THE EFFECTS OF UV PHOTOCATALYTIC ETHYLENE REMOVAL ON POSTHARVEST DISEASE PREVENTION, FRUIT PHYSIOLOGY AND STORAGE ENVIRONMENT

Alexander George Devlin Fletcher, BSc

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I dedicate this work to my parents.

Without their love and support in every aspect of my life,

I would not have been able to achieve my successes.

Thank you.

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ABBREVIATIONS

µg	--	micrograms
µl	--	microlitres
ACC	--	1-aminocylopropane-1-carboxylic acid
ACO	--	1-aminocylopropane-1-carboxylic acid oxidase
ACS	--	1-aminocylopropane-1-carboxylic acid synthase
AE	--	amplification efficiency
AUDPC	--	Area Underneath the Disease Progression Curve
AVG	--	aminoethoxyvinylglycine
BLAST	--	basic local alignment search tool
CA	--	controlled atmosphere
cDNA	--	complementary DNA
CFP	--	cyan fluorescent protein
cm	--	centimeter
Cnr	--	colourless non-ripening
CNSC	--	Container Nursery Stock Compost
d	--	days
DNA	--	Deoxyribonucleic acid
DoT	--	days of treatment
DPI	--	days post inoculation
EFE	--	ethylene-forming enzyme
ERF	--	ethylene response factor
GC	--	gas chromatography
h	--	hour
HPI	--	hours post inoculation
Hz	--	Hertz
JA	--	jasmonic acid

L	--	litre
m	--	meter
MA	--	modified atmosphere
MAP	--	modified atmosphere packaging
MEA	--	Malt Extract Agar
mg	--	milligrams
min	--	minutes
mm	--	millimeter
mM	--	millimolar
mRNA	--	messenger ribonucleic acid
MTA	--	5'-methylthioadenosine
N	--	newtons
nm	--	nanometers
Nor	--	*Non-ripening*
Nr	--	*never ripe*
PDA	--	Potato Dextrose Agar
PL	--	pectate lyase
ppb	--	parts per billion
ppm	--	parts per million
PR	--	pathogenesis-related
RH	--	relative humidity
rin	--	*ripening inhibitor*
RNA	--	Ribonucleic acid
ROS	--	reactive oxygen species
SA	--	salicylic acid
SAM	--	S-adenosyl-methionine
SDW	--	Sterile Distilled Water
TCI	--	tomato colour index

UV	--	Ultraviolet
V	--	voltage
W	--	Watts
wk	--	weeks

ABSTRACT

Ethylene, a naturally produced gaseous phytohormone, causes many detrimental effects during post-harvest fruit storage. A UV photocatalytic ethylene removal system, designed for use in post-harvest fruit and fresh produce storage and transport, was investigated to determine how it affects storage environment, fruit physiology and fruit resistance to various post-harvest plant pathogens.

UV photocatalysis was effective in completely oxidising ethylene from the storage environment. However, use of the technology was detrimental in increasing storage temperature and reducing storage RH. Reductions in the treated tomato fruits ripening rate were observed when experimental chambers were subjected to applications of external ethylene, indicating potential benefits of the treatment in increasing fruit shelf life. Treatment appeared to reduce hormone activated defence responses, whilst however also reducing ripening related susceptibility to *Botrytis cinerea* and *Penicillium expansum*, therefore demonstrating both positive and negative effects against the pathogens. UV photocatalysis was particularly effective against *Colletotrichum gloeosporioides* as treatment completely inhibited pathogen activity. This is likely as a result of a combination of ethylene removal and RH reductions preventing *C. gloeosporioides* conidial germination.

Future work must identify optimum UV photocatalytic treatment times to prevent anthracnose, whilst not proving detrimental to fruit physiology. Furthermore, technology must be applied to commercial fruit storage systems and examined to investigate how it affects fruit crops and the storage environment in real-world settings.

CHAPTER ONE

1 GENERAL INTRODUCTION

GENERAL INTRODUCTION

1.1 POSTHARVEST PATHOGENS

Due to their highly perishable nature and rich supply of nutrients, fruits are a suitable host for many fungal plant pathogens. Significant economic losses have occurred to many crop species globally due to yield losses induced by fungal disease (Dean *et al.,* 2012). According to the FAO, in developing countries, around 40% of losses occur at post-harvest and processing levels, due in large part to poor storage conditions leading to pest infestation and infection (FAO, 2011; Bayer, 2018). The majority of microorganisms to which fruit and vegetable crops are exposed are unable to invade plant tissue and cause disease. Fungal plant pathogens require a certain arsenal of tools to be able to infect a potential host and cause disease for the benefit of self-growth.

Fungal pathogens have a life cycle which goes through several stages to get to the production of decay symptoms. From a spore landing on a host surface, it must attach itself, germinate, penetrate the host (either by production of a penetration structure or directly through wounds) and activate pathogenicity factors in order to stimulate decay (Prusky, 1996). The purpose of the pathogen causing infection is to macerate host tissues in order to release nutrients which are required for sustained development. Successful colonization of host tissue will allow further fungal development by way of spore production on the host which will lead to the continued spread of disease. However, for a pathogen to be successful in its attack, it must overcome host defences under prevailing environmental and physiological conditions.

Fungal plant pathogens are usually categorised into two groups: biotrophs and necrotrophs. Biotrophic pathogens will attempt to invade a host without being detected and feed off living tissue within the host cells. Biotrophs, known as ‘conmen’ pathogens, appear to go unrecognised by the host as they divert photoassimilates and nutrients for their own development. Necrotophic pathogens will look to kill host cells and feed off nutrients that are released. Referred to as ‘thugs’, necrotrophs tend to utilise cell-wall-degrading-enzymes and/or toxins as weapons (Dickinson, 2003). Many postharvest pathogens can actually be categorised into a third group known as hemibiotrophs. A hemibiotroph utilises both a biotrophic lifestyle as well as a necrotrophic one. The fungus will initially attach to its host and exhibit a biotrophic lifestyle remaining undetected before turning aggressive and exhibiting a necrotrophic lifestyle. Some fungal species, such as those belonging to *Colletotrichum* spp., may attach to a host and go through a period of dormancy. This is known as the quiescent stage of infection (Prusky, 1996). The fungus will remain dormant until the host has reached a physiological stage whereby conditions are more favourable for the onset of pathogen virulence. This in postharvest pathogens is brought about by the ripening of fruit. At this stage of fruit development, the fungus will become necrotrophic to obtain nutrients from its host (Alkan & Fortes, 2015).

Previously, postharvest losses have been largely managed by the use of fungicides. The development of fungicides has greatly improved and extended the shelf lives of postharvest produce whilst reducing disease (Eckert & Ogawa, 1985, 1988). However, the use of fungicides in agriculture has come under much scrutiny in recent times. Pathogens have developed resistance to a number of key fungicides which previously provided effective control over them (Yourman & Jeffers, 1999; Fernández-Ortuño *et al.,* 2008; Leroch *et al.*,

2011; Hahn, 2014; Rupp *et al.,* 2017). Much research now focusses on fungicide resistance risk assessment in attempts to ensure that new fungicides are created which will remain free of pathogen resistance for as long as possible (Grimmer *et al.,* 2015). Moreover, there is public perception that pesticides are harmful to the environment and human health. Following this negative perception, governmental policies have promoted restricting the use of fungicides (Gullino & Kuijpers, 1994; Ragsdale & Sisler, 1994). It is therefore clearly of great importance to look for alternative means of controlling the pathogens.

1.2 *COLLETOTRICHUM GLOEOSPORIOIDES*

1.2.1 Host range and infection symptoms

Colletotrichum gloeosporioides is responsible for anthracnose disease of many plant species. The plant pathogenic genera, *Colletotrichum*, is responsible for causing disease on a huge variety of hosts from trees to grasses (Dean *et al.*, 2012; De Silva *et al.*, 2017). Specifically, *C. gloeosporioides* has a large and diverse host range having been associated with at least 470 different host genera (Sutton, 1980; Hyde *et al.*, 2009). Postharvest, tomato, pepper, mango, avocado, as well as a range of citrus fruits are all susceptible to the pathogen (Brown, 1975; Beno-Moualem & Prusky, 2000; Kefialew & Ayalew, 2008; Pedroso et al., 2011; Kim et al., 2014; Tucho et al., 2014). The fungus is known to be more abundant in tropical and subtropical regions as opposed to temperate ones. This is a factor as to why many of the fruits the pathogen is known to infect are grown and produced within these regions.

Initial infection of very young immature fruit may result in abortion (Cooper, 1993); however, *C. gloeosporioides* can infect a fruit at an immature unripe stage and remain asymptomatic until the fruit reaches maturity and ripening. The fungus can be described as a quiescent endophyte which exhibits a necrotrophic lifestyle after a latent period of infection (Latunde-Dada, 2001). On unripe, immature fruit, the pathogen undergoes a period of quiescence once initial germination has occurred. Upon ripening, the development of disease symptoms occurs rapidly starting with the appearance of small sunken black lesions. In tomatoes, the black lesions enlarge and aerial mycelial production from the lesions becomes apparent as disease develops. The infection begins to develop characteristic deliquescent orange-pink spore masses which burst through the fruit surface.

1.2.2 Infection process

Conidia can be spread over short distances by rain splash as well as overhead irrigation and infect other healthy plant tissue (Gautam, 2014). Following the dispersal of *C. gloeosporioides* conidia onto a susceptible host fruit, the propagule will begin to germinate. During conidial germination, the events that take place are similar for all *Colletotrichum* species (Perfect *et al.,* 1999): germination hypha will grow a short distance before forming a penetration structure known as a terminal appressorium.

Studies show that the phytohormone ethylene plays a role in stimulating *C. gloeosporioides* conidial germination and appressorial formation. Evidence of this is presented by Flaishman & Kolattukudy (1994), who showed that on wild type ethylene producing tomato fruits, *C. gloeosporioides* spores produced multiple appressoria; whereas on transgenic tomatoes and oranges, where the

fruits could not produce ethylene, exogenous ethylene was required for multiple appressorial formation. It was therefore concluded that the host's ripening hormone was used by the pathogen as a means as establishing the appropriate timing of invasion.

It is also reported that host surface wax plays a significant role in the induction of appressorial formation (Podila *et al.,* 1993). Although this study was performed only on avocado, the suggestion that wax compositions may serve as complex mediators for host-pathogen interactions may hold true for other *C. gloeosporioides* hosts. In Podila *et al.* (1993), isolated cultivar waxes stimulated appressorial formation from *C. gloeosporioides* spores, whereas non-host plant waxes did not. Moreover, the same waxes were unable to stimulate other *Colletotrichum* species appressorial formation. Following this research, it was established that host wax plays a greater role in stimulating appressorial formation than ethylene. This was evident as an ethylene inhibitor was unable to prevent wax induced appressorial formation (Flaishman & Kolattukudy, 1994). Further to this, it has been shown that conidia require priming in order to respond to wax and phytohormone host signals. Contact with a host's hard surface for approximately 2 hours induces Ca^{2+}-calmodulin signalling which primes conidia allowing a response to these host signals to germinate and form appressoria (Kim *et al.,* 1998).

Appressoria are necessary for successful host penetration. The infection peg formed by appressoria is used to penetrate pre-formed defensive barriers including the cuticle and underlying pectinaceous layer (Kim *et al.,* 2000). Prior to the formation of an infection peg by the appressoria, it is crucial that the structure adheres tightly to the host surface. In *Colletotrichum* species, hair like fimbriae are present at the surfaces of germ tubes and appressoria. It is

believed that these cells secrete components in the extracellular matrix, such as glycoproteins, which are responsible for adhesion (Perfect *et al.,* 1999; Deising *et al.,* 2000). The adhesion between appressoria and host is far stronger than adhesion between spore and host. This is necessary in order to counteract the force exerted onto the underlying host surface by the infection peg during penetration. Appressoria either use mechanical force and/or cell wall degrading enzymes to break down the hosts cell wall barrier (Deising *et al.,* 2000). It is likely that *C. gloeosporioides* uses the two methods in combination; primarily utilising turgor-generated physical force, whilst also using assistance from enzymes secreted as a response to host signals (Podila *et al.,* 1993; Wattad *et al.,* 1997; Kim *et al.,* 2000).

On unripe fruit, following penetration via an infection peg, *C. gloeosporioides* develops two distinct structures upon entering the quiescent stage. The first structures are dendritic like protrusions which form within the fruit cuticle. The pathogen also forms swollen hyphae which act to colonise the first epidermal cell layer but advance no further (Alkan & Fortes, 2015; Alkan *et al.,* 2015). On ripe fruit, *C. gloeosporioides* germination occurs in the same manner as on unripe fruit and undertakes a short biotrophic phase. However, due to the ripe fruit having a more accessible physiology than unripe fruit, the speed of infection is much more rapid and quiescent structures switch to necrotrophic growth immediately. This demonstrates developmentally cued hemibiotrophic growth in *C. gloeosporioides*. However, if *C. gloeosporioides* was to germinate at a wound site of a host, colonization adopts the necrotrophic stage immediately, skipping a biotrophic-like stage of growth (Alkan & Fortes, 2015; Alkan *et al.,* 2015). *Colletotrichum gloeosporioides* infection switches to a necrotrophic stage always upon ripening, degrading host tissue and producing symptoms of disease (Prusky, 1996; Prusky *et al.,* 2013).

1.2.3 Quiescence and Changing Lifestyles

Colletotrichum gloeosporioides is a pathogen which utilises different lifestyles throughout its infection process. As previously eluded to, if the pathogen was to land on an unripe crop, following conidial germination and appressorial formation, it would undergo a period of dormancy prior to switching to an aggressive necrotroph and producing infection symptoms on the host. This period of dormancy is known as the quiescent stage of infection (Prusky & Lichter, 2007; Prusky *et al.,* 2013).

During the quiescent stage of infection, due to close association between the colonizing fungus and its host, it is important that the pathogen has coping mechanisms for dealing with plant defence responses. *Colletotrichum gloeosporioides* has been reported to do this in various ways (Prusky & Lichter, 2007); one method revolves around the detoxification of antifungal compounds. Avocado fruit can form an antifungal compound 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (antifungal diene, AFD) which can promote *C. gloeosporioides* resistance. Declines in AFD have been attributed to declining epicatechin levels (Prusky *et al.,* 1988). Levels of AFD and epicatechin decrease as fruit ripen; a process associated with increasing susceptibility to the pathogen. A laccase enzyme from *C. gloeosporioides* has been reported to detoxify epicatechin resulting in declining levels of AFD. This leads to decreasing levels of resistance highlighting the pathogens ability to subdue host defence response playing a significant role in fruit pathogenicity (Guetsky *et al.,* 2005).

Localised production of reactive oxygen species (ROS) by the host at a site of *C. gloeosporioides* infection during quiescence in unripe fruit was found to be one of the earliest detectable defence responses to attempted penetration (Beno-Moualem & Prusky, 2000). However, in ripe susceptible fruits, there is no significant enhancement of ROS production. This is due to the fungus requiring ROS production from the fruit at an unripe physiological stage in order to inhibit fungal development therefore activating the quiescent phase (Beno-Moualem & Prusky, 2000). At the stage of ripening, *C. gloeosporioides* is able to secrete ammonia to alkalinize the infection site leading to a further decrease in ROS levels as well as a naturally increasing pH stimulated by ripening fruits. This allows for pectate lyase activity and a necrotrophic lifestyle as the pathogen comes out of the quiescent phase (Kramer-Haimovich *et al.,* 2006).

The switch from a biotrophic stage to a necrotrophic stage of fungal virulence infection involves a massive shift in fungal metabolism and up-regulation of pathogenicity factors (Alkan *et al.*, 2015). Fungal pathogenicity during necrotrophic colonization can be characterised by the massive activation of cell wall degrading enzymes (CWDEs) including fungal cellulases and pectinases (Alkan *et al.*, 2015). Furthermore, the secretion of ammonium as an elicitor is another tell-tale sign of a switch in *C. gloeosporioides* to necrotrophic growth. *Colletotrichum* species alkalinize by inducing large quantities of ammonium production (Alkan *et al.*, 2008; O'Connell *et al.*, 2012). In *C. coccodes*, it has been observed that ammonia is secreted in tomato infection to activate tomato NADPH oxidase, resulting in oxidative burst and the induction of a salicylic acid mediated defence responses. This results in host cell death through programmed cell death and enhanced necrotrophic colonization (Alkan *et al.*, 2009, 2012). The secretion of ammonia as an elicitor

in necrotrophic colonization of *C. gloeosporioides* to alkalinize host tissue has been shown to be necessary to induce the expression of several CWDEs including polygalacturonase and pectate lyase in order to stimulate tissue maturation (Alkan *et al.*, 2015).

The necrotrophic growth of the pathogen will induce the production of disease symptoms as described in section 1.2.1. The quiescent nature of *C. gloeosporioides* infection can prove particularly damaging to the postharvest market. It can lead to the harvesting of visually healthy fruits which have been infected by the pathogen and undergoing a period of dormancy. The transport of these infected, but seemingly healthy crops long distances provides economic cost when the fruits ripen and go to waste as disease symptoms develop.

1.2.4 Current control

Disease control of *C. gloeosporioides* is currently achieved mainly through chemical control. Cultural control is also important with the application of good orchard sanitation procedures and adequate handling practices to prevent fruit cuts and bruises which may benefit the pathogen being important (Kimaru *et al.*, 2018). Further to this, the use of *Streptomyces* sp. A1022 has demonstrated significant inhibitory effects against *C. gloeosporioides* as a biocontrol agent (Kim *et al.*, 2014). However, at present, chemical control is routinely used during fruit development using fungicides including Mancozeb, Benomyl, Propineb, Prochloraz, Metiram, Fluazinum and Thiabendazole (Smith *et al.*, 2011; Bill *et al.*, 2014b,a; Kimaru *et al.*, 2018).

1.3 *BOTRYTIS CINEREA*

Botrytis cinerea is a fungal plant pathogen which utilizes a necrotrophic lifestyle to attack over 200 species of crops worldwide (Williamson *et al.*, 2007). The causal agent of grey mould; *B. cinerea* is the most extensively studied necrotroph due to the availability of its genome sequence and a variety of molecular tools (Dean *et al.*, 2012). van Kan *et al.* (1997) demonstrated the ease of *B. cinerea* transformation to obtain knock out mutants to determine the importance of cutinase in the *B. cinerea* infection process. Gene silencing with *B. cinerea* is also possible, making it a particularly useful pathogen to work with (Patel *et al.*, 2008, 2010). Furthermore, the significant economic relevance of *B. cinerea* provides another reason for the amount of research performed with the pathogen.

Due to the nature of the pathogen and its broad host range, the economic costs of *B. cinerea* damage are very hard to estimate. Although the actual cost of *Botrytis* damage must be huge, there are no reliable figures available (Dean *et al.*, 2012). A serious quality issue lies with the reduction of fruit shelf life and flower vase life due to *B. cinerea* infection. The loss of produce before consumption by a consumer due to grey mould is often economically disregarded as a result of the product already being paid for and purchased. However, this may lead to prevention of the product being re-purchased at a later date due to a dissatisfied customer, leading to further loss. The same can be said of ornamental flowers if the product goes bad soon after purchasing. This kind of economic cost is difficult to estimate and therefore cannot be incorporated into total estimated economic costs of *Botrytis*. Official figures for the costs of grey moulds can therefore be considered as underestimates in the soft fruit and ornamental market (Dean *et al.*, 2012). Altogether, the global

cost of *Botrytis* control easily passes €1 billion/annum; and if quality loss during the retail chain is taken into account the that figure is very likely to be significantly higher (Dean *et al.*, 2012).

1.3.1 Disease symptoms

Botrytis cinerea gets its name of grey mould due to the symptoms of disease it produces. On tomato fruit, symptoms can be seen within a couple of days post inoculation as splits across the host cuticle surface. From this stage disease progresses rapidly as white mycelia begins to spill out of the open wound site as the split enlarges. As time passes the quantity of mycelia rapidly increases before the pathogen quickly begins to sporulate. The spores of *B. cinerea* are grey and hence 'grey mould'.

1.3.2 Life cycle and infection process

Botrytis cinerea can survive as either mycelia and/or conidia; or as sclerotia in crop debris for extended periods of time. Sclerotia developing within dying host tissue provides a melanised rind and β-glucans which encase internal mycelium. This acts to protect the pathogen over long periods of time by acting as a defence against UV radiation and microbial attack. Moreover, the sclerotia contain an internal nutrient reserve providing the 'hibernating' pathogen vital reserves for continued life (Blackhouse & Willetts, 1984). Sclerotia act within a crop as an early source of inoculum to produce multinucleate conidia and conidiophores. The mummified fruits, dead leaves and flowers contain masses of mycelia to produce conidia and initiate infections (Williamson *et al.*, 2007). At the sources of primary inoculum, conidia are ejected by conidiophores into air currents due to rapid declines in

humidity and a rise in temperature. Conidia will then travel airborne until landing on a susceptible host.

Once the conidia lands on a susceptible host, there is a weak initial adhesion prior to the formation of a fibrillary matrix that develops a germ tube and causes a strong adhesion prior to penetration (Dickinson, 2003). *Botrytis cinerea* can enter its host through wound sites, natural openings and by directly penetrating the host cell cuticle. When *B. cinerea* penetrates a host cell, it utilizes both mechanical pressure generated by the appressoria and enzymatic degradation of the host cell cuticle (McKeen, 1974; Williamson *et al.*, 2007). It is not feasible for the *B. cinerea* infection peg to penetrate the host cuticle by mechanical pressure alone as *B. cinerea* germlings do not contain a septum which seals the germ tube from the appressorium. This makes it impossible to produce the high osmotic pressures required to break through the cuticle (van Kan, 2006). Even though Doss *et al.* (2003) demonstrated that *B. cinerea* germlings contain melanin, the highly melanized wall required to sustain high physical pressure is not produced by the pathogens appressorium. Moreover McKeen (1974) using light and electron microscopy looking at *B. cinerea* infection on *Vicia faba* leaves, identified that the pathogen produced a blunt infection peg which made a clean passage way though the cuticle without leaving any cuts or tears. This indicated that the cuticle appeared to be enzymatically dissolved rather than mechanically broken.

Botrytis cinerea is considered a typical necrotroph which acts to co-opt its host into activating programmed cell death pathways in order to cause infection and feed off the nutrients released (van Kan, 2006; Van Baarlen *et al.*, 2007). *Botrytis cinerea* produces effector molecules involved in pathogenesis which

enable the pathogen to kill the host and decompose plant tissue for the purpose of conversion into fungal biomass. Various enzymatic determinants have been proven to be important in the infection for hosts by *B. cinerea*. Fungal pectinases are critical for breaking down the pectin rich anticlinal wall of epidermal cells during infection. The endopolygalacturonase BcPG2 has been shown to be very active during infection of bean and tomato leaves as *BcPG2* deletion mutants demonstrated delays in primary infections and caused reductions in virulence (Kars *et al.*, 2005). Cutinases and lipases are crucial for cell wall degradation also, including an enzyme known as BcSOD1 which is a superoxide dismutase that is active during appressorial cuticle penetration (Rolke *et al.*, 2004).

