonoid UV-B response and disease susceptibility were used to determine which phenolics, with a focus on flavonoids, may play a role in a UV-B-induced downy mildew defence using LC-MS and compound infiltration. Following UV-B-pretreatment, a subset of lettuce plants was taken for LC-MS, with the remainder infected with *Bremia lactucae.* LC-MS analysis revealed 10 UV-B-induced metabolites had high correlations with disease reduction. Of these, three correlated compounds were directly

infiltrated into plants (not light-treated) and the effect of the individual compounds on disease reduction was assessed. A compound; quercetin 3-O-(6″-O-malonyl)-β-D-glucoside, had a strong UV-B-induced correlation with reduction in conidia count. Upon infiltration, quercetin 3-O-(6″-O-malonyl)-β-D-glucoside also reduced *B. lactucae* conidia count in cultivar El Dorado. Chapter 4 provides strong evidence towards a role of UV-B-induced flavonoids in a decreased disease susceptibility.

4.2. Methods

4.2.1. Metabolomics methods

4.2.1.1. Plant growth

Lettuce (*Lactuca sativa*) plants were sown into black plastic trays, with a cell size of 3cm^2, containing 'Daltons Seedling Raising Mix' in a randomised pattern. Two experiments with LC-MS analysis were run. The first set (LC-MS-1) used lettuce cultivars; El Dorado, Iceberg, and Salinas [Richard Michelemore, UC Davis, USA]. The second set (LC-MS-2) used cultivars La Brilliante, Emperor, Grand Rapids, Calicel [Richard Michelemore, UC Davis, USA], Greenway [Yates, NZ], Faclon, Pedrola [Terranova seeds, NZ], Desert Storm. These cultivars expressed a range of responsiveness to UV-B-treatment as indicated by flavonoid level and susceptibility to downy mildew disease. This was shown for LC-MS-1 cultivars in Figures 2.11 (Chapter 2) and 3.5 (Chapter 3). LC-MS-2 cultivars were chosen from a screen for disease susceptibility (as conidia count) as well as UV-B-induced flavonoid levels (Table B.1). Of these, four cultivars (Great Lakes, Glendana, Vegas, and Pedrola) were completely resistant to *B. lactucae* sextext code IBEB-C 36-01-00 or EU-B 16-63-40-00. Pedrola was chosen to represent complete resistance to *B. lactucae* in the extended LC-MS-2 analysis. In the first LC-MS set (LC-MS-1), cultivar Iceberg had low susceptibility, Salinas: moderate susceptibility and El Dorado: high susceptibility. The disease screen indicated an even less susceptible (La Brilliante) and more susceptible (Emperor) cultivar than Iceberg and El Dorado respectively. These cultivars were included as new extreme examples of high or low susceptibility in LC-MS-2. The remaining cultivars had intermediate levels of disease susceptibility. In terms of metabolites, flavonoid response to UV-B was used to indicate likely LC-MS metabolite UV-B response range. Most cultivars experienced a similar increase between 43 and 47% increase, with Falcon and Calicel experiencing lower increases

(28% and 30% respectively). Emperor had the highest increase in flavonoids (50%). In order achieve a range of both metabolite response to UV-B and disease susceptibility as well as ease of growth e.g germination uniformity, Desert Storm, Falcon, Greenway, and Calicel were included in the cultivar set.

Following sowing, a single layer of grade 3 medium vermiculite [Auspari pty LTD, NSW] was spread over the tray. Sown trays were misted with water then placed in darkness at 14^{o}C for 48 h for vernalisation. Following vernalisation, plants were moved to a controlled temperature room (CTR) and grown for 14 days. The CTR had a temperature of 17^{o}C, and a 10 h photoperiod supplied by 215µmol $m^{-2}s^{-1}$ white light from FL58W/965 super daylight deluxe fluorescent tubes [Slyvania Premium Extra, China]. Water was applied daily to capillary matting underneath the trays. Separate plants were randomly designated for dualex measurements, LC-MS and disease assessment.

4.2.1.2. Light Treatments

Light treatments were applied through the use of a stationary LED array designed by Biolumic Ltd. Two-week-old CTR grown plants were treated with 215 µmol $m^{-2}s^{-1}$ of PAR light through red and blue LEDs plus either 0.5 µmol $m^{-2}s^{-1}$ UV-B light or no UV-B light (control) (Treatment D, Table 2.1) for a photoperiod of 10 h for three days. Following light treatments, plants were allowed 14 h' recovery time in darkness prior to infection with disease. Treatments were conducted in a 17^{o}C CTR with the LED array acting as a sole light source. Three repeats were completed for LC-MS set 1, only one repeat, spread over two treatments, was completed for LC-MS-2.

4.2.1.3. Quantitative phenolic measurements

A subset of plants from each cultivar from each treatment were designated for Dualex measures. A Dualex [Force A, Orsay; France] is a hand held, non destructive tool which uses light fluorescence and transmission through a leaf clip to estimate leaf chlorophyll content (Chl), epidermal UV-absorbance (Flv), anthocyanins and a Nitrogen Balance Index (NBI, patented) (Goulas et al., 2004). Dualex measures were taken at 0 h (just prior to treatment start), then every 24 h til inoculation (0, 24,48,72 h) on the first true leaf of each plant. Following inoculation dualex measures were taken at 1,7 and 12 days post-inoculation (DPI) on the first true leaf of inoculated plants regardless of the presence of disease symptoms. Dualex measurements were taken in LC-MS-1 experiments only (n =

14-15 per treatment per cultivar).

4.2.1.4. Metabolomic profiling

At 72H= h, between treatment end and inoculation, sample plants were frozen in packets of three in liquid nitrogen and stored at -80^{o}C. LC-MS-1 contained nine samples (three plants per sample) per cultivar per treatment. LC-MS-2 contained three samples (three plants per samples) per cultivar per treatment. A modified version of Wargent et al. (2015) was used to perform liquid chromatorgraphy - mass spectrometry (LC-MS). Foliar material was homogenized in liquid N_2 and weighed to an equal mass of 150 mg for each sample. Each of the powdered leaf samples was extracted overnight at 1 °C with 1.5 mL of methanol/MQ/formic acid (80/20/1 v/v/v). Samples were diluted with methanol before analysis by LC-MS. LC-MS-grade methanol was from Merek [Newmarket, Auckland, New Zealand]. Ultrapure water was obtained from a Milli-Q Synthesis system [Millipore, Billerica, MA, USA].

The LC-MS equipment used was the same system as Wargent et al. (2015). The LC-HRMS system was composed of a Dionex Ultimate® 3000 Rapid Separation LC and a micrOTOF QII mass spectrometer [Bruker Daltonics, Bremen, Germany] fitted with an electrospray ion source. The LC contained an SRD-3400 solvent rack/degasser, an HPR-3400RS binary pump, a WPS- 3000RS thermostated autosampler and a TCC-3000RS thermostated column compartment. The column used was C68 [Luna Omega C18 100x2.1 mm id, 1.6 um; Agilent, Melbourne, Australia] and was maintained at 40^{o}C. The flow rate was 0.400mL min^{-1}. Solvents were A = 0.2% formic acid and B = 100% acetonitrile which set up a gradient over 20 min. The gradient was set up as 90% A, 10% B, 0-0.5 min; linear gradient to 60% A, 40% B, 0.5-9 min; linear gradient to 5% A, 95% B, 9- 14 min; maintained at 5%A, 95%B. 14-18 min; linear gradient to 90%A, 10%B, 18-18.2 min; then returns to original conditions for next sample injection at 20 min. The injection volume was 1µL. Mass spectrum [micrOTOF QII] parameters were as Wargent et al. (2015).

Analysis of raw output was completed by XCMS online (Gowda et al., 2014) to determine molecular features labelled with accurate mass and retention time. XCMS also grouped features into peak groups which likely represent a singular metabolite. Feature groups and representative mass was confirmed by spectral data using MZMINE (Pluskal et al., 2010). Intensities of feature within a peak group were summed to determined feature area intensity. Feature area intensity was submitted to statistical tests, such as PCAs, ANOVA and t-tests

to determine differences between cultivars and treatments. Identification (formula and compound name) of features was determined using MSDIAL and MSFINDER(Lai et al., 2017; Tsugawa et al., 2016). Proposed identification by MSFINDER was given a score of confidence and confirmed by comparison of QC MS/MS spectral data (MZMINE) to published spectrum and published literature.

4.2.1.5. Pathogen inoculation and disease assessment

A subset of plants in LC-MS experiments (LC-MS-1 n= 20-24, LC-MS-2 n = 9-15) were assessed for downy mildew disease severity. Lettuce plants were misted with 10^5 conidia $mL^{-1}$of *B. lactucae* (sextext code IBEB-C 36-01-00 or EU-B 16-63-40-00) using a pressure sprayer until plants were saturated. Inoculated plants were kept in a misting tent at a temperature of 17°C and misted twice daily with water to encourage a high humidity. Disease was visually assessed using a damage scale (Table 4.1) or a sporulation scale (Table 2.3) daily from six DPI till 12 DPI on the sets of plants designated for disease assessment for each cultivar and treatment. Rating scales were based on observed disease development of downy mildew disease using two to four week old lettuce Casino seedlings infected with *B. lactucae* sextext code IBEB-C 36-01-00 or EU-B between 6 to 12 DPI.

Conidia counts were taken 12 DPI. Count samples consisted of one plant washed in 20mL of distilled water. Five millilitres of the resulting suspension was pipetted into a haemocytometer (0.1mL depth) [BRAND® counting chamber BLAUBRAND® Neubauer improved, Sigma - Aldrich] and conidia were counting in the four corner squares. The average of these four squares was multiplied by 10,000 to give a measurement in conidia mL^{-1}. Two technical repeats of conidia counts were completed for each conidia solutions and averaged together.

Table 4.1.: Damage scale: Describes the level of yellowing (excluding hypersensitive response) in lettuce (*L. sativa*) as a result of downy mildew disease.

Rating	Description
0	No visible yellowing
1	Yellowing on up to 25% of a single leaf
2	Yellowing >25% on a single leaf OR up to 25% on multiple leaves
3	Yellowing 25 - 50% on multiple leaves. Signs of wilting
4	Yellowing 50- 75% on multiple leaves. Heavy wilting on yellowed leaves
5	Yellowing 100% on multiple leaves OR leaf death/extreme wilting

4.2.2. Phenolic infiltration method

4.2.2.1. Plant growth

Lettuce (*L. sativa*) cultivars; El Dorado, Iceberg, and Salinas were sown into black plastic trays (repeats 1-3), with a cell size of 3cm^2, containing 'Daltons Seedling Raising Mix' in a randomised pattern. In repeat four only cultivars El Dorado and Salinas were sown. A single layer of grade 3 medium vermiculite [Auspari pty LTD, NSW] was spread on top. Sown trays were misted with water then placed in darkness at 14°C for 48 h for vernalisation. Following vernalisation, plants were moved to a controlled temperature room (CTR) and grown for 16 days to reach a similar age to plants at the end of a UV-B period of method Section 4.2.1. The CTR had a temperature of 17°C, and a 10 h photo-period supplied by 215µmol m^{-2}s^{-1} white light from FL58W/965 super daylight deluxe fluorescent tubes [Slyvania Premium Extra, China]. Water was applied daily to capillary matting underneath the trays.

4.2.2.2. Compound selection

The compounds 5-caffeoylquinic acid (chlorogenic acid) (CA), 3,5-dicaffeoylquinic acid (DCQA) and quercetin 3-O-(6"-malonyl-glucoside) (Q) were chosen from the list of metabolites identified in Section 4.4.5 as having significant negative correlations with disease severity as well as a strong certainty of identification. Standards of CA (https://www.sigmaaldrich.com /catalog/product/aldrich/c3878?lang=en&region=NZ) and Q (https://www.sigmaaldrich.com /catalog/product/sigma/16733?lang=en&region=US) were ordered from Sigma Aldrich, and DCQA from Carbosynth (https://www.carbosynth.com /carbosynth/website.nsf/(w-productdisplay)/1DB1FA22CAF00B3C48257ECB00243A6B).

Published studies on polyphenol content were used to determine a control content of Iceberg/Crisphead type lettuce plant as 3.78 mg/100 g fresh weight (FW) CA (Yamaguchi et al., 2003), 0.28mg/100g FW DCQA (Ribas-Agustí et al., 2011) and 1.85 mg/100 g FW Q (DuPont et al., 2000). Concentrations were adjusted for leaf weight, and infiltration volume of plants at 16 days old of each of the three cultivars and dilutions of the standards made to achieve a 1.5, 2.5 and 4 times increase in El Dorado, Iceberg and Salinas plants (Table 4.2). These fold changes are based on UV-B-induced increases to levels of CA, DCQA and Q of 1.2 to 2.6 fold 4.4.3. The higher fold increase (4) is to provide insight on how further increases to the compound could alter disease susceptibility.

Table 4.2.: Concentrations of each compound infiltrated into El Dorado, Iceberg and Salinas lettuce (*L. sativa*) plants to achieve 1.5, 2.5 or 4-fold increase in the compound compared to a standard Iceberg/Crisphead type lettuce plant.

Fold increase	Cultivar	Compound (µg/mL) Quercetin 3-O-(6"-malonyl-glucoside)	5-Caffeoylquinic acid (Chlorogenic acid)	1,3-dicaffeoylquinic acid
1.5	El Dorado	2.50	5.12	0.38
	Iceberg	2.44	4.98	0.37
	Salinas	2.59	5.29	0.39
2.5	El Dorado	7.51	15.35	1.14
	Iceberg	7.31	14.94	1.11
	Salinas	7.77	15.88	1.18
4	El Dorado	15.02	30.70	2.27
	Iceberg	14.62	29.87	2.21
	Salinas	15.55	31.76	2.35

4.2.2.3. Compound infiltration

Syringe infiltration is usually used for bacterial infiltration; however, was adapted in this case for infiltration of phenolic compounds. I used a similar leaf infiltration method to Kim and Mackey (2008). Plants (16 days post sowing) were placed in a humid environment for 30 min to encourage stomatal opening. The oldest leaf (leaf 1) of each plant was marked for infiltration with vivid at the base of the leaf. Infiltrations were carried out by injections of either water (mock) or the compound solution (Table 4.2) using a 1mL needle-less syringe into the back of the leaf at two points (one on each side of the rib). Plants were infiltrated till the entire leaf had changed colour indicating entry of the liquid (approx. 0.8 +/- 0.1 mL). Plants were allowed to rest 17 h (5 h light, 12 h dark) before inoculation. Due to delay in arrival of the Q standard, time limitations and limited plant numbers, the first two repeats tested compounds CA and DCQA only. Repeat 3 tested Q only and repeat 4 tested all three compounds (CA, DCQA and Q). Trays of plants were blocked by cultivar and compound, with the compound concentrations arranged in a Latin square within each block.

4.2.2.4. Disease assessment

Plants were misted with 10^5 conidia mL^{-1} of *B. lactucae* (sextext code IBEB-C 36-01-00 or EU-B 16-63-40-00) using a pressure sprayer until plants were saturated. Inoculated plants were kept in a misting tent at a temperature of 17°C and misted twice daily with water to encourage a high humidity. Disease of the infiltrated leaf and the entire plant were measured separately. Sporulation severity and incidence were visually assessed using

a leaf sporulation scale (Table 4.3) or a plant sporulation scale (Table 2.3) daily from 6 DPI till 8 DPI (n = 14-52 per treatment per cultivar).

Table 4.3.: Visual rating scale to measure sporulation severity of downy mildew on infiltrated lettuce (*L. sativa*) leaf (leaf 1).

Rating	Description
0	no sporulation
1	0-10 % leaf coverage
2	11- 20% leaf coverage
3	21-30% leaf coverage
4	31-40% leaf coverage
5	41-50 % leaf coverage
6	51-60% leaf coverage
7	61-70% leaf coverage
8	71-80% leaf coverage
9	81-90% leaf coverage
10	91-100% leaf coverage

Conidia counts were taken 8 DPI. Count samples consisted of the infiltrated leaf washed in 5 mL of distilled water. Five millilitres of the resulting suspension was pipetted into a haemocytometer (0.1mL depth) [BRAND® counting chamber BLAUBRAND® Neubauer improved, Sigma - Aldrich] and conidia were counted in the four corner squares. The average of these four squares was multiplied by 10,000 to give a measurement in conidia mL^{-1}(n = 14-52 per treatment per cultivar). In El Dorado tray 1, once the leaf conidia count had been taken, 15 mL of distilled water (total 20 mL) was added to the leaf suspension and used to wash the remainder of the plant. Conidia counts were taken of the plant suspension using a haemocytometer (0.1mL depth) in the same method (n = 9-30 per treatment).

4.2.3. Data analysis

IBM SPSS Statistics for Windows, Version 23.0 was used for quantitative summaries and statistical analysis of results.

Graphs were produced using Microsoft Excel 2016, IBM SPSS Statistics for Windows, Version 23.0, or Minitab 17. Significant differences between means of normally distributed data; such as conidia counts, feature area intensity, dualex measurements and absorbance measurements, were determined with t-tests or ANOVAs (LSD post hoc). Significant differences between non-normally distributed data, such as rating scales, used non-parametric

tests including Mann-Whitney U and Kruskall-Wallis Following Kruskall-Wallis tests, multiple comparisons were made using Mann-Whitney U. To avoid the occurrence of a type I error, Bonferroni correction was applied (Armstrong, 2014). To determine differences between incidence counts, Fisher's Exact test, two-tailed was used.

The % control calculated by:$(Treatment/Control)\times 100$.

Degree of Infection (DoI) was used to summarise the final value of sporulation intensity, as a percentage of the maximum score for rating scales based on Lebeda and Petrzelova (2010); Townsend and Heuberger (1943).

$DoI = \Sigma(n\times v)\times 100/(x\times N)$

n Number in each rating category

v Rating x scale range

x Scale range

N Total number assessed

Rating scales were normalised to improve comparability between repeats and treatments. The normalised scale was presented as a ratio of the mean of control plants at final DPI [12 in LC-MS sets, 8 in infiltration sets]

Normalised rating = rating/(mean control rating at 8/12 DPI)

This attempted to account for the differences in disease pressure, by expressing the value as a percentage of the maximum rating gained in each repeat.

Results and Discussion

4.3. UV-B-induced total flavonoid level correlates with disease reduction

4.3.1. UV-B reduced downy mildew disease in all cultivars

Three lettuce cultivars [El Dorado, Salinas, Iceberg] were assessed for basal disease susceptibility and disease reductions following UV-B-pretreatment. El Dorado had the highest basal susceptibility, followed by Salinas and Iceberg. Susceptibility level was indicated by overall sporulation severity ratings expressed as DoI over time (Figure 4.1) as well as conidia counts (Figure 4.2) of control plants.

UV-B-pretreated lettuce plants had a significantly lower distribution of normalised sporulation ratings at late stage disease (10 to 12 DPI) in Salinas and at 8 and 12 DPI in Iceberg (Daily Mann Whitney U test). Reductions to disease did not cause overlap between cultivar susceptibility groupings.

Conidia count was significantly reduced (t-test, $p<0.05$) in UV-B-pretreated lettuce plants compared to control plants of each cultivar (Figure 4.2). Iceberg displayed the largest reduction (55%, $p = 0.002$), followed by Salinas (45%, $p = 0.008$) than El Dorado (35%, $p = 0.014$). UV-B-pretreatment in these lettuce cultivars can successfully reduce late stage disease, with the strongest evidence provided by conidia count.

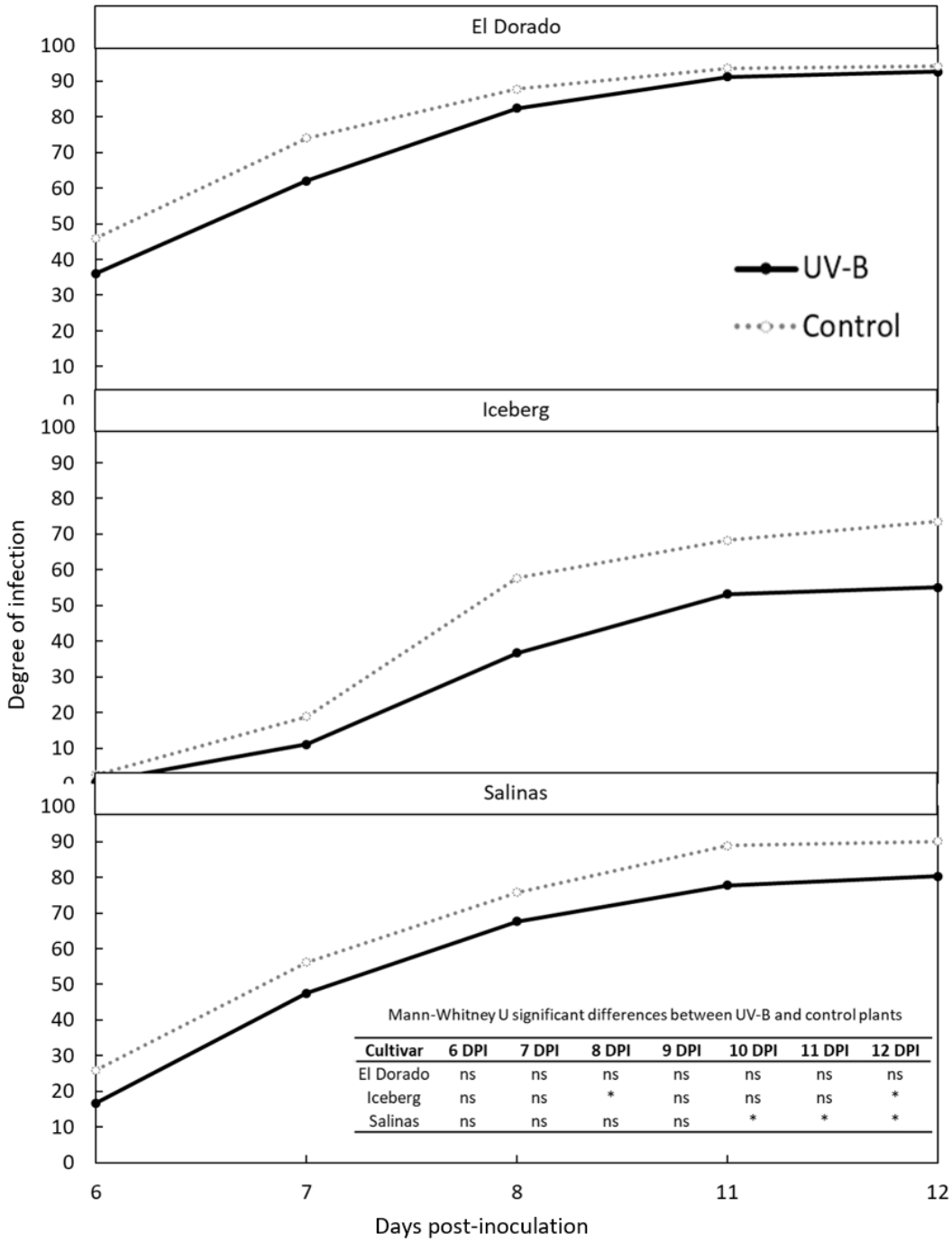


Figure 4.1.: The degree of infection (DoI) of downy mildew disease of UV-B-pretreated [black line] and control [grey line] plants of lettuce (*L. sativa*) cv. El Dorado, Iceberg and Salinas over time. Plants were treated with photosynthetically active radiation (PAR)+UV-B light or PAR only (control) for three days then inoculated with 10^5conidia mL^{-1} of *B. lactucae*. The Table shows significant differences in the distribution of normalised sporulation scale [Mann Whitney U; * = $p<0.05$, ns = non-significant) between UV-B and control plants from each cultivar on each disease day. DPI = days post-inoculation.

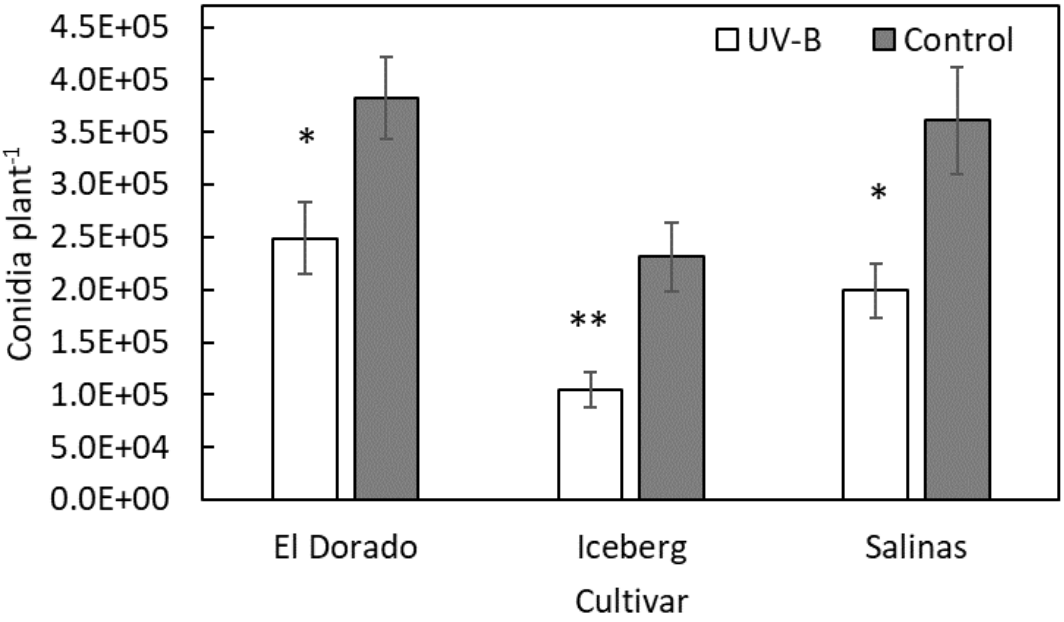


Figure 4.2.: The number of *B. lactucae* conidia harvested per lettuce (*L. sativa*) plant of El Dorado, Iceberg and Salinas was significantly lower in UV-B [grey bar] than control [white bar] plants. Plants were treated for three days of photosynthetically active radiation (PAR)+UV-B or PAR only (control) then inoculated with 10^5 conidia mL^{-1} of *B. lactucae.* At 12 days post inoculation-(DPI), plants were washed in water and the resulting conidia suspension was counted using a haemocytometer. Error bars are 1 SE. Asterisks indicate significant differences between control and UV-B of each cultivar according to a t-test where * = $p<0.05$ and ** = $p<0.005$.

4.3.2. UV-B-treatment increased flavonoid levels in all cultivars

Flavonoid levels were measured with a Dualex throughout the light (healthy plants) and disease (infected plants) period on the first true leaf of each selected plant. A dualex is a hand held, non destructive tool which uses light fluorescence and transmission through a leaf clip to estimate leaf chlorophyll content (Chl), epidermal UV-absorbance (Flv), anthocyanins and a Nitrogen Balance Index (NBI, patented) (Goulas et al., 2004). Flavonoid levels tended to increase over the light treatment period followed by a gradual decrease to levels similar to that at time 0 or lower during the disease period (Figure 4.3). A repeated measures ANOVA from 24 to 360 h indicated both cultivar ($p = 0.001$) and treatment ($p <0.0001$) had a significant effect on flavonoid level over the entire experiment, but not the cultivar-treatment interaction ($p = 0.30$). UV-B-treated plants have significantly higher (t-test, $p < 0.05$) flavonoid level than control plants of each cultivar at the following time points: El Dorado: 48 - 96 h, Iceberg: 48 - 240 h and Salinas: 72 – 168 h. At all other time points, there was no significant difference between flavonoid levels of UV-B-pretreated

and control plants of any lettuce cultivar.

An increase to flavonoid level is similarly induced by PAR + UV-B and PAR only (control) at 24 h (Figure 4.3). The earliest difference in flavonoids between control and UV-B occurs at 48 h (in El Dorado and Iceberg). At 48 h, UV-B-pretreated lettuce plants of El Dorado and Iceberg had significantly higher flavonoid levels than their respective controls. Therefore a higher induction of UV-B responsive flavonoids in UV-B-treated plants likely causes this difference at 48 h. Previous temporal studies of UV-B-induced flavonoids show initially (3 h) white light as well as UV-B increase flavonoid levels (Ballare et al., 1995). Only after 6 h do UV-B-specific induction of flavonoids occur (Ballare et al., 1995). These levels continue to increase for least 24 h. The experiment by Ballare et al. (1995) was completed on tomato seedlings and used a much higher dose (3.0 µmol $m^{-2}s^{-1}$) than my work which may explain the faster induction of flavonoids. Ballare et al. (1995)'s study supports evidence that both PAR and UV-B can cause overall flavonoid increases; however, UV-B is required for enhanced induction.