Cuticle penetration during *B. cinerea* infection triggers an oxidative burst (Schouten *et al.*, 2002). Oxidative burst involves the accumulation of H_2O_2 and rapid generation of superoxide; and is a characteristic feature of the hypersensitive response (Lamb & Dixon, 1997), which confers resistance against attack by biotrophic plant pathogens (van Kan, 2006). However, against the necrotrophic attack of *B. cinerea*, plant cell death due to the hypersensitive response leads to increased susceptibility (Govrin & Levine, 2000). The ability of *B. cinerea* to trigger oxidative burst during infection therefore aids in its necrotrophic attack.

Botrytis cinerea is able to produce low molecular weight phytotoxic compounds during attack with the best studied being ‘botrydial’ (Colmenares *et al.*, 2002; Williamson *et al.*, 2007). A number of botrydial genes have been identified and it has been observed that deletion of one of these genes resulted in severe virulence reductions of one *B. cinerea* strain, but not two others (Siewers *et al.*, 2005). These results indicated that whilst certain *B.*

cinerea strains may rely on botrydial to kill their hosts, others are able to produce additional toxins such as botcinolides (Reino *et al.*, 2004; van Kan, 2006).

1.3.3 Current Control

Botrytis cinerea is a particularly difficult pathogen to control due to the fact that infestation can occur at any stage during its hosts life cycle; from seedling stage up until the product ripens (Dean *et al.*, 2012). Furthermore, the ability of the pathogen to utilise various different modes of penetration and attack, the diverse variety of hosts as inoculum sources, and the ability of the pathogen to survive as conidia and/or mycelia, or for extended periods in crop debris as sclerotia, make *B. cinerea* very difficult to control (Williamson *et al.*, 2007).

The most common way to control *B. cinerea* is still the application of fungicides, despite the increasingly problematic issue of the pathogen developing resistance to commonly used fungicides (Yourman & Jeffers, 1999; Leroch *et al.*, 2011; Latorre & Torres, 2012; Hahn, 2014; Rupp *et al.*, 2017). At farmer level, controlling and preventing *Botrytis* is one of the major investments in terms of cost per treatment with on average (all crops, all countries), *Botrytis* protection costing around 40 Euro/ha (Steiger, 2007). In 2001, botryticides (fungicides specifically targeted against *Botrytis*), represented over 10% of the world fungicide market reaching a cost of €540 million (UIPP, 2002). Of the market for botryticides, 50% of the value is represented by the grape industry (wine and table), whilst solanaceous vegetables, strawberries, ornamentals and cucurbits each making up 5-9% (Steiger, 2007).

However, there is a continuing alarming increase in the occurrence *B. cinerea* populations demonstrating multi-resistance against grey mould fungicides. This represents a serious threat to the chemical control of *B. cinerea* (Rupp *et al.*, 2017). In order for adequate protection to be maintained against the pathogen, strict resistance management measures for fungicide use must be implemented in combination with the search for alternative disease control strategies with non-chemical products (Hahn, 2014). Alternative disease prevention strategies to conventional fungicides include the use of biocontrol agents, natural antimicrobials, physical treatments and disinfecting agents; and are reviewed by Romanazzi *et al.* (2016).

1.4 *PENICILLIUM EXPANSUM*

1.4.1 Host range and infection symptoms

Penicillium expansum is a filamentous fungus and the causal agent of blue mould. Widespread in the environment, *P. expansum* is considered one of the most important postharvest pathogens (Jurick *et al.*, 2011) capable of causing decay during postharvest handling and storage, resulting in significant yield and economic losses. Although the preferred hosts of *P. expansum* are pomiferous fruits, the pathogen is capable of causing disease on a wide and diverse range of hosts.

Blue mould (mature asexual conidia) spilling out the infection site of a host crop is characteristic of disease caused by *P. expansum*, although other symptoms include browning and sunken lesions. On tomato fruits, disease spreads internally as tissue softening spreading from the inoculation point. White mycelia will then begin to leak out of the wound. The white mycelia will

quickly turn blue over the next 24 -48 h as *P. expansum* asexual conidia mature.

1.4.2 Infection process

Penicillium expansum infection typically occurs as conidia penetrate through wounds and microwounds. Stem ends, open calyx tubes and lenticels are also common areas where infection might occur (Amiri & Bompeix, 2005).

Extensive tissue maceration caused by the necrotrophic *P. expansum* is as a result of a common mechanism of tissue acidification by the fungus (Prusky *et al.*, 2004). *Penicillium expansum* is able to produce organic acids in order to lower the ambient pH of the host environment, therefore providing the optimum environment for CWDE's, such as polygalacturonase, to operate (Yao *et al.*, 1996; Prusky *et al.*, 2004; Jurick *et al.*, 2011). Host acidification is achieved by the pathogen activating glucose oxidase 2 (gox2) in combination with catalysed glucose oxidation which results in the secretion of small molecules such as D-gluconic acid (GLA) (Prusky *et al.*, 2004; Hadas *et al.*, 2007). The release of these small acidic molecules reduce host tissue pH to around 3.5-4 which activates several polygalacturonases, inducing tissue maceration by pectin depolymerisation (Yao *et al.*, 1996; Sánchez-Torres & González-Candelas, 2003; Prusky *et al.*, 2004; Barad *et al.*, 2016b). A recent study by Barad *et al.* (2016a) reported that as well as GLA, *P. expansum* is able to secrete ammonia at the leading edge of apple fruit colonization: this would effectively increase pH during colonization. It was suggested that *P. expansum* bi-directional pH regulation is probably dependant on nutrient availability of the host. The transcription factor *pacC* modulates pH response factors in pathogens during fungal colonization. The same study (Barad *et al.*,

2016a), suggested that the *P. expansum pacC* gene was active under acidic conditions, and that ammonia accumulation by the pathogen in the host environment under acidic pH was a regulatory cue for *pacC* activation, contributing to pathogenicity, and leading to the accumulation of secondary metabolites such as patulin. Although the overall process remains poorly understood, pH modulation under the influence of both GLA and ammonia accumulation is important for understanding fungal response to differential gene regulation by pH (Barad *et al.*, 2016b).

1.4.3 Patulin production

Patulin is a mycotoxin which has strong antibiotic activity against bacteria (Stott & Bullerman, 1975). Furthermore, it has been shown to be toxic to animal cells and tissues (Escoula *et al.*, 1988) and deleterious to human health (da Rocha *et al.*, 2014), thus it is a potential health hazard in foods. *Penicillium expansum* is a main producer of patulin which makes the pathogen more dangerous to the postharvest market. Patulin has phytotoxic effects (Riley & Showker, 1991), and reports have indicated potential strong (Sanzani *et al.*, 2012) or mild (Barad *et al.*, 2014) pathogenicity contributions of the metabolite. Conversely, there have been reports which have suggested no contributions of patulin at all to pathogenicity and the infection process (Li *et al.*, 2015).

There are a number of factors which effect patulin production by *P. expansum* that consequently affect the accumulation of this toxic metabolite in fruits. Such factors include fruit cultivar and fruit pH responses (Damoglou *et al.*, 1985; Damoglou & Campbell, 1986; Morales *et al.*, 2008; Barad *et al.*, 2016a). Accumulation can be affected by storage conditions (Baert *et al.*, 2007). The

fact that postharvest storage conditions can affect patulin accumulation in fruits demonstrates a need that various storage methods should be tested to determine whether they can reduce *P. expansum* disease and patulin accumulation in order to increase food safety.

1.4.4 Current control

Previously, postharvest blue mould disease was mainly controlled by the application of chemical fungicides, in particular benzimidazoles (Eckert & Ogawa, 1988). Similarly to all postharvest pathogens however, *P. expansum* has developed high degrees of resistance against commonly used fungicides (Koffmann & Penrose, 1987). As a result, various alternative control methods have been tested including the use of different fungicides in combination (Errampalli & Crnko, 2004), biocontrol (Calvente *et al.*, 1999), the use of plant volatiles (Neri *et al.*, 2006; da Rocha Neto *et al.*, 2015), UVC hormetic treatments (de Capdeville *et al.*, 2002) and ethylene action prevention treatments (Li *et al.*, 2017).

1.5 FRUITS IN THE POSTHARVEST MARKET

Postharvest conditions contribute significant effects to the quality of fruit crops. This has been demonstrated with tomatoes, where it was observed that variations in temperature, light, UVC radiation and ethylene all affected tomato quality traits, with differences in quality continuing through 3 weeks of supermarket storage (Verheul *et al.*, 2015). Postharvest biology and technology research primarily seeks to improve the quality and quantity of fresh produce reaching consumers by reducing the quantity of waste, and spoiled products. Additionally, it must look to maintain the appropriate food

safety standards. In order to attain these goals, various strategies must be adopted. Integrated crop management systems should be applied to promote high yields without losing crop quality as a consequence; cultivars should be selected for long postharvest shelf lives following maturation, and to promote high quality nutritional values and flavours; and optimal postharvest handling and distribution practices should be maintained to ensure that fresh produce quality and food safety standards are kept high (Kitinoja *et al.*, 2011). Furthermore, it has been demonstrated that storage conditions such as temperature and relative humidity (RH) must be kept consistent to specified product guidelines (Hardenburg *et al.*, 1986), as the massive variations in temperature and RH previously observed in fruit and vegetable storage have resulted in reductions in product quality and increased product waste (Nunes *et al.*, 2009).

1.5.1 Current losses

Despite research programs and horticultural education in many countries, studies have shown that postharvest losses are still high, with little improvement observed in percentage losses from the 1970s to present day (Kitinoja *et al.*, 2011). High waste rates of postharvest fresh produce stems from the high perishability of fruit and vegetable crops. Losses of postharvest produce have been observed at harvesting, during packing and transportation, and during retail and wholesale marketing. Data produced by the Food and Agricultural Organization of the United Nations (FAO) showed that between industrialised countries and developing countries, there are little differences in food losses. However, in developing countries greater than 40% of food losses occur at postharvest and processing levels, whereas in industrialised countries, greater than 40% of food losses occur at retail and consumer levels

(FAO, 2011). These statistics highlight distinct differences between the issues faced by developing and industrialised countries. In industrialised countries, much of the postharvest waste at retail and consumer levels can be attributed to high standards of produce demanded by consumers. For example, 24.9% of waste in these industrialised regions is due to the expiry date of products such as ready to eat salads, with a further 15.1% attributed in part to bruising (Nunes *et al.*, 2009). In developing countries such as in Sub-Saharan Africa and South Asia, a recent study has observed that a lack of education about maintaining quality and food safety has resulted in physical and quality reductions due to rough handling, poor quality packages, poor temperature management, poor sanitation and the time required to reach the market (Kitinoja & AlHassan, 2012). It is for this reason that research should focus on developing the knowledge and understanding of postharvest management in developing countries, as well as develop science based, simple methods for postharvest handling (Kitinoja *et al.*, 2011).

1.6 TOMATO FRUIT

Behind potato, tomato (*Solanum lycopersicum*) is the second most important vegetable crop (FAOSTAT, 2015). Globally consumed, tomatoes are an important source of nutrients. Although other fruits contain higher contents of vitamins A and C, tomatoes are the major source of these nutrients due to their high per capita consumption (Klee & Giovannoni, 2011). Furthermore, phytochemicals from the fruits have been observed to have health-promoting benefits such as carotenoids (Mares-Perlman *et al.*, 2002), and flavanols. Flavanols are now being targeted in genetically modified tomatoes due to the potential health promoting benefits (Choudhary *et al*., 2016a).

Tomatoes represent an ideal model crop species for biologists. The availability of its genome sequence (The Tomato Genome Consortium, 2012) and several important ripening mutants from the crop make it a model system to study physiological, biochemical and molecular changes responsible for fruit development and subsequent susceptibility to pathogens. Furthermore, the ease of cultivation under greenhouse conditions, quick growing times and continuous truss and fruit production allow for high quantities of research to be performed using the crop.

1.6.1 Fruit ripening

Although considered a developmental stage, ripening is actually an 'arbitrary point of maximum palatability in a continuous process leading to the senescence of the entire organ' (Klee & Giovannoni, 2011). The evolution of fleshy fruits, and the genetics, regulation and control of fruit ripening are well characterised in scientific reviews (Klee & Giovannoni, 2011; The Tomato Genome Consortium, 2012; Seymour *et al.*, 2013a,b). Changes in expression of hundreds to thousands of genes are required to stimulate the biochemical processes necessary for ripening (Alba *et al.*, 2005). Coinciding with seed maturation, it is a highly coordinated and complex developmental process resulting in alterations to fruit texture, colour, flavour and aroma to facilitate seed dispersal. In tomato fruits, ripening initiates the transition from a firm, mature green physiology to a soft, red ripe one. The resulting ripe fruits are succulent, fleshy and attractive to animals so their consumption may allow for the dispersal of their seeds over great distances. During fruit ripening, changes in physiological characteristics are associated with various biochemical events; colour changes from green to red occur as chlorophyll is degraded and leaf-like xanophylls disappear, whilst there is an accumulation

in carotenoids such as lycopenes and carotenes (Fraser *et al.*, 1994; Su *et al.*, 2015); fruits soften due to increasing polygalacturonase activity (Tucker *et al.*, 1980), and flavour and aroma changes are caused by the increased accumulation of sugars, acids and volatile compounds. Additionally, during fruit ripening, fruit susceptibility to disease increases due to several physiological processes that occur (Alkan & Fortes, 2015). These include decreases in the amount of phytoalexins and phytoanticipins (Prusky, 1996) and declines in inducible host defence responses (Beno-Moualem & Prusky, 2000).

Fruits can be physiologically classified into two groups: climacteric and nonclimacteric. Nonclimacteric fruits include grapes, strawberries and citrus fruits where ethylene is not essential to fruit ripening. These nonclimacteric fruits do not exhibit increased respiration at the onset of ripening and ethylene does not stimulate fruit ripening. On the other hand, ethylene, and ethylene synthesis coordinates the ripening of climacteric fruits (Alexander & Grierson, 2002). Climacteric fruits include tomato, banana, avocado and apple and can be characterised by an increase in respiration, known as the ‘climacteric-rise’, in combination with an autocatalytic increase in ethylene production. The blocking of ethylene synthesis in climacteric fruits is capable of preventing ripening.

1.7 ETHYLENE

Ethylene is a simple unsaturated hydrocarbon consisting of two carbon atoms and four hydrogen atoms. As well as the role played in the ripening of climacteric fruits, ethylene is involved in numerous other aspects of plant development, growth and responses to biotic (such as pathogen attack) and

abiotic (such as wounding) stresses (Bleecker & Kende, 2000; Lin *et al.*, 2009). Root initiation, senescence, seed germination, shoot growth and flower development are all aspects of the plant life cycle regulated by the gaseous phytohormone. Ethylene is a readily diffusible gas which makes it an ideal stress hormone. Plants release ethylene in response to stresses in order to slow the rate of plant growth. When under stress, plants produce ethylene, but when the stress is removed, the gas rapidly diffuses (Klee & Giovannoni, 2011).

1.7.1 The biochemical synthesis of ethylene

Pioneering work by Yang & Hoffman (1984) led to the definition of the YANG cycle which defines the biochemical synthesis of ethylene. The biosynthesis of ethylene is initiated by the conversion of methionine to S-adenosyl methionine (SAM). This reaction occurs via SAM synthase which catalyses the adenolysation of methionine's sulphur atom. The resulting SAM is converted to aminocylopropane-1-carboxylic acid (ACC) and 5'-methylthioadenosine (MTA) by a γ-elimination (1,3-elimination) reaction (Yang & Hoffman, 1984). The process involves the metabolisation of SAM to MTA, which then gets incorporated into the methionine cycle to recover the sulphur atom and ACC, in a process catalysed by the pyridoxal phosphate dependant ACC synthase (ACS). Lastly, ACC is converted to ethylene by ACC oxidase (ACO) (formally known as ethylene-forming enzyme (EFE)) in the presence of oxygen, as summarised by Cara & Giovannoni (2008). In the aforementioned methionine cycle, otherwise referred to as the sulphur cycle, the MTA is regenerated to methionine through an intermediate of 5-methylthioribose. This methionine reproduction in the methionine cycle is very important in the maintenance of substantial ethylene production (Adams & Yang, 1977; Zhang *et al.*, 2017).

ACS activity has been considered the rate limiting step in the ethylene biosynthetic pathway (Yang & Hoffman, 1984). The gene encoding ACS was identified in work performed by Sato & Theologis (1989) where it was also shown that gene activation was induced by various inducers including wounding and the plant hormone auxin. In tomatoes, there are now at least eight characterised *ACS* genes with each one having a distinct pattern of stimulus and tissue specificity (Klee & Giovannoni, 2011). *ACO* encoding genes were identified by Hamilton *et al.* (1990), with later work identifying that there are a group of five *ACO* genes and enzymes which act to form ethylene during biosynthesis (Hamilton *et al.*, 1991; Barry *et al.*, 1996; Sell & Hehl, 2005). Certain *ACO* genes are ethylene-inducible, particularly in ripening fruits (Barry *et al.*, 1996).

1.7.2 Ethylene in fruit ripening

Work performed using naturally occurring tomato mutants, transgenic tomato fruits, and chemical inhibitors of ethylene biosynthesis and perception, have all demonstrated the absolute requirement of ethylene in the ripening of climacteric fruits. Reductions in ethylene synthesis resulting in delayed ripening have been observed when antisense genes have been used to target *ACS* (Oeller *et al.*, 1991) and *ACO* (Hamilton *et al.*, 1990). Additionally, it has been observed that the introduction of a bacterial ACC deaminase gene, which encodes an enzyme that metabolizes ACC to α-ketoglutarate, to tomato plants was effective in reducing ethylene synthesis and delaying ripening (Klee *et al.*, 1991). Furthermore, the use of silver ions, which inhibit ethylene action, have demonstrated the ability to delay fruit ripening (Hobson *et al.*, 1984; Tucker & Brady, 1987), whilst ethylene biosynthetic inhibitors, such as

aminoethoxyvinylglycine (AVG), are widely used in research to delay ripening and prevent ethylene synthesis.

A mutation known as *Never-ripe* (*Nr*) which occurs at ethylene receptors, interferes with ethylene perception causing ethylene insensitivity, resulting in ripening inhibition (Lanahan *et al.*, 1994; Wilkinson *et al.*, 1995). The fact that *Nr* mutations prevent ripening, even in the presence of ethylene, confirm that ethylene perception is essential for ripening. Further ripening mutants such as *nor*, *rin* and *Cnr* have proven very beneficial in understanding how the ethylene signal is transduced to cause fruit ripening (Giovannoni, 2007; Osorio *et al.*, 2011).

In order to be perceived, ethylene binds to receptors localised to the endoplasmic reticulum. Genetic analysis of these receptors in *Arabidopsis* (Hua & Meyerowitz, 1998) and tomatoes (Tieman *et al.*, 2000) has shown that they are negative regulators of ethylene signalling. Ethylene absence causes receptors to actively supress ethylene responses. Conversely, the binding of ethylene to a receptor removes suppression stimulating a response. In the absence of ethylene, repression of an ethylene response is due to the activation of the downstream negative regulator, CTR1. Once ethylene has bound to a receptor, CTR1 activation is inhibited (Hua & Meyerowitz, 1998), and in absence of its activation, a positive regulator, EIN2 is activated. EIN2s carboxyl terminal domain activates EIN3, a member of the EIN3/EIL transcription factor family (Chao *et al.*, 1997). The EIN3/EIL family activate transcription factors known as ethylene response factors (ERFs). ERFs are positive regulators which bind to the ‘GCC box’ promoter of genes regulated by ethylene to promote ripening, and all other ethylene mediated responses (Chang & Shockey, 1999; Bapat *et al.*, 2010).

Much research has been performed using 1-methylcylopropene (1-MCP) in fruit storage and ripening studies (Blankenship & Dole, 2003; Watkins, 2006). 1-MCP binds to ethylene receptors with 10 times more affinity than ethylene itself (Blankenship & Dole, 2003), thereby inhibiting the perception and action of ethylene. Numerous studies have demonstrated the effects of 1-MCP ethylene perception blocking in delaying/controlling fruit ripening (Jiang *et al.*, 1999; Hofman *et al.*, 2001; Palou & Crisosto, 2003; Liu *et al.*, 2005; Guillen *et al.*, 2006; Manenoi *et al.*, 2007; Tiecher *et al.*, 2013; Yang *et al.*, 2013; Amornputti *et al.*, 2014). The reductions in ripening rate caused by 1-MCP blocking ethylene perception on various different fruit crops demonstrates the importance of ethylene in climacteric fruit ripening.

Furthermore, a recent study has identified that sound waves are able to delay fruit ripening. It was identified that low frequency sound waves were able to reduce ethylene production and respiration rate by influencing ethylene biosynthetic and ripening related gene expression. This resulted in a delay in the transition of mature green tomato fruit to a red ripe stage (Kim *et al.*, 2015), further demonstrating the importance of ethylene in climacteric fruit ripening.

1.7.3 Ethylene in response to postharvest infection

Ethylene has been demonstrated to result in increases to both disease susceptibility and resistance. As a ripening hormone, it promotes susceptibility to pathogens; however, ethylene can provide resistance as a participant in hormone-activated defence responses (Glazebrook, 2005).

Ethylene, in combination with jasmonic acid (JA) has been reported to improve resistance against necrotrophic plant pathogens (Glazebrook, 2005; Spoel *et al.*, 2007; Spoel & Dong, 2008; Bari & Jones, 2009). Numerous studies using 1-MCP have resulted in increases in disease susceptibility as a result of treatment with the blocker of ethylene perception. On tomatoes, 1-MCP treatments have resulted in increased decay symptoms caused by *Alternaria* (Su & Gubler, 2012; Biswas *et al.*, 2014). In research performed with 'Shamouti' oranges, fruits treated with ethylene had reduced mould rot development from *Penicillium digitatum* and *Penicillium italicum*. The effects of 1-MCP treatment however, enhanced the development of these mould rots on the citrus fruits, as well as stem end rot caused by *Diplodia natalensis* (Porat *et al.*, 1999). On nonclimacteric strawberry fruits, 1-MCP treatment has been associated with an earlier onset and slight increase in rot development (Bower *et al.*, 2003). Increased susceptibility to *P. expansum* induced blue mould, and bitter rot caused by *C. acutatum* has been reported on apples as a result of 1-MCP standalone treatment (Janisiewicz *et al.*, 2003). Furthermore, Hofman *et al.* (2001) observed a slightly higher severity of rots on apple, avocado, papaya and mango fruits as a result of 1-MCP treatment. Increased severity in avocado body rots and stem end rots caused by *Colletotrichum* spp. and *Dothiorella* spp. respectively were also observed following treatment with 1-MCP (Adkins *et al.*, 2005). Perhaps, the observed increase in avocado rots were as a result of 1-MCP treatment reducing antifungal diene levels below the fungal inhibition threshold, as observed in work performed by Wang *et al.* (2006). The results from all these studies therefore demonstrate a requirement of ethylene in the maintenance of basic levels of disease resistance.