The delay in induction of UV-B responsive flavonoids is likely due to the process of UV-B-induced gene transcription and expression. It follows that maximum flavonoid levels were reached in this experiment during the disease period at 96 h in El Dorado and Salinas as induction of flavonoids is still occurring from gene expression at the light stage. Iceberg plants had a faster and more extreme induction of flavonoids than other cultivars. Iceberg plants reached a maximum flavonoid level at 72 h, with further UV-B-treatment providing no additional flavonoid induction.

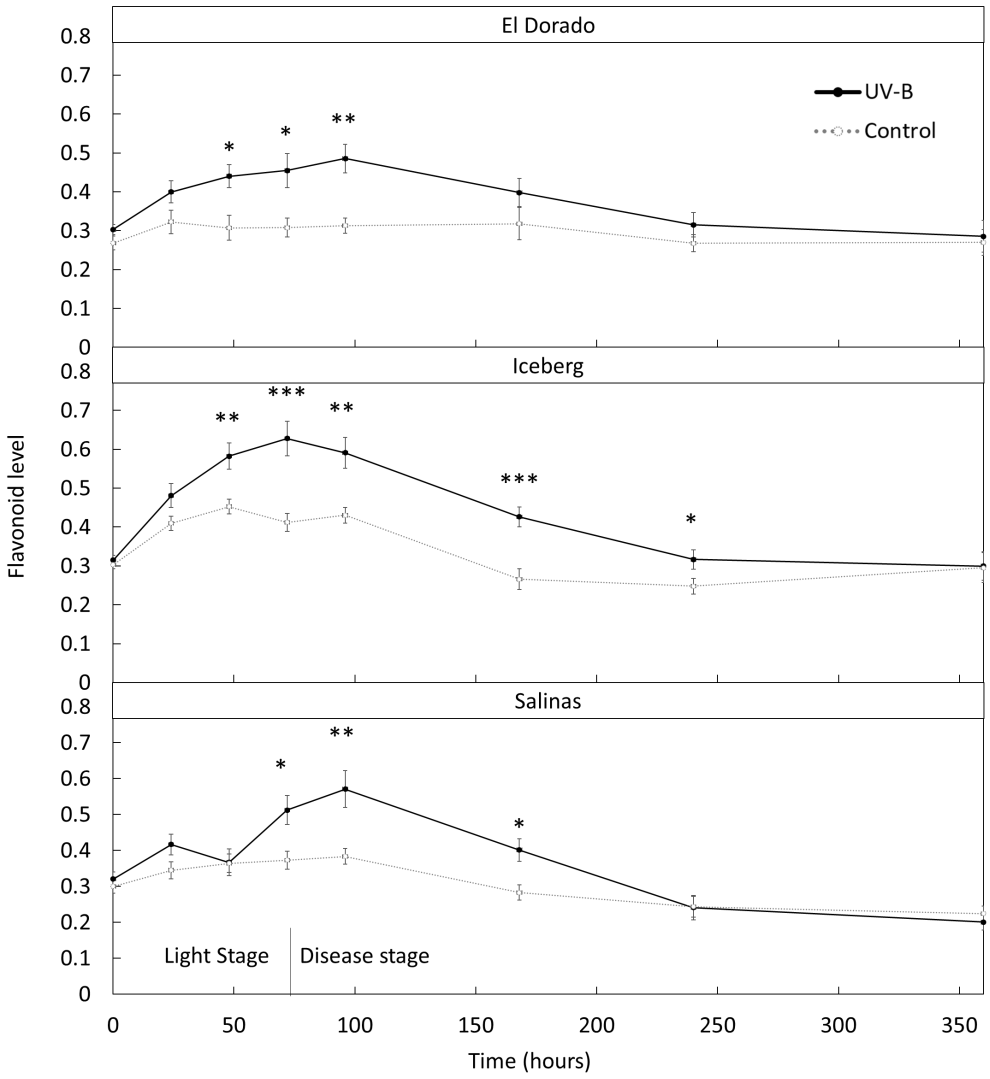


Figure 4.3.: Mean flavonoid level of UV-B [black line] and control [grey line] lettuce (*L. sativa*) plants of cv. El Dorado, Iceberg, and Salinas over time. Plants were treated with photosynthetically active radiation (PAR)+UV-B light or PAR only (control) for three days then inoculated with 10^5conidia mL^{-1} of *B. lactucae*. Flavonoid measurements were taken non-destructively with a dualex throughout the light (0-72 h) period and disease (96 - 360 h) period on the first true leaf of selected plants. Error bars indicate 1 S.E. Asterisks indicate significant difference between UV-B and control flavonoid levels according to daily t-tests where * = $p<0.05$, **=$p<0.005$, ***=$p<0.0005$.

A decrease in flavonoids in the disease period is most likely due to the removal of exposure to UV-B light. Iceberg is the most effective cultivar at maintaining flavonoid levels throughout disease period (til 168 h); however, by the end of disease period, UV-B-pretreated plants (of any cultivar) do not have significantly different flavonoid levels from control plants.

Salinas plants reached flavonoid levels below that of time 0 at the end of the disease

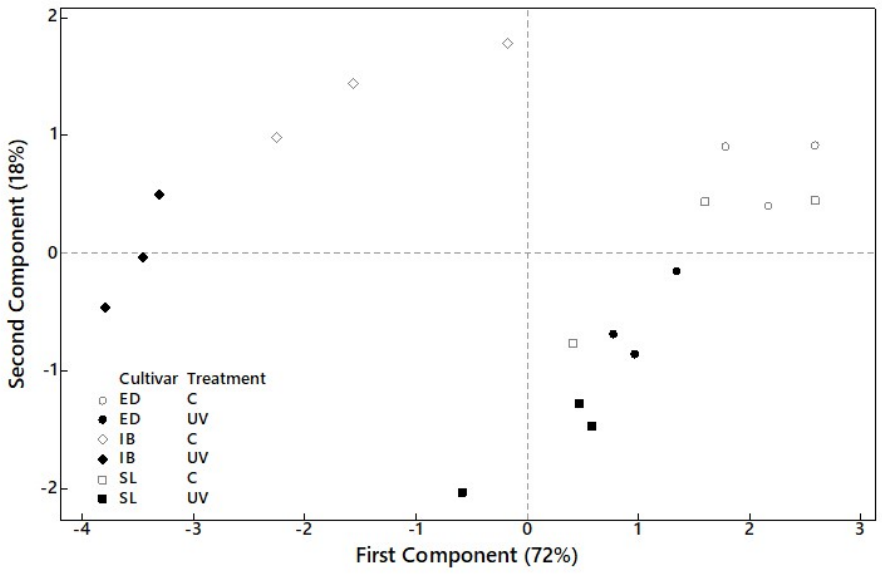
period. Previous studies have observed that flavonoids are down-regulated upon recognition of disease (Hahlbrock et al., 1981; Gleitz et al., 1991; Lo and Nicholson, 1998; McLusky et al., 1999), likely to partition resources to other defensive pathways (Schenke et al., 2011). Therefore there may be conflicting signals during the disease period in which UV-B induces flavonoid accumulation; however, disease infection induces down-regulation. The maintenance of flavonoids in the absence of disease can not be established for these cultivars from this experiment. However, given disease reduces flavonoid levels, I expect that UV-B-induced flavonoids would remain elevated for longer in the absence of disease.

4.3.3. There is a negative correlation between conidia count and flavonoid accumulation in UV-B-treated plants

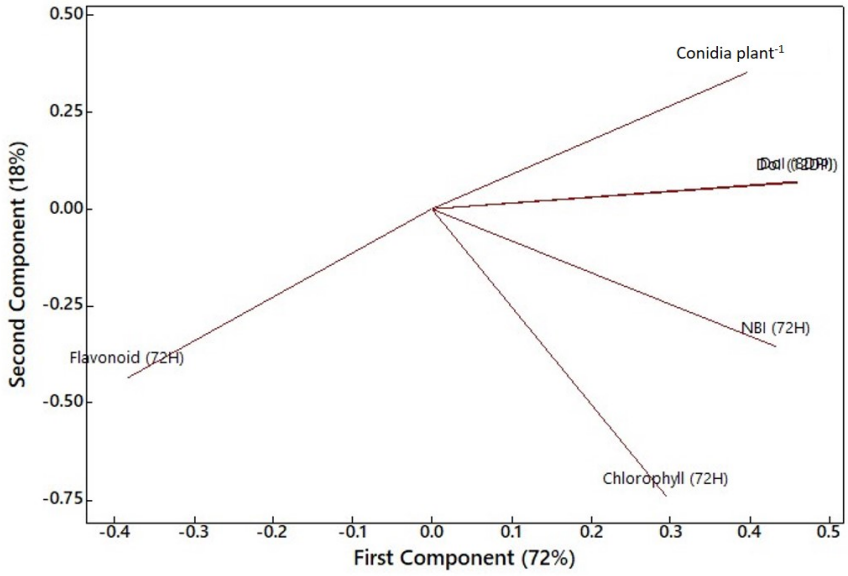
A principal component analysis of flavonoids, chlorophyll and NBI at 72 h and disease measures (DoI at 8 and 12 DPI and conidia count) revealed a strong negative relationship between flavonoid level and conidia count (Figure 4.4). Both cultivar and treatment drive this relationship as shown by separation along both the first (72% of total variance) and second (18% of total variance) component.

The scores along the first component separate Iceberg plants (negative) from the remainder of cultivars (positive) (Figure 4.4a). The first component also separates each cultivar individually by treatment (except Salinas repeat 1 control) where UV-B-treated plants have a lower score. There is separation of UV-B (lower score) and control plants along the second component within each cultivar as well (except Salinas repeat 1 control). The exception of a Salinas control (repeat 1) in both situations suggest it may be a possible outlier; however, an outlier plot did not support this with all points below the reference line.

Chlorophyll may also have a role in a disease defence with separation from conidia count along the 2nd component; however, as this only accounts for 18% of variance, flavonoid level at 72 h appears to have a greater impact on conidia count.



(a) Score plot of UV-B and control-treated lettuce cultivars based on pigment and disease measures. The first component accounts for 72% of the variation whilst the second component accounts for 18% of the variation. Cultivars: ED = Eldorado, IB = Iceberg, SL = Salinas. Treatments: C = control (photosynthetically active radiation (PAR) only), UV = UV-B +PAR.



(b) Loading plot of variables onto the 1st (72% of the variation) and 2nd (18% of the variation) component.

Figure 4.4.: A principal component analysis (PCA) evaluating the variables; flavonoids, chlorophyll, NBI at 72 h, DoI (8 DPI), DoI (12 DPI) and conidia count (12 DPI) of UV-B and control lettuce (*L. sativa*) plants of the cv. El Dorado, Iceberg and Salinas. Plants were treated with photosynthetically active radiation (PAR)+UV-B light or PAR only (control) for three days then inoculated with 10^5conidia mL^{-1} of *B. lactucae*. Flavonoid measurements were taken non-destructively with a Dualex throughout the light (0-72 h) period and disease (72 - 360 h) period. The resulting disease was assessed on a visual sporulation (S) scale and summarised as DoI as well as harvested for conidia count. NBI = nitrogen balence index, DoI = degree of infection, DPI = days post-inoculation.

Regression analysis showed flavonoid level at 72 h correlated negatively with both early (r = -0.688, p = 0.002) and late (r = -0,659, p = 0.003) stage sporulation ratings (DoI) as well as $\log_{10}$ conidia count (R= -0.812, p < 0.0005) (Figure 4.5). UV-B responsive flavonoids heavily drive this regression between $\log_{10}$ conidia count and flavonoid level. A regression analysis on control plants alone shows no significant regression (R= -0.491, p = 0.180); however, on UV-B plants alone, the regression is strengthened (R = -0.844, p = 0.004) (Figure 4.5). The higher level of flavonoids in UV-B-treated plants can be attributed to UV-B responsive flavonoids. Thus it can be argued that UV-B responsive flavonoids rather than general flavonoid contribute to the correlation and role in reduction of conidia count.

As flavonoid levels decrease over the disease period, preformed flavonoid defences and signals (phytoanticipins) at the point of inoculation (72 h) are likely the key time point for a maximum UV-B-induced flavonoid defence in plants. A significant negative correlation between conidia count ($\log_{10}$) and flavonoid level was present at 24 (R = -0.680, p = 0.002), 48 (R = -0.805, p <0.0001) and 96 (R = -0.756, p <0.0001) h. At these time points, preformed phenolics are still significantly higher in UV-B than control plants of most lettuce cultivars. At time points later then 96 h, where induced phenolics (phytoalexins) contribute to defence, the significant correlation is lost. The correlation between conidia count and flavonoid level was strongest at 72 h highlighting the importance of phytoanticipins in disease severity. This justifies further LC-MS analysis at 72 h.

4.3.4. Dualex flavonoid readings are a marker for LC-MS features

As LC-MS is an expensive and time consuming procedure, it would be extremely beneficial to use a quick non-destructive flavonoid assessment tool such as the dualex as an indicator of LC-MS features to evaluate the usefulness of LC-MS analysis. Dualex measures could provide an estimate of LC-MS feature intensity level of a treatment. Regression analysis was completed on all metabolic features identified by LC-MS (Table 4.5) and flavonoid levels as measured by a dualex (72 h).

Flavonoid levels at 72 h significantly correlated positively with total intensity of all LC-MS features (r = 0.517, p = 0.028), as well as feature IDs 7 (r = 0.611, p = 0.007), 17 (Quercetin-3-Glucuronide, r = 0.684, p = 0.002), and 18 (Kaempferol 3-glucuronide, r = 0.716, p = 0.001) (Figure 4.6). Higher flavonoid Dualex readings at 72 h would therefore indicate higher levels of total metabolites from LC-MS as well as the three metabolic

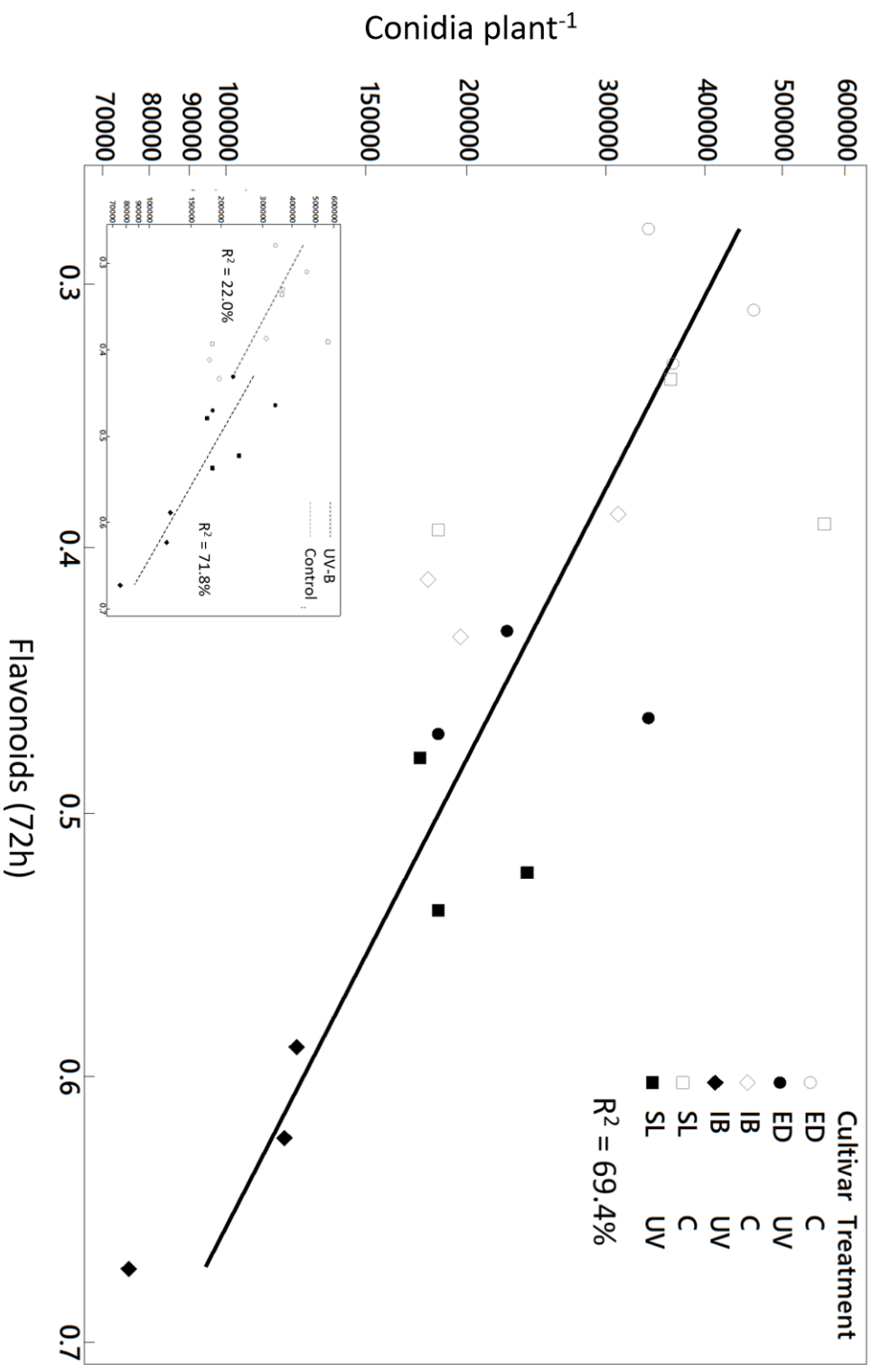


Figure 4.5.: $\log_{10}$ Conidia count of *B. lactucae* decreases as flavonoid level of infected lettuce (*L. sativa*) plants increases. Plants were treated for three days with photosynthetically active radiation (PAR)+UV-B or PAR only (control). At 72 h, plants were non-destructively measured for flavonoids using a Dualex and then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. At 12 days post-inoculation, plants were washed in water and the resulting conidia suspension was counted using a haemocytometer. UV-B [black points]-pretreated plants drive this response with the regression [dashed line] lost when control [white points] are considered separately [inset]. Scale axis labels apply to both main graph and inset. Cultivars are indicated by point shape [El Dorado (ED) = circle, Iceberg (IB) = diamond, Salinas (SL) = square]. Regression fit is indicated by the R^2 values.

features 7, 17 and 18. The fit (R^2) is much higher for UV-B plants (72 - 84%) than control plants (0.5 - 3%), when regression analysis is run by treatment. These regressions are therefore clearly driven by a UV-B effect. A more accurate indicator of LC-MS feature intensity is by considering UV-B-treatments which cause a large significant increase compared to the control as promising for high intensity of correlated LC-MS features.

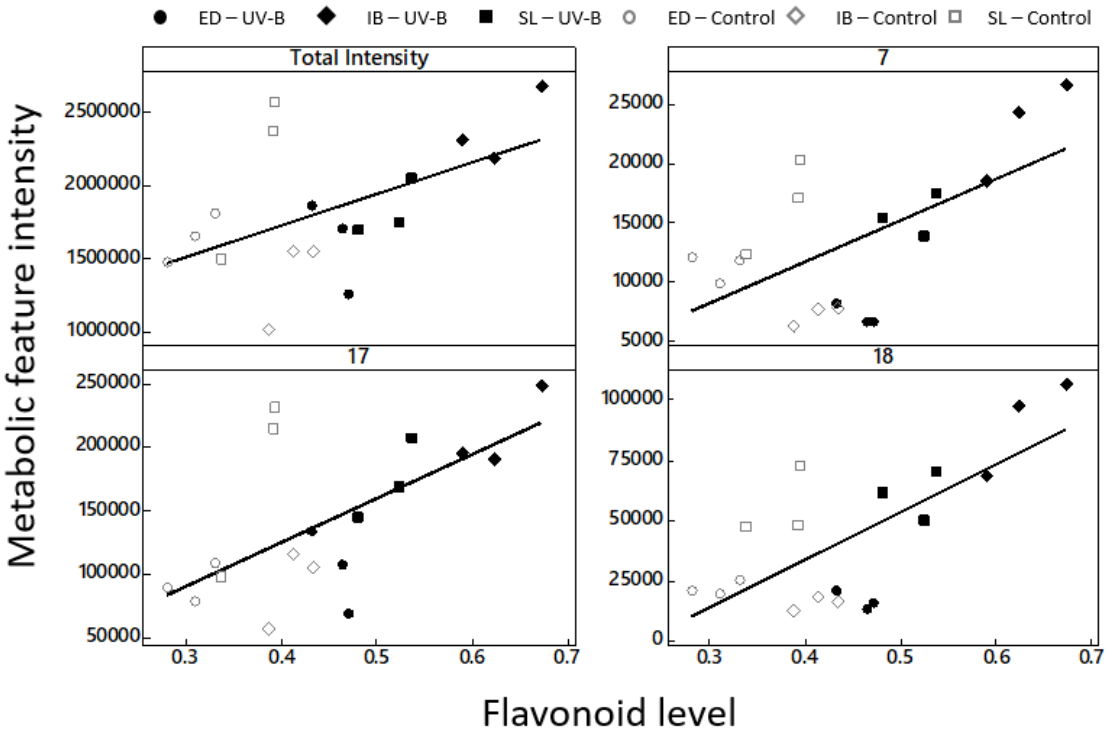


Figure 4.6.: Intensity of feature area [panel] against flavonoid level at 72 h. Lettuce (*L. sativa*) plants were treated with photosynthetically active radiation (PAR)+UV-B or PAR only (control) for three days. Immediately following the treatment period (72 h), a subset of plants were measured for flavonoid levels non-destructively using a Dualex. Another subset of plants were taken for LC-MS analysis at this same time-point. Treatment is indicated by colour [UV-B = black, control = white]. Cultivar is indicated by shape [El Dorado (ED) = circle, Iceberg (IB) = diamond, Salinas (SL) = square].

4.4. Ten metabolic features negatively correlate with disease severity in a UV-B dependent manner over three lettuce cultivars

To determine which flavonoids (and possibly other phenolics) contribute to the correlation with disease reduction found in Section 4.3.3, LC-MS analysis was completed. Lettuce plants [cv. El Dorado, Iceberg, Salinas] were treated with UV-B for three days then a subset frozen for LC-MS analysis (LC-MS-1). LC-MS analysis indicated UV-B influenced

metabolic features. I putatively identified several features, but others remain unknown. The remaining plants were inoculated with *B. lactucae* conidia and resulting disease severity assessed. Disease severity measures were then correlated with the intensity of major metabolic features from the LC-MS analysis. Strongly correlated features (r > +/- 0.5, P <0.05) may have a role in decreasing disease susceptibility in a UV-B-dependent manner.

4.4.1. The main pathways influenced by UV-B-treatment and cultivar were those in phenylpropanoid pathways

The pathways influenced by the interactions between UV-B and cultivar were determined using Mummichog version 1.1.6 (Li et al., 2013a) pathway analysis of LC-MS data (Table 4.4). The most affected pathways were those belonging to the phenylpropanoid pathway, including the flavonoid pathways; kaempferol glycoside biosynthesis (*Arabidopsis*), quercetin glycoside biosynthesis (*Arabidopsis*), luteolin glycosides biosynthesis, syringetin biosynthesis, anthocyanidin modification (*Arabidopsis*), and polyphenol ester pathway; chlorogenic acid (Figure 4.7). Sugar pathways stachyose biosynthesis as well as degradation, and ajugose biosynthesis II (galactinol-independent) were also significantly altered by treatment and cultivar interaction. The significant alterations to many flavonoids pathways are expected due to the significant increase to flavonoid levels found in my experiments. These sugars both belong to raffinose family oligoscaccherides (RFOs).

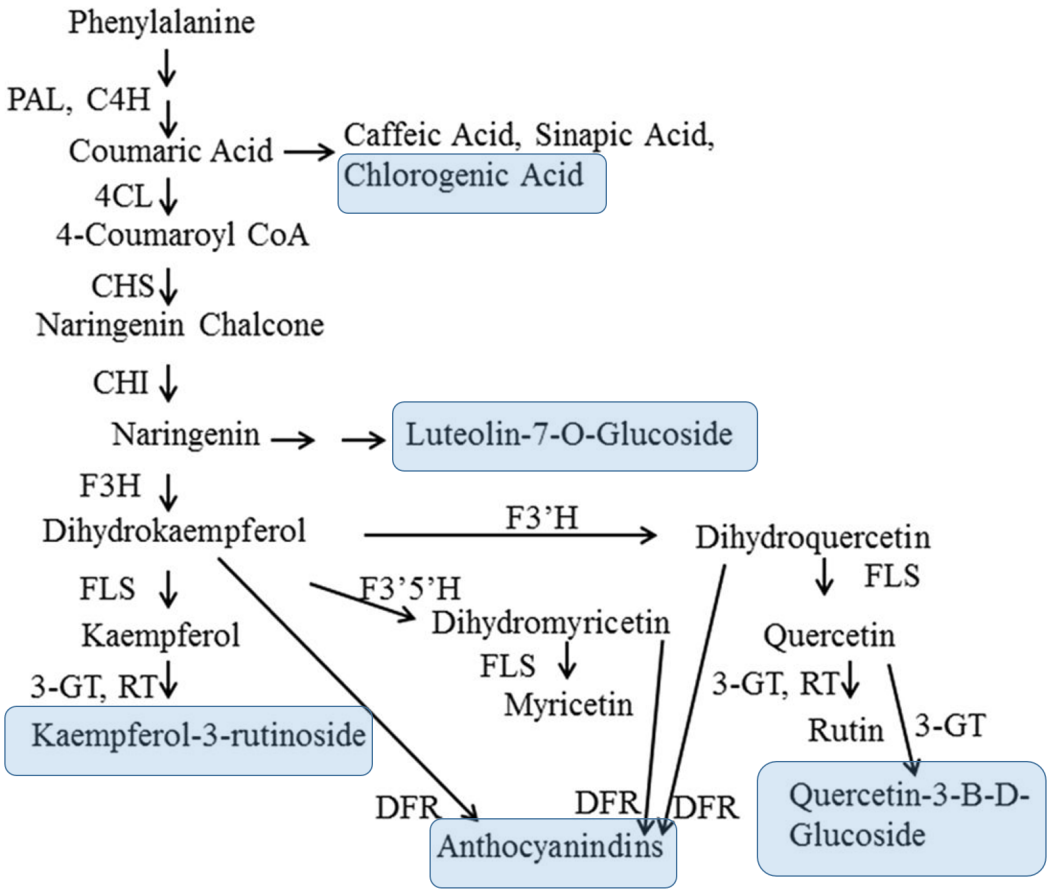


Figure 4.7.: The phenylpropanoid pathway with pathways influenced by UV-B and cultivar highlighted in blue. PAL: phenylalanine ammonia lyase; 4CL: 4-coumarate:coenzyme A ligase; C4H: cinnamate 4-hydroxylase; C3H: 4-coumarate 3-hydroxylase; CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone-3-hydroxylase; F3´H: flavonoid-3¢-hydroxylase; F3´5´H: flavonoid-3´5´-hydroxylase; FLS: flavonol synthase; DFR: dihydroflavonol reductase; 3-GT: flavonoid 3-O glucosyltransferase; RT: flavonoid 3-O-glucoside-rhamnosyltransferase. Image adapted from Lim and Li (2017).

Both phenylpropanoid and RFO pathways are responses to abiotic stress and are up-regulated by high light levels (Rossel et al., 2002). As an abiotic stress, UV-B likely has a major impact on the expression of these pathways. As was well established in the literature review (Section 1.3.3) the up-regulation of the phenylpropanoid is a typical response to UV-B exposure as a protective measure to the high energy light of UV-B. Flavonoids (kaempferol, quercetin, luteolin pathways) absorb UV-B light in the epidermis thus protecting deeper tissue and photosystems (Agati et al., 2013). Lignins (syringetin pathway) thicken the cell wall, thus reducing the level of radiation able to pass through (Hilal et al., 2004). Chlorogenic acid (polyphenol ester pathway) may have a role in UV-B protection as an antioxidant or as a precursor to lignin (Cle et al., 2008). Phenolic compounds are also well established to have a role in disease protection (Section 1.1.10.2).

It is likely that changes to RFOs are also a protective response to UV-B. Both stachyose and ajugose are RFOs (Sengupta et al., 2015) which are soluble sugars, with no energetic role, involved in stress tolerance mechanisms (Arbona et al., 2013). RFOs could control the balance of reactive oxygen species (ROS) created by UV-B exposure as either antioxidants or stress signals (Couée et al., 2006). Galactinol, and possibly other RFOs have been implicated in induced systemic disease resistance against *Botrytis cinerea* (Cho et al., 2010; Kim et al., 2008b). Although ajugose and stagyose are not specifically linked to disease resistance, they are part of the same RFO family.

As described in Section 4.4.2, cultivar also had a major effect on the basal metabolite level present and will influence significantly altered phenylpropanoid and sugar pathways.