On the other hand, ripening related susceptibility has been reported to increase as a result of ethylene action. The process of fruit ripening induces

increased susceptibility to plant pathogens due to the natural decrease in intrinsic resistance of fruits when they are ripe. The role of ethylene in climacteric fruit ripening therefore stimulates and accelerates this decrease in intrinsic resistance levels. Ripening reduces the amount of preformed phytoanticipins and phytoalexins originally present in unripe fruits (Prusky, 1996). In tomatoes, this has been seen with α-tomatine, present in high concentrations in mature green tomato peel. This saponin is inhibitory to fungi. However, it is undetected in mature ripe fruits. In avocado fruits, the major antifungal compound in unripe fruits was shown to be 1-acetoxy-2-hyroxy-4-oxo-heneicosa-12,15-diene (antifungal diene). During ripening, this preformed compound was observed to decrease tenfold to subfungitoxic concentrations (Prusky *et al.*, 1982). In unripe mango fruit, resistance to *Alternaria alternata* was observed due to a mixture of the antifungal compounds 5-12-cis-heptadecenyl resorcinol and 5-pentadecenyl resorcinol (Droby *et al.*, 1987). Research suggests that as the preformed resorcinols decrease to nontoxic levels during ripening leaving fruits more susceptible to disease (Prusky, 1996). Furthermore, ripening reduces inducible host defence responses, as observed in work performed by Beno-Moualem & Prusky (2000) where ROS production in unripe resistant fruit was twice as high as in ripe susceptible fruit.

Previous research has also suggested that ethylene is capable of inducing the onset of virulent infection from *C. gloeosporioides* and *Diplodia natalensis*. With *C. gloeosporioides*, it has been shown that ethylene produced by the host at ripening acted to terminate the appressorial dormancy during the quiescent stage of infection and stimulate the induction of the pathogens necrotrophic life cycle (Flaishman & Kolattukudy, 1994). This was also observed with *C. musae*, but not with *Colletotrichum* species that infect nonclimacteric fruit (Prusky, 1996). Furthermore, when *C. gloeosporioides*

spores were inoculated onto nonclimacteric tangerines, multiple appressoria formation was not observed, nor decay. Multiple appressoria formation and decay were only observed following ethylene de-greening treatment to the infected fruits (Brown, 1975; Brown & Barmore, 1977). Similar results were observed on citrus fruits with *D. natalensis* where exogenous ethylene was required in order to stimulate disease development and increase the incidence of stem-end rot caused by the pathogen (Brown & Lee, 1993). The exogenous ethylene was able to activate quiescent mycelia of *D. natalensis*. Additionally, exo-polygalacturonase and cellulose activity was enhanced by the ethylene which enabled fungal penetration and stem-end rot to occur (Prusky, 1996).

Furthermore, research conducted using 1-MCP treatments has demonstrated reductions in rotting caused by fungal pathogens, likely due to induced delays in fruit ripening and senescence. Reductions in fungal decay on tomatoes (Guillen *et al.*, 2006; Su & Gubler, 2012), apples (Saftner *et al.*, 2003; Li *et al.*, 2017), peaches (Liu *et al.*, 2005), plums (Menniti *et al.*, 2004), cut flowers (Seglie *et al.*, 2009) and mangoes (Xu *et al.*, 2017) have all been reported as a result of 1-MCP treatment. In work performed by Wang *et al.* (2006), 1-MCP treatment prevented decay development in early harvested avocado fruits, regardless of observed treatment induced reductions in antifungal diene. This demonstrates that disease prevention here is likely caused by delays in ripening and fruit softening due to the prevention of ethylene perception by 1-MCP treatment. In addition to work performed with 1-MCP, exogenously applied ethylene has also been observed to increase stem end rot disease severity on citrus fruits (Porat *et al.*, 1999).

Interestingly, research has demonstrated 1-MCP treatments proving beneficial or detrimental to fruit crops against disease, dependant on the concentrations

of the compound used. For tomatoes (Blanco-Ulate *et al.*, 2013), citrus fruits (Dou *et al.*, 2005) and strawberries (Ku *et al.*, 1999), studies have shown that low concentrations of 1-MCP improve resistance to decay, but high concentrations promote increased susceptibility. This indicates that maintenance of basic resistance against pathogens may require small quantities of endogenous ethylene due to its involvement in plant defence regulation (Marcos *et al.*, 2005; Alkan & Fortes, 2015).

The contrasting results observed support conclusions that ethylene plays opposing roles in defence against postharvest pathogens. As a ripening hormone, it can trigger the onset of disease, and promote susceptibility by inducing reductions in host intrinsic resistance. However, it can provide resistance against disease in hormone-activated defence responses (Glazebrook, 2005; Bari & Jones, 2009; Broekgaarden *et al.*, 2015). Therefore, whether ethylene action will cause increased resistance or susceptibility to disease is likely to be dependant on the timing of ethylene release and perception, and the quantity of ethylene present (Alkan & Fortes, 2015).

1.8 ETHYLENE CONTROL IN FRUIT STORAGE

1.8.1 Ethylene concentrations in fruit storage and transport

Research was conducted by Wills *et al.* (2000) over a three year period, where 700 ethylene measurements were conducted in fruit and vegetable holding areas, wholesale markets, domestic refrigerators and distribution centres. Results suggested that on average, the majority of products held at the

marketing step were subjected to ethylene concentrations between 0.015 and 0.1 ppm where respectively, 10% and 30% of potential postharvest life is lost.

Much greater ethylene concentrations were reported in mixed shipments on refrigerated container ships and control atmosphere apple stores (Lawton, 1991). In mixed cargos, 50 ppm of ethylene was detected emanating from apples which was also contaminating other areas which were believed to be isolated from the detrimental effects of ethylene. The same study identified that controlled atmosphere apple and pear stores were found to have ethylene concentrations up to 250 ppm.

Unfortunately, there are few published case studies investigating ethylene concentrations within the whole cold chain. Ethylene concentrations vary in postharvest storage and transport units depending on the crop stored, size of the unit and quantity of produce. Exogenously produced ethylene will also effect ethylene concentrations within various storage and transport systems. There is a requirement for studies which highlight ethylene levels currently in transport and storage spaces in the marketing chain (Keller *et al.*, 2013). Case studies of this nature would allow for more effective laboratory research into ethylene removal technologies and their effects and applicability to the postharvest storage and transport of fruit crops.

1.8.2 Negative effects of ethylene in fruit storage

In postharvest fruit and vegetable storage, it is a goal to avoid ethylene action (Martínez-Romero *et al.*, 2007). Although ethylene is necessary for the ripening of climacteric fruit crops, bringing about positive characteristics in texture, flavour and colour for human consumption, the phytohormone

provides many detrimental effects on the quality of fresh fruit and vegetable products.

Ethylene at any level can have a deleterious effect on produce, and the effect of ethylene on postharvest shelf life is linearly related to $\log_{10}$ concentrations of ethylene (Wills *et al.*, 2001). Different species of fruits and vegetables are found to have different sensitivities to ethylene. Some climacteric fruits, such as kiwi and apples, are highly ethylene sensitive at concentrations as low as 0.03 ppm (Martínez-Romero *et al.*, 2007). Interestingly, even though nonclimacteric fruits only produce very small quantities of ethylene, it has been reported that their ethylene sensitivity is greater. In nonclimacteric fruits, the threshold level of ethylene action has been reported to be less than 5 ppb (Wills *et al.*, 1999) along with the suggestion that any method of reducing postharvest ethylene accumulation in the vicinity of nonclimacteric produce, will increase postharvest life. Overall, reports indicate that no 'safe' ethylene level exists which will not have a deleterious effect on postharvest produce.

Asides from the previously discussed effects on pathogen induced postharvest fruit decay, ethylene has been known to contribute to over-ripening, reductions in shelf life, accelerated senescence, discolouration (browning and yellowing), excessive softening, abscission of flowers, sprouting of potatoes, and hastened toughening of vegetables (Saltveit, 1999). There are, however, factors which govern the response of plants to ethylene. Tissue sensitivity, concentration of ethylene, duration of exposure, atmospheric competition and temperature are all variables which can affect the detrimental effects of ethylene on plant tissue (Saltveit, 1999). As a result of this, much research has experimented with various methods of ethylene control postharvest to

promote high quality fruit and vegetable crops with longer shelf lives and increased pathogen resistance.

Previously tested methods of exogenous and endogenous ethylene control, including removal technologies, biosynthesis inhibitors and action inhibitors have been well reviewed by Martínez-Romero *et al.* (2007). As technology develops and our understanding of ethylene action, biosynthesis and perception increases, the development of potential new methods of ethylene control will continue to be experimented with. Here, current methods of ethylene control previously worked with are discussed.

1.8.3 Current ethylene control strategies

1.8.3.1 Inhibition of ethylene biosynthesis

Ethylene production has been supressed by inhibiting the activities of ACS and ACO enzymes.

With regards to ACS inhibition, AVG treatments have been most investigated, with a commercial product, ReTain® (Valent BioSciences Corp., Libertyville, Illinois, USA) containing 15% w/w AVG registered for use on apples in the USA (Martínez-Romero *et al.*, 2007). AVG has demonstrated an effective means of inhibiting ethylene production in pre- and post- harvest treatments.

Examples of ACO inhibitors include cobalt (Co^{2+}) and ethanol and acetaldehyde vapours. Co^{2+} has been observed to inhibit ethylene production and ripening in banana fruits (Dominguez *et al.*, 1998). However, due to the compound being a toxic heavy metal, it is not allowed to be used in the food

industry. Furthermore, acetaldehyde has been added to a list of known carcinogenic materials, therefore limiting its use in commercial postharvest application (Martínez-Romero *et al.*, 2007).

1.8.3.2 1-methylcylopropene (1-MCP) ethylene perception inhibition

As previously discussed, 1-MCP provides ethylene inhibition at a receptor level due to its ability to bind to ethylene receptors with more affinity than ethylene itself. Silver ions, applied as silver thiosulphate, also inhibit ethylene action at receptor level. Ethylene receptors contain a copper ion which co-ordinates ethylene binding (Rodríguez *et al.*, 1999). Applied silver ions may act by occupying the binding site occupied by copper, and interacting with ethylene preventing its action (Knee, 1995). However, unlike 1-MCP, silver cannot be used in foods as silver is a heavy metal.

Unsaturated cyclic olefins inhibit ethylene action by binding to fruit and vegetable ethylene receptors. In the treatment of ethylene responsiveness in plant material, 1-MCP is considered an almost ideal compound when applied as a gas (Sisler, 2006). This is due to it being nontoxic, active in low concentrations and protective for extended time periods (Keller *et al.*, 2013). The non-toxicity of 1-MCP, ability to maintain and extend postharvest quality of both climacteric and nonclimacteric produce, and the fact that it is a safe postharvest chemical in the agro-food industry has led to worldwide interest in the compound (Blankenship & Dole, 2003; Watkins, 2006). The benefits of 1-MCP in delaying fruit ripening and shelf life extension, and effects against postharvest decay, have already been discussed in section 1.7.2 and section 1.7.3 respectively. As a result, a commercial product, Smartfresh® (Agrofresh,

PA, USA) uses 1-MCP as an active ingredient for use in postharvest fruit and vegetable storage.

1.8.3.3 Controlled atmosphere (CA) storage and modified atmosphere packaging (MAP)

Controlled atmosphere (CA) storage requires simultaneously increasing CO_2 and decreasing oxygen concentrations to alter and maintain atmospheric composition different from that of air (Keller *et al.*, 2013). Optimum concentrations of CO_2 and oxygen lower ethylene production rates, reduce ethylene action, delay ripening and senescence, and retard pathogen decay (Saltveit, 2003). This is because low oxygen is capable of reducing ethylene synthesis, whilst elevated CO_2 levels can reduce sensitivity to ethylene.

Modified atmosphere (MA) storage is a type of CA storage. However, where CA systems actively manage storage atmosphere compositions, MA systems use the respiration of the products and passive technology to generate a required atmosphere (Keller *et al.*, 2013). However, the high cost of CA and MA storage technologies has meant that commercial application has been limited, despite the fact that the treatments have been shown to be effective in extending postharvest shelf life.

Modified atmosphere packaging (MAP) refers to a technique of sealing respiring fruits and vegetables in polymeric film packages to modify oxygen and CO_2 levels within the package atmosphere (Ahvenainen, 1996; Keller *et al.*, 2013). Whilst CA and MA storage refers mainly to larger storage rooms and containers, MAP refers to small packaging units. Use of MAP in shipping

containers, pallets and consumer packaging levels is growing (Keller *et al.*, 2013).

1.8.3.4 Biofilters

Biofiltration is a technology where biological catalysts are being used to reduce the detrimental effects of ethylene. Elsgaard (Elsgaard, 1998, 2000) reported the first satisfactory operational stability and sufficient efficiency in removing ethylene concentrations to near the threshold limit for plant hormone response (Martínez-Romero *et al.*, 2007). Following this, research performed with *Pseudomonas* packed with activated carbon caused 100% ethylene removal meaning the technology could reduce ethylene concentrations below the threshold limit for plant hormonal response (Kim, 2006). Biofiltration technology has been reported to be efficient in cold temperatures, have good operational stability, and have a high capacity for ethylene removal. Furthermore, a recent study using bananas has demonstrated that biofiltration technology could extend fruit shelf life (Moghadam *et al.*, 2015). However, further research needs to be performed to confirm the efficacy of these systems in reducing ethylene concentrations from fruit and vegetable storage facilities to provide the beneficial effects of ethylene removal.

1.8.3.5 Ethylene oxidisers

Ethylene oxidation is a destructive technology that causes irreversible ethylene removal. The process of this oxidation requires an oxidant, which can break down ethylene into CO_2 and H_2O. The circulation of air inside a storage facility to circulate past the oxidising agent is crucial for most oxidation technologies.

The most widely used ethylene oxidation technology is with potassium permanganate ($KMnO_4$). Due to the high toxicity of $KMnO_4$ however, it cannot come into contact with foods. Due to this, sachets, films and filters are required along with inert substrates (i.e, perlite, silica gel, alumina) for the commercial use of $KMnO_4$ technology. Many commercial $KMnO_4$ based ethylene oxidation technologies exist and are used in packages, storage facilities, or during transportation (Martínez-Romero *et al.*, 2007). The products work by the substrates adsorbing ethylene, and permanganate oxidising it. Research has demonstrated ethylene removal by $KMnO_4$ resulting in delays in ripening of various fruit crops including mangoes (Illeperuma & Jayasuriya, 2002) and avocados (Zamorano *et al.*, 1994), with a recent study also demonstrating the treatment preserving the quality attributes of tomato fruits (Wabali *et al.*, 2017). However, it has been reported that large quantities of the adsorbent would be required for use in large packages containing high quantities of ethylene, putting a question mark over the use of this technology in horticultural produce (Wills & Warton, 2004). A further disadvantage with this treatment is the requirement for frequent replacement of $KMnO_4$ beads as $KMnO_4$ is rapidly consumed and must be replaced for continued ethylene removal (Keller *et al.*, 2013).

1.8.3.6 Ethylene adsorbents

A surface phenomenon where particles are held on the surface of a solid material, is called adsorption. The adsorption of any molecule depends on a variety of parameters. These include: gas composition, temperature, the adsorbate concentration, and partial pressure.

Activated carbons and zeolites are the main compounds used as ethylene adsorbers. With activated carbons, it has been shown that the granular form has the greatest ethylene adsorption capacity (≥80%) in comparison to powdered and fibre forms at ≥80% and ≥40% adsorption capacities respectively (Martínez-Romero *et al.*, 2007).

Publications on ethylene adsorption in horticultural product storage, and the effects of treatment on the produce, are scarce. This is despite considerable interest in the method for food technology processes. The lack of studies on different ethylene adsorbents with horticultural products has significantly hindered the use of the technologies in horticultural commodities (Keller *et al.*, 2013).

1.9 UV PHOTOCATALYTIC ETHYLENE SCRUBBING

Research conducted throughout this thesis has focused on an external method of ethylene removal known as UV photocatalysis. Photocatalytic oxidation of ethylene requires semi-conductor materials with suitable bandgaps as catalysts. However, as opposed to conventional catalysts which require heat energy for activation, energy is supplied by light (photonic activation). Amongst tested semi-conductors, titanium dioxide (TiO_2) is currently the most efficient and common. TiO_2 is considered attractive due to its high quantum yields leading to high photocatalytic efficiency, stability against photocorrosion and chemicals, insolubility in water and low costs (Keller *et al.*, 2013). Upon activation by UV light, hydroxyl radicals and superoxide radicals are generated by TiO_2 at its surface. These act as oxidising agents which convert organic pollutants to CO_2 and H_2O (Maneerat *et al.*, 2003; de Chiara *et al.*, 2015). This oxidation also occurs to ethylene, as

it was previously demonstrated that in the presence of oxygen, ethylene could be completely oxidised to CO_2 on a powdered TiO_2 surface (Park *et al.*, 1999).

There is an increasing amount of research demonstrating the effects of UV photocatalytic ethylene oxidation proving beneficial in prolonging fresh fruit and vegetable quality. Research has demonstrated increasing fruit shelf lives due to reductions in ripening rate caused by UV photocatalysis (Maneerat *et al.*, 2003; de Chiara *et al.*, 2015), as well as reductions to fungal and bacterial pathogens due to the antimicrobial effects of TiO_2 (Kim *et al.*, 2003; Maneerat & Hayata, 2006; Chawengkijwanich & Hayata, 2008; Bodaghi *et al.*, 2013).

There are currently commercial photocatalytic devices available which claim to be designed for the benefit of fruit storage, such as BIO-KES® (Kes Science and Technology, USA) and Titan Aire® (Catalyx Technologies LLC, USA). However, significant research needs to be performed both in laboratories and on an industrial scale in order to demonstrate performance for industrial adoption (Keller *et al.*, 2013). Performance of the technology needs investigating in various storage and transport conditions, whilst taking into account critical variables including atmospheric composition, storage duration and the type and quantity of commodity (Keller *et al.*, 2013). This is the basis behind the research undertaken in this study: to determine whether an industrially available UV photocatalytic ethylene scrubber can provide benefits in fruit and vegetable storage.

Previous work demonstrating positive effects of UV photocatalysis on fruit storage has localised treatments to the fruit crops, with TiO_2 powders and covered films present in the storage location of fruits, and with UV radiation applied at the same place. These methods, although representative of the

effects of UV photocatalysis on fruit crops and microbes, are not representative of the available technologies for fruit and vegetable storage. The Catalyx Technology (USA) product experimented with here for use in fresh produce storage environments has UV photocatalytic action happening within a light sealed steel box (as described in section 2.2). Within the UV photocatalytic ethylene scrubber, fans operate to induce airflow around the storage chamber and transfer exogenous and endogenously produced ethylene through the photocatalytic system to remove the phytohormone from the environment.

If research demonstrates UV photocatalytic technologies to be beneficial in prolonging fruit and vegetable quality postharvest, then the technology could prove beneficial over other methods of ethylene control. This is because the technology requires minimal maintenance once it has been set up. Due to the photocatalytic material not being consumed during oxidation, replacement and disposal of depleted treatment units is not required (Keller *et al.*, 2013). Compared to other methods of ethylene control, such as use of potassium permanganate, where the compound requires frequent replacement, UV photocatalysis requires very little maintenance. The only maintenance required is the occasional replacement of UV lighting. Furthermore, the technology can be applied to storage units of both large and small size and is capable of removing high and low quantities of ethylene from a storage environment (Keller *et al.*, 2013).

1.10 RESEARCH AIM AND OBJECTIVES

The aim of this research was to determine whether a UV photocatalytic ethylene removal system, designed for use in postharvest fruit and fresh

produce storage and transport environments, could be effective in both increasing fruit shelf life whilst decreasing the onset of disease caused by post-harvest pathogens.

The aims of this research were to be tested by performing experimental research based around the following objectives:

1. Determine how UV photocatalytic ethylene scrubbing would affect storage atmosphere by examining temperature and humidity levels; with a view to how they could affect fruit physiology and pathogen activity. Furthermore, confirm that the scrubber actively removes ethylene from the environment.

2. Measure colour evolution and changes in firmness and weight of fruit subject to treatment to determine if ethylene scrubbing treatment effects fruit ripening and consequently shelf life potential.

3. Examine the effects of treatment on incidence and severity of diseases caused by three prevalent postharvest pathogens: *B. cinerea*, *P. expansum*, and *C. gloeosporioides*.

4. Perform molecular analysis of tomato defence related gene expression in response to ethylene scrubbing treatment and disease caused by *C. gloeosporioides*. Furthermore, analyse *C. gloeosporioides* pathogenicity gene expression to determine how treatment effects the externally applied spores.

CHAPTER TWO

2 GENERAL MATERIALS AND METHODS

GENERAL MATERIALS AND METHODS

2.1 INTRODUCTION

This chapter will outline materials and methods used throughout experimentation. Methods described in this chapter will have remained consistent in all experiments where the specified material was utilised and any exceptions will be stated in the following results chapters where specific experimental methods are listed.

2.2 UV PHOTOCATALYTIC ETHYLENE SCRUBBER

The Catalyx technologies, FRESH+ EC-60 model UV Photocatalytic ethylene scrubber was provided by Nutricycle LTD (UK). The unit has a dimension size of 122 x 15 x 15 cm and, as a guideline, can filter the air of a room the size of up to approximately 425 m^3. This figure is based on 70% free air volume with a target of 6-8 air changes per day. This figure is a guideline as it is dependent on variations in ethylene production and sensitivity. The EC-60 runs at an airflow of 1.7 m^3/min and is constructed out of stainless steel. Total lamp power is 150 Watts and the lamps run at a wavelength of 254 nm meaning the machine utilises UV-C light to activate the catalyst. The catalyst used in the EC-60 model is a FRESH+ proprietary formulation. Machine power is at 120/220 V, 50/60Hz and includes a receptacle plug with a 2 m cord. Figure 2.1 shows the inside of the ethylene scrubber.

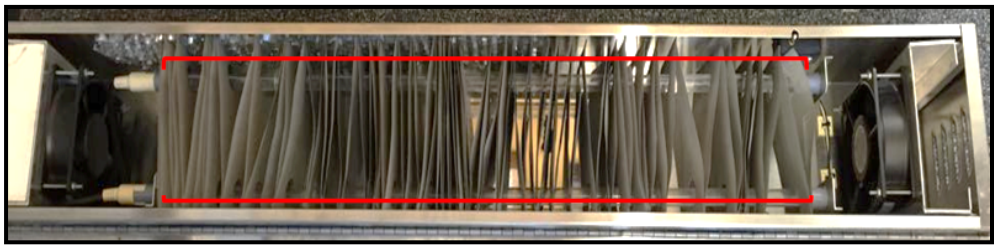


Figure 2.1 The Catalyx Technologies FRESH+ UV Photocatalytic ethylene scrubber opened up. Indicated by red brackets are the UVC lamps which run along the aluminium mesh covered with a FRESH+ proprietary formulation catalyst. This catalyst, once activated by the UV lamps, oxidises ethylene to carbon dioxide and water. At either end of the scrubber is a fan which pumps gas in the environment through the system.