Table 4.4.: Mummichog analysis LC-MS (Liquid chromatography–mass spectrometry) features indicating pathways influenced by treatment and cultivar. LC-MS samples were taken following three days of photosynthetically active radiation (PAR) + UV-B or PAR only (control) treatment on lettuce (*L. sativa*) cv. El Dorado, Iceberg and Salinas.

Pathways	Overlap size	Pathway size	p-value
stachyose biosynthesis	4	4	0.002
kaempferol glycoside biosynthesis (*Arabidopsis*)	5	7	0.004
quercetin glycoside biosynthesis (*Arabidopsis*)	6	10	0.005
luteolin glycosides biosynthesis	3	3	0.005
syringetin biosynthesis	3	4	0.013
chlorogenic acid biosynthesis I	3	4	0.013
ajugose biosynthesis II (galactinol-independent)	3	4	0.013
chlorogenic acid biosynthesis II	3	4	0.013
anthocyanidin modification (*Arabidopsis*)	2	2	0.024
phenylpropanoid biosynthesis	3	5	0.027
stachyose degradation	3	5	0.027
flavonoid biosynthesis (in equisetum)	4	9	0.048
flavonol biosynthesis	3	6	0.049

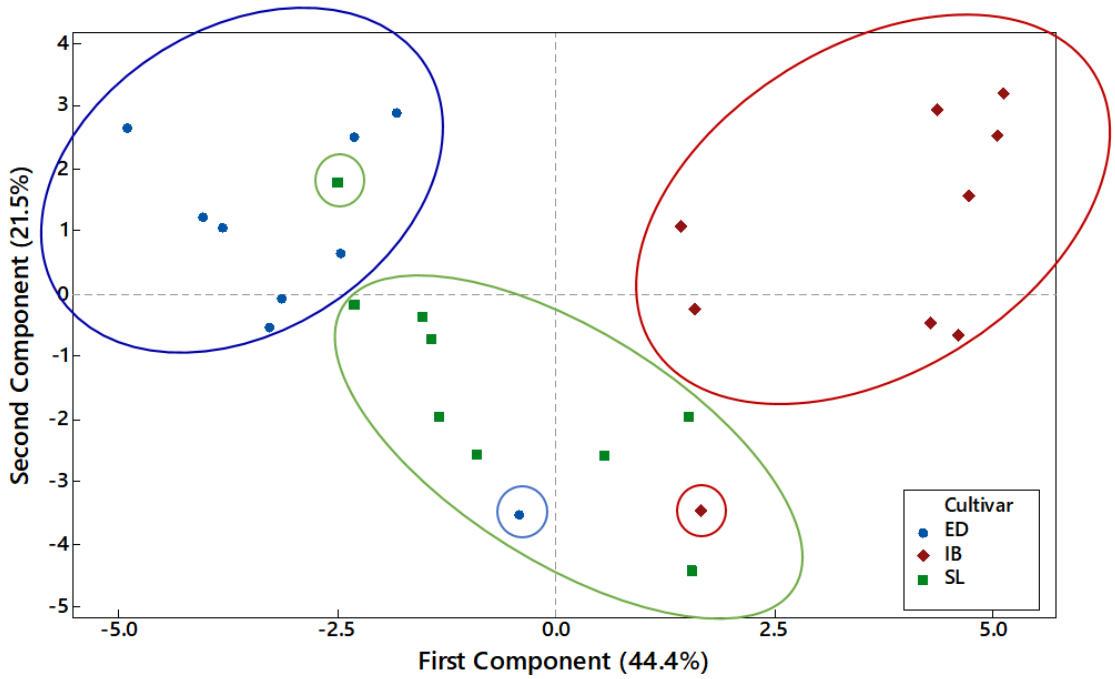
4.4.2. Cultivar had a major impact on level of metabolite expressed

Lettuce cultivars have different basal levels of metabolites. In control plants, an ANOVA indicated that 61% of LC-MS feature intensities significantly differed between cultivars (Between Groups significance $p<0.05$). Upon PCA of significantly altered features, the scores separated points into cultivars along the first and second component (Figure 4.8a).

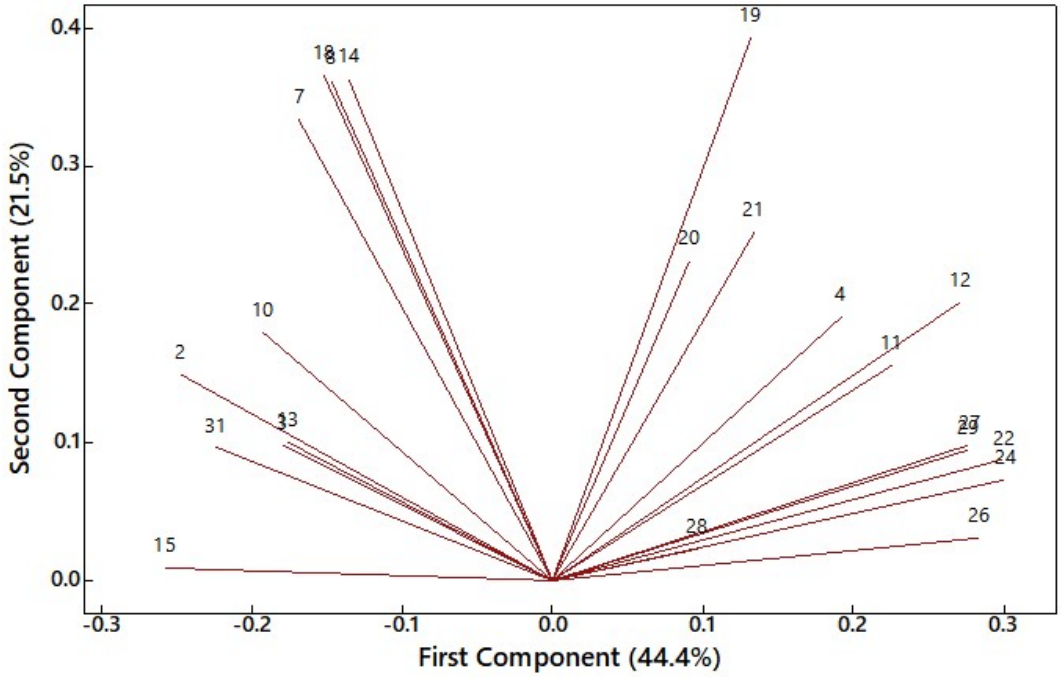
Cultivars formed relatively clean groups, with one exception for El Dorado and Iceberg (grouped with Salinas) and one exception for Salinas (grouped with El Dorado). The first

component (44.4% of variation) accounted for most of the separation between cultivar groups. El Dorado presented the most negative scores along the first component. Salinas fell in the middle of the first component with a group centre near 0. Iceberg had the most positive first component scores. El Dorado and Salinas groups had similar positive values along the second component (21.5 % of variation); however, differed from Salinas.

The separation of cultivars can be attributed to a number of features (Figure 4.8b). Feature IDs 12, 22, 24, 26, 37 and 29 (Table. 4.5) have the strongest positive scores along the 1st component, whilst features 2 and 15 have the most negative. The intensities of these features therefore likely have a large influence of the separation of the three cultivars along the first component. In terms of the influence on both first and second component, features 7, 8, 14 and 18 have a strong influence on El Dorado whilst features 19, 20, and 21 have a strong influence on Iceberg. The remainder of the features separate El Dorado and Iceberg from Salinas along the second component only.



(a) Score plot of lettuce cultivars El Dorado (ED), Iceberg(IB) and Salinas(SL) based on significantly different metabolic features



(b) Loading of scores of lettuce cultivars based on metabolic features

Figure 4.8.: Principal components analysis of lettuce (*L. sativa*) cultivars [El Dorado (ED) = blue, Iceberg (IB) = red, and Salinas (SL) = green] based on loadings onto significantly different (ANOVA, $p<0.05$) LC-MS feature groups. Plants were sampled for LC-MS after two weeks of growth under white fluorescent tubes [FL58W/965 super daylight deluxe] followed by three days of red and blue LEDS. Cultivar groups have been circled in their respective colour.

4.4.3. Putative identities of the major metabolic features in lettuce

Analysis of raw LC-MS data with XCMS (Gowda et al., 2014) revealed 1630 metabolic features (combinations of m/z and retention time). These formed 188 peak/feature groups. Using MZ mine (Pluskal et al., 2010) and MS-DIAL (Tsugawa et al., 2015) features with intensities over 1E3 were confirmed through comparison against the blank and examination of m/z patterns. The resulting 36 features (chromogram Figure 4.9, Table 4.5) were then given putative identities using MS-FINDER or database searches to match precursor MS and resulting MS/MS spectrum using METLIN (Guijas et al., 2018) and MoNA (http://mona.fiehnlab.ucdavis.edu/). These putative identities are given. In cases in which compounds have identified by spectral analysis of lettuce in previous literature references are provided. The majority of the putatively identified compounds were phenylpropanoids, especially flavonoids. Several later eluting compounds are likely to be lipids; however, identity is uncertain.

4.4.4. UV-B increased the abundance of many metabolic features present in lettuce

The metabolic features found in lettuce (*L. sativa*, cv. El Dorado, Iceberg and Salinas) were expressed at different intensities (Figure 4.10). The most intense features, indicating the highest abundance, were feature IDs 2, 6, 17, 18, 19 28, 31, 34, 35 and 36 (Table. 4.5). Although intensity indicates quantity, it does not indicate importance of the corresponding metabolite, as metabolites may be required in different amounts to cause a response. Following a UV-B-treatment, many features (3, 4, 5, 11, 15, 22, 24, 25, 26, 27, 29, 31, and 35) experienced little or no change in feature intensity of UV-B-treated compared to control lettuce plants (Figure 4.10). Many of these compounds, which were unaffected by UV-B, were altered by cultivar, which is discussed in Section 4.4.2. Several features (6, 9, 10, 13, 18, and 28) exhibited an overall higher intensity in UV-B-treated plants of each cultivar compared to control (pattern 1). Features which experienced a general UV-B increase had a range of putative identities including a phenolic acid, flavonoid and terpene.

Other features (16,17,19, 20 and 21) follow a pattern of both cultivar and UV-B effect (pattern 2). Pattern 2 features had higher feature intensity in Iceberg than El Dorado and Salinas in control plants. In UV-B-treated plants, all cultivars have a higher feature intensity than control plants. Putative identities of compounds which fall into pattern 2

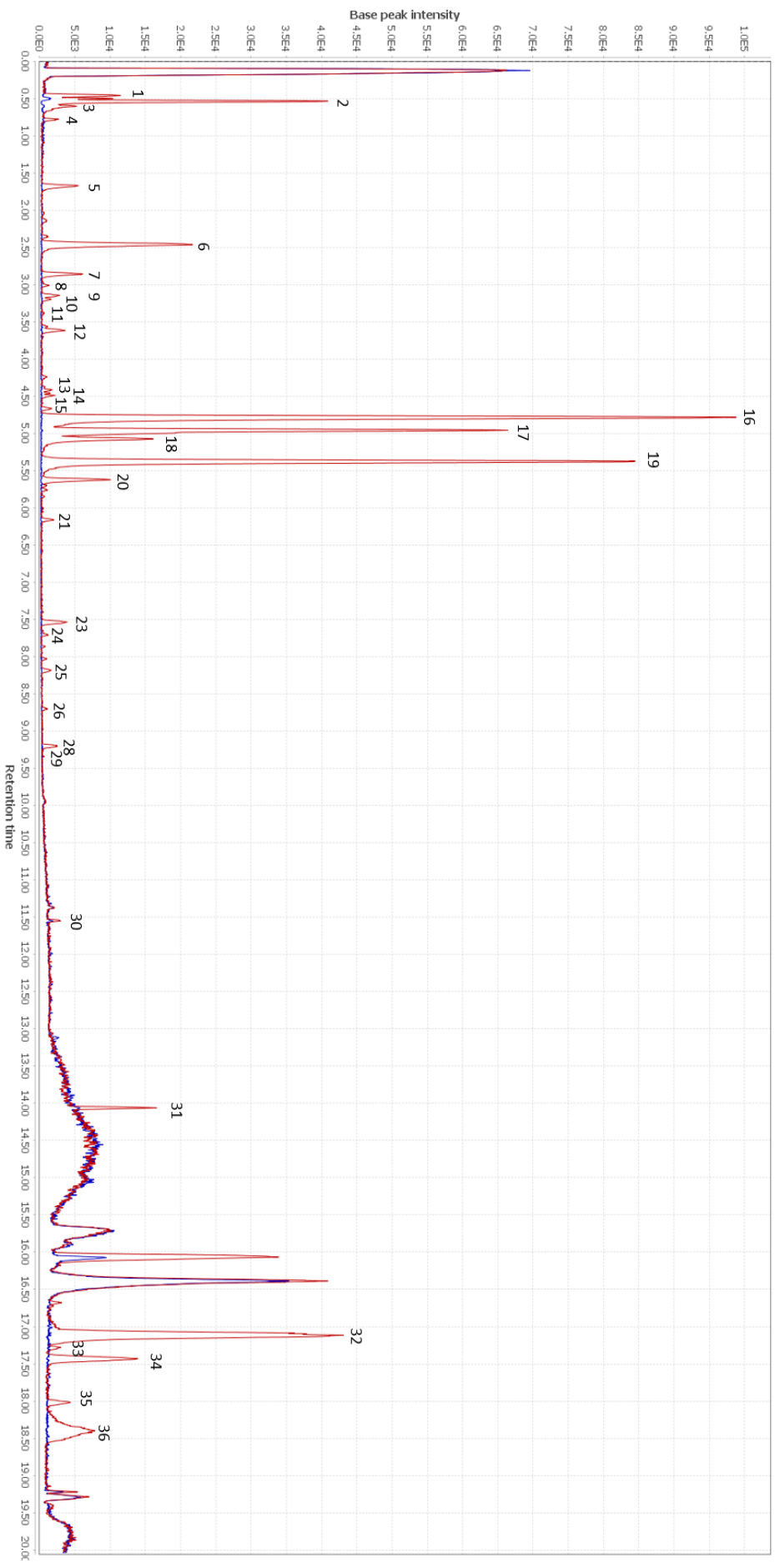


Figure 4.9.: Base feature chromatogram of feature intensity over retention time of an example sample (UV-B-treated Iceberg, rep 4) [red] and blank [blue] to represent features present in LC-MS (Liquid chromatography–mass spectrometry) data. LC-MS samples were taken from lettuce (*L. sativa*) cv. El Dorado, Iceberg and Salinas lettuce plants after three days of photosynthetically active radiation (PAR) + UV-B or PAR only (control) treatment. Numbers indicate true major features.

Table 4.5.: Putative identification of LC-MS (Liquid chromatography–mass spectrometry) feature groups. Lettuce (*L. sativa*) plants were treated with photosynthetically active radiation (PAR)+UV-B or PAR only for three days. Following treatment, samples were taken for LC-MS analysis with a negative polarity. Feature number is given as feature identifier throughout this Chapter. Retention time (RT) is given for LC-MS set 1 and set 2. Putative identification of adducts is given, with M-H mass charge (m/z) noted. If no evidence for M-H is found, then the highest intensity m/z is provided. Predicted formula and structure identity was determined using MS-DIAL and MS-FINDER. The corresponding error and score are given from these programs. In the case (**) where identity was determined through databases (Metlin and Mass Bank), no score is given. Two features (*) which strongly identify as chlorogenic acid were present. Features with grey coloured texts had low intensities, or were present in only some samples, so have been labelled as real features, but certainty is reduced. Identification of some compounds were confirmed by previous studies in lettuce. These are labelled as 1 : Ribas-Agustí et al. (2011), 2: Garcia et al. (2016), 3: García et al. (2017), 4: Sessa (2000), 5: Yang et al. (2018), 6: Ferreres et al. (1997) and 7: Romani et al. (2002).

Feature ID	RT LC-MS1	RT LC-MS2	M-H M/Z	Predicted Formula	Error [mDa]	Score (max = 5)	Predicted compound	Score (Max = 10)	Most abundant adducts present (m/z:adduct)	Reference
1	0.46	0.48	304.91	Unknown			Unknown		174.9527, 304.9147, 418.8948, 548.8559, 678.8115, 808.7748	
2	0.54	0.56	341.12	C12H22O11	-1.16	3.42	Sucrose	6.12	377.0863, 439.0882	
3	0.61	0.63	191.02	C6H8O7	-0.37	3.30	Citric acid	6.08	133.0082, 421.0027, 275.0219	
4	0.78	0.68	405.03	C18H14O9S	-0.62	2.69	Unknown		436.9673, 383.0479, 426.9673	
5	1.69	1.70	311.05	C13H12O9	-0.74	3.11	Caffeoyltartaric acid	5.22	149.0116 (M-162), 179.0400, 623.0938 (2M-H)	1, 2, 3,
6	2.47	2.63	353.10	C16H18O9	-0.19	3.14	Chlorogenic acid *	5.52	191.0635 (M-162), 707.1901 (2M-H), 1061.2890 (3M-H), 729.154 (2M-2H+Na)	1, 2, 3, 5, 6, 7
7	2.88	-	725.13	Unknown			Unknown		787.0558	
8	3.01	-	711.14	Unknown			Unknown			
9	3.16	3.12	353.09	C16H18O9	-0.89	3.33	Chlorogenic acid *	5.91	191.0637 (M-162)	1, 2, 3, 5, 6, 7
10	3.21	3.17	373.15	C17H26O9	1.71	3.21	Deoxyloganin	5.47		
11	3.39	3.31	431.18	Unknown			Unknown		198.9358, 283.2703	
12	3.65	3.56	295.05	C13H12O8	-0.36	3.30	Caffeoylmalic acid	5.79	133.0152 (M-162) 591.1070(2M-H)	1, 2
13	4.43	4.28	471.19	C22H32O11	-1.01	3.12	Possible Phenolic glycoside			
14	4.50	4.43	463.09	C21H2O12	-1.30	3.37	Quercetin 3-galactoside	6.13	611.2421	5, 6, 7
15	4.67	4.54	427.20	Unknown			Unknown			
16	4.79	4.98	473.08	C22H18O12	-0.45	3.25	Dicaffeoyltartaric acid / Chicoric acid	5.43	311.0487 (M-162), 947.1581 (2M-H)	1, 2, 5, 6, 7
17	5.00	5.09	477.33	C21H18O13	-0.14	2.98	Quercetin-3-Glucuronide (Miquelianin)	**	311.0396, 301.0363	1, 5, 6 , 7
18	5.09	5.25	461.07	C21H18O12	-1.25	3.39	Kaempferol 3-glucuronide	6.06	923.1616 (2M-H), 311.0479, 285.0407(M-H-C6H8O6), 483.0544(M-2H+Na)	
19	5.39	5.50	549.09	C24H22O15	0.76	3.13	Quercetin 3-O (6-malonyl)-glucoside	5.37	505.0993 (M-H-CO2), 1099.1945 (2M-H), 611.0154 (M-iospropH), 1143.12, 1122.2, 1153.103, 1396.706	1, 5, 6, 7
20	5.62	5.70	515.13	C25C24O12	-0.90	3.35	3,5-Dicaffeoylquinic acid	5.59	353.0959 (M-162), 1031.2572 (2M-H)	1,2, 5, 6, 7
21	6.16	6.29	533.09	C24H22O14	0.87	**	Luteolin 7-O (6" malonyl glucoside)	**	489.1109 (M-H-CO2)	
22	6.91	6.77	415.18	C20H32O9	-2.14	3.16	Ethyl 7-epi-12-hydroxyjasmonate glucoside	5.09	327.0833	
23	7.54	7.56	481.12	C25H22O10	-0.58	2.87	lactucopicrin 15-oxalate	**	963.2419 (2M-H), 409.1382 (M- CO2CO-H), 257.0914 (M- 4-hydroxyphenylacetyl - H)	2,4, 5
24	7.72	7.35	347.18	C16H28O8	-1.86	3.11	Unknown			
25	8.21	8.16	581.17	C30H30O12	-3.55	3.09	Epicatechin 3-O-(2-trans-cinnamoyl-beta-D-allopyranoside)	5.02		
26	8.71	8.34	349.19	C16H30O8	-0.11	3.17	methyl 9-(alpha-D-galactosyloxy)nonanoate	5.24	417.2174	
27	9.01	8.69	461.24	C22H38O10	2.52	3.10	Unknown			
28	9.22	8.83	377.18	Unknown			Unknown		809.2898, 1187.4960, 755.3709 (2M-H), 1177.5026, 778.3550, 291.1820, 333.1916 (M-H-CO2), 799.2918, 485.1123, 391.1956	
29	9.35	9.05	417.21	C24H34O6	-3.80	3.07	Unknown			
30	11.55	-	293.18	Unknown			Unknown		236.1049, 221.1540	
31	14.08	13.63	271.19	Unknown			Unknown		371.1165, 565.3711, 471.0350, 199.1696	
32	17.15	16.14	819.53	Unknown			Unknown		836.5200, 891.4600	
33	17.29	16.23	714.55	C40H77NO9	3.46	2.51	Nitrogen containing lipid	**	760.5576 (M+FA-H), 777.5508 (M+CHOONH4-H), 832.4901	
34	17.44	16.45	959.60	Unknown			Unknown		976.5923, 1027.5907, 1031.5367	
35	18.00	16.66	821.55	Unknown			Unknown		893.4773, 838.5316	
36	18.39	17.00	837.48	C40H72N7O18P3S	-5.54	2.64	Nitrogen containing lipid	**	937.4068, 905.4744, 922.4683	

are all phenolic compounds including phenolic acids (chicoric acid and 3,5-Dicaffeoylquinic acid) or flavonoids (Quercetin-3-Glucuronide, Quercetin 3-O (6-malonyl)-glucoside and Luteolin 7-O (6" malonyl glucoside)). Pattern 2 is of interest as it is forms a pattern antagonistic to that of disease in which Iceberg has a lower disease severity than both El Dorado and Salinas, then following UV-B-treatment, all disease is reduced (Figure 4.2). This makes features with pattern 2 quite promising in terms of negative correlations with conidia count.

Another common instance (features 2, 7, 8 and 14) of combined cultivar and UV-B effect is an elevation in feature intensity in El Dorado plants, as well as increased levels of all cultivars following UV-B-treatment (pattern 3). Putative identities of features which formed pattern 3 were largely unknown; however, two compounds were putatively identified as sucrose and Quercetin 3-galactoside. Although there were many features driven by both El Dorado and UV-B effect (pattern 3), as El Dorado was the most susceptible cultivar, these features do not form a pattern of interest. As increases to these features were higher in more susceptible cultivars, they are unlikely to have a role in disease defence.

UV-B resulted in the decrease of feature intensity in a number of features (30, 33, 34 and 36); however, was less common than UV-B up-regulation. Some features had individual cultivar characteristics and therefore did not fall into a pattern. This includes increases to a feature intensity in one cultivar with no change (feature 12) or a decrease (feature 1, 23 and 32) in the others.

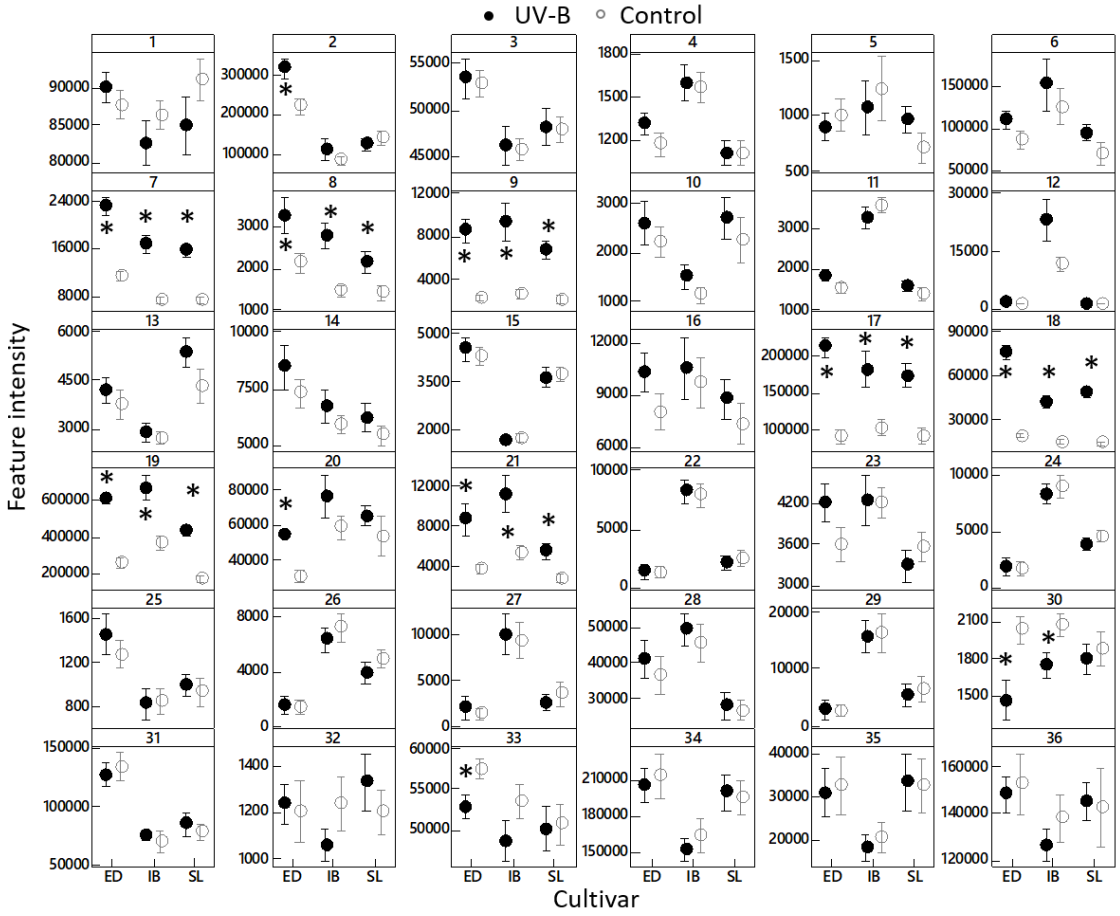


Figure 4.10.: Intensity of all identified features (Table 4.5) in UV-B [black] and control [white] plants of lettuce (*L. sativa*) cv.; El Dorado (ED), Iceberg (IB) and Salinas (SL). Plants were treated with photosynthetically active radiation (PAR)+UV-B or PAR only (control) for three days. Following treatment, samples were extracted for Liquid chromatography–mass spectrometry (LC-MS) analysis. Significant differences between UV-B and control plants of each feature [panel] are indicated by an asterisks (t-test, * = $p<0.05$). Error bars are 1 S.E.

4.4.5. Several UV-B-induced metabolites had a strong negative correlation with disease severity

A bivariate correlation analysis was run to determine relationships between disease severity and metabolite level (as feature intensity) across all cultivars and treatments. The significant correlations with a Pearsons correlation (r) over 0.5 (positively or negatively) between conidia count and feature intensity are shown in Table 4.6. All significant correlations (except feature ID 33) were negative indicating increases in feature intensity correlate with decreases in disease severity. The feature intensity of feature 33; however, had a positive correlation with not only conidia count, but also disease ratings as DoI at

early (8 DPI) and late (12 DPI) stage disease. The strongest negative correlations with conidia count were at features 11, 19, and 20. Feature 11 had relatively low intensity values, with a noisy mass spectrum, so confidence in accuracy of feature values is not as strong as other feature groups. Features which correlated with conidia count also tended to correlate with early and late DoI values, with the strongest correlations to sporulation rating as features 11, 22, and 24.

Table 4.6.: Correlation statistics between disease severity measures and feature intensity across cultivars and treatments. Lettuce (*L. sativa*) cultivars [El Dorado, Iceberg and Salinas] were treated with photosynthetically active radiation (PAR)+UV-B or PAR only (control) for three days. Following treatment, samples were taken for Liquid chromatography–mass spectrometry (LC-MS), with the remaining plants inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. Disease symptoms were assessed with a rating scale (represented by degree of infection (DoI)) and harvested for conidia count at 12 days post-inoculation (DPI). A Bivariate correlation analysis was carried out on all LC-MS features and disease symptoms. Information on feature IDs is provided in Table 4.5. Significant correlations ($p<0.05$) with a pearsons value over +/- 0.5 between feature intensity and conidia count are shown in the Table. Significance level is indicated by asterisks where * = $p<0.05$, ** = $p<0.005$, and *** = $p<0.0005$ or ns (not significant).