2.3 EXPERIMENTAL CHAMBER PARAMETERS

Two identical perspex chambers were built for experimentation. Chambers measured 80 x 120 x 60 cm with a detachable front panel. During experimentation the detachable panel was fixed on and sealed using a rubber pile tape draught seal (Wickes). Each chamber contained paper tray shelving units to increase the quantity of tomatoes that could be treated. An equal amount of 'humidity boxes' were placed at the base of each chamber to maintain a high humidity level within the chambers during experimentation. 'Humidity boxes' contained blue paper towels regularly saturated with sterile water. Full treatment and control chambers can be seen in Figure 2.2

The treatment chamber was attached to the UV ethylene scrubber by Manrose Aluminium Ducts (Wickes, UK). Figure 2.3 shows the connection from the treatment chamber to the ethylene scrubber. The connection remained sealed through further rubber pile tape draught seals between ducts and chambers and by sealed casing placed around the ethylene scrubber to prevent any loss of air. Aluminium duct tubing was also attached to the control chamber (see Figure 2.2) in order to duplicate the treatment chambers volume as much as possible. Excess duct tubing was used (Figure 2.3) to prevent heat generated by the lamps of the ethylene scrubber affecting the outcome of results. Excess tubing was required to allow air to cool from leaving the scrubber before circulating back into the chamber where experimental samples were stored. With the aluminium ducts attached, overall chamber volume came to approximately 605,000 cm^3.

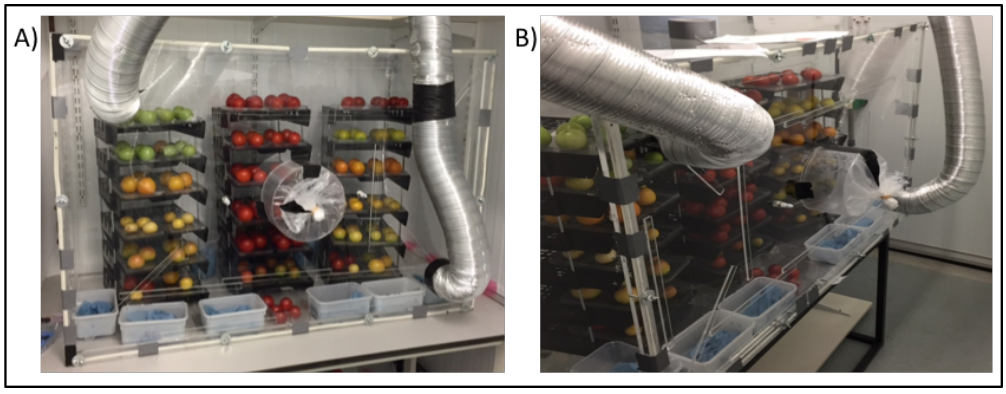


Figure 2.2. Full capacity sealed chambers. Internal shelving units are holding experimental fruit samples. Humidity boxes are at the base of each chamber. Extending from the detachable front door is an aluminium duct. A) Treatment chamber connected to ethylene scrubber. B) Control chamber. Both chambers were located within the same experimental growth room.



Figure 2.3. Aluminium duct connection from treatment chamber to ethylene scrubber. Image shows excess length of tubing duct used in order to allow treated air to cool.

2.4 GROWTH ROOM CONDITIONS

All experimentation took place within a culture growth room. This was an ideal location to control room conditions and keep pathogens growing out of the main lab areas. Growth room temperature was maintained at 16°C and lights were generally kept off. Lights were only switched on when results were being taken within in the room.

2.5 TOMATO FRUITS

Tomatoes used for experimentation were either sourced from seed stores from Sutton Bonington Campus (the University of Nottingham) or acquired from partners at APS Salads (UK).

2.5.1 Growth on campus

Tomato varieties Shirley, Moneymaker, Ailsa Craig as well as Ailsa Craig ripening mutant varieties: *rin* (*ripening-inhibitor*), *Nor* (*non-ripening*), *Nr* (*Never ripe*) and *Cnr* (*Colorless non-ripening*) were all grown in glasshouses at Sutton Bonington Campus (the University of Nottingham). Seeds were sown into 12 cell trays containing Levington M3 high nutrient compost and manually watered daily. After approximately 1-2 wks growth, plants were transferred to 9 cm pots (approximately 0.36 L volume) with added M3 compost for a further 2-3 wks. At this stage, plants were transferred to 7.5 L pots containing Levington CNSC compost for continued growth and attached to an automated watering system. Plants were supplemented with water four times a day for between 2-10 min a time depending on weather conditions. Plants were grown at approximately 22°C during the day and approximately 18°C overnight under

glasshouse conditions. Natural light was supplemented with 400 W high pressure sodium lighting to provide a total 16h photoperiod between the months of November to April.

2.5.1.1 Harvesting of fruit

Fruits were harvested on the same day as experimentation began. Days post anthesis was used to determine fruit maturity and recorded by dating and tagging flower trusses. Originally, 40 days post anthesis was used as the time frame to harvest fruit at a mature green stage. However, weather fluctuations caused this method to be unreliable. Therefore, fruit were harvested at a mature green stage when fruit tagged 1-3 days before them had started to break in colour. Ripening could be detected approximately 6-8 wks post anthesis.

Red ripe fruit were harvested between 7-10 d following colour break from a mature green stage. At this stage, red pigmentation had fully developed, but the fruit were still firm. Once colour break was seen on a fruit it was marked with a colour coded sticker indicating the day in which it broke. The day in which the fruit turned red ripe was again dependant on weather conditions. During hotter drier spells, fruit ripening from a mature green to red ripe developmental stage occurred at a quicker rate than during colder wetter periods.

Ripening mutant Ailsa Craig varieties (*rin*, *nor*, *Nr* and *Cnr*) were harvested at 40 d post anthesis. Due to the individual mutations inability to undergo colour break 40 d post anthesis was used for a ‘mature green’ fruit. Mutant varieties were therefore harvested at between 47-50 d post anthesis for ‘red ripe’ fruit.

Ailsa Craig fruits which were growing at the same time were used as a reference point for the developmental stage the mutant fruits were at.

2.5.2 Sourcing from APS Salads

Externally sourced fruit varieties cv. Mecano and Brioso tomatoes were grown in glasshouses at APS Salads (UK) prior to delivery at ambient temperature to Sutton Bonington Campus (the University of Nottingham) within 24 h of harvesting. Mature green and red ripe tomato fruit were sorted and any fruit showing surface damage were discarded. Furthermore, fruit showing colour deviation from the desired developmental stage or lack of uniformity of size were also discarded.

2.6 Experimental randomization of fruit

Tomato fruits were assigned random treatment groups for every experiment. Microsoft Excel software was used to randomly assign each tomato into either the ethylene scrubbing or control chamber. The same process was used for experiments with more treatment groups.

2.7 Fruit surface sterilization

Fruits were surface sterilized following harvest to prevent naturally occurring or foreign microbes already present on the tomato surface contaminating the fruits or effecting results.

Tomatoes were submerged in a 2% sodium hypochlorite solution (Honeywell). Three washing steps then followed to clear the fruit of any bleach solution.

Bleach was washed off the tomatoes in three sterile water baths. Tomatoes were then left to dry for approximately 1 h within a sterile flow cabinet.

2.8 PATHOGENS

Three pathogens were used during experimentation: *Botrytis cinerea*, *Penicillium expansum* and *Colletotrichum gloeosporioides*.

2.8.1 Origin of pathogens

Botrytis cinerea, originally isolated from a plant of the genus *Rosa*, was supplied in culture from the University of Nottingham's fungal collection. *Penicillium expansum* was also supplied from the same culture collection. *Colletotrichum gloeosporioides* (isolate CBS 100471) was provided by the Westerdijk Fungal Biodiversity Institute (the Netherlands) from the CBS-KNAW collection.

2.8.2 Confirmation of Specificity

Specificity of each pathogen used for experimentation was confirmed following *in vitro* growth. Tissue from each pathogen was taken from agar growth plates and the DNA was extracted using a DNeasy Plant Mini Kit (Qiagen) following the manufacturer's guidelines. Extracted DNA was then subject to polymerase chain reaction (PCR) using fungal ITS primers (see Table 2.1). Fungal ITS primers were used as part of a master mix (see Table 2.2) at a concentration of 0.3 μM per reaction. DNA extracted from each sample was added to individual master-mixes at a quantity of 1 μl to make up a total of 30 μl per PCR reaction tube. A Flexigene (Techne) PCR machine was used to run the

samples on the following cycle; an initial denaturation at 94°C for 2 min followed by 34 cycles of 94°C for 30 s, 53°C for 1 min and 72°C for 1 min as denaturation, amplification and elongation steps respectively. A final elongation step of 72°C for 5 min followed the 34 cycles before samples were left to cool at 4°C.

Table 2.1 Fungal ITS Primers

Primer	***Sequence 5'-3'***
Forward	GTTTCCGTAGGTGAACCTGC
Reverse	ATATGCTTAAGTTCAGCGGGT

Table 2.2 PCR Master-mix Reagents

Product	***Quantity per sample (µl)***
Mango Mix	15
Forward Primer	1
Reverse Primer	1
Hyclone Water	12

PCR products were then ran on a 1.5% agarose gel to confirm presence of product. A QIAquick® PCR Purification Kit was then used to purify DNA following manufacturer's guidelines. Purified products were sent to Eurofins Genomics (Europe) for sequencing. Specificity of product was confirmed using NCBI basic local alignment search tool (BLAST) analysis.

2.8.3 Maintenance of fungal material

For long term storage, stock cultures of *B. cinerea*, *P. expansum*, and *C. gloeosporioides* were all stored at 4°C on potato dextrose agar (PDA) slopes. New stocks for each slope were prepared every 3-6 months. *Botrytis cinerea* stock spore solutions were also stored in a -80°C freezer following passing of disease over three generations of tomato fruit to increase disease virulence. Frozen spore solutions were amended with 20% glycerol prior to freezing.

Culture plates of *B. cinerea* and *P. expansum* were maintained on PDA at room temperature until sporulation. *Colletotrichum gloeosporioides* cultures were plated on malt extract agar (MEA) plates and stored in the dark in a 27°C Gallenkamp incubator until sporulation.

2.8.3.1 Culture Media

Both PDA and MEA were Oxoid products and prepared according to manufacturer's recommendations. All culture media were supplemented with Streptomycin sulphate salt (Sigma-Aldrich) at 133 mg/L and Penicillin G sodium salt (Sigma-Aldrich) at 33 mg/L prior to plating.

2.8.4 Preparation of conidial suspensions

Each pathogen sporulated *in* vitro at different days post sub-culturing. Spore solutions of *B. cinerea*, *P. expansum* and *C. gloeosporioides* were made from, 10 to 18 d old cultures, 5 to 10 d old cultures and 6 to 8 d old cultures respectively.

From each pathogen, calibrated spore solutions were made using the same method. Petri dishes were flooded with 10 ml sterile distilled water (SDW) and supplemented with 20 μl Tween 20. Spores were gently agitated to be released prior to being filtered through a layer of Miracloth. The Petri dish was again flooded with 10 ml SDW, this time without Tween 20. Any remaining spores were again agitated and filtered through Miracloth. The solution was then vortexed vigorously to release conidia from conidiophores. The spore solution was centrifuged at 184 g for 10 min in a Centaur 2 (MSE). Following centrifugation, the supernatant was discarded and the pellet re-suspended in SDW. The solution was again vortexed and centrifuged at 184 g for further 10 min and the supernatant was discarded. The pellet was re-suspended in SDW and a haemocytometer was used to obtain the desired spore concentration for inoculations.

For *B. cinerea*, inoculum spore concentrations varied dependant on the physiological stage of the fruit. Red ripe fruit were inoculated with a spore concentration of 1×10^5 spores per ml. Mature green fruits were inoculated with 1×10^6 spores per ml due to lower levels of disease susceptibility.

All *P. expansum* inoculations occurred with a spore concentration of 1×10^6 spores per ml. *Penicillium expansum* inoculations were only performed on fruit harvested at a red ripe stage or mature green fruit left to ripen in either experimental chamber prior to inoculation. This was due to complete disease resistance of mature green fruit to *P. expansum* shown in preliminary work.

Colletotrichum gloeosporioides inoculations took place with a spore concentration of 2×10^5 spores per ml. This spore concentration was used for fruits inoculated at both red ripe and mature green stages.

2.9 TOMATO FRUIT INOCULATIONS

Type of inoculation used was dependant on the pathogen being used. For *B. cinerea* and *P. expansum* inoculations, fruit were wounded prior to inoculation, whilst *C. gloeosporioides* inoculations were applied as a spray.

2.9.1 Wound inoculations

A hypodermic needle, 3 mm in depth, was sterilized and used to wound the fruit. A pipette was used to inoculate the wound site with 5 μl of specified inoculum mixture (see 2.8.4). Two disease inoculation points were used per tomato on opposing sides to one another.

2.9.2 Spray inoculations

Spore solutions were pipetted into glass misting spray vials. Tomatoes were mounted and spray vials were used to inoculate one side of each tomato from 10 cm away. Inoculations occurred within a Class 2 flow cabinet. Each tomato received 3 pumps of the spray vial to give the side inoculated an even spread of spore solution.

2.9.3 Mock inoculations

Mock inoculations took place using the corresponding method of inoculation as used with the pathogen in the given experiment. Mock inoculations were performed using the same SDW used to make up spore inoculums. The purpose of these inoculations was to confirm firstly, that the water used for spore inoculum was not contaminated, and secondly, that disease symptoms

recorded from pathogen inoculation sites were as a result of disease and not inoculation method. For wound inoculated tomatoes, mock inoculations took place on the same fruit. A mock inoculation site was placed in-between the two spore inoculation sites. For spray inoculation experiments, separate mock inoculated fruits were used.

2.10 TOMATO FRUIT DISEASE SCORING

Due to differing methods of fruit inoculation, the method of quantifying disease *in vivo* varied between pathogens.

2.10.1 Wound inoculation

Disease was recorded by measuring total lesion diameter including all sunken lesions splitting and tissue maceration. Diameter was recorded at the longest point of the lesion using digital Vernier callipers. Disease was recorded between 3 and 5 d post inoculation (DPI) and used to calculate AUDPC (see 2.10.3).

2.10.2 Spray inoculations

A method of quantifying anthracnose disease caused by *C. gloeosporioides* was created by modifying the Pedroso *et al.* (2011) diagrammatic scale for sweet-pepper anthracnose (see Figure 2.4). The scale is used to compare disease as seen on tomatoes within each section (A-H) in Figure 2.4. Summaries of symptoms associated with each disease score can be seen Table 2.3.

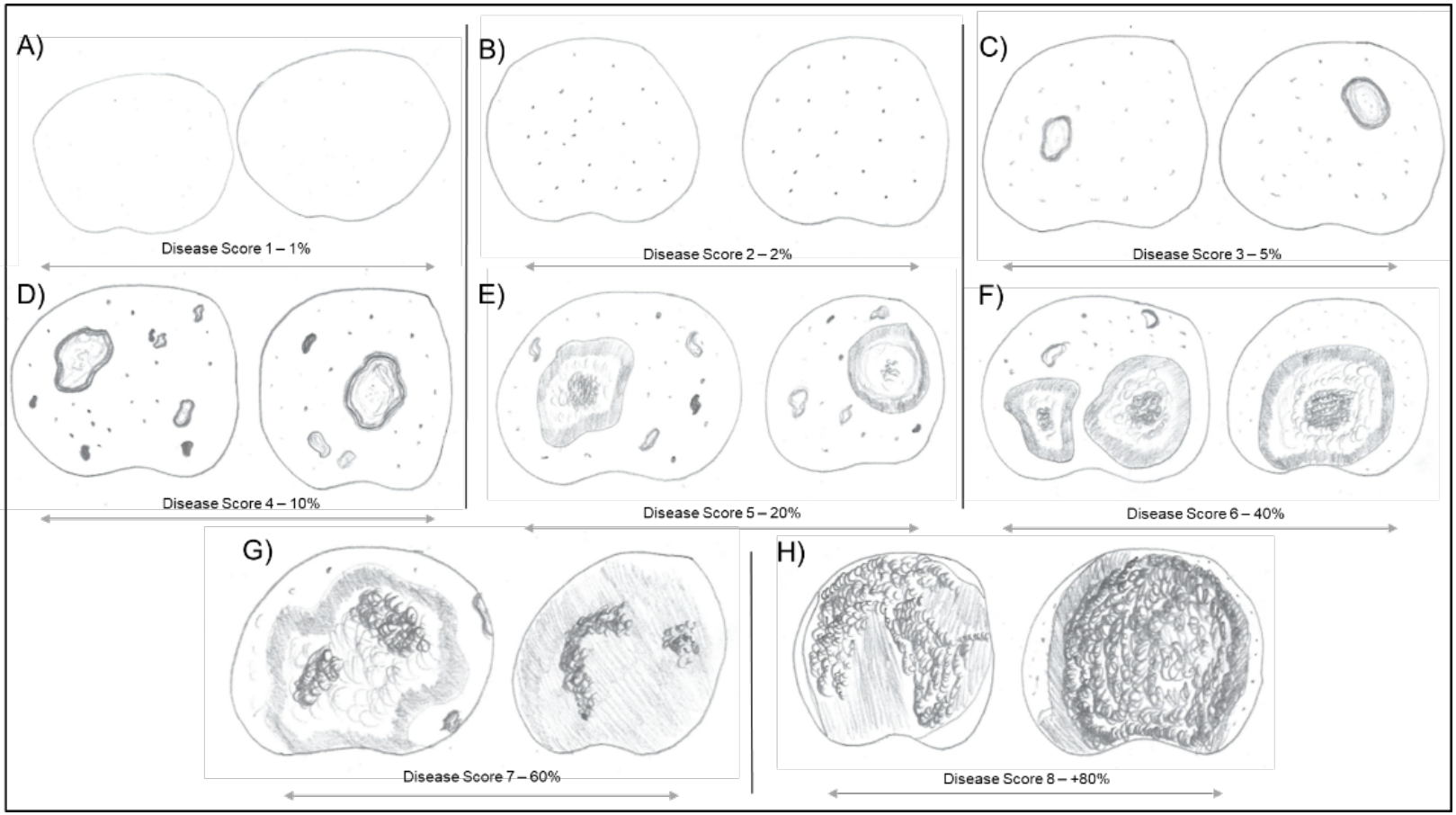


Figure 2.4.Diagramatic scale of anthracnose disease scaled used to score anthracnose disease on tomatoes

.

Table 2.3 *Colletotrichum gloeosporioides* disease score associated symptoms.

Disease Score	***Symptom Summary***
0	Healthy tomato exhibiting no symptoms of disease. Epidermal colour should remain consistent with no signs of small black lesions. Epidermal layer should be smooth and firm with no pitted morphology.
1	Small black lesions begin to appear but remain faint. Lesions are sparse across inoculated tissue.
2	Black lesions darken with skin pigmentation more obvious. Surface coverage of lesions has increased from disease score one. Slight pitted morphology on the epidermis noticeable.
3	Continued pitted morphology of developing lesions. Smaller lesions appear to becoming loaded into one area as well as larger lesions beginning to form. Internal softening obvious as larger lesions.
4	Surface area of larger lesions increases with further internal spread of disease causing increased softening of infected site. Appearance of further developing lesions possible.
5	Further softening around lesion sites due to internal disease spread. Orange/pink mycelial/spore leakage present on the surface of larger lesions. Severe disease (tissue softening and/or pathogen leakage on surface) now covering around 30% of inoculated tomato site.
6	Around 50% of inoculated area now affected by tissue softening and/or pathogen leakage from fruit epidermis.
7	Tissue of inoculated area now almost completely covered tissue maceration induced softening and or pathogen leakage from fruit epidermis.
8	Severe disease symptoms covering vast majority of infected area. Orange/pink spore masses engulfing inoculated region of fruit with underlying softening obvious due to concaving tomato and soft feel of fruit.

2.10.3 Area underneath the disease progression curve

Measurements of both lesion diameter from *B. cinerea* and *P. expansum* disease and recorded disease scores from *C. gloeosporioides* infection were used to calculate area underneath the disease progression curve (AUDPC) (Shaner & Finney, 1977; Campbell & Madden, 1990; Jeger & Viljanen-Rollinson, 2001), (see Equation 2.1).

$$\mathrm{AUDPC} = \sum_{i}^{n-1} ((\boldsymbol{y_i} + \boldsymbol{y_{i+1}})/\boldsymbol{2})(\boldsymbol{t_{i+1}} - \boldsymbol{t_i}) \qquad \text{Equation 2.1}$$

Area Underneath the Disease Progression Curve.

Area underneath the disease progression curve formula where *n* = total number of observations, *i* = at the 'ith' observation, *y* = disease scored recorded and *t* = time (Shaner & Finney, 1977; Scott *et al.*, 2017).

2.11 STATISTICAL ANALYSIS OF DATA

The statistical software package SPSS 24 (IBM) was used to analyse data. Independent T Tests were performed on the data when comparing results between treated and control chambers. A Levene's test for homogeneity of variances was used to assess whether equal variances could be assumed in the statistical analysis. Further statistical analysis on data included non-parametric testing involving an independent Mann-Whitney U test. Where further treatment groups were used, one-way ANOVA with Tukey's post-hoc

testing was performed. Statistical significance throughout this research is defined as $p \leq 0.05$.

CHAPTER THREE

3 EFFECTS OF THE ETHYLENE SCRUBBER ON CHAMBER AND STORAGE ENVIRONMENT

EFFECTS OF THE ETHYLENE SCRUBBER ON CHAMBER AND STORAGE ENVIRONMENT

3.1 INTRODUCTION

Throughout experimentation, conditions within testing chambers were monitored and recorded to determine how the ethylene scrubbers' mode of action could affect fruit storage conditions. From both the ethylene scrubbing (treatment) and control chamber, ethylene levels (ppm), temperature (^{o}C) and relative humidity (%) were recorded. This was to examine firstly; how any differences in these three parameters between chambers would interact with the fruits and pathogens to affect results; and secondly, whether any differences in storage conditions between chambers were consistent and therefore a causative effect of the ethylene scrubber.

3.2 MATERIALS AND METHODS

3.3 ETHYLENE LEVELS

Two methods of monitoring ethylene levels were used throughout experimentation. A portable ethylene analyser was used in each experiment to confirm that the ethylene scrubber was consistently removing ethylene and that ethylene levels were greater in the control chamber. Gas Chromatography was also performed to test the accuracy of the ethylene analyser and to reaffirm that the ethylene scrubber was functioning correctly.

3.3.1 Portable ethylene analyser

A Felix Instruments (USA) F-900 Portable Ethylene Gas Analyser was used throughout every experiment to monitor ethylene levels within both chambers. The F-900 was used to provide accurate real-time measurements of ethylene gas concentrations. The instrument utilises an electrochemical sensor which is able to detect ethylene at levels as low as 0.04 ppm.

Following attachment to a sample chamber, subject gas is taken up by the analyser. The gas travels across the surface of the electrode and diffuses into the electrochemical cell where ethylene is oxidised. The oxidation of ethylene in this manner creates a current which is measured and interpreted as the concentration of ethylene.

The real benefit of this method of ethylene analysis is that it allows real-time continuous monitoring of ethylene levels within a given environment. Therefore, it is possible to track changing levels of ethylene over a continuous time period. Furthermore, the F-900 ethylene analyser allows for incredibly sensitive onsite ethylene detection.

3.3.2 Gas chromatography

A Bruker Scion 456-Gc gas chromatographer was used to confirm the readings present from the portable ethylene analyser. Due to initial problems seen with the analyser (section 3.3.5), it was important to confirm that its following outputs were correct in showing ethylene removal from the treatment chamber. Gas chromatography (GC) was the method selected to double check ethylene levels within the two chambers.