Feature ID	DoI 8DPI		DoI 12DPI		Spore Count	
	Pearson Correlation (r)	Sig. (2-tailed)	Pearson Correlation (r)	Sig. (2-tailed)	Pearson Correlation (r)	Sig. (2-tailed)
9	-0.47	*	-0.41	ns	-0.51	*
11	-0.72	**	-0.70	**	-0.62	*
17	-0.35	ns	-0.30	ns	-0.52	*
19	-0.53	*	-0.52	*	-0.68	**
20	-0.73	**	-0.68	**	-0.61	*
22	-0.75	***	-0.79	***	-0.55	*
24	-0.75	***	-0.77	***	-0.56	*
27	-0.66	**	-0.69	**	-0.53	*
29	-0.62	*	-0.68	**	-0.52	*
33	0.52	*	0.48	*	0.55	*

Visualisation of these correlations as linear regressions in scatter graphs provide some insight into how cultivar and treatment affect these significant correlations (Figure 4.11). Regressions of features 9, 17, and 19 (Table 4.5) are influenced by treatment. Control plants group in the top left due to a high conidia count and low feature intensity whilst UV-B group in the bottom right with a low conidia count and high feature intensity (opposite pattern for feature 33). When the two treatments are combined, this causes a strong correlation. However, when separated into treatments, several features (9, 1, and 19) lack a correlation with conidia count within control or UV-B. Other features (11, 22, 23, 24, 27 and 29) retained a significant negative correlation when only the UV-B-treated plants are considered. Feature 11 regression is also influenced by cultivar effect, largely due to the high flavonoid level and low conidia count of Iceberg plants. With the removal of Iceberg,

correlation of feature 11 and conidia count remains significant (r = -0.757, p = 0.004). The equations of the linear slope are similar between all negative regressions.

The role of cultivar and treatment in correlations between disease assessment (8 and 12 DPI DoI, conidia count) and feature intensities was further investigated through a PCA (Figure 4.12). UV-B and control plants are heavily separated diagonally across the first (60.3% of variation) and second (20.7% of variation) component (Figure 4.12a). This diagonal separation between treatments is driven between the negative correlation between disease and features 9, 17, 19, 20 (Figure 4.12b, Feature IDs; Table 4.5). These features therefore likely have a major role in a UV-B-induced disease defence.

The remaining features (11, 21, 23, 24, 27, 29) form a group high in both the first and second component. These negatively correlate with disease severity (and feature 33) along the first component only and therefore are likely informative about the separation of Iceberg from the other cultivars. Whilst these features might be important for disease defence, they are more likely to be driven by the low disease susceptibility of Iceberg plants rather than a UV-B-treatment.

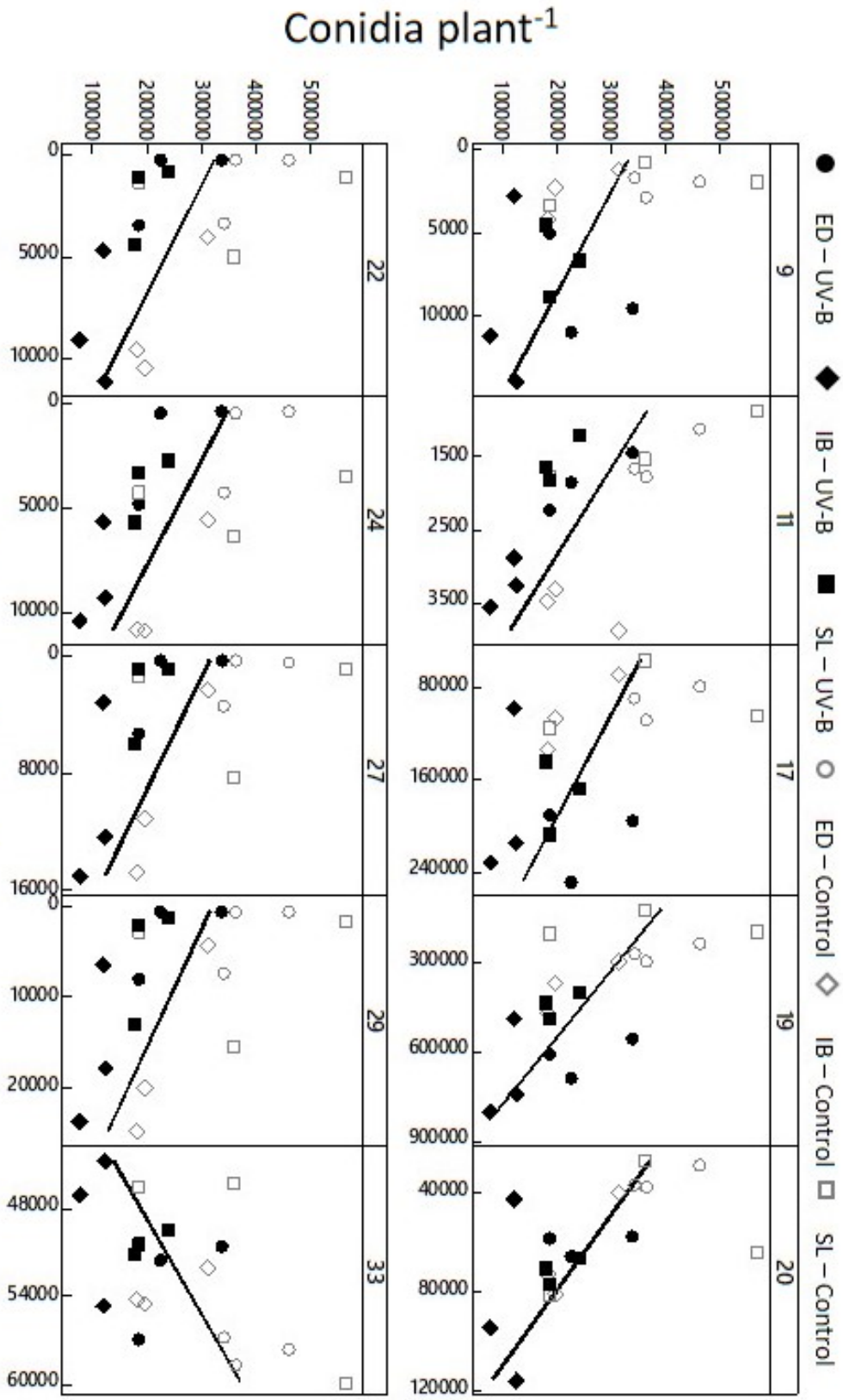
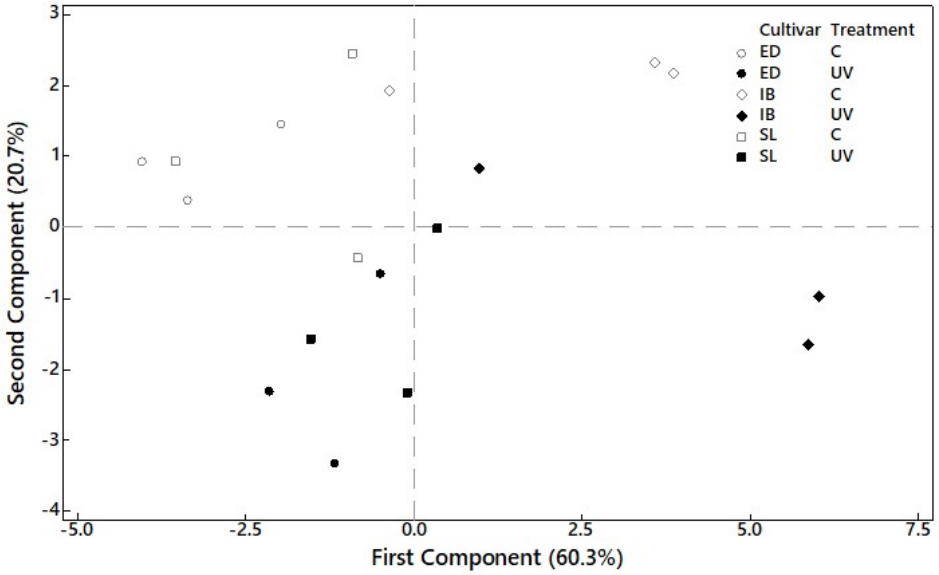
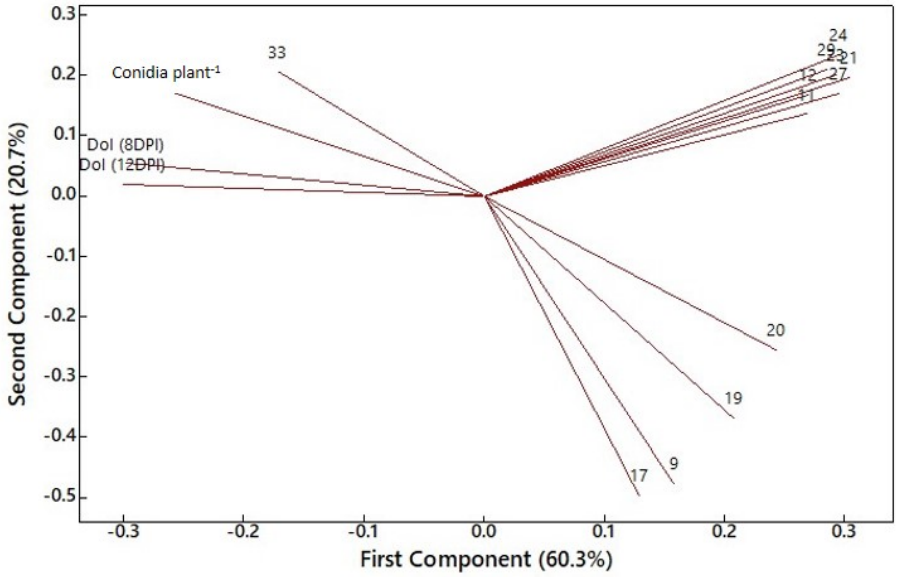


Figure 4.11.: Scatter graphs of the feature area intensity against conidia count of feature groups identified by their feature number [panels]. Lettuce (*L. sativa*) plants were treated with photosynthetically active radiation (PAR) +UV-B or PAR only (control) for three days. Following treatment, samples were taken for Liquid chromatography–mass spectrometry (LC-MS) and the remaining plants were inoculated with 10^5 conidia mL^{-1} *B. lactucae*. At 12 days post inoculation, plants were harvested for conidia count and the resulting data correlated with LC-MS feature intensity. Colour indicates treatment [UV-B = black, control = white] and shape indicates cultivar [El Dorado (ED) = circle, Iceberg (IB) = diamond, Salinas(SL)= sqaure]. Linear regression is shown by a black line.



(a) Score plot of treatment [UV-B =black, control = white] and cultivar [El Dorado (ED)=circle, Iceberg (IB)=diamond, Salinas (SL)=square] based on disease assessment variables (degree of infection at 8 and 12 dasy post-inoculation and conidia count) as well as significantly correlated feature groups.



(b) Loadings of scores of treatments and cultivars onto disease assessment and significantly correlated features

Figure 4.12.: A principal component analysis evaluating disease assessment and significantly correlated metabolite features of UV-B [Black] and control [white] lettuce (*L. sativa*) plants of the cv. El Dorado (ED) [circle] , Iceberg (IB) [diamond] and Salinas (SL) [square]. Plants were treated with UV-B light for three days. Following treatment, samples were taken for Liquid chromatography–mass spectrometry (LC-MS) and the remaining plants were inoculated with 10^5 conidia mL^{-1} *B. lactucae*. Disease was assessed for visual sporulation rating at 8 and 12 days post-inoculation (DPI). At 12 DPI, plants were also harvested for conidia count and the resulting data correlated with LC-MS feature intensity.

4.4.5.1. Putative identities of correlated features have roles in both UV-B and disease protection

Previous studies have identified roles in both UV-B protection and disease defence of many of the putative identities of LC-MS features which correlate with a reduction in conidia count. These studies provides further evidence towards the identification of the metabolite as well as proposing a reasoning for the correlation. Of the significantly correlated features; 11, 24, 27 and 29 were unable to be confidently identified (Table. 4.5)

Feature 9 was putatively identified as chlorogenic acid. Chlorogenic acid is commonly up-regulated by UV-B light (Lavola, 1998; Hagen et al., 2007; Lancaster et al., 2000; Romani et al., 2002) and CA accumulation has been associated with increased protection against UV light (Cle et al., 2008). Chlorogenic acid has also been associated with disease resistance. Cultivars with higher resistance to rot fungus (*Monilinia fructicola*) had a higher level of chlorogenic acid compared to susceptible cultivars in peach (Bostock et al., 1999), bilberry (Koskimaki et al., 2009), maize (Atanasova-Penichon et al., 2012) and potatoes (Ngadze et al., 2012). Although chlorogenic acid itself is non-toxic to pathogens, it can be converted into compounds with higher anti-fungal activity (Fawcett and Spencer, 1968) which would likely contribute towards a higher resistance. Chlorogenic acid derivative; 3,5-Dicaffeoylquinic acid (isochlorogenic acid) (feature 20) was significantly higher in lettuce grown outdoors (UV-B present) than glasshouse lettuce (UV-B absent) which may be due in part to UV-B levels, but is likely influenced by other variations between these two growth environments (Romani et al., 2002). Moglia et al. (2008) show dicaffeoylqunic acids in artichoke can be increased by UV-C light; however, other observations of UV-B induction are not readily available. 3,5-Dicaffeoylquinic acid has been implicated in disease defence in sunflower as well as insect defence in lettuce (Cole, 1984; Andary et al., 1996). In leaf disk assays by Zhu et al. (2004), both chlorogenic acid and 3,5-Dicaffeoylquinic acid had direct antimicrobial activity on bacterial and fungal pathogens. As quantification of UV-B-induced levels of these compounds was not completed in this work, the levels required for antimicrobial activity and those induced by UV-B cannot be compared. UV-B-induced chlorogenic acid 3,5-Dicaffeoylquinic acid may have role in reducing susceptibility through recruitment of other defences or direct antimicrobial activity.

Quercetin and derivatives are well established as up-regulated by UV-B light (Kuhlmann

and Muller, 2009; Josuttis et al., 2010; Higashio et al., 2005; Tattini et al., 2000; Scharff et al., 2012; Bidel et al., 2015; Becker, 2016). Although features 17 and 19 (Quercetin-3-Glucuronide and Quercetin 3-O (6-malonyl)-glucoside respectively) may not be specified in all these cases, such overwhelming evidence for quercetin up-regulation supports that these compounds are likely also up-regulated by UV-B. Quercetin (and some derivatives) have also been implicated in increased disease resistance (Tao et al., 2010; Jia et al., 2010; Sanzani et al., 2010). Several studies have suggested a mode of action of Quercetin and derivatives. For example, quercetin-3-galactoside has been shown to inhibit germtube elongation of *Botrytis cinerea in vitro* (Tao et al., 2010). Other studies suggest that quercetin has no direct anti-fungal action (Sanzani et al., 2008), but acts to enhance host resistance such as application of quercetin onto apples enhancing resistance to *P. expansum* (Sanzani et al., 2010). A possible role of quercetin is enhancing resistance as a pro-oxident (Jia et al., 2010). Quercetin, although more commonly known as an anti-oxident, can increase H_2O_2 levels resulting in the activation of defensive responses such as PR1 and PAL induction (Jia et al., 2010).

Feature 22 was putatively identified as Ethyl 7-epi-12-hydroxyjasmonate glucoside. Hydroxyjasmonates have been identified in lettuce in previous studies (García et al., 2017); however, not this particular type. The identification score was over 5 (out of 10); however, confidence in identification is lower for feature 22 than other compounds. UV-B induction of hydroxy jasmonate glucosides is not well documented. Hydroxy jasmonate glucosides are part of the jasmonate family, but their specific role in signalling and disease is unknown (Gidda et al., 2003). Ethyl 7-epi-12-hydroxyjasmonate glucoside may have a role in a UV-B-induced disease defence; however, confirmation of identity with a standard would be required to have confidence in the identification of feature 22.

4.5. Several UV-B-induced correlations between disease and phenolics are conserved over an increased cultivar range

Strong correlations between UV-B-induced metabolic features and disease reduction were found over lettuce cultivars El Dorado, Iceberg and Salinas (Section 4.4.5). To determine if these correlations are conserved in lettuce, an additional seven cultivars [La Brilliante, Emperor, Grand Rapids, Calicel, Greenway, Faclon, Desert Storm] underwent a similar

method of UV-B-treatment, metabolite analysis (LC-MS-2) and disease assessment. An additional completely resistant cultivar, Pedrola, was included to determine if UV-B affected any metabolic features key for cultivar dependent resistance. Due to time constraints, only one repeat (spread over two treatments) was completed for the additional seven cultivars. Metabolic features which negatively correlate with all 10 cultivars (LC-MS-1 and LC-MS-2) likely have a stronger role in a UV-B-induced disease defence as are conserved across all cultivars.

4.5.1. Disease severity was affected by cultivar and treatment in additional seven cultivars

Disease severity (as conidia count) was significantly affected by treatment (ANOVA, $p < 0.0001$) and cultivar (ANOVA, $p < 0.0001$) individually but not the interaction between cultivar and treatment (two-way ANOVA, $p = 0.063$) resulting in no significant difference between *B. lactucae* conidia count in UV-B-pretreated lettuce plants compared to control in any cultivars (t-test, $p > 0.05$) (Figure 4.13). The small sample size (n = 9-15) may have hidden potential differences as the variation is reasonably low for each cultivar and treatment, and differences between UV-B and control plants are large for some cultivars e.g 60% decrease in conidia count in UV-B-treated La Brilliante plants.

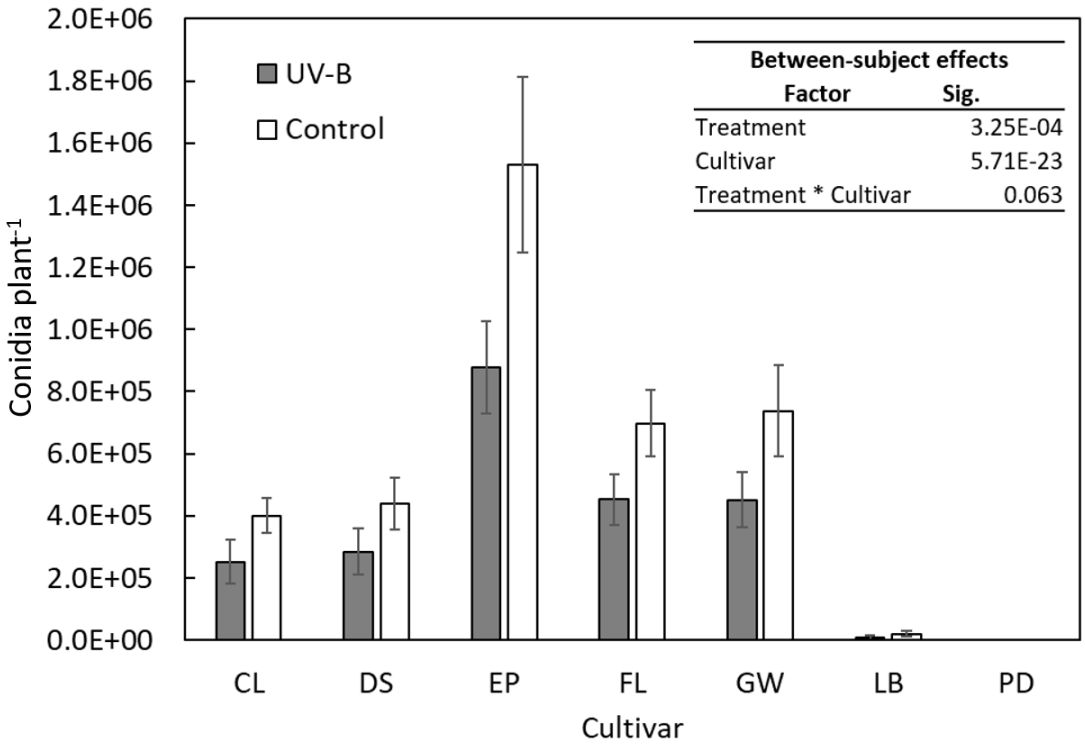


Figure 4.13.: Count of *B. lactucae* conidia harvested from UV-B [white bar] or control [grey bar] lettuce (*L. sativa*) plants of cv. Calicel (CL), Desert storm (DS), Emperor (EP), Falcon (FL), Greenway (GW), La Brillinate (LB) and Pedrola (PD). Plants were treated with photosynthetically active radiation (PAR) + UV-B or PAR only (control) for three days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae* . At 12 days post-inoculation, plants were washed in water and the resulting suspension counted. Error bars indicate 1 S.E. Table shows two-way ANOVA of between-subject effects on conidia count per plant.

As the extended cultivar list had a range of different sized plants, the conidia count was also analysed per gram fresh weight (Figure B.1). However, this data provided no additional information, with no significant difference between conidia counts of UV-B and control plants of any cultivar (two-way ANOVA, $p = 0.88$). A significant cultivar (ANOVA, $p < 0.0001$) and treatment (ANOVA, $p = 0.006$) effect on conidia count was maintained when conidia counts were analysed per g of fresh weight.

4.5.2. Expression of metabolite levels varied significantly according to cultivar

The effect of cultivar on metabolic feature intensity was greater in LC-MS-1 experimental set (3 cultivars) then the LC-MS-2 experimental set (7 cultivars). In LC-MS-2, 56% of features significantly differed between cultivars (ANOVA, $p<0.05$). The significantly different features were entered into a PCA analysis (Figure 4.14). Cultivars are not easily separated into groups based on the score plot with a spread of the repeats for each cultivar along either the first or second (or both) components. Often two repeats of a cultivar

are grouped, with the third repeat separated (e.g. Desert Storm, Falcon, La Brilliante and Pedrola). As samples were separated over two treatments in a 2-1 split per cultivar, experimental differences may have caused the separation. Emperor forms the closest grouping of repeats into a cultivar along the first and second component. Calicel, Desert Storm, and Pedrola, are grouped into cultivars by the second component only.

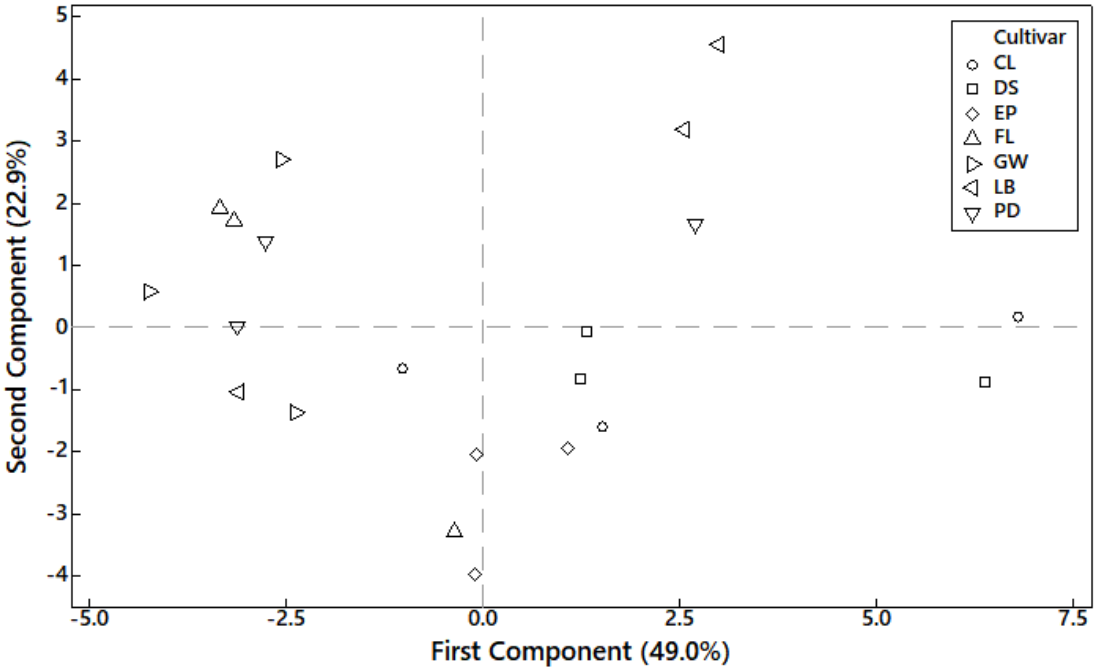


Figure 4.14.: Scores of lettuce (*L. sativa*) cv. [Calicel (CL), Desert storm (DS), Emperor (EP), Falcon (FL), Greenway (GW), La Brillinate (LB) and Pedrola (PD)] based on loadings onto significantly different (ANOVA, $p<0.05$) Liquid chromatography–mass spectrometry (LC-MS) features. Plants were sampled for LC-MS after two weeks of growth under white fluorescent tubes followed by three days of red and blue LEDs.

4.5.3. LCMS set 1 and 2 showed similar metabolites between cultivars but at difference intensities

The same protocol was used for LC-MS-1 and LC-MS-2 experiments with different cultivars and number of plants. All features except 7, 8 and 30 were found in LC-MS-2 (Figure 4.5); however, feature intensities for LC-MS-2 were higher than LC-MS-1. A PCA reveals that intensities of the LC-MS features and disease assessment (8 and 12 DPI DoI, conidia count) separate the cultivars from the two experimental sets along the first component (Figure 4.15). As there were no cultivars in common between experiments, it is unsure if the separation is due to cultivar differences or experimental differences. The first component accounts for 54.1% of the variation, so if the separation is caused by

experimental difference, than comparison across both LC-MS sets would be inappropriate, and the experiments should instead be analysed individually.

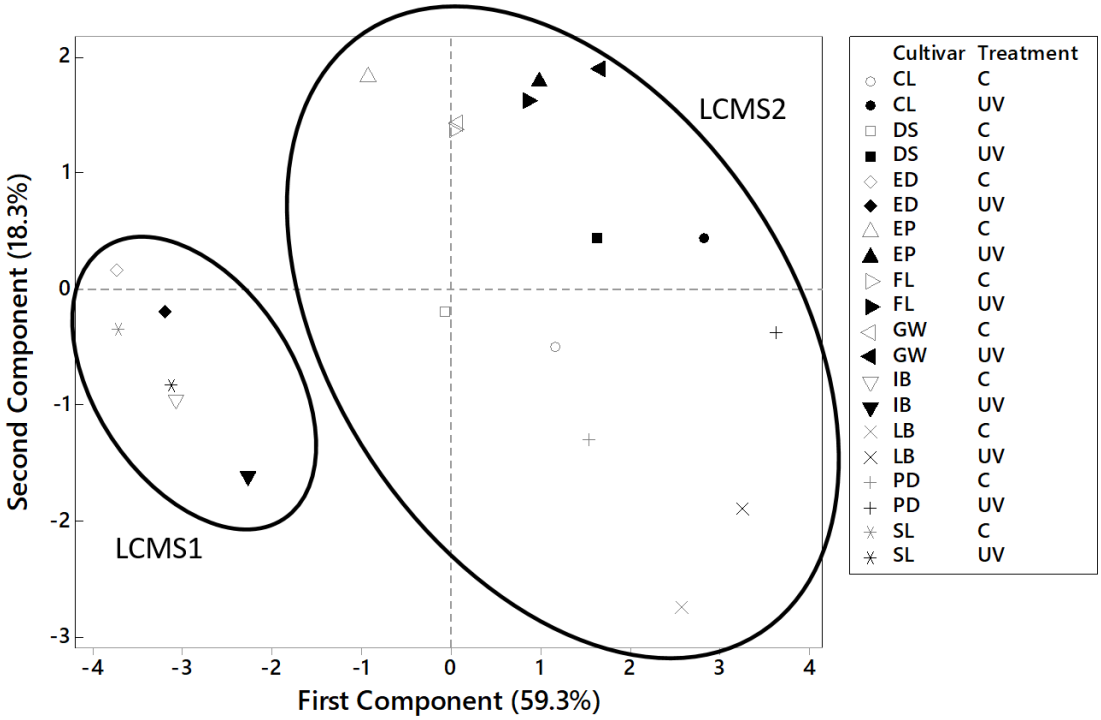


Figure 4.15.: Score plot of all lettuce (*L. sativa*) cultivars from LC-MS-1 and LC-MS-2 separated by disease assessment and Liquid chromatography–mass spectrometry (LC-MS) feature intensities. Colour indicates treatment [UV-B (UV) = black, control (C) = white]. Lettuce cv. [Calicel (CL), Desert storm (DS), Emperor (EP), Falcon (FL), Greenway (GW), La Brillinate (LB) and Pedrola (PD)] were sampled for LC-MS after two weeks of growth under white fluorescent tubes followed by three days of red and blue LEDs. Shape indicates cultivar. The different LC-MS sets are circled.

A comparison of the internal standard injected at the start of each LC run (mixture of isopropanol/formic acid/NaOH) between experimental set showed LC-MS-2 was significantly higher than LC-MS-1 (t-test, $p < 0.0005$) (Figure 4.16). This standard was injected at the same concentration in both LC-MS runs, and therefore, if LC-MS runs were the same than they should have similar feature intensities. As they are significantly different, it indicates these LC-MS runs are different. Data from these experimental sets should therefore be analysed separately.

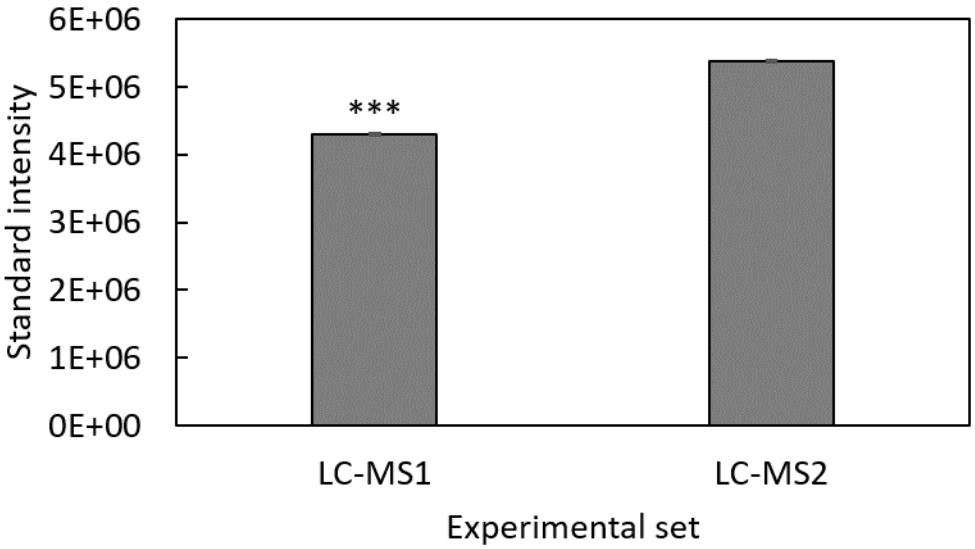


Figure 4.16.: Feature area intensity of a standard injected at the same concentration was significantly higher (t-test, p<0.0005) in Liquid chromatography–mass spectrometry set 2 (LC-MS-2) than LC-MS set 1. Error bars indicate 1 S.E.

4.5.4. Many feature groups were influenced by UV-B in additional cultivars

UV-B increased the intensity of features 10, 18 19 and 21 (Table. 4.5) in all additional cultivars compared to control (t-test, $p < 0.05$) (Figure 4.17). Feature 17 was higher in UV-B-treated plants of all cultivars except Emperor and Falcon (t-test, p<0.05). Although not significant for all cultivars features; 4 (Calicel, $p = 0.048$), 5, 8, 9, 11 (Calicel, $p = 0.007$), 16, and 28, appeared to have higher intensities overall in UV-B compared to control plants of each cultivar. A few features (25, 33 (Falcon, $p = 0.048$), 34, 35, 36) decreased in feature intensity following UV-B-treatment; however, were also non-significant differences. As differences were not significant, it can be argued that these features did not change. However, as there was a small sample size ($n = 3$) per cultivar per treatment, trends are worth mentioning, as significance may have been hidden by sample size. Many features (2, 12, 13, 15, 20, 22, 23, 26, 27, 29, and 31) underwent no significant or visual change in feature intensity following UV-B-treatment compared to control. Other features had a cultivar dependent UV-B effect. For example, features 1 and 14 were higher in UV-B-treated plants of cultivar Pedrola but lower in cultivar La Brilliante. Other features with cultivar dependent UV-B effects included 3, 24, and 32.

Inclusion of new cultivars strengthens the evidence towards some features (and their corresponding metabolites) as highly UV-B responsive. Across all ten cultivars (LC-

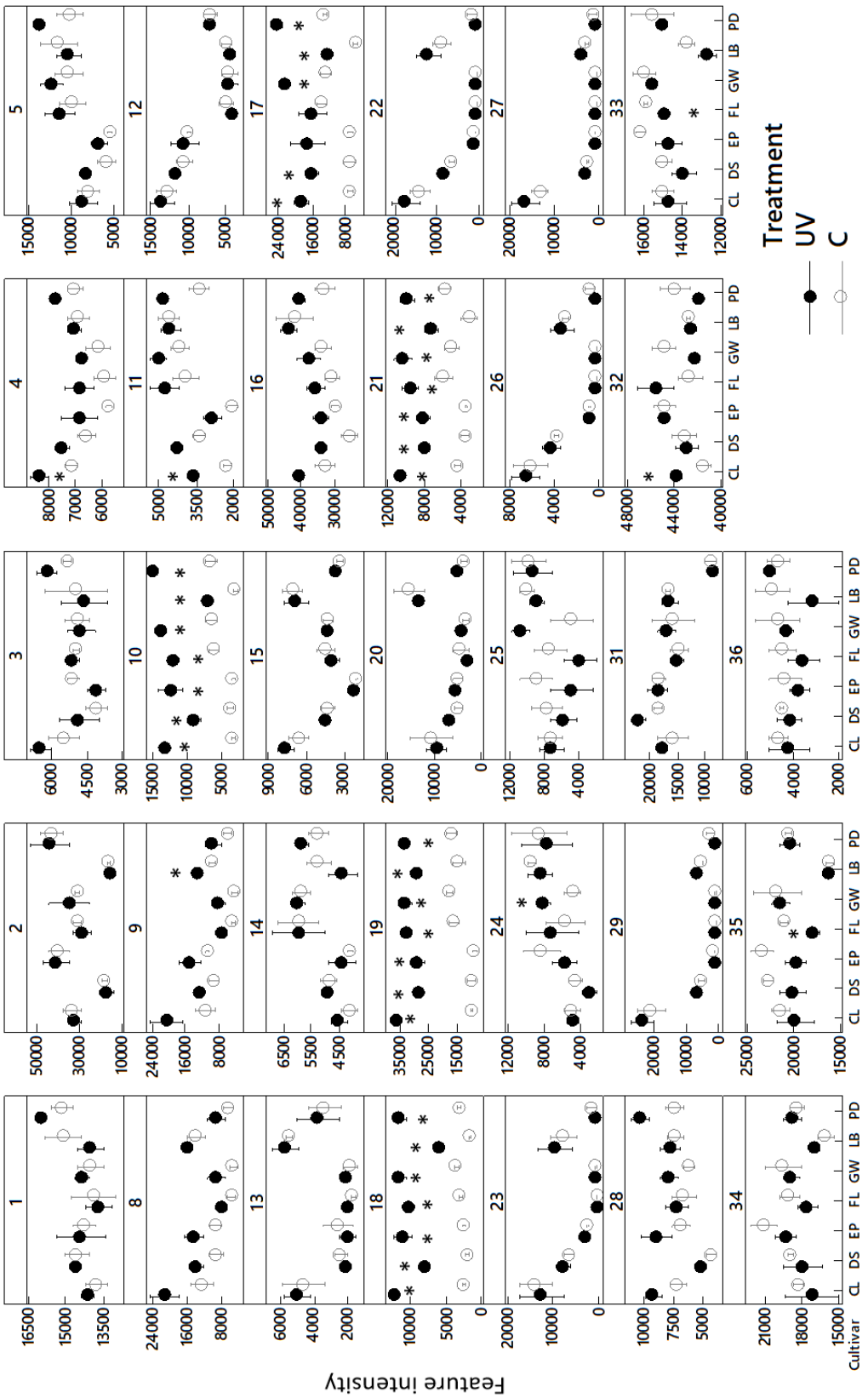


Figure 4.17.: Intensity of all identified features (Table 4.5) in UV-B (UV) [black] and control (C) [white] plants of lettuce (*L. sativa*) cv. Calicel (CL), Desert storm (DS), Emperor (EP), Falcon (FL), Greenway (GW), La Brillinate (LB) and Pedrola (PD). Plants were treated with photosynthetically active radiation (PAR)+UV-B or PAR only (control) for three days. Following treatment, samples were extracted for Liquid chromatography–mass spectrometry (LC-MS) analysis. Significant differences between UV-B and control plants of each feature [panel] are indicated by an asterisks (t-test, * = $p<0.05$). Error bars are 1 S.E

MS-1 and LC-MS-2), features 8, 9, 10, 16, 17, 18, 19, 21 and 28 were higher in UV-B compared to control plants (only significant in some cases). As these features underwent a similar expression pattern under UV-B-treatment, it can be argued that the corresponding metabolites are UV-B responsive.

4.5.5. Six metabolomic features correlated with disease reduction in additional cultivars

A regression analysis was run between feature intensity and conidia count of all features found in LC-MS (Table 4.5), and the resulting correlations compared to those found in LC-MS-1. Of the ten significant correlations stronger than -/+ 0.5 in LC-MS set 1 (Table 4.6), four were also significant in LC-MS set 2; features 11, 20, 22 and 33 (Table 4.7). An additional two features (15 and 16) had a significant negative correlation with conidia count which was not present in LC-MS-1. Although fewer significant correlations were found in LC-MS-2, those which were significant also had a higher pearsons correlation (negative or positive) indicating a stronger relationship. As four significant correlations were present in both LC-MS sets, a negative correlation between these features and conidia count is likely conserved across lettuce cultivars.

Table 4.7.: Correlation statistics between *B. lactucae* conidia count and feature intensity across lettuce (*L. sativa*) cultivars [Calicel, Desert Storm, Emperor, Falcon, Greenway, and La Brilliante] and treatments. Lettuce cultivars were treated with photosynthetically active radiation (PAR)+UV-B or PAR only (control) for three days. Following treatment, samples were taken for Liquid chromatography–mass spectrometry (LC-MS), with the remaining plants inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. Plants were harvested for conidia count at 12 days post-inoculation. A bivariate correlation analysis was carried out on all LC-MS features and disease symptoms. Significant correlations ($p<0.05$) with a pearsons value over +/- 0.5 between feature intensity and conidia count are shown. Significance level is indicated by asterisks where * = $p<0.05$, ** = $p<0.005$, and *** = $p<0.0005$, ns = non-significant.

	LC-MS 1		LC-MS 2	
Feature ID	Pearson Correlation (r)	Sig. (2-tailed)	Pearson Correlation (r)	Sig. (2-tailed)
11	-0.618	*	-0.625	*
15	0.281	ns	-0.818	**
16	-0.395	ns	-0.596	*
20	-0.611	*	-0.620	*
22	-0.552	*	-0.622	*
33	0.549	*	0.762	**

Scatter graphs of conidia count plotted against feature intensity of significantly correlated LC-MS-2 features (Figure 4.18) provides visual evidence that cultivar effect causes most of these correlations with both UV-B and control samples from a cultivar having similar coordinates. Features 11, 16 and 33 regressions may be more heavily influenced by UV-B with some clustering of treatments.

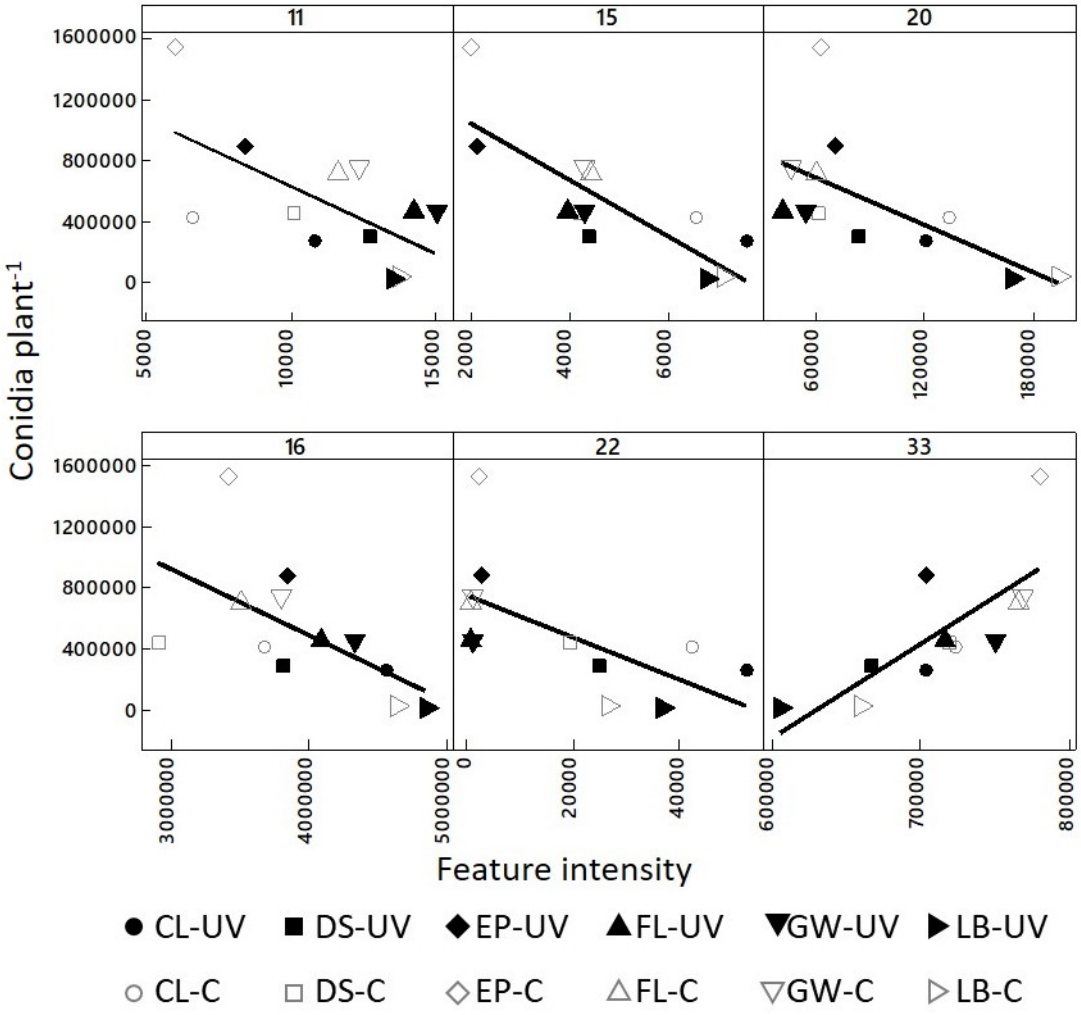
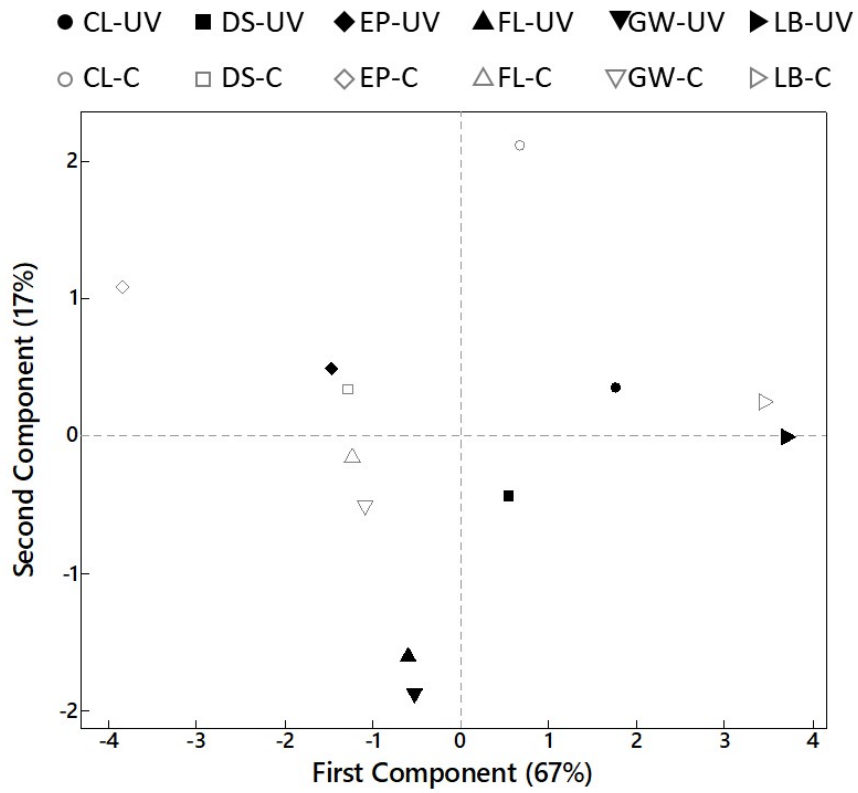
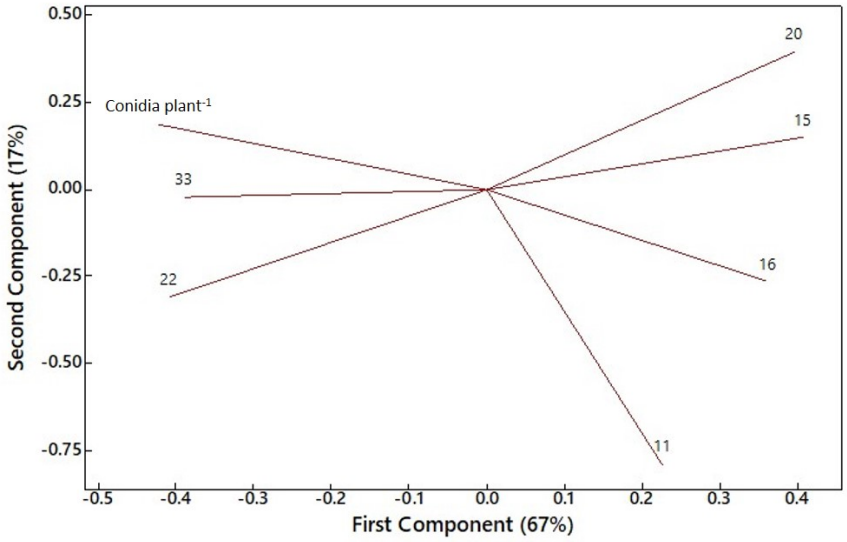


Figure 4.18.: Scatter graphs of the feature intensity against *B. lactucae* conidia count of significantly correlated features [panel]. Lettuce (*L. sativa*) cv. [Calicel (CL), Desert storm (DS), Emperor (EP), Falcon (FL), Greenway (GW), La Brillinate (LB) and Pedrola (PD)] were treated with photosynthetically active radiation (PAR)+UV-B (UV) or PAR only (C = control) for three days. Following treatment, samples were taken for Liquid chromatography–mass spectrometry (LC-MS), with the remaining plants inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. Plants were harvested for conidia count at 12 days post inoculation. A Bivariate correlation analysis was carried out on all LC-MS features and disease symptoms. Significant correlations ($p<0.05$) with a pearsons value over +/- 0.5 between feature intensity and conidia count are shown in the Table 4.7.

A PCA confirms trends from the scatter graphs with separation of cultivars along the first component from highest (Emperor) to lowest (La Brilliante) susceptibility cultivars (Figure 4.19). Keeping in mind there is only one point per treatment per cultivar, the first component moderately separates some specific cultivars (Calicel, Desert Storm, Emperor) and slightly separates the remainder (Falcon, Greenway, La Brilliante). There is higher separation by treatment along the second component (all cultivars bar La Brilliante); however, this only accounts for 17% of variation. In comparison to cultivar effect, treatment effect is relativity less important. Loading of scores onto variables (Figure 4.19) shows feature 16 (and 11 to a lesser extent) had the strongest negative correlation with conidia count along the first and second component. Features 20 and 15 negatively correlate with conidia count along the first component. Feature 33 and 22 correlate positivity along the first component; however, are separated weakly by the second component. As the heavy treatment grouping was not present in the score plot, the loading does not provide evidence towards which features may be important to a UV-B-related correlation.



(a)



(b)

Figure 4.19.: A principal component analysis evaluating disease assessment and significantly correlated metabolite features of UV-B-pretreated [UV = black] and control [C = white] lettuce (*L. sativa*) plants of cv. Calicel (CL) = circle, Desert Storm (DS) = square, Emperor (EP) = diamond, Falcon (FL) = up triangle, Greenway (GW) = down triangle. La Brilliante (LB) = right triangle. Plants were treated with UV-B light for 3 days. Following treatment, samples were taken for Liquid chromatography–mass spectrometry and the remaining plants were inoculated with 10^5 conidia mL^{-1} *B. lactucae*. Disease was assessed for visual sporulation rating at 8 and 12 days post inoculation (DPI). At 12 DPI plants were also harvested for conidia count.

4.5.5.1. Correlated features have been observed to have antimicrobial features in previous studies

Of the two additional features which correlate with disease reduction in LC-MS-2 cultivars, only feature 16 could be identified (chicoric acid). Chicoric acid is only present in some plant orders (including the lettuce order; Asterales) (Lee and Scagel, 2013) resulting in few studies available. Chicoric acid has been reported to be up-regulated in lettuce by UV-B light in previous studies (Romani et al., 2002; Almeida De Oliveira, 2016); however, these were both in red lettuce. Caffeic acid derivatives, such as chicoric acid have been noted to have antimicrobial activity in leaf disk assays against bacterial and fungal pathogens (Gatto et al., 2011; Zhu et al., 2004). To add, chicoric acid was increased by fungal elicitor in *E.purpurea (Hudec et al., 2007)* indicating that it may have a role in fungal disease responses. A UV-B induction of chicoric acid may therefore increase the antimicrobial activity of the lettuce plant resulting in the observed correlation.

4.5.6. Difference in metabolites of resistant and susceptible cultivars may provide insight into phenolic disease defence mechanisms

LC-MS-2 experiments included a cultivar (Pedrola) which was resistant to downy mildew disease. Comparison of metabolite features induced in Pedrola to the highly susceptible cultivar Emperor highlighted six features (10, 15, 17, 19, 21 and 31) which were differentially expressed (Figure 4.20). Feature 10 had the most substantial difference (119% higher in Pedrola) between Pedrola and Emperor. Three features (17, 19 and 31) were present in high abundance in both lettuce cultivars (<100,000 intensity), feature 10 had a moderate abundance (>10,000 intensity) and features 15 and 21 had low abundance (<5000 intensity). All of these features except 31 were significantly higher in Pedrola than Emperor. There were no features which were present in Pedrola which were not present in Emperor, indicating that if secondary metabolites are involved in the resistance, it is a matter of metabolite concentration rather than presence.

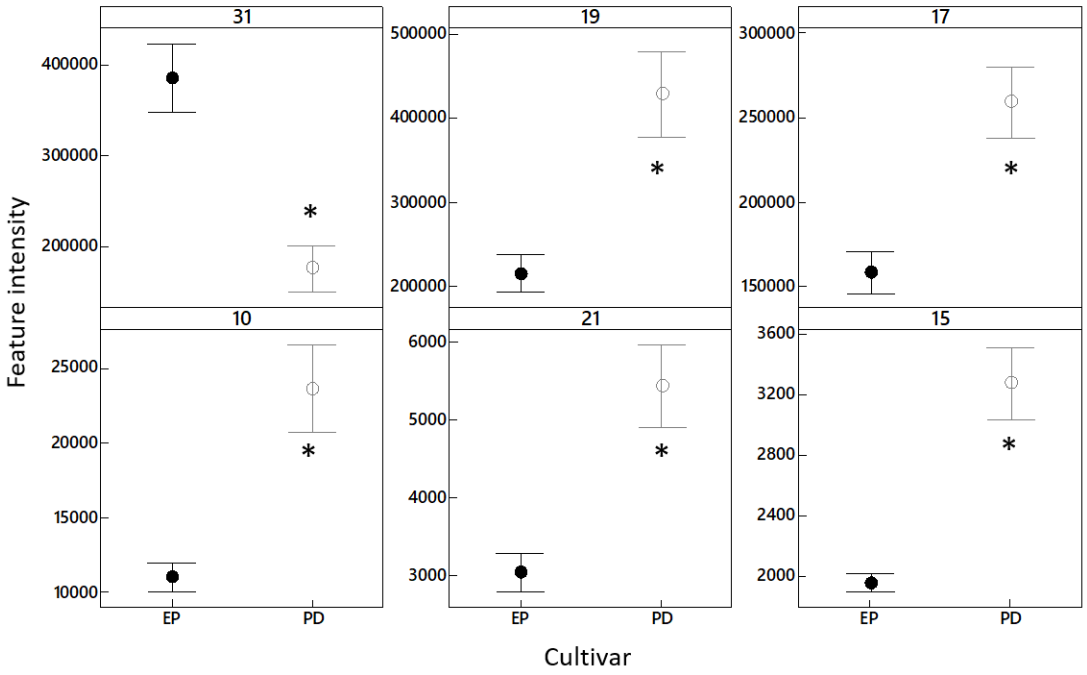


Figure 4.20.: Intensity of features in high susceptibility lettuce (*L. sativa*) cv. Emperor (EP) [black] and completely resistant Pedrola (PD) [white]. Plants were sampled for Liquid chromatography–mass spectrometry (LC-MS) after two weeks of growth under white fluorescent tubes followed by three days of red and blue LEDS. Error bars are 1 S.E. Asterisks indicate significance between Emperor and Pedrola where *= $p<0.05$ according to a t-test.

UV-B light treatment can also induce several features which were higher in a resistant cultivar. UV-B increased intensity of features 19 and 21 in all cultivars (Figure 4.10 and 4.17). Features 15 and 31 were unaffected by UV-B-treatment. Intensity of feature 10 was increased in UV-B-pretreated plants of cultivars in LC-MS-2 but not in LC-MS-1. Intensity of feature 17 was increased by UV-B in all cultivars except Emperor and Falcon.

As determined by previous correlation analysis (Table. 4.6 and 4.18), features 17 and 19 have a significant negative correlation with conidia count over LC-MS-1 cultivars and feature 15 has a negative correlation with conidia count over LC-MS-2 cultivars. Features 17 and 19 may contribute to increased *B. lactucae* resistance in a UV-B dependent manner. They both have a higher intensity in a resistant cultivar, are increased by UV-B light (in majority of cultivars) and correlate with disease reduction. Although feature 15 has a negative correlation with disease, it is unaffected by UV-B in all cultivars and so may contribute to disease resistance but is unlikely to be driven by a UV-B-treatment.

4.6. Infiltration of UV-B-induced phenolics can alter disease susceptibility

Phenolic compounds were identified in Section 4.4.5 as strongly negatively correlated with disease reduction. Three compounds [chlorogenic acid (CA), 3,5-dicaffeoylquinic acid (DCQA) and quercetin 3-O-(6″-O-malonyl)-β-D-glucoside (Q) (Figure 4.21)] which had strong certainty of identification and strong disease correlations were infiltrated into lettuce cultivars El Dorado, Iceberg and Salinas. Three concentrations of each compound were used to achieve a 1.5, 2.5 or 4-fold increase in the compound compared to a standard crisphead/iceberg type lettuce plant. Following infiltration, the plants were inoculated with *B. lactucae* and the resulting downy mildew symptoms were assessed. These experiments attempted to link the correlations with the ability of the compound to reduce disease severity.

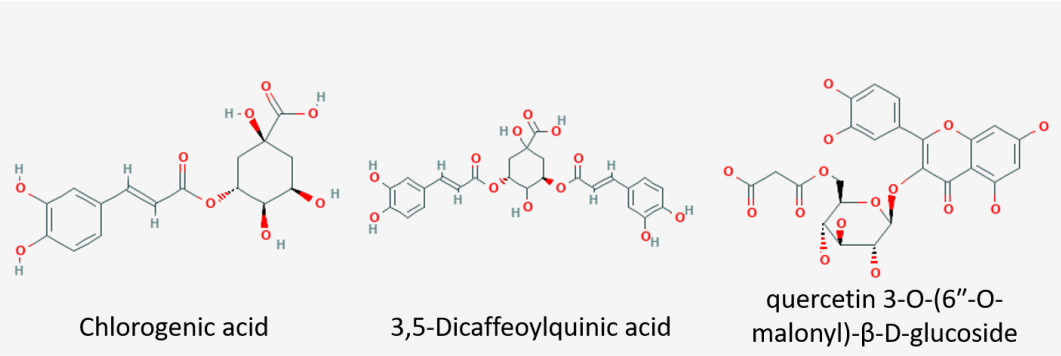


Figure 4.21.: Structures of Chlorogenic acid (CID=1794427, 2020), 3,5-dicaffeoylquinic acid (CID=6474310, 2020) and quercetin 3-O-(6″-O-malonyl)-β-D-glucoside (SID=329751366, 2020).

4.6.1. Compound infiltration decreased disease incidence at early or late stage disease depending on cultivar

Following the majority of treatments, there was no significant difference (Fisher's exact test, $p > 0.05$) in disease incidence between control/mock and plants infiltrated with a compound based on leaf rating. It is interesting to note that the act of infiltration did not affect disease incidence. Plants infiltrated with water (mock) did not significantly differ (Fisher's exact test, $p < 0.05$) from plants which were not infiltrated (control) at any time point. At 6 DPI, El Dorado plants had a reduced level of disease incidence following

infiltration with DCQA at 2.5-fold (Fisher's exact test, p = 0.012) or Q at 4-fold (Fisher's exact test, p = 0.024) compared to mock (Figure 4.22). At 6 DPI, disease incidence decreased with increasing Q concentration. This trend is also present at 7 DPI to a lesser degree, but lost by 8 DPI.