3.3.3 Method of portable ethylene analyser use

The F-900 portable ethylene analyser was connected to either chamber for ethylene measurements via Viton tubing and quick connects provided by Felix Instruments (USA). Prior to measurements, every day the ethylene sensor was zero calibrated. Calibration was achieved through use of potassium permanganate ($KMnO_4$) to scrub ethylene from the gas within the analyser and set zero.

The analyser ran in measurement cycles containing a measurement stage and a cleaning stage. During the measurement stage, ethylene concentrations within the specified chamber were calculated. The cleaning stage was utilised to clean the ethylene sensor between each ethylene measurement and bring the sensor back down to detecting 0 ppm ethylene. This is why we see the ethylene peaks present on all of the below portable ethylene analyser figures.

In a daily analysis of ethylene within each chamber, following zero calibration, the ethylene analyser was attached to one chamber at a time for a minimum of two measurement cycles per chamber.

3.3.4 Method for performing gas chromatography reading

Prior to sample testing, the GC machine was calibrated to ethylene concentrations as high as 10 ppm. This was achieved by individually injecting ethylene gas at concentrations of 0.5 ppm, 1 ppm, 5 ppm and 10 ppm into the gas chromatographer and measuring the resulting peak area output. The peak

areas were plotted onto a graph and trend line was added to the results to give an equation which we would then use to calculate sample ppm from peak areas.

Three gas samples per chamber were extracted on the day of GC. One millilitre syringes were used to extract the samples before the syringe was instantly sealed with a rubber bung until analysis. Each sample syringe was run individually by injecting the sample into the gas chromatographer for a 5 min run time. Resulting peaks were analysed using the software package: Compass CDS v 3.

3.3.5 Issues with portable ethylene analyser

Initially the F-900 potable ethylene analyser proved to be a problematic piece of equipment to use. Although it appeared to confirm that ethylene concentration was far greater in the control chamber than the treatment chamber, the exact concentrations provided could not be trusted (Figure 3.1).

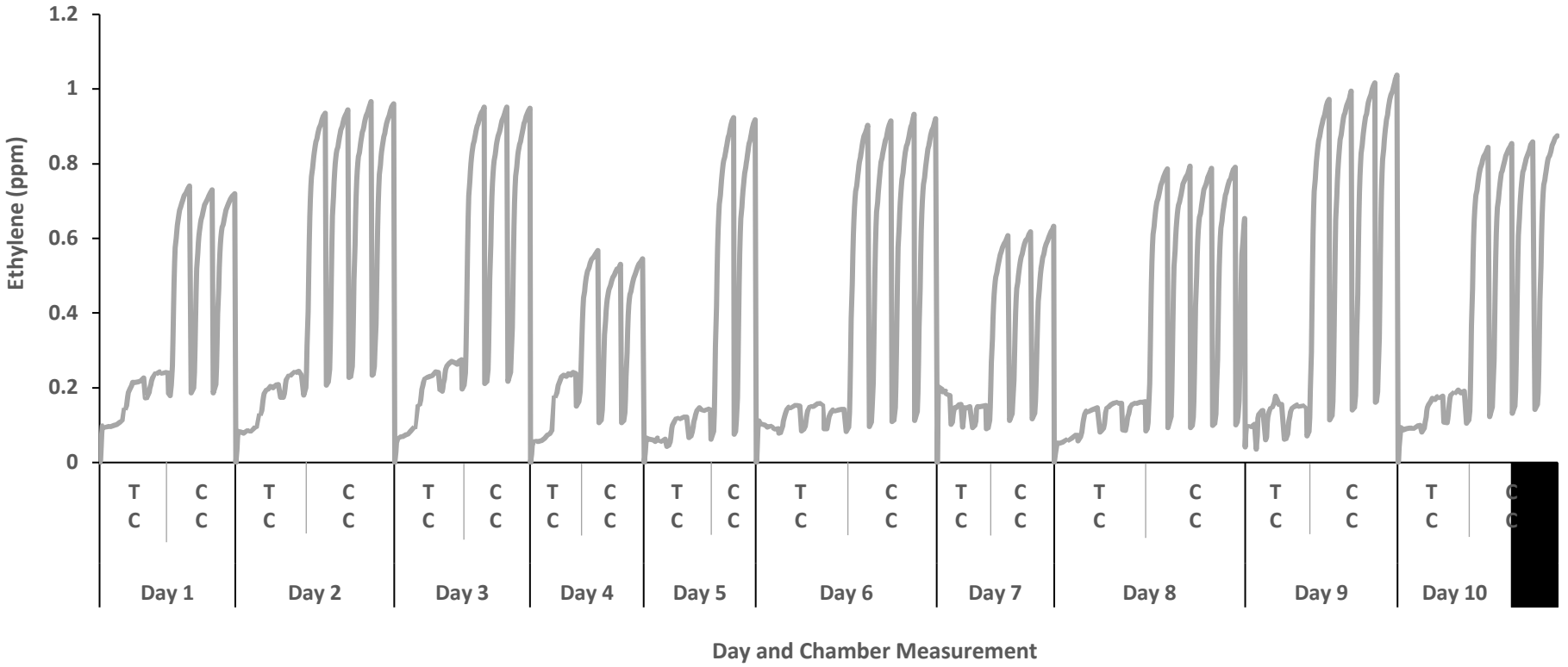


Figure 3.1 Portable Ethylene analyser output showcasing results related issues. These specific measurements were taken between 02/09/2016-04/10/2016. Each day represents a day worth of measurements. TC represents measurement cycles from the ethylene scrubbing (treatment) chamber and CC represents measurements from the control chamber.

As previously stated, Figure 3.1 clearly represents the fact that the concentration within the control chamber is greater than within the treatment chamber. However, due to the resulting output of the ethylene analyser, the exact concentrations of ethylene (ppm) within each chamber cannot be determined. As can be seen in Figure 3.1, there has been an issue with the cleaning stage of the measurement cycle. The cleaning stage is supposed to bring the ethylene sensor back down to 0 ppm so that the following measurement stage starts from 0 ppm. This process had clearly not been working correctly. Therefore, the results in ethylene ppm as seen in Figure 3.1 are likely to be higher than the actual concentration of ethylene detected within each chamber. In Figure 3.2, Figure 3.3 and Figure 3.4, the cleaning stage has functioned correctly, with each measurement cycle starting from 0 ppm. Therefore, the detected ethylene concentration in these experiments are far more reliable than what was detected in initial experiments. This is one of the reasons why confirmation of ethylene levels between chambers was required with the gas chromatography (GC) method (section 3.3.2). With the issues being witnessed with the portable ethylene analyser, it was important to confirm with another method that: A) ethylene was consistently and effectively removed from the treatment chamber and B) ethylene levels were greater in the control chamber.

3.4 RESULTS

3.4.1 Ethylene analyser

Where there were no obvious issues with ethylene analyser outputs, results across all experiments were consistent. Very little to no ethylene was detected within the treatment chamber, whereas ethylene was constantly detected in

the control chamber. An example of ethylene outputs can be seen in Figure 3.2.

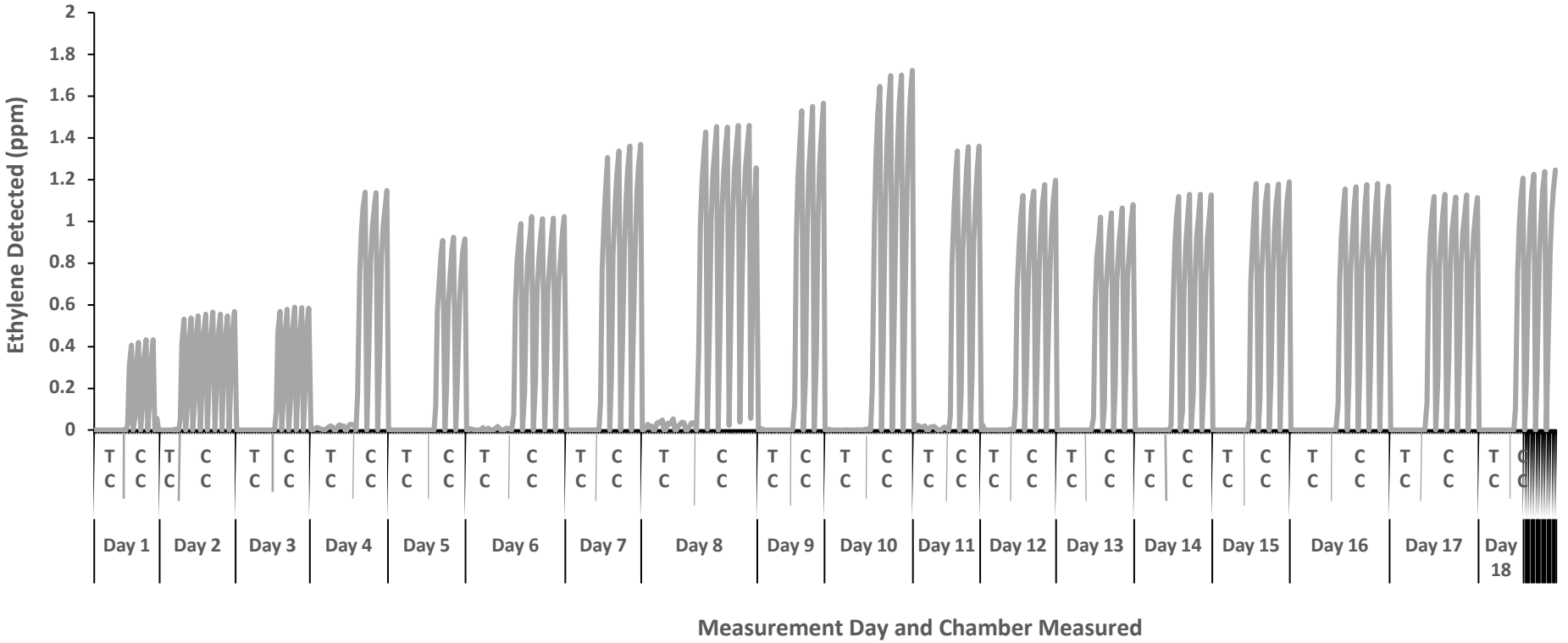


Figure 3.2 Ethylene analysis during molecular experimentation (chapter 7) (05/10/2017 – 27/11/2017). Each day represents a day worth of measurements. TC represents measurement cycles within the treatment chamber and CC represents measurements within the control chamber.

Similarly to what was seen in Figure 3.1, Figure 3.2 clearly demonstrates little to no ethylene detection within the ethylene scrubbing chamber across the entire period of experimentation. This result has been consistent across all experiments performed. Due to the consistent ethylene detection within the control chamber, these results confirm that the ethylene scrubber has been working efficiently to remove ethylene from the treatment chamber environment.

Figure 3.2 demonstrates varying levels of ethylene detection within the control chamber across the measurement period. Varying ethylene levels within the control chamber have also been consistent across experimentation. There are a few possible reasons for this. Firstly, due to the quick turnover time of experiments completed throughout this research, the quantity of tomatoes within the chambers changed from day to day. Adding or removing tomatoes would have significant affects on the levels of ethylene produced and therefore present within the chamber. Secondly, tomatoes would have been added to the chambers at various physiological stages. Fruits are known to produce greater levels of ethylene as they ripen to continue their development (Janssen *et al.*, 2014). Therefore, starting an experiment with mature green tomatoes is likely to yield low levels of ethylene production within the chambers until the tomatoes begin to ripen. At the point of ripening, irreversible autocatalytic ethylene synthesis causes ethylene levels to increase within the chambers. Figure 3.2 can be used as a good example to explain both of these points. During the molecular experiments, tomatoes were placed into the chambers at a mature green stage which is why there are low ethylene levels at days 1, 2 and 3. However, as the tomatoes begin to ripen ethylene levels gradually start to increase. Furthermore, at day 7, the second molecular experiment was initiated, so another batch of tomatoes was added

to the chambers, which caused further increases in ethylene concentrations within the control chamber.

Mechanical or human error may also have been a reason why ethylene reductions were observed at times throughout research. If there was a failure in the sealing of chambers, then it would have been possible for ethylene concentrations to decrease due to the gas diffusing out of the chamber into the outer environment. Furthermore, opening of the chambers to retrieve or add in new samples would have allowed the diffusion of ethylene into the outer environment decreasing concentrations within the chamber.

3.4.1.1 Ethylene injection tests

As can be seen in Figure 3.1 and Figure 3.2, ethylene levels remain quite low even within the control chamber. This is due to the chamber sizes only being able to hold a certain number of fruit samples, whilst still being big enough in size so that ethylene produced by the fruits only produces relatively low concentrations of ethylene within the chambers. Within the postharvest market, storage and transport systems would contain significantly greater quantities of fruit and fresh produce than what was used in these experiments. As a consequence of this, there would be variations in the ethylene concentrations postharvest fruits and vegetables are subjected to (Lawton, 1991; Wills *et al.*, 2000). Therefore, it was important to examine how effective the ethylene scrubber was against higher ethylene concentrations. Furthermore, this would allow examination of the rate at which high levels of ethylene were removed from the ethylene scrubbing chamber.

For a period of time, experiments were run whereby external ethylene was injected into both treatment and control chambers. During this period, 10 ml of external ethylene (≈16.5 ppm) was injected into both 605 l chambers daily, Monday to Friday. Portable ethylene analyser measurements were completed prior to the ethylene injections. Ethylene injections were performed immediately after ethylene analysis with the portable ethylene analyser. Any fruit sample experiments where ethylene injections were taking place will be highlighted throughout the thesis.

A selection of ethylene concentrations within each chamber during a period of ethylene injections can be observed in Figure 3.3.

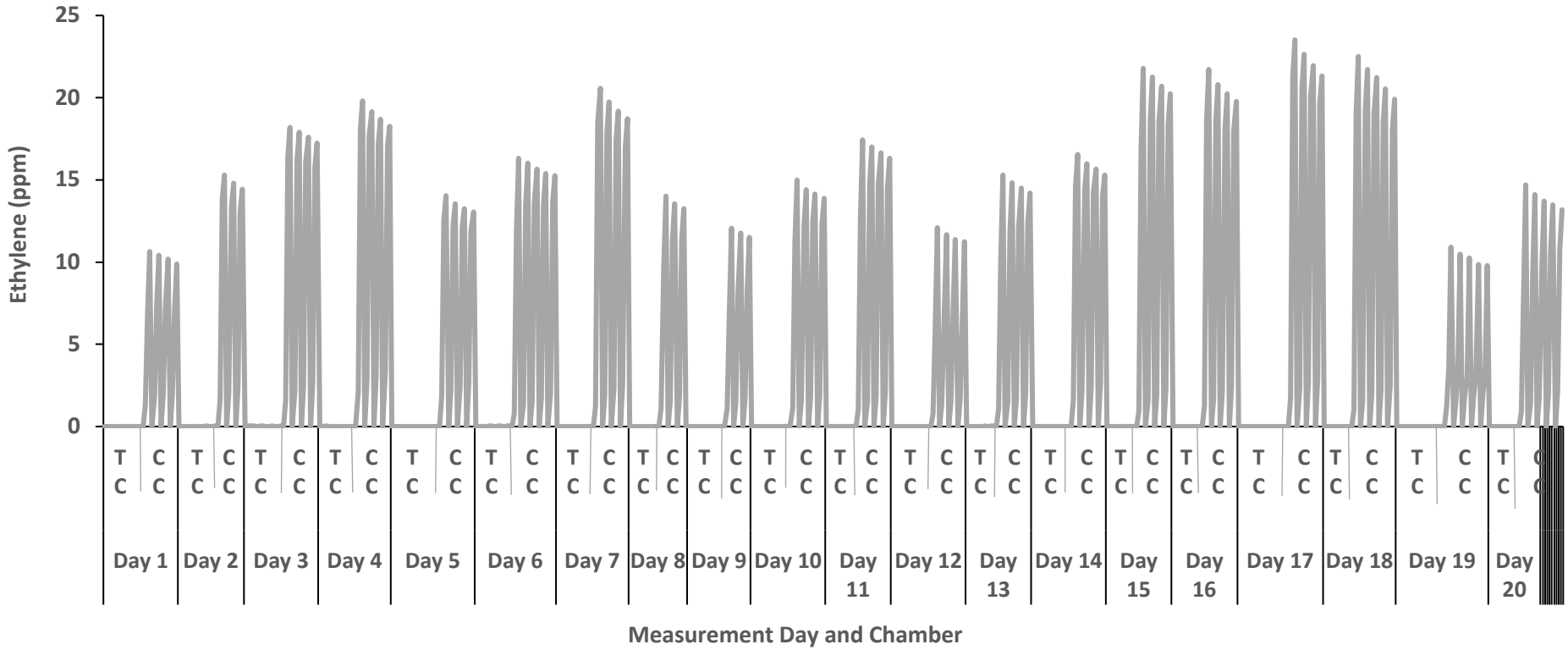


Figure 3.3. A selection of portable ethylene analyser outputs from a period of ethylene injections into either chamber between 17/07/2017 – 14/09/2017. External ethylene at a quantity of 10 mL was injected daily Monday to Friday following ethylene analysis. TC represents measurement cycles from the treatment chamber. CC represents measurement cycles from the control chamber.

Figure 3.3 shows that the UV photocatalytic ethylene scrubber had no issues in removing the added external ethylene from the treatment chamber. The concentration of ethylene in the control chamber was approximately ten times greater in Figure 3.3 with the ethylene injections than was recorded in Figure 3.2 where the chambers were devoid of external ethylene. Within the treatment chamber however, the result was the same as what was seen in Figure 3.2. Little to no ethylene was detected demonstrating the ethylene scrubbers' capacity to remove increasing quantities of ethylene from an environment.

It would still be good to test the ethylene scrubber against significantly larger quantities of ethylene. However, it is known that the process of photocatalytic oxidation is capable of removing high or low quantities of ethylene from large or small storage units (Keller *et al.*, 2013). Work performed here demonstrates the ability of the UV photocatalytic ethylene scrubber to remove increasing levels of ethylene from an environment. Because the ethylene scrubber utilised in this work operates by photocatalytically oxidising ethylene to remove it from the environment, previous research coupled with the observed results here would suggest that the UV photocatalytic ethylene scrubber is capable of removing significantly greater quantities of ethylene from a storage environment than what was tested here.

In additional experiments to get an understanding of how quickly ethylene was removed, the portable ethylene analyser was attached to the treatment chamber immediately after ethylene injection. Results can be seen in Figure 3.4.

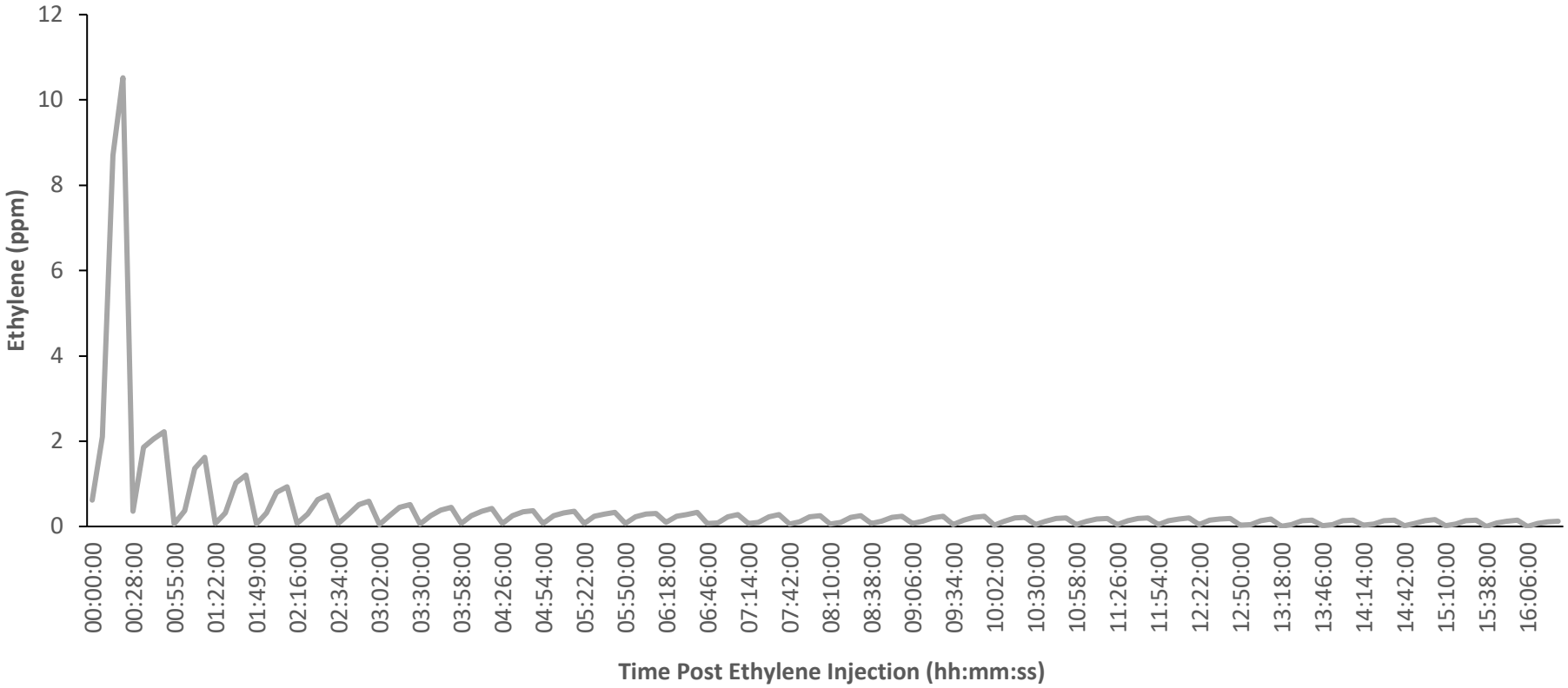


Figure 3.4. Ethylene scrubbing (treatment) chamber ethylene levels immediately following a 10 mL ethylene injection as detected by the portable ethylene analyser.

As demonstrated in Figure 3.4, the rate of ethylene removal by the UV photocatalytic ethylene scrubber is rapid. Less than an hour after the first ethylene measurement, just a fifth of the injected ethylene remained present within the chamber. Following this initial removal, ethylene levels continued to gradually decrease and remain very low. These results prove that the UV photocatalytic ethylene scrubber is a quick and effective method of removing external ethylene within a given environment.

3.4.2 Gas chromatography

All ethylene samples subjected to GC analysis were taken from experiments where there were no ethylene injections taking place. It was not possible to analyse ethylene concentrations during the period of ethylene injections into either chamber (section 3.4.1.1). This was due to the GC machine only being calibrated to 10 ppm ethylene concentrations and ethylene injections into the control chamber were likely to produce ethylene concentrations greater than this. Therefore, the accuracy of the equation used to determine ethylene ppm following GC would have likely been unreliable.

Firstly, results observed with the GC machine matched what was seen with the portable ethylene analyser in showing that there was little to no ethylene present within the treatment chamber (Figure 3.5). On the treatment chamber graph, there is no peak present around 3 minutes into the measurement run. In comparison, there is a peak present at around 3 minutes in the control chamber indicating the presence of ethylene, confirming that ethylene has effectively been removed from the treatment chamber as a result of the UV photocatalytic ethylene scrubber.

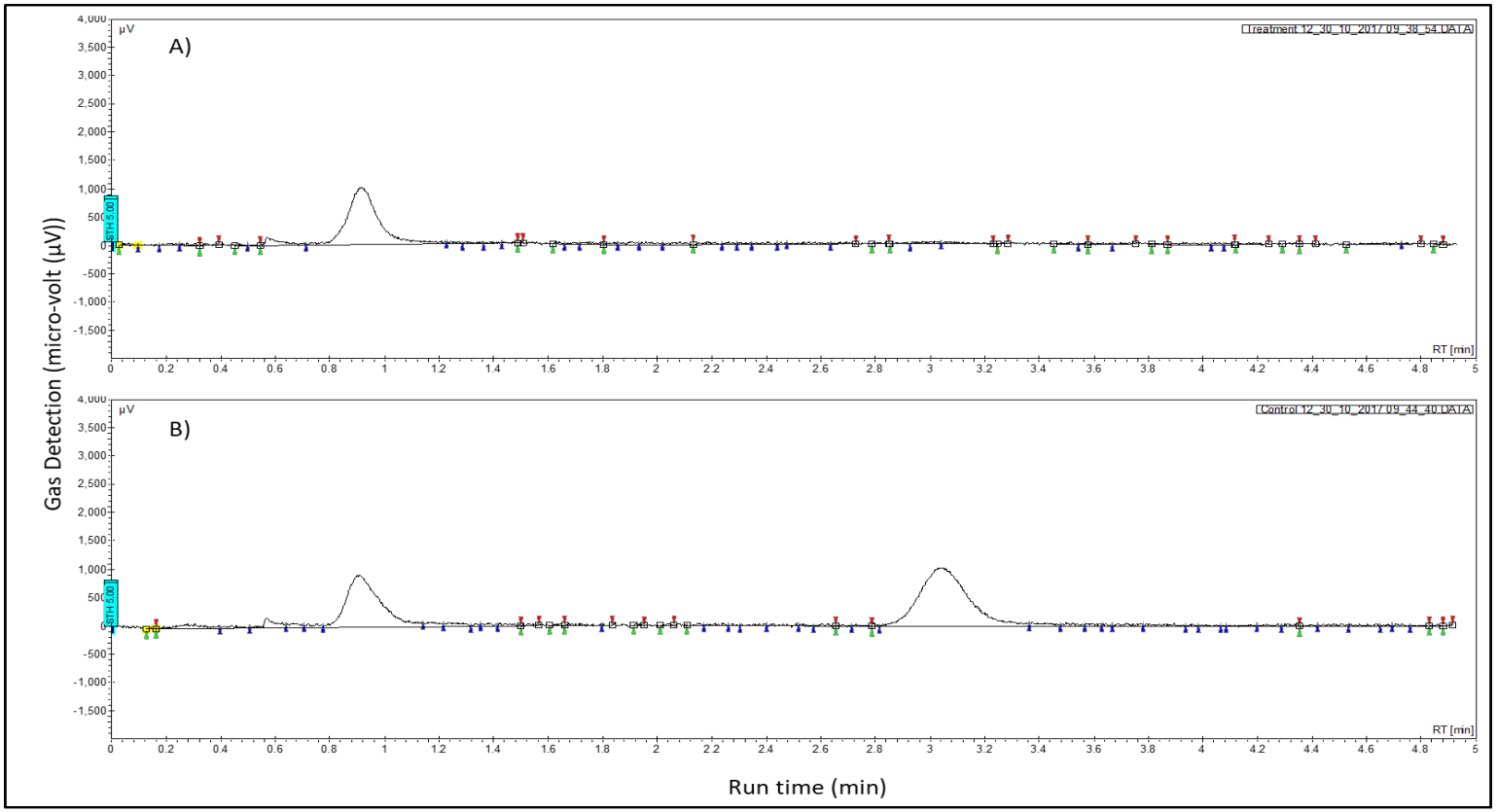


Figure 3.5. Output from GC generated by the Compass CDS v 3 software package from chamber samples extracted on the 30/10/2017. A) GC output from a treatment chamber sample. B) GC output from a control chamber sample. Ethylene detection is presented as a peak around the 3 min mark. Peak area (µV.min) was used to calculate ethylene concentration (ppm).

It was observed that GC calculated ethylene concentrations from within the control chamber differed from those obtained by the portable ethylene analyser. Across the days where both GC measurements and portable ethylene analyser measurements occurred, greater concentrations of ethylene were always observed in the GC outputs (Table 3.1).

Table 3.1. A comparison of the detected ethylene (ppm) concentrations within the control chamber as generated by both the F-900 portable ethylene analyser and the Bruker Scion 456-Gc gas chromatographer. Results presented were obtained by averaging the three sample outputs from the GC and by averaging the peak measurements from the measurement cycles of the portable ethylene analyser and presented with ± SEM. The correlation coefficients between ethylene detection methods are shown for each individual days measurements also.

	Date of Measurements			
	10.10.2017	***23.10.2017***	***30.10.17***	***13.11.2017***
Portable Ethylene Analyser (ppm)	0.55 ± 0.006	1.34 ± 0.017	1.84 ± 0.009	1.18 ± 0.006
Gas Chromatography (ppm)	0.98 ± 0.165	1.96 ± 0.144	3.12 ± 0.184	2.51 ± 0.078
Correlation Coefficient	0.999	-0.18	-0.941	0.930

At all four dates where ethylene analysis took place using both methods of ethylene detection, ethylene concentrations detected from GC were greater than what was detected using the portable ethylene analyser. This means it is not possible to say for certain what the exact ethylene concentrations are within the control chamber. However, what can be confirmed is the consistent removal of ethylene from within the treatment chamber. Results show that

neither GC nor the portable ethylene analyser were able to detect ethylene consistently within the treatment chamber.

3.4.3 Overall ethylene results

Results presented from both the portable ethylene analyser and GC clearly demonstrate the ability of the UV photocatalytic ethylene scrubber to consistently remove ethylene from an environment. This could prove beneficial to the post-harvest market. Ethylene can cause significant losses to post harvest produce such as fruits, vegetables and ornamentals. The harmful impacts of ethylene can cause over-ripening, increased senescence, physiological disorders, reduced shelf life, increased softening and increased susceptibility to plant pathogens (Saltveit, 1999). These effects have been estimated to cause product losses as high as 10-80% (Kader, 2003; Keller *et al.*, 2013).

The differing levels of ethylene detected in the control chamber between the two described methods of ethylene analysis demonstrate issues with one or both of the methods. Without testing a third method of ethylene analysis, it is difficult to determine whether the portable ethylene analyser or GC is providing the most accurate ethylene reading within the control chamber. GC is a commonly used and trusted method of ethylene detection from plants (Cristescu *et al.*, 2013). Therefore, it can be assumed that the GC results are presenting the most accurate ethylene concentrations. Furthermore, when the issues encountered with the portable ethylene analyser are considered (section 3.3.5), it further suggests that the GC method of ethylene detection is more reliable. Further methods of ethylene detection are well described by Cristescu *et al.* (2013) and Janssen *et al.* (2014), including laser-based

sensors (photoacoustic detection), electro-catalytic sensor technology and non-dispersive infrared spectroscopy, and with more time, testing of these methods might have been useful.

Regardless of the exact amount of ethylene present within the control chamber, the absence of ethylene detection within the treatment chamber should act to stimulate differences in results between fruit samples. Ethylene levels at less than 1 ppm are capable of stimulating the ripening process and affecting the fruit physiology of climacteric fruits (Inaba & Nakamura, 1988; Janssen *et al.*, 2014). Therefore, ethylene reductions to undetectable levels within the ethylene scrubbing chamber should affect treated fruit samples and result in variations between treatment and control groups. Results presented here also demonstrate the rate at which ethylene is removed from the treatment chamber (Figure 3.4). Concentrated external ethylene is removed quickly from the environment as a result of ethylene scrubbing.

3.5 EFFECTS OF THE UV PHOTOCATALYTIC ETHYLENE SCRUBBER ON TEMPERATURE AND RELATIVE HUMIDITY

Temperature is the most important factor known to influence the impact level of other factors affecting storage conditions (i.e. ethylene concentration, relative humidity and atmospheric composition) (Keller *et al.*, 2013). Here, the effects of the UV photocatalytic ethylene scrubber on temperature were monitored throughout experimentation.

Relative humidity (RH) within a fruit and fresh produce storage environment influences decay development, physiological disorders and uniformity of ripening. Low RH levels are associated with increasing ethylene production

rates, the induction of water stress, and the drying out of fruit surfaces resulting in weight loss. Due to these influences, high RH's are beneficial for the storage of fruits and vegetables (Kader & Rolle, 2004; Keller *et al.*, 2013). According to Kader & Rolle (2004), the appropriate RH range for the storage of vegetables varies between 90-100%, and for the storage of fruits, 85% to 95%.

A sensor, used to monitor temperature (°C) and relative humidity (RH) (%), was placed in both chambers for the duration of the research. Temperature and RH levels were continuously recorded and the results used to determine if the action of the ethylene scrubber could affect the temperature and relative humidity within a fresh produce storage environment.

3.5.1 Temperature variations between chambers

Throughout this research, it was observed that the treatment chamber was continuously slightly warmer than the control chamber (Figure 3.6). Although the difference between chambers was never large, the results do suggest that the action of the UV photocatalytic ethylene scrubber causes increasing temperatures within the storage environment.

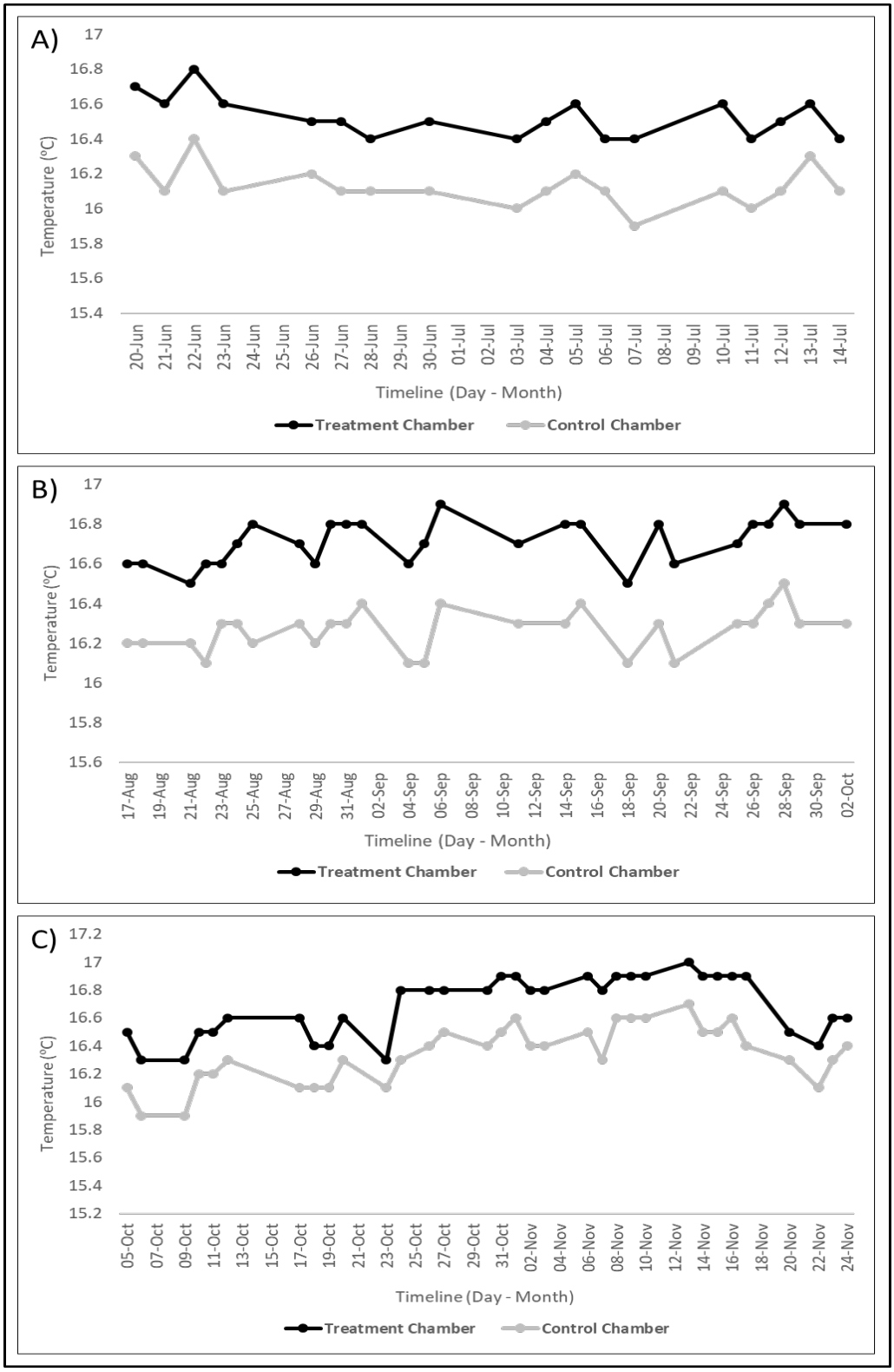


Figure 3.6. Temperature recordings (°C) from both chambers across three timespans during experimentation. A) Temperature recordings 20/06/17 – 14/07/17. B) Temperature recordings 17/08/17 – 02/10/2017. C) Temperature recordings 05/10/17 – 24/11/17.

As can be seen in Figure 3.6, the temperature within the treatment chamber was always between 0.1 - 0.5 ^{o}C warmer than in the control chamber. It is evident from the pattern of the graphs that growth room fluctuations in temperature affected both chambers in the same manner. However, the changing temperature in the growth room did not affect the difference in temperature between chambers demonstrating that the difference was down to the ethylene scrubber alone. The consistency of these results across all research performed infers a slight warming effect of the ethylene scrubber.

It was possible that the added heat within the treatment chamber could have been due to chamber locations within the growth room. Throughout all research conducted, the treatment and control chambers could not be swapped around in the growth room due to spacing issues. Therefore, to determine whether the temperature difference between chambers was as a result of the scrubber or the location of the chambers within the room, the sensors were placed into the empty chambers when the ethylene scrubber was turned off. The resulting outputs from both sensors were continuously the same. This result therefore confirms that the observed temperature differences between chambers (Figure 3.6) was a direct result of the action of the UV photocatalytic ethylene scrubber.

3.5.1.1 Temperature discussion and conclusions

The increase in temperature observed as a result of the UV photocatalytic ethylene scrubber is likely to be as a result of the UV lamps. The lamps generate a lot of heat which is why excess piping was incorporated to attach the scrubber to the chamber (section 2.3). The heat generated from the lights

is most probably being blown by the fans from the scrubber, through the piping to the storage chamber.

Increases in temperature, as well as affecting storage factors, cause significant effects on the physiology and quality of fruits and fresh produce (Shin *et al.*, 2008; Mekonnen, 2017). Higher temperatures also increase ethylene production in fruits, increasing metabolism and reducing shelf life (Keller *et al.*, 2013). It is therefore important that the specific storage guidelines of postharvest produce, with regards to temperature, are followed precisely in order to ensure fruits remain at the highest quality. Although results presented here only demonstrate a very small increase in temperature compared to the control chamber, the continued exposure of produce to this temperature increase could prove to be a problem. Therefore, considerations to storage unit layouts would need to be taken into account should the application of a UV photocatalytic ethylene scrubber provide the maximum possible benefits to the fruit storage environment. Otherwise the potential benefits of ethylene removal could be negated by increases in temperature.

3.5.2 Relative humidity variations between chambers

Results across all experimentation demonstrated a constant effect of the action of the ethylene scrubber on treatment chamber humidity. Some results to demonstrate this can be seen in Figure 3.7. Throughout the research performed, RH within the control chamber remained consistent at 99%. Within the treatment chamber however, RH consistently fluctuated and decreased suggesting an effect of UV photocatalysis.

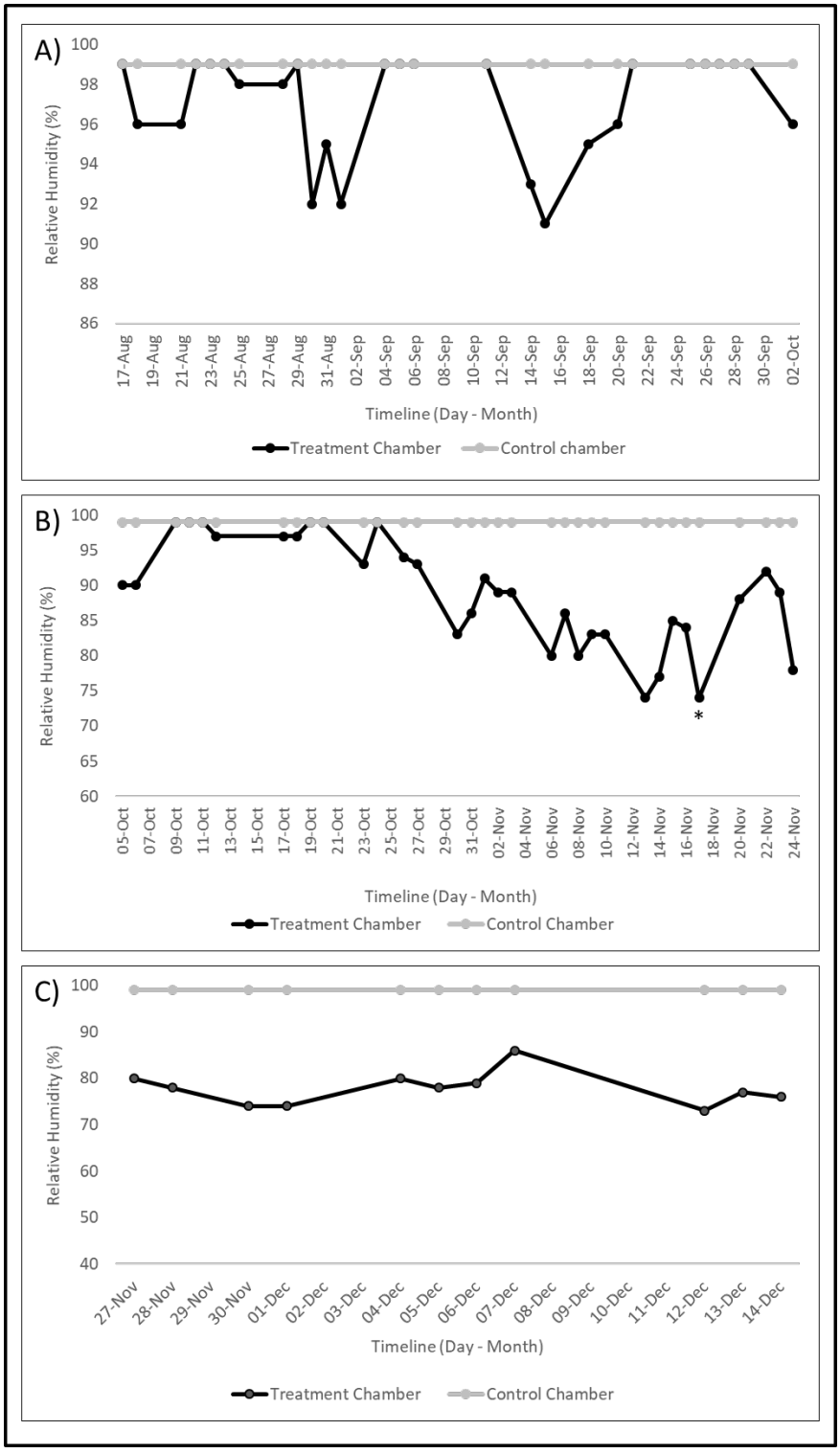


Figure 3.7. Relative humidity (%) recordings from both champers from three timespans across experimentation. A) Recordings from 17/08/17 – 02/10/17. B) 05/10/17 – 24/11/17. C) 27/11/17 – 14/12/17. The asterisk (*) present in graph 'B' represents water being added to the humidity baths within both chambers.

Figure 3.7 clearly demonstrates the differences in RH levels recorded between the treatment and control chamber. Due to the consistency of the humidity levels within the control chamber, it can be confirmed that the fluctuations seen within the treatment chamber are solely as a result of the action of the UV photocatalytic ethylene scrubber.

Throughout this research, RH in the treatment chamber was always decreasing in comparison to the control chamber. Figure 3.7 demonstrates three periods of the ethylene scrubber affecting RH within the treatment chamber. Figure 3.7(a) shows RH fluctuations not causing major drops in humidity with changing levels ranging from 90-99%. However, during the timespan of Figure 3.7(a), efforts were being made to maintain high levels of humidity within the treatment chamber because of previously observed reductions in humidity. Extra humidity boxes were placed in the treatment chamber and continuously topped up in order to perform experiments where differences in RH between chambers would hopefully not affect results. Even with these attempts to keep humidity levels high, constant fluctuations and drops in humidity can be observed. This clearly shows the strong continuous effect of ethylene scrubbing on storage RH. Had it not been for the attempts to maintain high humidity with the treatment chamber, Figure 3.7(a) would have most likely looked similar to Figure 3.7(b).

Figure 3.7(b) shows what was observed most frequently throughout the research, the continuation of decreasing RH levels as a result of the ethylene scrubber. Although there are small fluctuations throughout the timespan, there is an overall negative correlation of RH. Even when water was added to the humidity boxes within the chamber to increase humidity, it is observed in the treatment chamber that RH levels begin to drop again. Due to these

observations being the most frequently observed, it can be concluded that the ethylene scrubbers' mode of action continuously decreases RH within an environment.

Figure 3.7(c) shows a maintained low RH within the treatment chamber in comparison to the control chamber. This further demonstrates that the action of the ethylene scrubber results in humidity reductions within a storage environment.

3.5.2.1 Relative humidity discussion

Reductions in RH as a result of ethylene scrubbing technology could be deemed as being somewhat surprising. In the process of ethylene oxidation by photocatalysis, ethylene, along with oxygen are converted to carbon dioxide and water (Martínez-Romero *et al.*, 2007; Keller *et al.*, 2013). Therefore, it could be assumed that the action of UV photocatalysis would either maintain or even increase RH due to the production of water vapour during the process. The reasons for these observed decreases in humidity as a result of ethylene scrubbing are thus not completely clear. It could be hypothesised that that the action of the fans within the ethylene scrubber could act to reduce the RH within the treatment chamber. The constant trafficking of air through the system would increase air flow and could be removing excess moisture through evaporation (Andatech, 2017). A good test to confirm whether the action of the fans reduces humidity within the chamber would be to attach an ethylene scrubber to the control chamber without any of the ethylene removal equipment inside it. This way you would have the same fan mechanisms present within the control chamber set up without any ethylene removal. If humidity was affected in the same manner as is seen within the treatment

chamber, it could be concluded that the fans are the reason for humidity reductions.

Results may affect the results of both fruit physiology as well as affect the pathogens ability to cause infection. As previously mentioned, low RH levels can impact on fruit physiology, quality and shelf life. Low RH could however, have a positive effect on pathogen prevention. For example, *C. gloeosporioides* requires humidity levels as high as 95% for conidial germination and successful infection (Estrada *et al.*, 2000). Therefore, it is possible that the reductions in humidity caused by the ethylene scrubber may have a role in disease prevention. However, this trait would only be beneficial if the resulting low RH produces no detrimental effects on fruit quality.

If the ethylene scrubber was to be applied to storage facilities, considerations would need to be made so that the effects of the scrubber on humidity don't cause detriment to fruits. The ability of low humidity levels of dry out and cause physiological disorders would provide issues to the postharvest market.