At 8 DPI, Iceberg plants had significantly higher disease incidence than mock plants following infiltration with 2.5-fold DCQA (Fisher's exact test 0.008). It is possible however, that this increase may be a misrepresentation caused by small sample size.

At 8 DPI, disease incidence of Salinas plants was lower when infiltrated with 4-fold DCQA (Fisher's exact test, p = 0.048) or 2.5-fold Q (Fisher's exact test, p = 0.007) than mock-infiltrated plants. Compound effect was not conserved across cultivars, with decreases in incidence occurring from DCQA and Q infiltration at different time points and concentrations in different cultivars.

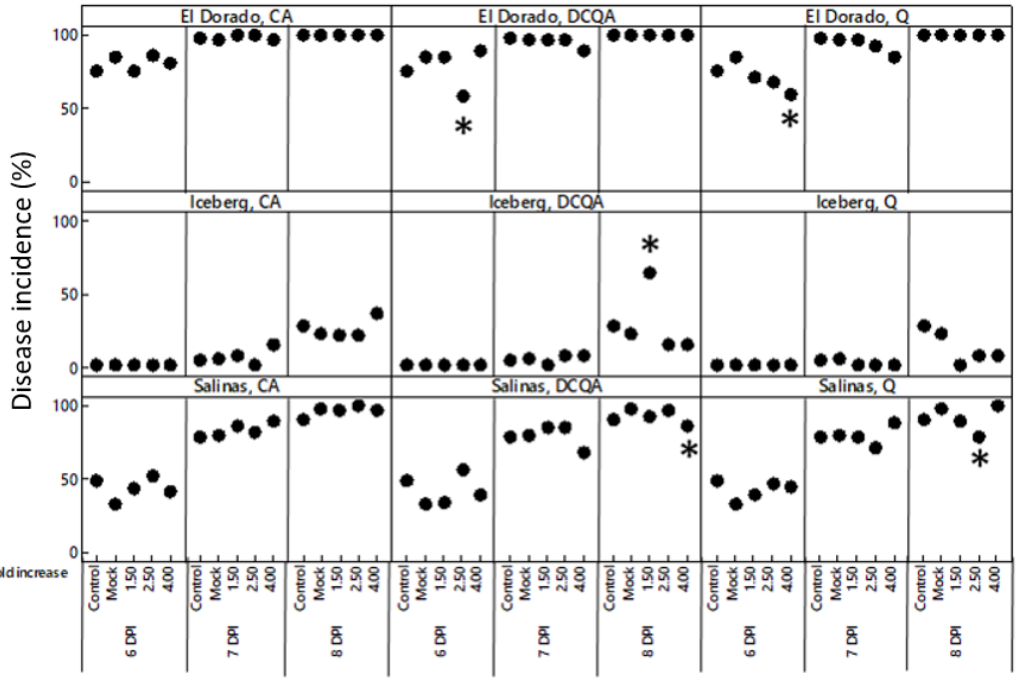


Figure 4.22.: The incidence of disease as a percentage of total plants infected across lettuce (*L. sativa*) plants with various concentration (fold increase) of compound (CA = chlorogenic acid, DCQA = 3,5-dicaffeoylquinic acid, Q = Quercetin 3-O-(6″-O-malonyl)-β-D-glucoside), mock (water) infiltrated or control plants (no infiltration) at 6 ,7 and 8 days post-inoculation (DPI). Lettuce plants [cv. El Dorado, Iceberg, Salinas] were grown to 17 days old then infiltrated with a concentration of phenolic compound with a needleless syringe to reach a 1.5, 2.5 or 4-fold increase compared to a standard Iceberg-type lettuce plant. Plants were then inoculated with 10^5 conidia mL^{-1} of *B. lactucae* and the resulting disease incidence measured over time. Asterisks indicate a significant difference in number of plants infected compared to mock [Fishers exact test, * = $p<0.05$].

Based on leaf rating data, all El Dorado plants reached 100 % infection regardless of compound type and amount infiltrated by 8 DPI. Most Salinas plants (93%) were infected by 8 DPI; however, only a small portion of Iceberg plants (21%) were infected by 8 DPI. The disease results from cultivar Iceberg were less robust then other cultivars when comparing all plants (n = 14 per treatment) due to skewing by uninfected plants (ratings = 0, conidia count = 0), or by low sample numbers if analysing infected-only plants. Due to the comparatively larger sample size of the other cultivars (n range per repeat; El Dorado = 26 - 53, Salinas = 25 - 52), conclusions drawn from these cultivars should hold more weight than conclusions drawn from Iceberg plant data.

4.6.2. Leaf sporulation severity is weakly affected by infiltration of phenolic compounds

The sporulation severity of the infiltrated leaf over time was indicated by a rating scale from 0 (no sporulation) to 10 (leaf covered in sporulation). Leaf sporulation was most severe in lettuce cultivar El Dorado followed by Salinas then Iceberg plants (Figure 4.23). Incidence levels confirmed susceptibility of cultivars found previously in this project (Section 4.3.1). Iceberg had almost negligible leaf sporulation severity with a maximum DoI of only 17% of the theoretical maximum rating reached by 8 DPI. The effect of compound infiltration varied between cultivars. Differences in DoI were not statistically tested; however, significant differences in distribution of normalised ratings are noted (Mann Whitney U test).

Chlorogenic acid (CA) had no significant effect on Iceberg or Salina plants; however, a 1.5-fold increase in CA resulted in a significantly higher leaf rating distribution (Mann Whitney U, p = 0.036) in El Dorado at 8 DPI (Figure 4.23) compared to mock.

3,5-dicaffeoylquinic acid (DCQA) infiltration significantly decreased normalised leaf rating distribution at a concentration of 2.5-fold at 6 DPI (Mann Whitney U, p = 0.036) and 7 DPI (Mann Whitney U, p = 0.030) in cultivar El Dorado. Iceberg plants; however, increased in normalised leaf rating distribution when infiltrated with DCQA (1.5-fold, 8 DPI : Mann Whitney U, p = 0.004). DCQA may have a role in decreasing leaf rating in El Dorado, but not Iceberg lettuce leaves.

Quercetin 3-O-(6″-O-malonyl)-β-D-glucoside (Q) infiltration had no significant effect on El Dorado and Iceberg leaf rating; however, infiltration of 2.5-fold of Q reduced the distribution of normalised leaf ratings at 8 DPI in Salinas lettuce leaves. Compound infiltration

does not alter leaf sporulation rating in majority of cases. Compound concentrations which do effect leaf sporulation are cultivar and concentration specific with no apparent general trends.

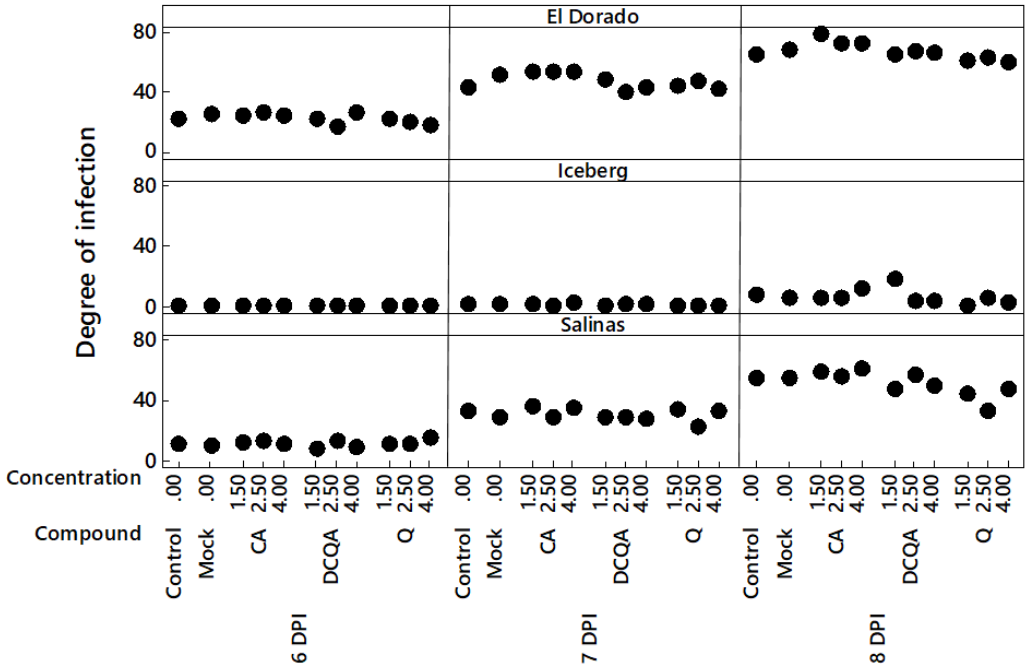


Figure 4.23.: The degree of infection (DoI) of the infiltrated leaf over time based on leaf rating scale across lettuce (*L. sativa*) plants with various concentration (fold increase) of compound (CA = chlorogenic acid, DCQA = 3,5-dicaffeoylquinic acid , Q = Quercetin 3-O-(6″-O-malonyl)-β-D-glucoside) infiltrated. Lettuce [cv. El Dorado, Iceberg, Salinas] plants were grown to 17 days old then infiltrated with a concentration of phenolic compound with a needleless syringe to reach a 1.5, 2.5 or 4-fold increase compared to a standard Iceberg-type lettuce plant. Plants were then inoculated with 10^5 conidia mL^{-1} of *B. lactucae* and the resulting disease sporulation severity of the infiltrated leaf was assessed using a rating scale between 6 and 8 days post-inoculation (DPI). The rating scale was summarised as a degree of infection for the population.

4.6.3. Entire plant sporulation rating was affected by infiltration of one leaf

Although only one leaf was infiltrated with a phenolic compound, the sporulation rating was also taken of the whole plant (0 = no sporulation, 9 = heavy sporulation on multiple leaves). As with leaf ratings, sporulation severity was highest in cultivar El Dorado followed by Salinas than Iceberg (Figure 4.24). The effect of compound infiltration varied between cultivars. Differences in DoI were not statistically tested; however, significant differences in distribution of normalised ratings are noted (Mann Whitney U test).

CA tended to result in an increase in plant sporulation severity in Salinas. CA-infiltrated

Salinas plants had a significantly higher distribution of normalized plant ratings at a 2.5-fold increase at 8 DPI (Mann Whitney U, $p = 0.006$) as well as at a 4-fold increase at 6, 7 and 8 DPI (Mann Whiteny U, $p = 0.023$, 0.026 and 0.018 respectively) compared to mock. CA also increased plant rating distribution at a concentration of 1.5-fold in El Dorado compared to mock (Mann Whitney U , $p = 0.044$) at 8 DPI.

An infiltration of 4-fold DCQA decreased normalised plant rating distribution at 7 DPI in El Dorado (Mann Whitney U, $p = 0.029$) but had no effect on plant sporulation other time points, concentrations or cultivars.

Q decreased plant rating in El Dorado at a concentration of 2.5-fold at 8 DPI (Mann Whitney U, $p = 0.049$); however, Q tended to increase plant rating in Salinas. Q significantly increased sporulation distribution in Salinas plants infiltrated with fold increases of 1.5 at 8 DPI (Mann Whitney U, $p = 0.030$), 2.5 at 6 DPI (Mann Whitney U, $p = 0.029$) and 4 at 6, 7 and 8 DPI (Mann Whitney U , $p < 0.0001$, $p = 0.004$ and $p < 0.000$ respectively) compared to mock. It is interesting to note that although plant sporulation was increased by Q in Salinas, leaf sporulation was decreased indicating different responses at a leaf and plant level.

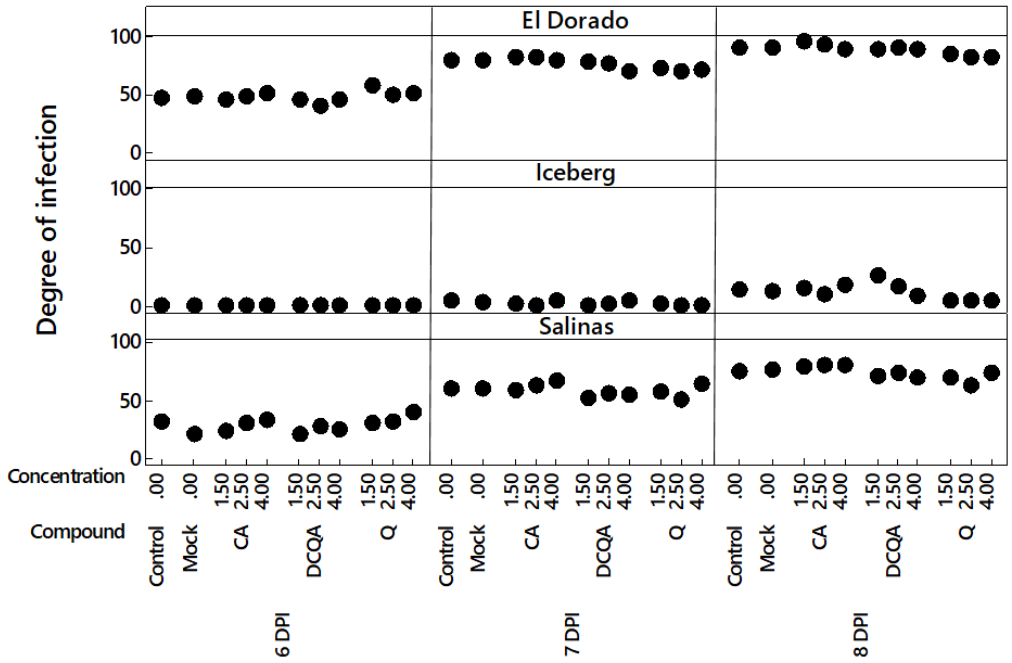


Figure 4.24.: The degree of infection (DoI) of the infiltrated plant over time based on plant ratings across lettuce (*L. sativa*) plants with various concentration (fold increase) of compound (CA = chlorogenic acid, DCQA = 3,5-dicaffeoylquinic acid , Q = Quercetin 3-O-(6″-O-malonyl)-β-D-glucoside) infiltrated. Lettuce [cv. El Dorado, Iceberg, Salinas] plants were grown to 17 days old then infiltrated with a concentration of phenolic compound with a needleless syringe to reach a 1.5, 2.5 or 4-fold increase compared to a standard Iceberg-type lettuce plant. Plants were then inoculated with 10^5 conidia mL^{-1} of *B. lactucae* and the resulting disease sporulation severity of the entire plant was assessed using a rating scale over 6 to 8 days post-inoculation (DPI). The rating scale was summarised as a Degree of infection for the population

4.6.4. Leaf conidia can be reduced by infiltration of phenolic compounds

Infiltrated leaves were individually washed at 12 DPI, and the resulting conidia suspension counted (Figure 4.25). Iceberg plants were unaffected by infiltration of any compound. This is likely due to the low level of susceptibility of Iceberg plants resulting in too low conidia numbers for the sensitivity of the conidia count assay to test.

Infiltration with CA resulted in no change to leaf conidia count in Iceberg and Salinas leaves regardless of concentration compared to control and mock. El Dorado leaves infiltrated with 1.5 or 2.5-fold of CA had a significantly higher leaf conidia count than both control (49, 48 % increase) and mock leaves (27, 26 % increase) (ANOVA LSD, $p < 0.0005$, $p = 0.024$ respectively).

Infiltration with DCQA had different effects in El Dorado than in Salinas. In El Dorado,

leaves infiltrated with 2.5-fold of DCQA had higher leaf conidia than in control (39% increase) but not mock. Other concentrations of DCQA in El Dorado were not significantly different. In Salinas, all infiltrations of DCQA had a reduced leaf conidia count compared to control (20, 19, 37 % decrease in 1.5, 2.5 and 4-fold respectively) and mock (19, 18, 36 % decrease in 1.5, 2.5 and 4 x respectively); however, only DCQA at a 4-fold increase was significantly lower (ANOVA LSD, control; $p = 0.034$, mock; $p = 0.032$). Infiltration of Salinas leaves with DCQA shows a trend of decreasing conidia count with increasing DCQA concentration, where the highest level tested (4-fold increase) was the only high enough dose to result in a significant difference.

Both El Dorado and Salinas leaf counts were decreased by addition of Q at 2.5-fold compared to mock (decrease of 25% in El Dorado, 39% in Salinas) (ANOVA LSD, $p = 0.029$, 0.024 respectively). In El Dorado, infiltration of 4-fold Q also resulted in a significant reduction of leaf conidia compared to mock (ANOVA LSD, $p = 0.001$). In El Dorado, conidia count decreases with increasing Q concentration. However, in Salinas, leaf conidia decrease is more of a threshold response in which a 2.5-fold increase in Q is the peak concentration for reduction of leaf conidia, with lower or higher concentrations causing a lesser reduction.

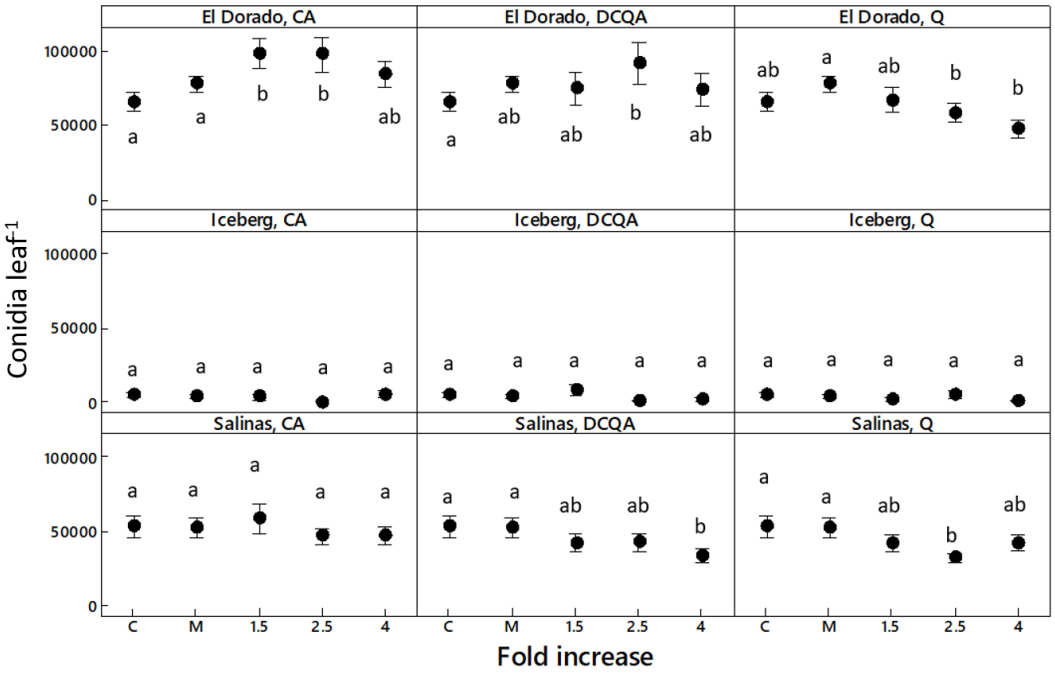


Figure 4.25.: *B. lactucae* conidia counts harvested from an infiltrated leaf across lettuce (*L. sativa*) plants with various concentration (fold increase) of compound (C = control (no infiltration), M = mock (water), CA = chlorogenic acid, DCQA = 3,5-dicaffeoylquinic acid, Q = Quercetin 3-O-(6″-O-malonyl)-β-D-glucoside) infiltrated of lettuce cv. El Dorado, Iceberg and Salinas. Lettuce plants were grown to 17 days old then the oldest true leaf infiltrated with a concentration of phenolic compound with a needleless syringe to reach a 1.5, 2.5 or 4-fold increase compared to a standard Iceberg-type lettuce plant. Plants were then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. At 12 days post-inoculation the infiltrated leaf was washed in water and the resulting conidia suspension counted. Error bars are 1 S.E. Letters indicated significance groupings within each compound and cultivar (ANOVA LSD, $p<0.05$).

4.6.5. Quercetin 3-O-(6″-O-malonyl)-β-D-glucoside infiltration reduced plant conidia count in cv. El Dorado

As well as leaf conidia counts, a subset of the entire El Dorado plants were also washed, and the resulting conidia suspension counted (Figure 4.26). CA (1.5-fold) increased plant conidia count compared to control plants (ANOVA LSD, p = 0.048) but not mock. Infiltration of DCQA resulted in plants which did not significantly differ from control or mock. Infiltration with Q decreased plant conidia compared to mock plants at a 1.5 and 4-fold increase (ANOVA LSD, p = 0.011, 0.004 respectively). Plant conidia did not follow a concentration trend, with a similar decrease from Q infiltration in all fold increases.

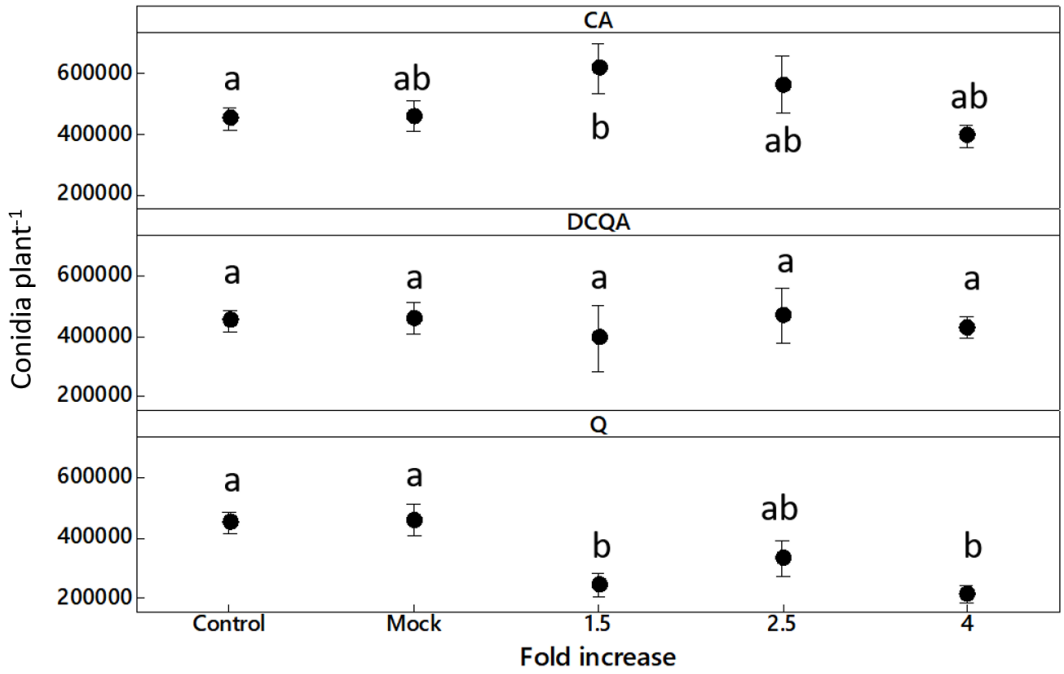


Figure 4.26.: *B. lactucae* conidia counts harvested from lettuce (*L. sativa*) cv. El Dorado plants with various concentration (fold increase) of compound (CA = chlorogenic acid, DCQA = 3,5-dicaffeoylquinic acid, Q = Quercetin 3-O-(6″-O-malonyl)-β-D-glucoside) infiltrated into one leaf. Lettuce plants were grown to 17 days old then the oldest true leaf infiltrated with a concentration of phenolic compound with a needleless syringe to reach a 1.5, 2.5 or 4-fold increase compared to a standard Iceberg-type lettuce plant. Plants were then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. At 12 days post-inoculation the entire plant was washed in water and the resulting conidia suspension counted. Error bars are 1 S.E. letters indicated significance groupings within each compound and cultivar (ANOVA LSD, $p<0.05$).

4.6.6. Cultivar differences may be due to differences in membrane permeability and natural compound levels

Syringe infiltration directly injects liquid into the plant apoplast (Kataria, 2017; Katagiri et al., 2002). Syringe infiltration is most commonly used for infiltration of bacterial suspensions for disease assays (Liu et al., 2015; Katagiri et al., 2002) or agroinfiltration for genetic transformations (Wroblewski et al., 2005; Vaghchhipawala et al., 2010). Agroinfiltration for transformation has been shown to be successful in lettuce, with transformation throughout the leaf lamina and transformed gene expression in 80% of mesophyll cells (Wroblewski et al., 2005). This indicates the infiltrated suspension is reasonably evenly distributed throughout the leaf, giving confidence that the infiltration of phenolic compounds in this experiment resulted in relatively even compound levels throughout the leaf lamina also.

The success of infiltration may depend on lettuce cultivar. For example, cultivars which have more open stomata, and increased membrane permeability are more likely to easily and evenly uptake the infiltrated solution. Those which resist infiltration, are more likely to be damaged by the process and have a less even distribution of infiltrated compound. Previous studies have not been completed on lettuce cultivars El Dorado, Iceberg and Salinas infiltrations; however, anecdotal notes from this experiment found that El Dorado plants underwent the infiltration process with the least resistance. Leaf conidia count data showed that El Dorado was also most commonly observed to have altered conidia count by compound infiltration. This may be due to a more successful infiltration from cultivar characteristics.

Cultivar effects may also be due to basal levels of infiltrated compounds or other proteins and metabolites affected by infiltrated compound levels as well as basal susceptibility. As noted in Section 4.4.2, El Dorado, Iceberg and Salinas, have different basal levels of LC-MS features corresponding to CA, DCQA, and Q. Therefore, addition of these compounds is more likely to influence cultivars with lower levels due to the relative increase. To add, cultivars may have different basal levels of downstream components of pathways involving these compounds which would result in different efficiency of use and therefore different levels of infiltration effect.

4.6.7. Infiltration of one leaf can alter susceptibility of the entire plant

The effect of phenolic compound infiltration was assessed on the entire plant as well as the infiltrated leaf. Plant disease assessment can indicate if infiltration of one leaf with a compound may have a systemic effect. Comparison of the trends in conidia count change in both the leaf and plant of El Dorado as a percent of the control can give some insight on the occurrence of a systemic effect.

4.6.7.1. Infiltration can be a stressful event for the leaf

Two controls were included in this experimental; no infiltration (control) or infiltration with distilled water (mock). The infiltration method involves injection of a volume of liquid into the leaf. This may be a stressful event for the leaf due to possible physical damage to the leaf caused by contact with the syringe, as well as increased pressure of additional liquid. The stress of infiltration may increase susceptibility. For example, physical damage may increase ease of penetration by the pathogen. Although methods for syringe infiltration of

bacteria stipulate that the syringe should not leave any marks on the leaf surface, this is not always the case (Liu et al., 2013). Any wounding caused by the syringe would induce a defence response which influences disease defence pathways and may alter susceptibility of the plant (Leon et al., 2001). It is also possible infiltration may cause dilution of other defensive compounds due to the additional liquid.

Mock plants that undergo the infiltration method; however, receive no additional phenolic compound, so should undergo a similar stress experience to compounds infiltrated with phenolic compound. In comparison to control leaves, mock leaves do not have significantly different number of leaf conidia (Figure 4.25), or distribution of leaf ratings (Figure 4.23). This indicates that the infiltration does not significantly affect the susceptibility of a leaf to disease. However, in El Dorado a significant difference in leaf conidia was observed between a 4-fold increase of Q to mock but not control. This indicates that although there is no significant difference between control and mock plants, there may still be an effect on disease susceptibility caused by infiltration which may be masked if leaves infiltrated with compound were compared only to control.

The effect on susceptibility of infiltration method is contained to the infiltrated leaf. Mock-infiltrated El Dorado plants had a similar conidia count to control plants; however, mock-infiltrated leaves had a higher conidia count than control leaves (Figure 4.27). Although infiltration may affect the susceptibility of the leaf, it has little effect on the susceptibility of the entire plant. Lack of systemic effect provides evidence towards syringe damage of the infiltrated leaf increasing susceptibility.

4.6.7.2. Systemic effect of infiltration was dependent on the compound

Infiltration had different plant-wide effects on disease severity depending on the compound. CA increased conidia count in both the leaf and plant at a fold increase of 1.5 and 2.5 (Figure 4.27). Increase to conidia count was larger in the infiltrated leaf than the entire plant. A reduction of effect from plant to leaf indicates that other leaves in the plant are not affected (or weakly affected) by the infiltrated compound. As plant conidia count includes the infiltrated as well as non-infiltrated leaf, the conidia count increase is likely driven by the infiltrated leaf, but the increase is weakened through averaging over non-infiltrated leaves which are similar to control plants. This is also the case with infiltration of DCQA at 2.5 or 4-fold, in which leaf infiltration increases conidia count, but at the same concentration,

plant conidia count is similar to control.

Q infiltration resulted in a plant-wide response. All fold increases of Q resulted in a reduction in conidia count in the infiltrated leaf (except 1.5 which resulted in no change) and the entire plant (Figure 4.27). The reduction in conidia count was greater in the entire plant than the infiltrated leaf indicating amplification of effect of the compound. Amplification of effect, could be caused by transport of Q to other leaves or signals induced by Q travelling to other leaves, resulting in reduced susceptibility of the entire plant.

In two cases; DCQA at a 1.5-fold increase and CA at a 4-fold increase, a slight reduction in conidia count occurred at a plant level; however, the infiltrated leaf had an increased conidia count compared to control. In these cases, the compounds are affecting the leaf and the entire plant but in different ways. Or the compound could be reducing conidia count in both leaf and plant, but the increased susceptibility caused by infiltration damage masked a decrease in the leaf conidia count.

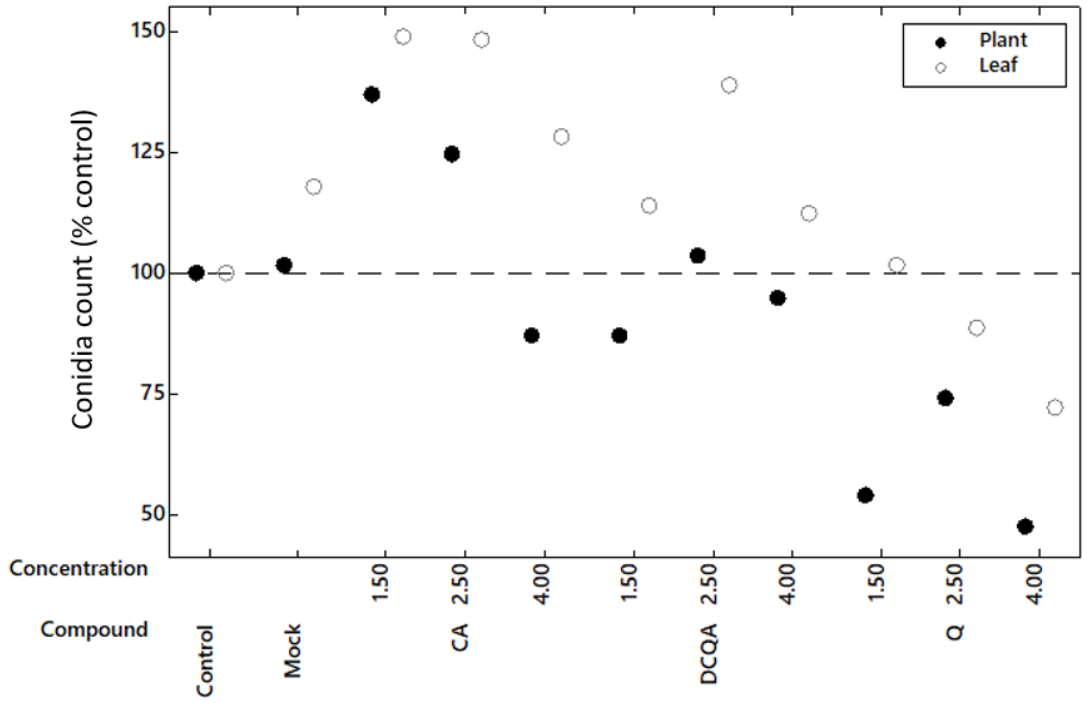


Figure 4.27.: *B. lactucae* conidia counts of lettuce (*L. sativa*) cv. El Dorado plants and leaves infiltrated with a compound (control (no infiltration), mock (water), CA = chlorogenic acid, DCQA = 3,5-dicaffeoylquinic acid , Q = Quercetin 3-O-(6″-O-malonyl)-β-D-glucoside) as a percent of the control. Lettuce plants were grown to 17 days old then the oldest true leaf infiltrated with a concentration of phenolic compound with a needleless syringe to reach a 1.5, 2.5 or 4-fold increase compared to a standard Iceberg-type lettuce plant. Plants were then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. At 12 days post-inoculation the infiltrated leaf or plant was washed in water and the resulting conidia suspension.

4.6.8. Infiltration with Quercetin 3-O-(6″-O-malonyl)-β-D-glucoside was the most promising compound to decrease disease susceptibility

The effect of each phenolic compound was highly dependent on cultivar. Q was the most promising phenolic compound across the three cultivars to decrease disease susceptibility. All concentrations of Q reduced leaf and plant conidia (compared to mock) in El Dorado, with the greatest reductions by Q at a 4-fold increase. Q (2.5-fold) also resulted in a decrease in leaf conidia count in Salinas. Both DCQA and Q caused decreases to overall disease susceptibility in Salinas. However, as DCQA resulted in a significant increase in leaf conidia count in El Dorado (2.5-fold), DCQA cannot reduce disease across cultivars. CA resulted in increases in disease severity or no change in El Dorado and Salinas, so

infiltration is not beneficial for disease reduction. Q reduces disease in the most cultivars at the largest range of concentrations. Although unable to cause large reductions in disease, Q may have a role as an antimicrobial agent.

4.6.9. Infiltration of Quercetin 3-O-(6″-O-malonyl)-β-D-glucoside yields similar conidia count reductions to UV-B-treatment

The UV-B-treatment in Section 4.4.3 increased levels of CA, DCQA and Q by 1.2 to 2.6 fold (Table. 4.8). In order to imitate this increase spread, infiltration fold increases of 1.5 and 2.5-fold were used. A fold increase of 4 was also included to indicate the effect if UV-B-pretreatment could further increase the levels of these compounds. Although LC-MS indicated the relative increase of each compound by UV-B, these compounds were not increased in isolation. This means the comparison of individual phenolic changes and resulting conidia count in the metabolomics data to the effect of infiltration of a singular compound (at a similar fold change to the metabolomics data) is not a direct comparison. With this limitation in mind, the infiltration of individual compounds can still provide some insight into the role the compound may have in a UV-B-induced disease defence.

Regression analysis of the metabolomics data (LC-MS-1) indicated that conidia count decreased as CA, DCQA and Q levels increased over all cultivars [El Dorado, Iceberg and Salinas] (Section 4.4.5). This suggests that these three compounds may contribute to a UV-B-induced disease defence. Due to possible inaccuracy caused by low sample number and low susceptibility of cultivar Iceberg, the leaf conidia counts of El Dorado and Salinas alone are considered against metabolomic data in Table 4.8.

Infiltration of CA did not support the role of CA in the UV-B-induced disease defence suggested by the correlation found in LC-MS analysis (Table 4.8). Conidia count was increased in cultivar El Dorado at all CA levels. A low concentration (1.5-fold) of CA increased leaf conidia count in Salinas; however, higher concentrations (2.5 and 4-fold) resulted in a small (11%) decrease. The infiltration increase closest to that of UV-B (1.5-fold) saw an increased disease in El Dorado and Salinas. Therefore, although there is a negative correlation between UV-B-induced CA and conidia count in the metabolomics experiment, CA alone does not appear to have a major positive reaction on disease reduction at the levels found in a UV-B-treated plant. To explain the correlation, it is possible that CA alone does not have a major influence on disease reduction and requires increases to other compounds to reduced disease. For example, CA is a precursor to lignin, so may

have a role in structural defence. However, infiltrated CA is unlikely to be incorporated into cell walls and aid in prevention of disease penetration between the time of infiltration and inoculation. Two metabolic features which strongly identified as CA where found in lettuce (Table 4.5). Only one of these correlated with disease reduction (feature 9). It is possible the other feature (6) was more representative of CA, and as this did not correlate with disease reduction, as expected, direct infiltration of the compound should have little effect on disease susceptibility.

The effect of infiltration of DCQA was cultivar dependent. El Dorado leaf conidia count was not affected by DCQA infiltration at the lowest (1.5) and highest (4) fold increase (Table. 4.8). The mid infiltration of 2.5-fold, resulted in a moderate increase in conidia count (18%). All concentrations of DCQA reduced leaf conidia count in Salinas, with the greatest reduction at a concentration of 4-fold. DCQA may play a role in Salinas as part of a UV-B-induced defence; however, is unlikely to act alone.

Infiltration of Q provides the most promising evidence for a role in UV-B-induced disease defence. In metabolomics data, Q had the strongest negative relationship with conidia count (Section 4.4.5). Infiltration of Q alone at similar levels to that of a UV-B increase (2.5-fold) resulted in a decrease in conidia count similar to that of a UV-B-induced decrease (+/- 10%) (Table. 4.8) in both El Dorado and Salinas. Although infiltration data can not directly speak to the UV-B effect, it does provide evidence that at levels induced by my UV-B-treatment, Q can cause a decrease in conidia count similar to that of the UV-B-treatment.

Table 4.8.: Comparison of decreases to *B. lactucae* conidia count on lettuce (*L. sativa*) cultivars El Dorado and Salinas following a UV-B-treatment to direct infiltration of phenolic compounds [chlorogenic acid (CA), 3,5-dicaffeoylquinic acid (DCQA) and Quercetin 3-O-(6″-O-malonyl)-β-D-glucoside (Q)]. UV-B induction of compounds was measured by Liquid chromatography–mass spectrometry (LC-MS) analysis of lettuce plants following three days of photosynthetically active radiation (PAR) +UV-B-treatment compared (fold increase) to PAR only. A subset of plants were inoculated with 10^5 conidia $mL^{-1}$of *B. lactucae* and after 12 days, washed in water and the resulting conidia suspension counted. Effect of direct infiltration of compounds was measured by infiltrating one leaf per plant with a concentration of the compound using a needleless syringe to result in a 1.5, 2.5, or 4-fold increase compared to a standard Iceberg-type lettuce. Plants were inoculated and conidia counts taken in a similar method; however, the conidia count on the infiltrated leaf alone was taken. Conidia count data is shown as a % of the control, which is PAR-treated diseased plants (UV-B-induced) or mock (water) infiltrated leaves (infiltration).

Cultivar	Compound	UV-B induced		Fold increase of infiltrated compounds		
				1.5	2.5	4
		Fold incr.	% Control Spore Count	% Mock Spore count		
El Dorado	CA	1.3	65%	127%	126%	109%
	DCQA	1.78		97%	118%	95%
	Q	2.34		86%	75%	61%
Salinas	CA	1.37	55%	113%	89%	89%
	DCQA	1.24		81%	82%	64%
	Q	2.62		80%	61%	81%

Although this protocol indicates that increased levels of DCQA and Q may contribute to increased disease defence, it does not indicate the exact mechanism. It is possible these compounds are directly toxic to the pathogen, aid in strengthening of the protective barriers or act as defensive signalling molecules. Previous studies describing the roles of these compounds in disease are discussed in Section 4.4.5.1.

Chapter 5

Discussion and Conclusions

Downy mildew caused by *Bremia lactucae* is a major disease in lettuce (*Lactuca sativa*) production. Current control measures are limited to breeding for resistance and chemical sprays. This project examined the use of UV-B-pretreatments to reduce downy mildew disease in lettuce. If successful, UV-B-pretreatments have potential to be used as a tool in an integrated pest management system. I hypothesised that :

1. UV-B-pretreatments can reduce disease severity in lettuce against *B. lactucae*
2. UV-B-induced phenolics in part mediate this reduced disease severity phenotype

The findings from this thesis generally tended to support these hypotheses (Table 5.1). UV-B-pretreatment reduced downy mildew disease severity in a range of lettuce cultivars. Severity reductions were commonly measured as a reduction in *B. lactucae* conidia count; however, reductions to sporulation rating and disease related damage also occurred in a cultivar-dependent manner. UV-B-pretreated plants took longer to display disease symptoms indicating an increased latent period. The increased latent period was caused by an increased time required for establishment of *B. lactucae* hyphae throughout a plant leaf (Section 3.3).

Reduction in disease severity (as conidia count) negatively correlated with UV-B-induced flavonoid level. LC-MS analysis of UV-B-induced metabolites indicates features which correlate with disease reduction to largely be phenolic compounds including phenolic acids and flavonoids. Three compounds which have a high negative correlation with conidia count were infiltrated directly in lettuce leaves. Quercetin 3-O-(6-O-malonyl)-b-D-glucoside infiltration resulted in a decreased leaf conidia count in cultivars El Dorado and Salinas;

however, lacked reductions in other disease parameters. Therefore UV-B-induced phenolics may reduce disease severity but are not responsible for the entire reduced susceptibility phenotype.

5.1. UV-B-pretreatments used in this project reduced disease at similar levels to previous studies

UV-B-induced disease reductions observed in this project fell within a reasonable range of previous observations (Table 5.2). As UV wavelengths can have varying biological effectiveness (Aphalo et al., 2012), and the intensity per wavelength is not recorded in previous studies, it is predictably challenging to compare my UV treatments to previously published UV treatments.

Wargent et al. (2006) performed a similar study to this work, in which Lettuce cv. Rex plants were treated with UV-B from fluorescent tubes and then inoculated with *B. lactucae* conidia (Table 5.2). Conidia counts were taken for nine days following inoculation. UV-B doses ranged from zero to 12 kJ m^{-2} d^{-1} as weighted by the plant action spectrum of Caldwell (1971). A greater overall reduction in conidia count (11.9 KJ $m^{-2}day^{-1}$: 74% reduction) was observed by Wargent et al. (2006) than in my work. As both studies showed a trend (stronger in Wargent et al. (2006)) of decreasing conidia count with increasing UV-B dose, this higher disease reduction is likely caused by a daily dose which was higher than doses used in my work. However, as the light treatment systems varied greatly, doses between studies cannot be accurately compared. Wargent et al. (2006) used fluorescent tubes to supply UV light with cut-off filters to limit the spectrum to the desired waveband. I used UV-B LEDs which can provide a very narrow waveband. My UV-B LEDs have a sharp irradiance peak at the high biologically important wavelength of 300nm with almost negligible irradiance levels +/- 10nm. Therefore, plants used in Wargent et al. (2006) received a larger range of UV-B wavelengths, which may have different biological effectiveness and may induce slightly different UV-B response pathways than my work. To add, the lettuce age and cultivar, growth system and infecting *B. lactucae* strain differed between studies. Despite differences in experimental design, both studies strongly support the ability of UV-B-pretreatment to reduce susceptibility of lettuce to downy mildew disease. Disease reduction in a lettuce-downy mildew system is highly conserved, and is

Table 5.1.: Evidence from this project supporting a phenolic mediated downy mildew disease reduction caused by *B. lactucae* in lettuce (*L. sativa*) following UV-B exposure.

Hypothesis	Research objective	Research outcome	Section
1	What is the optimal UV-B dose (300nm) for disease reduction in lettuce cv. Casino?	0.5 µmol $m^{-2}s^{-1}$ reduced disease rating, damage and spore count (58%)	2.3
	Is a UV-B disease defence conserved across a range of lettuce cultivars?	0.5 µmol $m^{-2}s^{-1}$ reduced spore count (35-62%) in an additional 4 cultivars	2.4, 4.3.1
	Do spore count reductions alter subsequent lettuce downy mildew infections?	Spore count was reduced when infecting/infected plant or both were UV-B-pretreated	2.6
	Is *B. lactucae* growth altered in UV-B-pretreated lettuce plants?	UV-B-pretreated plants contained hyphae with a reduced density and width	3.3
2	Are there similarities between gene expression following biotrophic disease and UV-B treatment?	Flavonoid synthesis related genes were important to *Hpa* resistance and up-regulated by UV-B in *Arabidopsis*	3.5.1
	Do total flavonoids have a role in a UV-B-induced disease defence?	Flavonoids significantly increased in all cultivars by UV-B and correlated with a reduced spore count	3.4, 4.3
	Which compounds are up-regulated by UV-B in multiple lettuce cultivars?	Phenolic compounds (flavonoids and phenolic acids) are higher in UV-B treated plants of many cultivars	4.4.1, 4.4.4, 4.5.4
	Do any UV-B up-regulated compounds contribute to a correlation with disease reduction ?	Ten or six (depending on cultivars) metabolites correlate with decreased spore count	4.4.5, 4.5.5
	Can highly correlated compounds directly reduces disease severity in lettuce downy mildew?	A quercetin compound can reduce spore count in cv. El Dorado and Salinas when directly infiltrated into a leaf	4.6.5
Application	Can a commercial light treatment system reduce downy mildew disease in lettuce?	Sporulation rating and spore count (40-54%) were reduced in cultivars following a commercial treatment	2.5.1

more effective at higher doses (as weighted by a plant action spectrum).

UV-B-pretreatments have also been noted to decrease susceptibility to the necrotrophic fungal pathogen *Botrytis cinerea* in *Arabidopsis thaliana* (Table 5.2). Demkura and Ballare (2012) observed a decrease in lesion size (~60%) in *Arabidopsis* leaves treated with UV-B from fluorescent tubes then inoculated with *B. cinerea*. The experimental system used by Demkura and Ballare (2012) varied greatly in terms of plant, disease type, light source and growth conditions from my work; however, resulted in a fairly substantial decrease in disease. Therefore, the ability of UV-B to decrease disease susceptibility is fairly well conserved across these variables. Lesion size is a description of disease damage. My work noted UV-B-pretreatment reduced the number of plants displaying disease damage in a lettuce-downy mildew system. Although highly different systems, these two studies provide evidence that UV-B-pretreatments can increase the tolerance of plants to disease through reduction of damage caused by the pathogen (or by the immune response).

A UV induced disease defence is not specific to the UV-B waveband. The higher energy waveband of UV-C with the key wavelength of 254nm, has has been observed to reduce susceptibility to downy mildew (*Hyaloperonospora parasitica*) in *Arabidopsis* and *B. cinerea* in lettuce (Table 5.2). UV-C light is not present in ambient sunlight (Tilbrook et al., 2013); however, can still weakly induce photoreceptor UVR8 which controls the majority of UV-B photomorphogenic responses (Christie et al., 2012). The efficiency of UV-C recognition by UVR8 is much lower than that of UV-B (Christie et al., 2012) with a large portion of UV-C signalling occurring through direct absorption by DNA or production of reactive oxygen species (ROS) (Urban et al., 2016). Different disease assessment methods of downy mildew, as well as plant-disease system were used by (Kunz et al., 2008) than in my project. However, it appears that UV-C can more successfully induce disease reduction against biotrophic oomycete pathogens than UV-B light. A larger disease reduction was achieved (Table 5.2), as well as induction of a hypersensitive response (HR), indicating effector triggered immunity (ETI) has also been induced following UV-C pretreatment (Kunz et al., 2008). Whilst my UV-B-treatment could reduce susceptibility, likely through increases to defensive compounds, it appears UV-C can induce a resistance response through activation of immunity. UV-C response pathways tend to be UVR8-independent whilst my 300nm UV-B is more likely to induce UVR8-dependant pathways (Christie et al., 2012). Therefore induction of HR and resulting increased disease reduction may be due to greater induction

of UVR8 independent UV signalling pathways.

5.2. UV-B light decreases disease susceptibility through up-regulation of the phenylpropanoid pathway

Previous studies suggest a switch between growth and defence lifestyles (Ballare et al., 2012) or increases to defensive barriers through increases to sinapate-style lignin (Demkura and Ballare, 2012) may mediate UV-B-induced disease defences. Although not directly linked to disease reduction, UV-B increases several compounds in plants which have been implicated in increased defence to disease. This includes UV-B induction of secondary metabolites such as glucosinolates, and flavonoids (Mewis et al., 2012), defensive hormones; salicylic acid (Surplus et al., 1998) and Jasmonic acid (Caputo et al., 2006), pathogenesis related (PR) proteins (Fujibe et al., 2000) and ROS (Hideg et al., 2013). My work attempted to provide more insight into mechanism by investigating the role of secondary metabolites in a UV-B-induced disease defence.

5.2.1. UV-B-induced flavonoids correlate with reduced downy mildew disease severity

This project provided evidence towards a role of UV-B-induced flavonoids in an increased defence against disease. In lettuce (*L. sativa*), flavonoid levels were significantly higher in UV-B-pretreated plants than control plants at the time of inoculation (Section 4.3.2). However, once plants were infected with *B. lactucae* conidia, the difference between flavonoid levels of UV-B and control plants decreased. At the time of inoculation, flavonoid levels negatively correlate with disease severity levels (conidia count), indicating as flavonoid levels increase at inoculation, disease severity decreases at later time pointers (Figure 4.5). This correlation was strongly influenced by UV-B responsive flavonoids with a loss of correlation when control plants alone were considered. Therefore it can be concluded UV-B-responsive flavonoids negatively correlate with a reduction in conidia count. As both flavonoid levels decreases and a significant correlation is lost during the disease period, it can be further concluded that flavonoids present at the time of inoculation, i.e. phytoanticipins, contribute to the disease defence, rather than phytoalexins at later disease stages. UV-B-induced flavonoids present at the point of infection can therefore slow the

Table 5.2.: Summary of UV-induced tolerance against disease in plants in this project compared to previous studies. Dose descriptions by Wargent et al. (2006) and Demkura and Ballare (2012) are biological weighted using the plant action spectrum of Caldwell (1971). Greyed example shows reduction caused by 0.5 µmol $m^{-2}s^{-1}$[7.03 KJ $m^{-2}d^{-1}$ unweighted] in lettuce (*L. sativa*) cv. Casino from dose response experiments in this project.

Reference	Waveband	Type	Dose	Plant	Disease	Susceptibility effect
Section 2.3.5	UV-B (300nm)	UV-B LEDs	7.03 KJ m^{-2} d^{-1}	*Lactuca sativa*	*Bremia Lactucae*	58% Reduction in spore count
Wargent & Paul (2006)	UV-B (<290nm) and UV-A	TL40 UV-B tubes	11.9 KJ m^{-2} d^{-1}	*L. sativa*	*B. Lactucae*	74% Reduction in spore count
Demkura & Ballare (2012)	UV-B	TL100W/01 UV-B tubes	5.5 KJ m^{-2} d^{-1}	*Arabidopsis thaliana*	*Botrytis cinerea*	~60% Reduction in lesion size
Kunz, et. al. (2008)	UV-C (254nm)	germicidal tube	500 J m^{-2}	*A. thaliana*	*Hyaloperonospora parasitica*	~80% Reduction in conidiophores
Vàsquez, et. al. (2017)	UV-C (254nm)	Spectroline, Model ZQJ-254 tube	0.85 kJ m^{-2}	*L. sativa*	*B. cinerea*	10–20% reduction in lesion size

establishment of downy mildew disease resulting in an increase in the incubation period, delay in disease and reduced conidia count.

There are conflicting thoughts on the role of flavonoids in a UV-B-induced disease defence. A study by Demkura and Ballare (2012) showed *Arabidopsis* mutants which had reduced function of chalcone sysnthase (*tt4-1*) and therefore deficient in flavonoid synthesis maintained a UV-B-induced reduction to lesion size by *B. cinerea.* In this case, UV-B-induced flavonoids were not required for disease resistance. The conflicting conclusions drawn by Demkura and Ballare (2012) and my study may be due to the different plant and disease systems. *B. cinerea* is a necrotrophic fungus whilst *B. lactucae* is a biotrophic oomycete. Necrotrophic pathogens tend to release toxic substances and enzymes to kill the plant cells and extract nutrients from dead material (Glazebrook, 2005). Biotrophic pathogens extract nutrients from living plant tissue so must enter the plant less destructively, mostly occupying extracellular space (Hahn and Mendgen, 2001). Therefore, biotrophic and necrotrophic pathogens have different invasion strategies and are defeated by different plant defences. In response to biotrophic pathogens, less toxic antimicrobial compounds, such as flavonoids, might have a larger role.

Analysis of transcriptomics data from previous studies (Table 3.1) supports the role of chalcone synthase in a UV-B-induced disease defence against biotrophic pathogens. *Arabidopsis* treated with low and high UV-B light both increased chalcone synthase transcripts ($\log_2$ FC of 4.4 and 5.3 respectively). Chalcone synthase was also up-regulated in an incompatible infection of *Arabidopsis* with *Hyaloperonospora arabidopsidis* (casual agent of downy mildew), but not in a compatible infection ($\log_2$FC 0.9 and -0.1 respectively). These separate studies indicate that in *Arabidopsis* chalcone synthase is important for resistance against *Hpa* and is up-regulated by UV-B light, providing evidence towards a role of flavonoids in a UV-B-induced biotrophic disease defence. Whilst UV-B-induced flavonoids may contribute to defence against biotrophic pathogens, they are less important to necrotrophic pathogen defence.

5.2.1.1. Quercetin derivatives are important for a UV-B-induced disease defence

Many metabolic features of lettuce were identified by LC-MS analysis as flavonoids (features 14, 17, 18, 19, 21; Table 4.5). Of these two; quercetin-3-Glucuronide (feature 17) and quercetin 3-O-(6″-O-malonyl)-β-D-glucoside (feature 19) had a strong significant negative

correlation with *B. lactucae* conidia count (Section 4.4.5). These are both quercetin derivatives. Further evidence towards the role of quercetin (and quercetin derivatives) in a UV-B-induced disease response was shown in the transcriptomics analysis (Section 3.5.1.1). The enzyme flavonoid 3´-hydroxylase (F3'H) converts kaempferol into quercetin resulting in an increased quercetin to kaempferol ratio (Peer et al., 2001). F3'H was up-regulated in an incompatible but not a compatible infection between *Arabidopsis* to downy mildew caused by *H. arabidopsidis*. F3'H and therefore increased quercetin are important to downy mildew resistance in *Arabidopsis*. F3'H is also up-regulated by low UV-B light indicating that UV-B can increase the ratio of quercetin:kaempferol in a manner that is important to defence against downy mildew.

Quercetin 3-O-(6″-O-malonyl)-β-D-glucoside had the strongest negative correlation with conidia count. Direct infiltration of quercetin 3-O-(6″-O-malonyl)-β-D-glucoside into lettuce leaves (no light treatment) reduced *B. lactucae* conidia count in cultivar El Dorado (at a 2.5 and 4-fold increase) and Salinas (at a 2.5-fold increase) compared to leaves infiltrated with water (Figure 4.25). Therefore, at amounts similar to that of UV-B induction, quercetin 3-O-(6″-O-malonyl)-β-D-glucoside alone can reduce *B. lactucae* conidia count. This work provides strong evidence that quercetin derivatives, in particular quercetin 3-O-(6″-O-malonyl)-β-D-glucoside is likely involved in the mechanism of a UV-B-induced disease defence.

As discussed in Section 4.4.5.1, quercetin and derivatives have been associated with increased disease resistance in previous studies. However, a clear mode of action of quercetin in decreasing disease is not well established. Some studies suggest quercetin derivatives can have direct antimicrobial activity against bacterial and fungal pathogens (Rauha et al., 2000; Zhu et al., 2004; Gatto et al., 2011). This activity has been attributed to inhibition of pathogen germ-tubes or increases to the ROS response (Section 4.4.5.1; Tao et al., 2010; Jia et al., 2010) whilst others propose no direct antimicrobial effect (Sanzani et al., 2008). Therefore, UV-B-induced up-regulation of quercetin may have a direct antimicrobial effect on invading *B. lactcuae* conidia resulting in delayed symptoms and reduced conidia count.

5.2.1.2. UV-B-induced quercetin: mechanism or marker of defence?

In my work, infiltration of quercetin 3-O-(6″-O-malonyl)-β-D-glucoside alone reduced disease severity; however, it is still uncertain if this was due to a direct effect or recruitment

of other defensive pathways. As quercetin derivatives have been noted to have antimicrobial effects against a range of plant pathogens, it is likely quercetin 3-O-(6″-O-malonyl)-β-D-glucoside and quercetin-3-glucuronide also have this antimicrobial activity. UV-B induction of quercetin derivatives in the epidermal layer would increase the amount of antimicrobial phytoanticipins in these outer cells. Upon confrontation with a pathogen, the preformed defences of a UV-B-pretreated plant would be higher resulting in the delay in infection observed in my experiments. Although it can be concluded that quercetin compounds could reduce disease severity in a UV-B independent manner, it is unlikely to be solely responsible for the UV-B-induced disease defence.

As the mode of action of quercetin 3-O-(6″-O-malonyl)-β-D-glucoside is uncertain, it can be suggested that it is also a marker for a UV-B-induced disease defence. Secondary metabolites are commonly up-regulated as part of a successful disease defence response. Increases to one of the key metabolites to increased defence (in my work; quercetin 3-O-(6″-O-malonyl)-β-D-glucoside) can be indicative that the entire defensive capability of plant has increased. For example, flavonoids are known to interfere with auxin transport and contribute to a more compact plant (Brown et al., 2001). Increases to the flavonoid; quercetin 3-O-(6″-O-malonyl)-β-D-glucoside, may aid in the signal process in which plants switch between vegetative and defensive growth described by Mazza and Ballare (2015). Thus increases to flavonoid level results in overall increased defence of the plant, but is not directly being caused by the flavonoid itself. Although the marker may not be directly responsible for the entire increased disease defence, it is usually increased when the overall increased defence is higher.