3.6 OVERALL CONCLUSIONS

Due to the numerous negative effects of ethylene during the postharvest storage of fruits and vegetables, methods of ethylene control and removal continue to be tested as they could play crucially important role in fruit storage and transport systems. Temperature and RH are significant factors in fruit storage which should strongly impact the design and application of ethylene removal methods (Nunes *et al.*, 2009; Keller *et al.*, 2013).

Here it has been demonstrated that the UV photocatalytic ethylene scrubber consistently removed varying ethylene levels from a storage environment. Confirmation of this result was achieved by using two methods of ethylene detection. Neither GC nor an electrochemical sensor were able to detect ethylene within the ethylene scrubbing chamber demonstrating the ethylene scrubbers' capabilities to induce consistent, effective ethylene removal within a storage environment.

This however comes at the cost of the treatment producing potentially negative effects on storage temperature and RH levels. The increasing temperatures and decreases in RH are likely to result in detrimental effects on the fruit physiology. Therefore, should the UV photocatalytic ethylene scrubber ever be applied on a large scale to the storage units of fruit and fresh produce, the treatment would need to be applied in such a way that its effects on temperature and RH could be negated and not cause detriment to the stored produce.

CHAPTER FOUR

4 THE EFFECTS OF UV PHOTOCATALYTIC ETHYLENE SCRUBBING ON FRUIT PHYSIOLOGY AND RIPENING

THE EFFECTS OF UV PHOTOCATALYTIC ETHYLENE SCRUBBING ON FRUIT PHYSIOLOGY AND RIPENING

4.1 INTRODUCTION

Ethylene is known to initiate the ripening of climacteric fruits and it is universally accepted that ripening initiation occurs quicker at higher ethylene concentrations and increased exposure times (Wills *et al.*, 1998). As well as ripening initiation, there is a linear relationship between ethylene concentrations and ripening, with increasing ethylene levels reducing the time taken for fruit to ripen (Wills *et al.*, 2001). Commercially, ethylene has been used at extremely large concentrations, as high as 1000 μl l^{-1} (ppm), in order to promote the ripening of numerous climacteric fruits including tomatoes, avocados, bananas, mangoes, honeydew melons, kiwifruit and stone fruits (Saltveit, 1999).

Ethylene plays a significant role in altering the visual appearance of fruits. The stimulation of ripening by ethylene enhances the appearance of many fruits bringing them to an appearance where a consumer would consider them of high quality. Ethylene promotes and accelerates chlorophyll degradation and loss in many fruits making the produce attractive for consumers (Fraser *et al.*, 1994; Saltveit, 1999). Ethylene is the cause of citrus de-greening (Shimokawa *et al.*, 1978), chlorophyll loss in bananas which promotes yellow colouration (Golding *et al.*, 1998; Saltveit, 1999), and the induction of carotenoid synthesis from leaf like xanothophylls in tomato (Fraser *et al.*, 1994; Su *et al.*, 2015). Ethylene also brings about beneficial softening of tissue during fruit ripening, having been reported to directly regulate the transcription of softening related

genes in fruits, with increasing concentrations of ethylene giving rise to increased fruit softening (Hayama *et al.*, 2006). The softening of fruit is crucial for consumer consumption as it brings about favourable characteristics in texture.

However, the benefits of ethylene in the ripening process can be outweighed by the numerous detrimental effects of ethylene on fruit physiology. Within the storage environment, ethylene accelerates the ripening process causing: accelerated senescence, excessive fruit softening, sprouting in potatoes, discolouration (e.g. browning or yellowing), toughening of vegetables and decreased fruit and vegetable shelf lives (Saltveit, 1999; Martínez-Romero *et al.*, 2007; Hussain *et al.*, 2010). Fruits, vegetables and flowers respond to the presence of ethylene through ethylene receptors on their surface (Alexander & Grierson, 2002; Lacey & Binder, 2014; Gallie, 2015). The actuation of an ethylene receptor detecting ethylene promotes further ethylene production by the fruit in a positive feedback autocatalytic process of ethylene production in climacteric fruit (Saltveit, 1999; Cara & Giovannoni, 2008; Van de Poel *et al.*, 2012). The continued production and detection of ethylene accelerates the fruits aging and deterioration and therefore decreases its shelf life.

It is for these reasons that manipulating postharvest ethylene action is an important goal in the postharvest market. Numerous methods of ethylene removal and control have been tested, with literature demonstrating commercial and conventional techniques and technologies utilised to control ethylene action (Martínez-Romero *et al.*, 2007). Commonly used methods of ethylene control are ethylene scavengers such as potassium permanganate ($KMnO_4$) (Vermeiren *et al.*, 1999). The use of 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception, has also shown promise in ethylene

removal with the product having been commercialised for a number of years (Blankenship & Dole, 2003).

Photocatalytic ethylene degradation is an emerging and promising technology for use in the storage and transfer of fresh produce (Hussain *et al.*, 2010; Keller *et al.*, 2013). It offers advantages over other methods of ethylene removal due to its low running costs and continuous effectivity. Here we analysed the effects of a FRESH+ EC-60 UV photocatalytic ethylene scrubber on tomato fruit physiology and ripening over time. Results were used to determine whether the ethylene removal caused by the ethylene scrubber could reduce the rate of ripening to extend the shelf life of fruits whilst maintaining a quality product for consumers.

4.2 MATERIALS AND METHODS

4.2.1 Colour examination

A calibrated CR-200 Chroma meter (Konica Minolta) in L*a*b* mode was used to conduct colour measurements. Readings were taken at three points across the tomato and averaged to get an accurate colour representation of the entire fruit surface. Readings were taken at various time points dependant on the experiment taking place (as specified in results section 4.4.1), and used to calculate tomato colour index (TCI) (Lopez Camelo & Gomez, 2004; Scott *et al.*, 2017) (Equation 4.1).

$$TCI = \frac{2000 \times a^*}{L^* \times \sqrt{(a^{*2} + b^{*2})}}$$

Equation 4.1

Tomato colour index (TCI) formula where L = lightness, a = red – green and b = blue – yellow values (Scott *et al.*, 2017).

Lower TCI values indicate a greener tomato, whilst higher TCI values indicate a redder, riper tomato.

4.2.2 Fruit firmness analysis

Flesh firmness was measured using a TA.XT plus Texture Analyser (Stable Micro Systems) with results being expressed in Newtons (N). For fruits beginning examination at a mature green stage, measurements were taken at three evenly spaced equatorial regions across the fruit throughout the course of experimentation. The resulting outputs from the three equatorial measurements were averaged to give and overall firmness measurement of the fruit. For fruits beginning examination at a red ripe stage, one polar measurement, as well as three equatorial measurements at evenly spaced regions across the fruit were recorded throughout experimentation and used to calculate the geometric mean giving an overall fruit firmness measurement. A polar measurement could not be applied to mature green fruit due to the fruits being too firm at harvest, meaning the texture analyser was unable to accurately process the result.

The tomato varieties Shirley, Moneymaker and Meccano were subject to a 4 mm compression setting on the texture analyser to assess fruit firmness. The Brioso tomato variety was subject to a 2 mm compression as the tomatoes are smaller in size.

Fruit firmness was recorded at various time points dependant on the experiment taking place (as specified in results section 4.5).

4.2.3 Weight measurements

Scales, set to one decimal place, were used to measure fruit weight in g. The results were used to calculate the change in weight from time point to time point for each fruit.

4.3 RESULTS

4.4 EFFECTS OF UV PHOTOCATALYTIC ETHYLENE SCRUBBING ON FRUIT COLOUR

Various methods of ethylene removal are known to delay or inhibit tomato fruit ripening and colour evolution (Blankenship & Dole, 2003; Guillen *et al.*, 2006; Martínez-Romero *et al.*, 2007; de Chiara *et al.*, 2015). The delays in colour evolution and ripening associated with ethylene removal can prove beneficial to the postharvest market by increasing fruit shelf life.

Throughout this research, to determine the ripening progression of tomato fruit samples, colour evolution was monitored (Hobson *et al.*, 1983; Lopez Camelo & Gomez, 2004; Scott *et al.*, 2017). Colour is the key external indicator of ripening progression and therefore an ideal physiological characteristic to use to compare ripening rates between treatment and control chambers.

4.4.1 Colour evolution between chambers

Overall, without supplementation of external ethylene into both chambers, the colour evolution of tomato fruits remained relatively unaffected by ethylene scrubbing treatment. Tomato fruits of all varieties did not ripen at a significantly different rate in the treatment chamber compared to the control chamber (Figure 4.1). A demonstration of the similarity in ripening rates between treated and control samples can be observed in Figure 4.2.

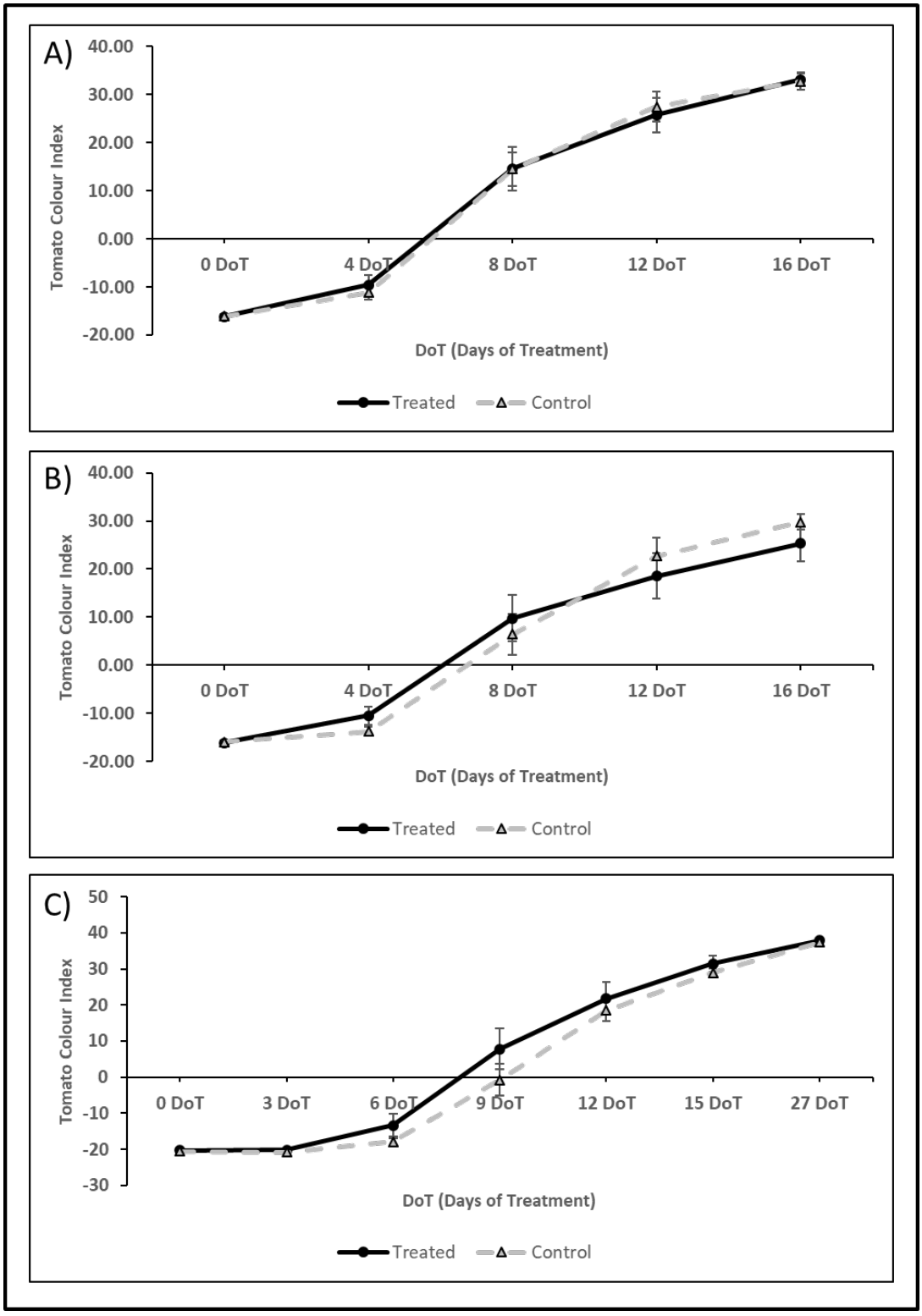


Figure 4.1. The mean TCI of tomato fruit samples overtime stored in either the ethylene scrubbing chamber or control chamber. Error bars represent SEM. A) Shirley variety tomato results compiled from two replicate experiments. B) Moneymaker variety tomato results compiled from two replicate experiments. C) Meccano variety tomato results.

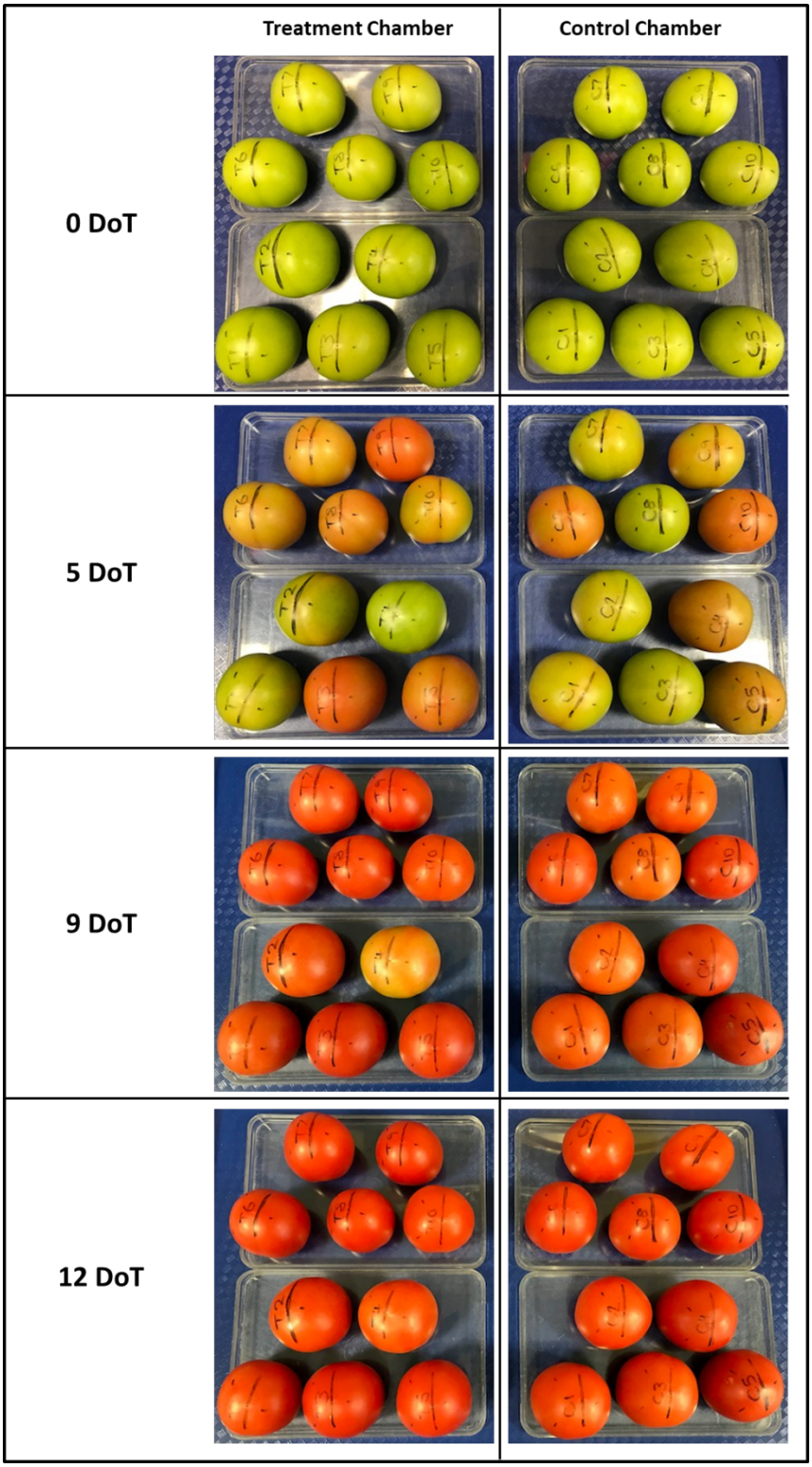


Figure 4.2. Images taken of originally mature green Shirley variety tomatoes at harvest (0 DoT) over time following storage in either the ethylene scrubbing treatment chamber or control chamber. DoT (Days of treatment/storage).

Both Figure 4.1 and Figure 4.2 clearly demonstrate a lack of effects of ethylene scrubbing treatment on the colour evolution of tomato fruits from a mature green stage. Across varieties, little to no difference in colour evolution was observed between chambers.

This result was surprising as it would be expected that removal of the ripening hormone ethylene, could delay or potentially inhibit the onset of fruit ripening from a mature green stage (Blankenship & Dole, 2003; Guillen *et al.*, 2006; Martínez-Romero *et al.*, 2007; de Chiara *et al.*, 2015). However, perhaps the low amounts of ethylene present in the air were sufficient enough to stimulate the onset of ripening from a mature green stage. As previously discussed, any level of ethylene present within in the air is capable of having a ripening effect on fruit and stimulating physiological activity (Wills *et al.*, 2001; Keller *et al.*, 2013).

The UV photocatalytic ethylene scrubber acts by removing ethylene externally, therefore ethylene present within the air, or ethylene that is produced by the fruits can be immediately picked up by ethylene receptors present on the fruits surface. The 1-MCP method of ethylene removal however, acts by 1-MCP binding to the fruit's ethylene receptors so ethylene is unable to bind (Blankenship & Dole, 2003; Martínez-Romero *et al.*, 2007). This is why is it is likely that the UV photocatalytic ethylene scrubbing method of ethylene removal is unable to inhibit fruit ripening from a mature green stage like 1-MCP has been reported to do. Interestingly, it has been reported by Maneerat *et al.* (2003) that photocatalytic oxidation of ethylene using TiO_2 was able to delay the ripening of mature green tomato fruits by four weeks. This however, could be down to a slightly varied method of applying photocatalytic oxidation used to what is presented here, where Maneerat *et al.* (2003) applied TiO_2

photocatalytic activity at a much closer proximity to the tomato fruits meaning ethylene could be destroyed at the same place at which it was produced.

Although the ethylene scrubbing method presented here was clearly unable to prevent the onset of fruit ripening, it was hypothesised that the treatment may be able to reduce the rate of ripening if there was the potential for higher levels of ethylene within each chamber. As reported by Wills *et al.* (2001), decreases in postharvest life are linearly related to increasing ethylene concentrations. Therefore, if increased ethylene concentrations were being actively removed from the treatment chamber, the rate of ripening might be reduced in treated fruits compared to fruits left to ripen in the increased ethylene concentrations of the control chamber.

4.4.1.1 The effects of ethylene injections on colour evolution

The injection of 10 ml of external ethylene (method described in section 3.4.1.1) into each chamber was capable of stimulating significant differences between treated and control samples TCI across various time points during storage. These results were again consistent across different tomato varieties (Figure 4.3) and a demonstration of the on fruit effects can be observed in Figure 4.4.

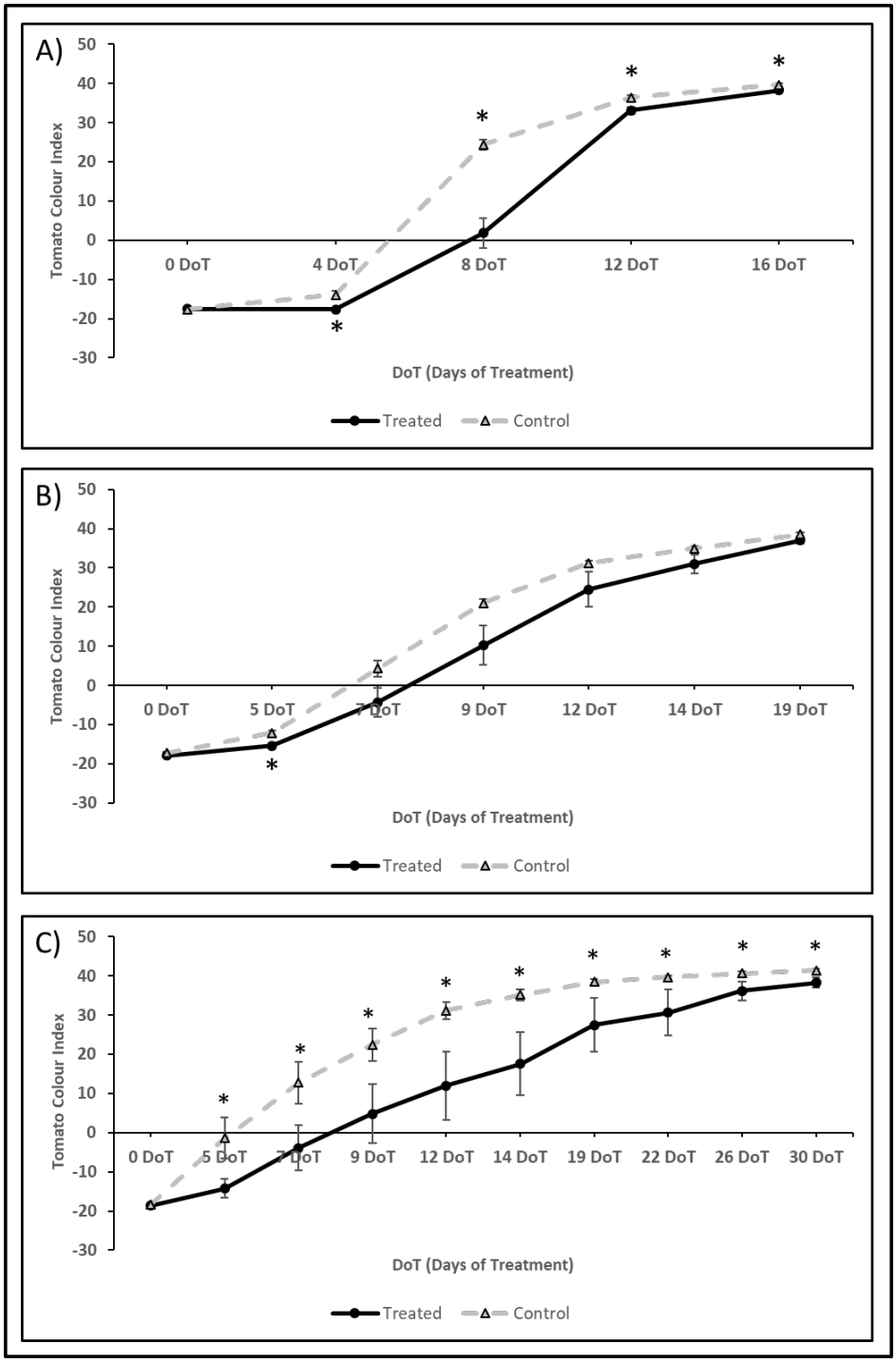


Figure 4.3. The average Tomato Colour Index over time of fruit samples either subjected to ethylene scrubbing treatment or storage within the control chamber. During these periods of storage, daily Monday to Friday 10 ml ethylene injections into each chamber were taking place. Error Bars represent SEM. Asterisks (*) represent a significant difference ($p < 0.05$) in sample average TCI on the specified day. A) Shirley variety tomato results. B) Meccano variety tomato results. C) Brioso variety tomato results.