Discovery of a marker is incredibly useful. Markers can be used for faster treatment development. In typical disease defence treatment development, a plant would be treated with a UV-B recipe (3-7 days), followed by inoculation with a pathogen and disease assessment (7-12+ days). However, if quercetin 3-O-(6″-O-malonyl)-β-D-glucoside levels at the conclusion of the treatment phase indicate if a treatment is likely to result in decreased disease severity (ie. act as a marker), then only promising treatments will be carried forth into the pathogen inoculation and assessment phase. This will save time, as treatments which lack changes to quercetin 3-O-(6″-O-malonyl)-β-D-glucoside following treatment will not be continued, thus reducing treatment evaluation by 7-12+ days. Marker-assisted treatment evaluation will increase the efficiency of treatment development through increased

evaluation speed and reduced resources wastage.

5.2.2. A range of phenolic compounds likely contribute to the UV-B-induced disease defence

Strong evidence was provided for a role of quercetin 3-O-(6″-O-malonyl)-β-D-glucoside in a UV-B-induced disease defence; however, this only correlated with disease reduction in cultivars El Dorado, Iceberg and Salinas. To add, infiltrations of quercetin 3-O-(6″-O-malonyl)-β-D-glucoside resulted in decreases in disease of cultivars El Dorado and Salinas only. In an increased range of cultivars, as well as these cultivars, a range of non-flavonoid phenolic compounds also significantly negatively correlated with disease reduction. As well as flavonoids, caffic acid derivatives such as chlorogenic acid (LC-MS-1), chicoric acid (LC-MS-2) and 3,5-Dicaffeolquinic acid (LC-MS-1 and 2) significantly negatively correlate with conidia count. These have been reported to have antimicrobial properties in previous studies (Gatto et al., 2011; Zhu et al., 2004), and in this work direct infiltration with 3,5-Dicaffeolquinic acid alone reduced conidia count in some cases. The slopes of all negative regressions found in my work were similar indicating no stand out metabolic feature as the main contributor to conidia count decreases. Rather, it is more likely, these correlated phenolic compound (or up/downstream compounds) act to improve the overall hardiness of the lettuce plant as a form of protection against the high energy of UV-B light. The increased hardiness also acts to reduce susceptibility to other stresses such as pathogen attack. Although phenolics were the focus of my investigation, other pathways not investigated in this study may also contribute to the overall increased hardiness and resulting increased disease defences of a UV-B-treatment.

5.3. UV-B light treatments have potential to be a useful disease control tool in horticulture

The ability of a UV-B-pretreatment to reduce subsequent *B. lactucae* disease severity has potential to be useful as a horticultural tool to decrease susceptibility of lettuce plants to downy mildew disease. In terms of commercial use, a delay in disease and reduction of conidia count likely are the most useful benefits.

A typical conclusion from experiments was a UV-B-induced delay in disease. This was

observed as a significant decrease in disease incidence (Section 2.3.1) as well as hyphal density (Section 3.3) of UV-B-pretreated plants at early stage disease; however, not at late stage disease. Therefore, it is likely that the UV-B response is increasing defences at the establishment stage of the pathogen resulting in an increased latent period. Delay of infection is of agricultural importantance. Increase in incubation period of a pathogen can reduce the risk of epidemics by reducing the rate in which inoculum is produced (Buonomo and Cerasuolo, 2015). Although, in this case, UV-B only delays infection by one to three days, downy mildew disease is polycyclic, therefore, over a season, this delay can reduce the number of disease cycles that can occur. This delay creates a larger window for the epidemic to be recognised and an increased opportunity for additional control measures to be applied. Delays in infection can also be important at a smaller scale for maintaining yield. Production of biomass is greater in healthy plants than diseased plants. Therefore, the longer the plant remains in a healthy state, the higher the potential for biomass accumulation (Peterson and Higley, 2000; Seem, 1988). Although, the economic implication of a one day delay in lettuce disease is unknown, Tan et al. (2008) estimates that a one week delay in disease in organic potato cultivation at one ton per hectare per day could result in over $1500 per hectare.

In all UV-B experiments, UV-B-pretreated plants had a reduced *B. lactucae* conidia count compared to control plants. As conidia count indicates the level of inoculum produced from an infected plant, a reduction in conidia count indicates a reduction in inoculum. UV-B-pretreated plants infected with disease produce a lower amount of inoculum and therefore have a lower potential for infection of healthy plants (Fall et al., 2016). As explored in Section 2.6, this reduction in conidia count is sufficient to reduce disease severity in plants infected from an infected UV-B-pretreated plant. This reduction in disease severity is amplified when both the inoculum plant and the secondary plant have been UV-B-pretreated. UV-B-pretreatment can aid in the reduction of disease spread, by reducing the inoculum produced over each infected UV-B plant.

The combination of delay in infection and reduction of inoculum can aid in slowing down disease progression and spread. UV-B-pretreated plants would tend to take longer to become infected and produce a lower amount of inoculum. This creates a greater opportunity for detection of disease and additional control measures to be effectively used. UV-B-pretreatments would also fit well in an integrated pest management system. UV-B-

pretreatments have high potential for nursery or glasshouse crops, which are commonly more susceptible to disease due to proximity, higher humidity, younger age and a more controlled environment (Poorter et al., 2016). To add, a glasshouse adds the infrastructure required to attach a light array for the logistics of completing a treatment. As glasshouse crops cost more to produce, use of UV-B to reduce disease is a highly feasible option. Use of UV-B-pretreatments would help reduce disease pressure whilst in the glasshouse, and have the potential to continue to provide a disease defence for plants which are transplanted into a field.

5.3.1. The longevity of a UV-B-induced defence is uncertain

The longevity of a UV-B-induced defence is unknown. UV-B may induce a short term defence which is only effective immediately following treatment, or may cause longer lasting effects which provide a life long decreased susceptibility. In all UV-B experiments, plants were confronted with disease 14 h after the end of a UV-B-treatment with final harvest at 11 or 12 days post-inoculation. A harvest time of 12 DPI was chosen in order to capture the severity of only one disease cycle. The lettuce-*Bremia* system used was observed to require seven days for sporulation to begin. In order to avoid assessment of re-infected plants from sporulating tissue, harvest for conidia count had to taken prior by 14 DPI to account for the 7 day latent period. To add, by 12 DPI, many plants were reaching a maximum sporulation rating, with some leaves becoming necrotic. In the secondary inoculation experiments, many plants reached a maximum rating by 11 DPI, and so plants were harvested for conidia at this time point instead. If leaves were to die, sporulating tissue would no longer grow on the dying leaf due to the biotrophic nature of *B. lactucae*. In this case, although disease was more severe, conidia count would be reduced due to less sporulating tissue. It would be interesting to allow the experiment to continue to run to determine if UV-B-pretreated plants can overcome disease, and if not, at what point the UV-B-induced defence is no longer effective. As disease assessment techniques used in these experiments were designed to asses one disease cycle with no leaf death, a different approach would be required to assess the longevity of disease protection.

The other question on longevity arises from the uncertainty of when disease infection will occur. As mentioned, in these experiments, plants were inoculated at 14 h after the UV-B-treatment had ended. However, disease infections are unpredictable, and in a

commercial system disease will not consistently occur directly following a UV-B-treatment. The permanence of a UV-B-induced disease defence is unknown and it would be useful to determine the maximum time between UV-B-treatment and disease inoculation for the UV-B-treatment to still reduce susceptibility. Throughout this project, evidence towards a delay in disease, likely due to preformed phenolics, appears to have a major role in the UV-B disease defence. UV-B-responsive phenolic (represented by flavonoids) level can be roughly used as an indication of presence of a UV-B defence. A significant difference in flavonoid level between UV-B-treated and control plants is present at 96 h after the end of the light treatment (168 h total) at the latest (Figure 4.3). It could be hypothesised that the UV-B effect therefore lasts until 168 h, and some level of UV-B-induced disease defence would be present for plants confronted with a pathogen up until this point. However, flavonoid data was taken on diseased plants. Previous studies observed that disease can down-regulate flavonoid levels (Schenke et al., 2014, 2011), indicating that disease may dampen the UV-B-induced phenolic effect, and therefore in the absence of disease, an induced UV-B effect may be longer lasting than 96 h post-treatment. An experiment in which plants are treated with UV-B then infected with *B. lactucae* at a range of time points post-treatment would provide a time line for the time of effectiveness of a UV-B-treatment.

A third scenario not tested in this project is a situation in which lettuce plants are infected with the pathogen during a light treatment. As well as inducing a response in plants, UV-B light can effect the microbe and their reproductive conidia. Plant pathogens, in particular biotrophic fungi or oomycetes tend to be most vulnerable to UV-B light at conidia stage (Raviv and Antignus, 2004). UV-B light is commonly noted to be detrimental to pathogen survival with decreases to germination, or death of conidia Braga et al. (2001); Mizubuti et al. (2000); Wu et al. (2000); de Menezes et al. (2015); Suthaparan et al. (2012); Cheng et al. (2014). UV-B light can also reduce hyphal development as well as sporulation of established pathogens (Fourtouni et al., 1998; Ensminger, 1993). UV-B has been noted to reduce virulence of *Podosphaera pannosa* by 90% in roses for as long as the treatment occurs. Other pathogens, mostly necrotrophic or saprophytic fungi, can have stimulated growth, such as increases in conidia/conidia production, by UV-B and near UV light (Alves et al., 1984; Mooney and Yager, 1990; Fourtouni et al., 1998; Zhang et al., 2009; Avalos and Estrada, 2010). Therefore, the direct effect of UV-B light is dependent on the pathogen. *B. lactucae*, the case study in this project, has been observed in a previous

study to be inhibited by light which includes UV-B resulting in a reduction of sporangium viability (Wu et al., 2000). Therefore, in this case, and most biotrophic infections, a UV-B-treatment would likely have a dual effect of increasing a plant's resistance to disease as well as decreasing the virulence of the pathogen, likely resulting in a further reduction in disease severity. In other cases where UV-B can stimulate pathogen growth, it would depend on whether pathogen stimulation was out-weighed by the induced plant defences to determine the outcome of increased resistance. This would heavily depend on not only treatment, but the plant and pathogen's responsiveness to UV-B light.

5.3.2. Alterations to experimental design are required to test the application of a UV-B disease control tool

All experiments were carried on lettuce seedlings (2-3 weeks old). This is indicative for a nursery environment; however, downy mildew disease can infect a lettuce plant at any stage before harvest. The growth habits and susceptibility of an iceberg type lettuce are very different at seedling and head stage. To add, all successful experiments were carried out in controlled temperature room with ideal lettuce growth conditions. By providing such an optimal environment for growth, the plant had little priming for stress, and therefore a UV-B-treatment may have been more impactful. In order to determine if a UV-B-treatment can maintain an increased defence from a nursery environment to the field; field trials which run from seedling (glasshouse) to harvest (field) would be required.

A glasshouse trail was attempted; however, was unsuccessful due issues with pests, uniformity and the light treatment system (Section 2.5.2). Suggestions were given on how to improve experimental design to overcome these issues. A field trial could be run similar to that of *B. lactucae* fungicide trails by the Pacific Ag group [Spreckles, CA, USA] (Richardson, 2018). A plot (30ft) with 40 inch beds would be set up at the start of the lettuce season (early spring). Highly susceptibility lettuce plants, such as cultivars Emperor or El Dorado [Richard Michelemore, UC Davis, USA], would be planted in one row on the one side of each bed. These highly susceptibility plants are sacrifice plants which are encouraged to become diseased with downy mildew. If disease does not occur on the first set of sacrifice plants, a second row will be planted on the bed until a solid infection is present (removing the first row as necessary to maintain one row of sacrifice plants per bed). Two week old seedlings (glasshouse-raised) will undergo a BioLumic commercial

UV-B-treatment as prescribed by BioLumic [www.biolumic.com]. Following treatment, plants will be transplanted into the plot to form a second row (Figure 5.1). UV-B and control plants will be randomly distributed throughout the "treatment" rows within the plot. The treatment plants will be allowed to become naturally infected from the sacrifice plants. A spore trap (http://www.burkardscientific.co.uk/ agronomics/hirst_spore_sampler.htm) may be used to measure the amount of inoculum present in the plot. Resulting disease on treatment plants will be assessed as incidence (the number of lesions on each leaf) and severity (visual rating scale). The plants will be grown to harvest, with the number of harvest-able lettuce heads within each size group recorded. Six repeats at multiple trial locations should be completed. If this field trial design suggests difference in susceptibility of UV-B and control plants, further experiments similar to secondary infection experiments (Section 2.6) but in a field environment should be completed.

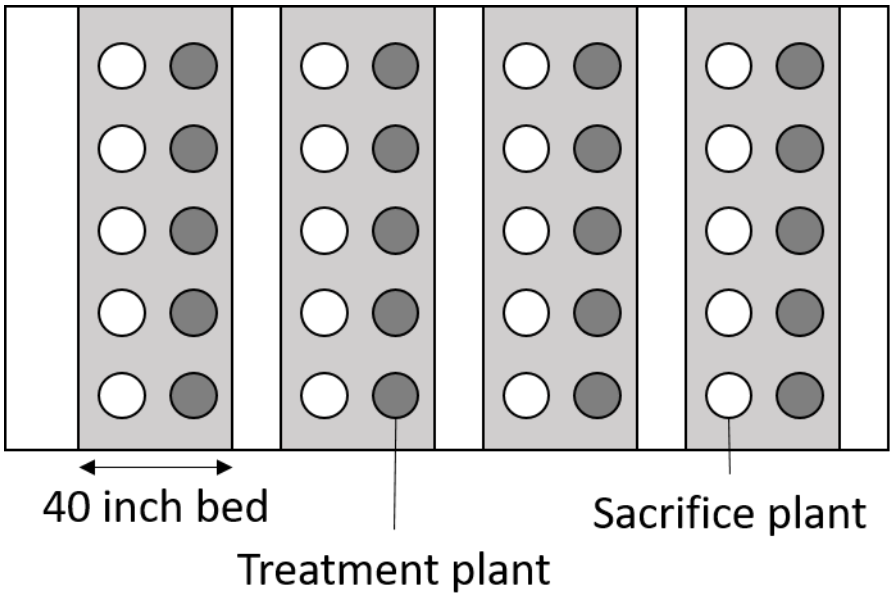


Figure 5.1.: Layout of lettuce plants into disease sacrifice plant or treatment (UV-B or control) plant rows.

5.4. Further investigation into UV-B-induced disease defence is required

This project produces strong evidence towards the ability of UV-B to reduce disease severity in lettuce cultivars against *B. lactucae* and proposes a role of quercetin flavonoids in the increased defence. However, there is still much that is unknown on the breadth of a

UV-B-induced defence, as well as the extent of the role of flavonoids.

Although the optimal treatment of 0.5 μmol $m^{-2}s^{-2}$(300nm) as well as a 280nm treatment was successful in decreasing disease, further tweaking of treatment could further improve disease reduction (Table 5.3). Dose response experiments indicated the highest does of the 300nm treatment (0.5 μmol $m^{-2}s$) resulted in the highest and most consistent decreases to disease severity. Although this was true for lettuce infected with downy mildew disease, to examine the range of effectiveness of this treatment, it should be trailed on a range of crop plants and disease systems. It will be useful to determine if a UV-B-treatment is specific to lettuce downy mildew or if it can decrease biotrophic disease in a range of crop plants, or even a range of disease types. Disease reduction tended to increase with increasing UV-B dose suggesting higher doses of a 300nm treatment could decrease disease severity further. As UV-B dose is also dependent on duration and wavelength, this dose could potentially be increased by altering these variables as well as fluence rate. As discussed by Mazza and Ballare (2015), a UV-B-induced disease defence may be caused by the interaction between signals from UV-B light as well as background light such as far-red light resulting in a switch between shading and defence responses. Therefore, the disease reduction effect of UV-B recipe could be enhanced by alterations of background light also. As background light is so important, use of the same light source between growth of plants and treatment, such as red and blue LEDs as a source of PAR throughout the plant's life will help isolate the UV-B effect. In some experiments, such as those investigation flavonoids (Section 4.3.2), the switch between lighting systems (fluorescent tubes to LEDs) resulted in a flavonoid increase in control plants as well as UV-B plants. By using LEDs for growth as well as treatment, plants will be acclimatised to LED light conditions and the effect of adding UV-B only can be analysed.

As discussed in this Chapter (Section 5.3.1), the longevity of a UV-B-induced defence is unknown. To further categorize this response, further experiments on disease timing should be completed. The longevity is important, as it will provide a guideline on how often a UV-B-treatment is required to increase increased disease protection, or if the protection is permanent. The level of protection provided following a treatment therefore needs to be further investigated (Table 5.3).

The mechanistic cause of a decreased disease susceptibility requires much more investigation. Mechanistic investigation not only increases fundamental knowledge on a plant's

Table 5.3.: Summery of next steps in development of UV-B as a tool for priming plants against biotrophic diseases.

Goal	Steps
Treatment development	• Higher doses of a stationary 300nm treatment • A range of UV-B wavelengths and treatment durations • Alterations to background light, such as intensity, the red:blue ration, as well as the addition of far-red light • Alterations to growth conditions prior to treatment, such as use of LEDs for growth as well as treatment • Test the ability of UV-B to reduce disease susceptibility in different crops and diseases
Longevity of disease reduction	• Time between treatment and infection, including infection during treatment • Duration of increased defence past 12 DPI
Mechanistic investigations	• UV-B treatment and disease assessment of UVR8 and flavonoid knock out mutants • Study direct effects of UV-B induced compounds on pathogen growth • Transcriptomics studies of differential expression UV-B treated and higher resistance plants
Commercial adaption	• Development of disease assessment tools for glasshouse grown plants, as well as different maturity stages • Field trials using a BioLumic treatment

response to UV-B light but may help with further treatment development. By increasing understanding of how UV-B is inducing a protective response, markers of resistance can be targeted in treatments. For example, if flavonoids are known to be important in reducing disease in plants, UV-B-treatments which increase flavonoids can be targeted as promising disease reducing treatments. Mechanistic investigation may also identify alternative disease control tools. If a UV-B-induced gene or compound is strongly linked with disease reduction, it can be used as a breeding target for improved qualitative resistance. If the compound has high disease reducing activity, it may even have potential as a direct spray as a stand alone disease control treatment. In order to identify these UV-B-induced defensive traits further mechanistic investigation, such as transcriptomics or antimicrobial tests of identified compounds is required (Table 5.3).

This project showed that UV-B can decrease disease in a completely control environment; however, this varies commercial growth of lettuce. In order to test the commercial application of a UV-B-induced disease defence, commercial adaptation of UV-B-pretreatments, experiments in which plants are grown to harvest is required. A proposed field trial was outlined in Section 5.3.2). A treatment which results in disease reduction in both glasshouse and field trials (Table 5.3) is required to push UV-B priming for disease control into a commercial application.

5.5. Conclusions

The UV-B-treatments used in this project can reduce susceptibility to downy mildew disease caused by *B. lactucae* in lettuce (*L. sativa*). This reduction in disease severity is correlated with increases to phenolic compounds, in particular quercetin flavonoids. Although, some insights into mechanism of induced defence are given, this is still a largely unexplored area. UV-B-pretreatments have high potential to be a commercial tool for disease control, and further work will advance the opportunity for commercial adaptation as well as optimization of treatments for different crop-disease systems.

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Appendices

Appendix A

Additional Chapter 3 data

A.1. Additional expression data of genes influenced by UV-B and biotrophic disease

Table A.1.: Logfold change of Salicylic acid synthesis-related genes in *Arabidopsis* plants treated with UV-B or infected with the biotrophic pathogen *Hpa* in a compatible or incompatible interaction.

Locus	Type	Name	Low UV-B	High UV-B	Incompatible	Compatible
at3g11480	hormonesynthesis-degradation	BSMT1, ATBSMT1	-1.62	2.81	-0.41	-1.08
at3g44860	hormonesynthesis-degradation	FAMT	0.55	3.21	1.68	0.46
at5g56300	hormonesynthesis-degradation	GAMT2	-0.51	0.27	-1.37	-3.04
at5g55250	hormonesynthesis-degradation	IAMT1	-0.80	-1.34	1.07	0.40
at3g21950	hormonesynthesis-degradation	methyltransferase	-0.38	-1.83	0.13	0.08
at5g04370	hormonesynthesis-degradation	NAMT1	-0.21	-2.89	-2.72	-1.76
at2g14060	hormonesynthesis-degradation	S-adenosyl-L-methionine:carboxyl methyltransferase family protein	1.60	-0.27	-0.12	-0.19
at1g68040	hormonesynthesis-degradation	S-adenosyl-L-methionine:carboxyl methyltransferase family protein	0.58	-1.13	-1.51	-1.15
at1g66690	hormonesynthesis-degradation	S-adenosyl-L-methionine:carboxyl methyltransferase family protein	-0.87	6.58	0.10	0.41

Table A.2.: Log_2 fold change of receptor genes in *Arabidopsis* plants treated with UV-B or infected with the biotrophic pathogen *Hpa* in a compatible or incompatible interaction.

Locus	Type	Name	Low UV-B	High UV-B	Incompatible	Compatible
at1g47890	Receptors	AtRLP7	-1.09	1.36	1.10	-0.18
at4g04220	Receptors	AtRLP46	0.26	1.08	1.88	0.78
at2g32680	Receptors	AtRLP23	-2.15	1.39	3.21	2.01
at5g45240	Receptors	TIR-NBS-LRR class	-1.14	-1.91	-1.29	-1.13
at3g24900	Receptors	AtRLP39	1.36	1.14	3.72	2.43
at3g23120	Receptors	AtRLP38	1.36	2.18	1.97	1.01
at3g25010	Receptors	AtRLP41	0.04	1.26	4.12	2.33
at3g11080	Receptors	AtRLP35	-0.08	2.89	2.01	2.45
at3g11010	Receptors	AtRLP34	-0.08	1.86	2.84	1.05
at1g65390	Receptors	ATPP2-A5	0.96	2.43	-1.26	-0.53
at2g32140	Receptors	transmembrane receptor	0.92	4.16	2.17	0.20
at2g20142	Receptors	toll-Interleukin-Resistance (TIR) domain family protein	-0.20	1.10	1.37	0.15
at3g28890	Receptors	AtRLP43	0.66	1.45	0.62	-1.01
at1g17600	Receptors	SOC3	1.98	0.86	1.24	0.85
at1g72910	Receptors	TIR-NBS class, putative	1.79	1.56	1.37	0.82
at3g46730	Receptors	CC-NBS class, putative	-1.14	-1.10	-1.69	-0.02
at1g50180	Receptors	CC-NBS class, putative	0.25	5.23	1.67	0.76
at5g41750	Receptors	TIR-NBS-LRR class, putative	0.12	1.31	1.08	0.35
at4g08450	Receptors	TIR-NBS-LRR class, putative	-0.65	-2.17	1.27	-0.53
at3g25510	Receptors	TIR-NBS-LRR class, putative	0.72	3.10	2.11	-0.12
at5g46260	Receptors	TIR-NBS-LRR class, putative	0.30	1.50	1.30	1.18
at1g57630	Receptors	toll-Interleukin-Resistance (TIR) domain family protein	0.76	6.16	3.38	1.77
at1g66090	Receptors	TIR-NBS class, putative	0.30	1.22	1.86	2.05
at2g16870	Receptors	TIR-NBS-LRR class, putative	0.33	2.57	2.63	2.02
at4g09430	Receptors	TIR-NBS-LRR class, putative	0.84	-1.38	-2.61	-1.79
at3g14460	Receptors	LRRAC1	-1.20	-0.84	0.16	-2.45
at1g63870	Receptors	TIR-NBS-LRR class, putative	1.88	0.08	0.00	1.64
at5g40100	Receptors	TIR-NBS-LRR class, putative	0.10	-1.18	0.73	1.52
at1g72910	Receptors	toll-Interleukin-Resistance (TIR) domain family protein	1.79	1.56	1.37	0.82
at2g17430	Receptors	MLO7	0.02	-1.22	-0.97	-1.30
at3g24982	Receptors	ATRLP40	2.30	2.90	1.61	-0.48

Appendix B

Additional Chapter 4 data

B.1. Characteristics of lettuce cultivars selected for LC-MS-2

Table B.1.: UV-B responsiveness (shown as UV-B flavonoid level as a % of control plants) and disease susceptibility for selection of lettuce (*L. sativa*) cultivars in Liquid chromatography–mass spectrometry (LC-MS-2). FW = fresh weight (g)

Cultivar	Flavonoid index (% control)	Spore count	Spore count/FW
Falcon	128%	4.7E+05	2.0E+06
Desert storm	146%	5.3E+05	4.0E+06
Caicel	130%	2.9E+05	3.3E+06
Emperor	150%	1.1E+06	3.0E+06
La Brilliante	138%	1.7E+04	2.7E+05
Greenway	147%	4.6E+05	1.5E+06
Grand Rapids	143%	7.5E+04	6.4E+05

B.2. Conidia count per gram of leaf tissue of the cultivars used in LC-MS-2

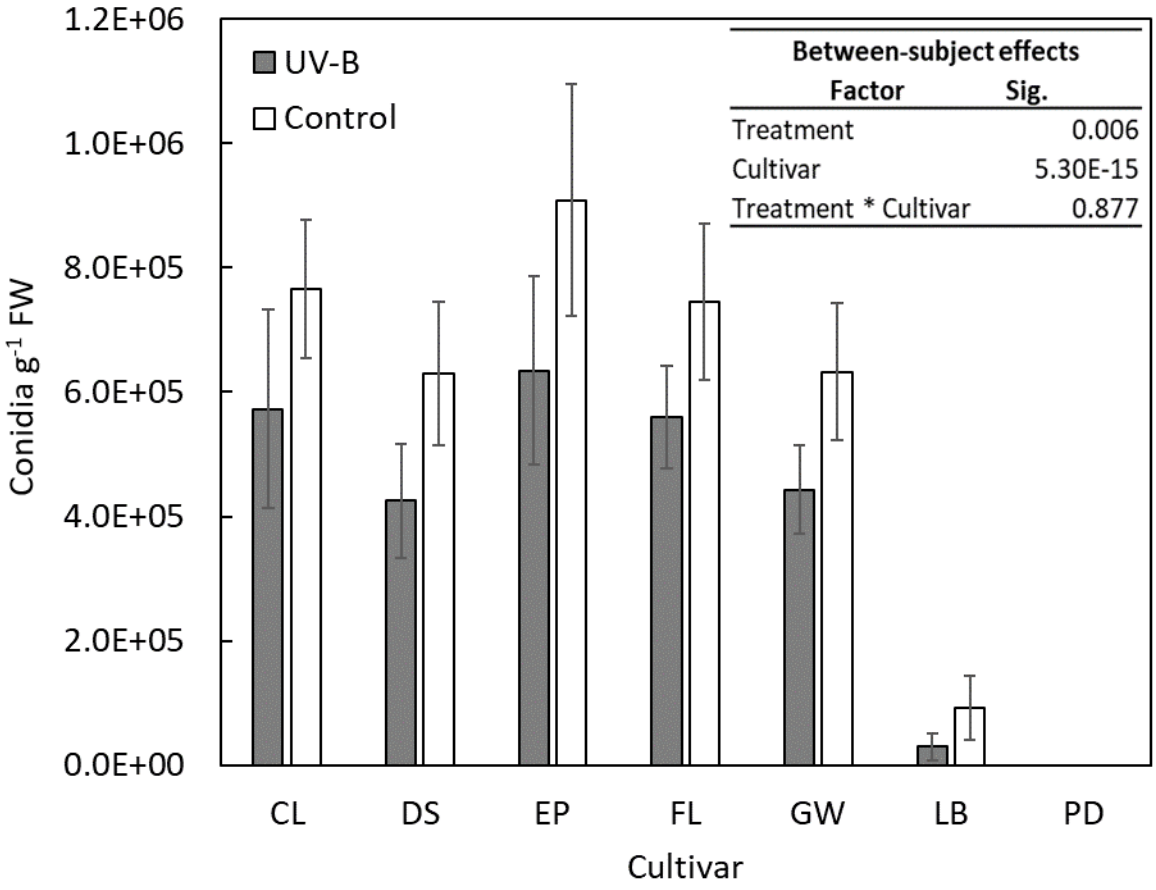

Figure B.1.: *B. lactucae* conidia count per gram of fresh weight (FW) on UV-B [grey bar] or control [white bar] lettuce (*L. sativa*) plants of cv. Calicel (CL), Desert storm (DS), Emperor (EP), Falcon (FL), Greenway (GW), La Brillinate (LB) and Pedrola (PD). Plants were treated with photosynthetically active radiation (PAR) + UV-B or PAR only (control) for three days then inoculated with 10^5 conidia mL^{-1} of *B. lactucae*. At 12 days post-inoculation, plants were washed in water and the resulting suspension counted. Error bars indicate 1 S.E. Table shows two-way ANOVA of between-subject effects on conidia count per gram of fresh weight.