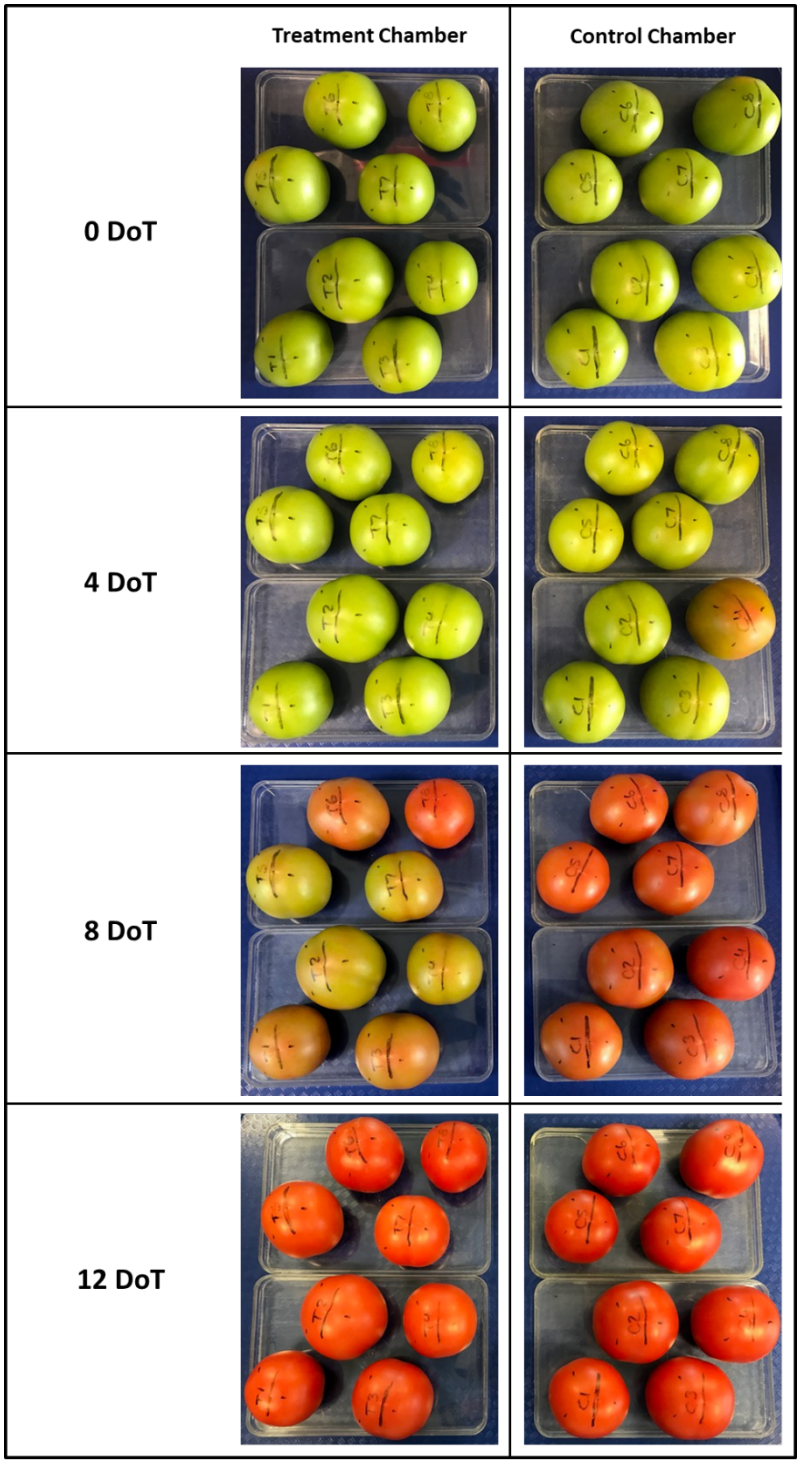


Figure 4.4. Images taken of originally mature green Shirley variety tomatoes at harvest (0 DoT) over time during storage in either the ethylene scrubbing treatment chamber or control chamber. During this period of fruit storage, daily Monday to Friday 10 ml ethylene injections into each chamber were taking place. DoT (Days of treatment/storage).

As can be seen in Figure 4.3, across varieties, there is a clear ripening delay from a mature green stage to a red ripe stage with fruits stored in the treatment chamber. Therefore, it can be confirmed that the ethylene scrubbers' ability to remove increasing levels of ethylene delays the rate of ripening in fruit storage. Figure 4.4 shows the effects of ethylene scrubbing on fruit ripening clearly at 8 DoT. It can be observed that all fruits stored in the control chamber are at a light red stage in colour development, whereas the treated tomatoes vary in colour stage between breaker and light red.

However, whilst it can be noticed that the delay in colour evolution doesn't last too long, this delay of a few days could extend the shelf/storage life of fruits for these few days. This would be particularly beneficial in fresh produce travel environments when fruits have to travel for days before reaching the desired location.

It can be observed in Figure 4.3 that the delay in ripening caused by ethylene scrubbing occurs very early in fruit storage. Following an initial delay in the treatment chamber, ripening then appears to happen at a similar rate between chambers. This suggests that ethylene scrubbing has an effect on the tomato fruit by delaying the initial onset of fruit ripening. It has been reported that once climacteric fruit ripening is initiated, internal ethylene concentration within fruits increases to saturation levels and exogenous application of ethylene has no further promotive effect on ripening (Saltveit, 1999). Furthermore, once the process of autocatalytic ethylene production and ripening occurs in fruits, reduction in external ethylene concentration has no effect on ripening as it cannot reduce internal fruit ethylene concentrations due to the skin and flesh's large diffusion resistance (Saltveit, 1999). This supports the results that are observed here where an initial delay in the onset of ripening due to ethylene

scrubbing can be seen, however once initiated, ripening appears to occur at a similar rate to what is observed in the control chamber.

Increasing ethylene concentrations have increased the ripening rate of control chamber stored fruits similarly to the effects reported by Wills *et al.* (2001), when comparing Figure 4.1(a) to Figure 4.3(a) and Figure 4.1(c) to Figure 4.3(b) for Shirley and Meccano variety tomatoes respectively. Using the Lopez Camelo & Gomez (2004) ripening index values for Shirley variety tomatoes at 8 DoT, without ethylene injections the TCI value is 14.51, indicating a pink-light red visual colour, whereas with ethylene injections, the TCI value is 24.37, indicating a red visual colour. For Meccano variety fruit stored without ethylene injections at 9 DoT, the average TCI value is -0.72, indicating a turning to pink visual colour, whereas with ethylene injections, the average TCI value of fruit was around 20.94 indicating a light red to red visual colour. These results clearly demonstrate that higher ethylene concentrations lead to quicker ripening rates.

4.4.1.2 Effects of ethylene scrubbing on colour uniformity

Occasionally, the effects of ethylene scrubbing treatment appeared to cause non-uniform colour evolution between treated fruit samples. It was observed that fruit within the control chamber appeared to ripen at similar rates; however, on a couple of occasions, fruit samples within the treatment chamber demonstrated different ripening rates. An example of this can be seen in Figure 4.5.

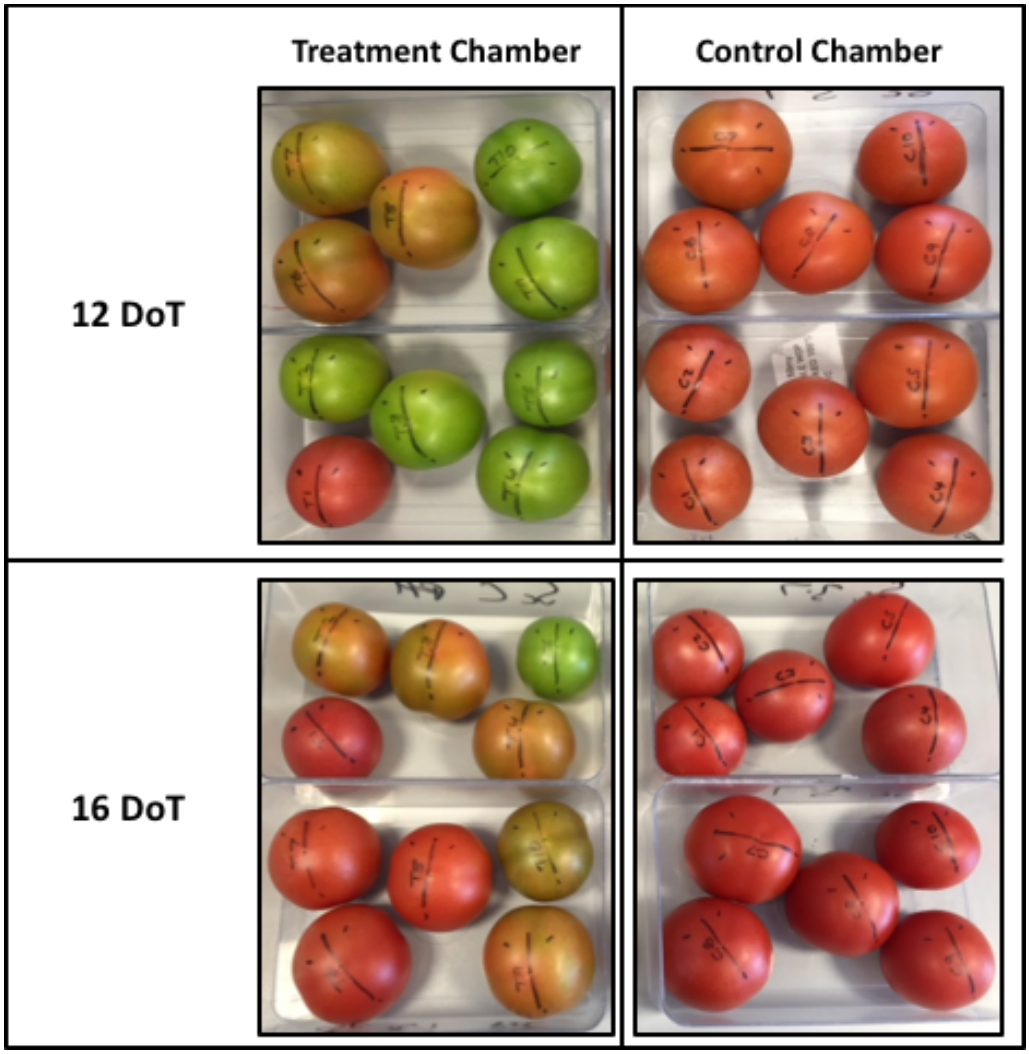


Figure 4.5. The occasional effects of ethylene scrubbing treatment on ripening uniformity of tomato fruit samples. Image depicts originally mature green Shirley variety tomato fruits left to ripen during storage in either the treatment chamber or control chamber. Images taken of fruits are 12 and 16 DoT (days of treatment/storage).

As can be seen in Figure 4.5, tomatoes stored in the ethylene scrubbing treatment chamber had little colour uniformity across the sample group. Tomato colour ranged from a mature green stage to a red ripe stage. By comparison, within the control chamber, fruit were uniform in colour between light red to red. The reason behind this experimental anomaly is unknown, although it has previously been observed that low ethylene concentrations (<0.1 ppm) in fruit storage have resulted in poor and uneven ripening (Geeson *et al.*, 1986). However, this experimental outcome was rare in this research.

Interestingly these results (Figure 4.5) were observed when no ethylene injections were taking place. The results represent a delay in ripening due to ethylene scrubbing treatment. However, different results were observed in repeat experiments as seen in Figure 4.1. What is observed in Figure 4.5 supports the results described by Maneerat *et al.* (2003), where the effects of photocatalytic oxidation seen here are delaying/inhibiting the onset of fruit ripening. It is a shame that these results were not repeatedly consistent.

4.5 EFFECTS OF UV PHOTOCATALYTIC ETHYLENE SCRUBBING ON FRUIT FIRMNESS (TEXTURE)

Ethylene is known to increase the rate of fruit softening, therefore reducing the shelf life of fruit and fresh produce. Ethylene removal has been credited to delay/inhibit fruit softening (Blankenship & Dole, 2003; Maneerat *et al.*, 2003; Guillen *et al.*, 2006), which could therefore lead to an increase in shelf life. Here, the rate of fruit softening in tomatoes subjected to UV photocatalytic ethylene scrubbing treatment was compared to fruit samples in the control chamber. Fruit firmness is related to the stage of fruit ripeness (Hobson *et al.*,

1983); therefore, fruit firmness was measured to examine the ripening progression of fruit between chambers.

4.5.1 Treatment effects

Throughout all physiological experiments, ethylene scrubbing demonstrated little to no significant effects on the rate of fruit softening. Across numerous tomato varieties as well as in fruits beginning storage at both mature green and red ripe physiological stages, UV photocatalytic ethylene removal failed to consistently cause significant changes to the rate of fruit softening (Figure 4.6 and Figure 4.7).

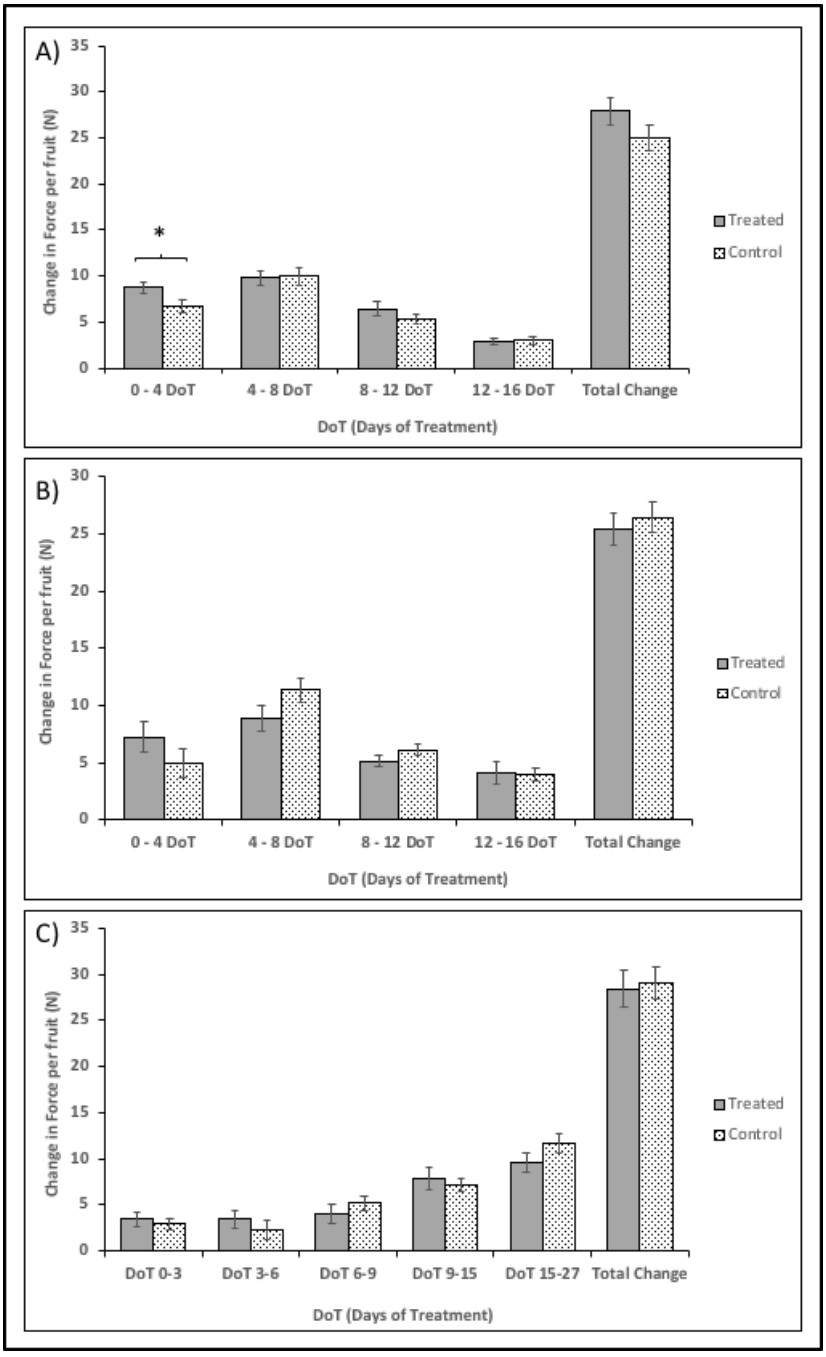


Figure 4.6. Mean change in force (N) required to compress mature green tomato varieties the specified amount (section 4.2.2). Total change represents the total change force required from the first day of storage to the last recorded day. Error bars represent SEM. An asterisk (*) represents a significant difference ($p < 0.05$) between results in the given time period. A) Shirley variety tomato results compiled from two replicate experiments. B) Moneymaker tomato results compiled from two replicate experiments. C) Meccano variety tomato results.

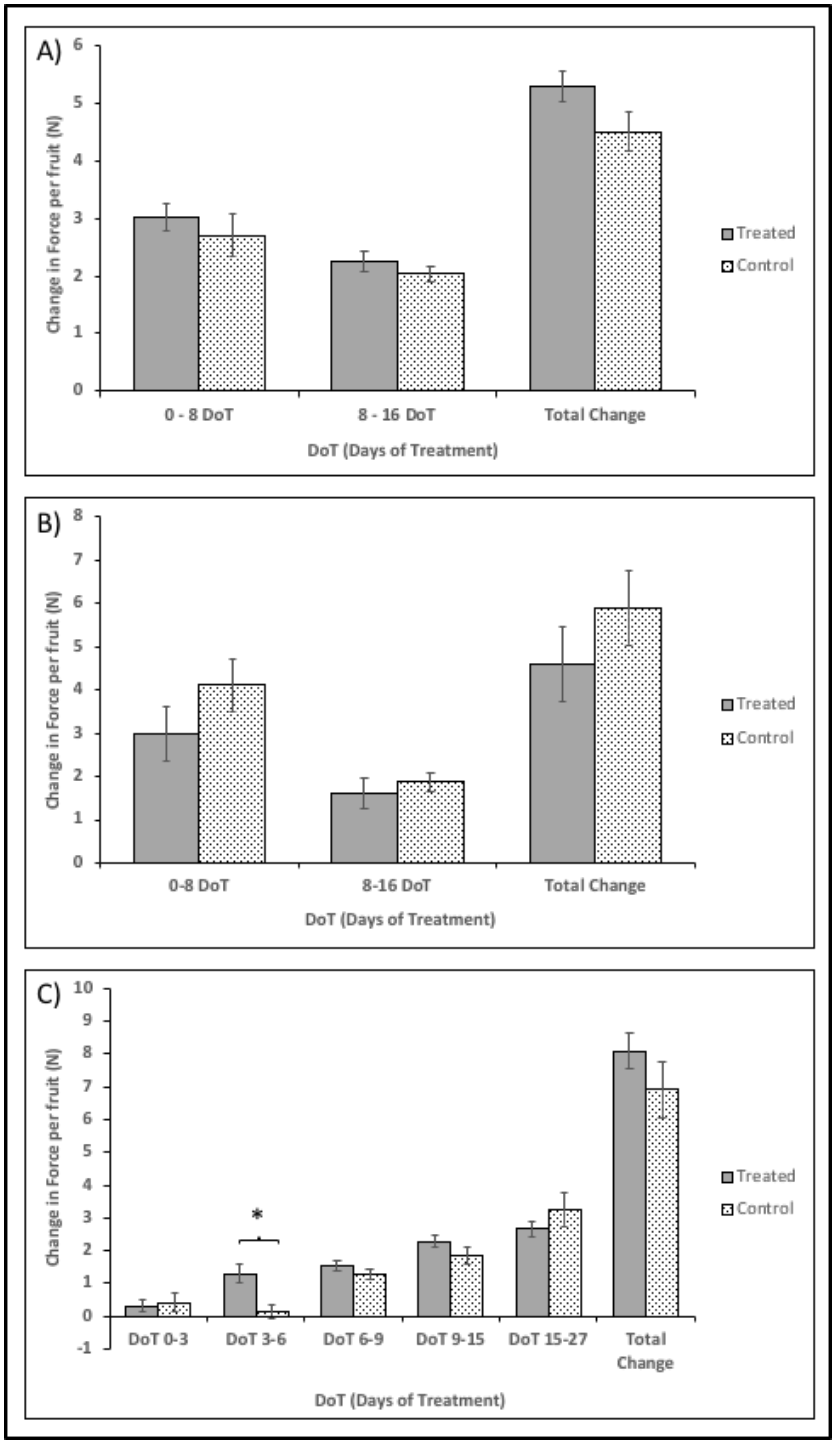


Figure 4.7. Mean change in force (N) required to compress red ripe tomato varieties the specified amount (section 4.2.2). Total change represents the total change force required from the first day of storage to the last recorded day. Error bars represent SEM. An asterisk (*) represents a significant difference ($p < 0.05$) between results in the given time period. A) Shirley variety tomato results compiled from two replicate experiments. B) Moneymaker tomato results. C) Meccano variety tomato results.

Results presented in Figure 4.6 and Figure 4.7 show that the UV photocatalytic ethylene scrubber is causing no consistent changes in the rate of fruit softening in both mature green and red ripe tomato fruits. This suggests that the ethylene scrubber is having no effect on reducing the rate of ripening or extending the storage and shelf life of fruits.

The results presented here contradict what has previously been observed in fruit softening due to TiO_2 photocatalytic ethylene decomposition. In work performed by Maneera*t et al.* (2003), it was shown that the photocatalytic removal of ethylene was able to delay fruit softening as well as colour development and the ethylene climacteric peak. Furthermore, other methods of ethylene removal, such as the use of potassium permanganate, as well as methods of ethylene perception blocking such as the use of 1-MCP and AVG, have also been reported to delay/inhibit fruit softening and prolong postharvest life (Blankenship & Dole, 2003; Keller *et al.*, 2013).

As can be seen in Figure 4.6 and Figure 4.7, occasionally, fruit stored within the treatment chamber would soften at a faster rate than fruits stored within the control chamber. This could be as result of the humidity reductions caused by the ethylene scrubbing treatment. Low RH brought about by the action of the ethylene scrubber could stimulate water loss within the tomato (Keller *et al.*, 2013), which consequently would reduce the firmness of the fruit.

As described in section 4.4.1, it was hypothesised that higher concentrations of ethylene around fruits within the control chamber would stimulate an increased rate of ripening compared with fruits stored in the ethylene scrubbing chamber. It was observed that the greater ethylene concentrations within the control chamber was able to induce colour evolution and ripening in

fruits at an earlier stage during storage compared to treatment chamber fruits. Fruit softening was also examined during periods of ethylene injection to determine whether similar results would be identified.

4.5.1.1 Effects of ethylene injections on fruit softening

Ethylene injections had a similar effect on fruit softening differences stimulated between the treatment and control chambers as they did on colour evolution. In mature green fruits across varieties, an increase in fruit softening in control chamber fruits was observed in comparison to treated fruit very early after storage. This effect however was not consistent throughout fruit storage as treated fruit quickly returned to softening at a similar rate to the control fruit (Figure 4.8).

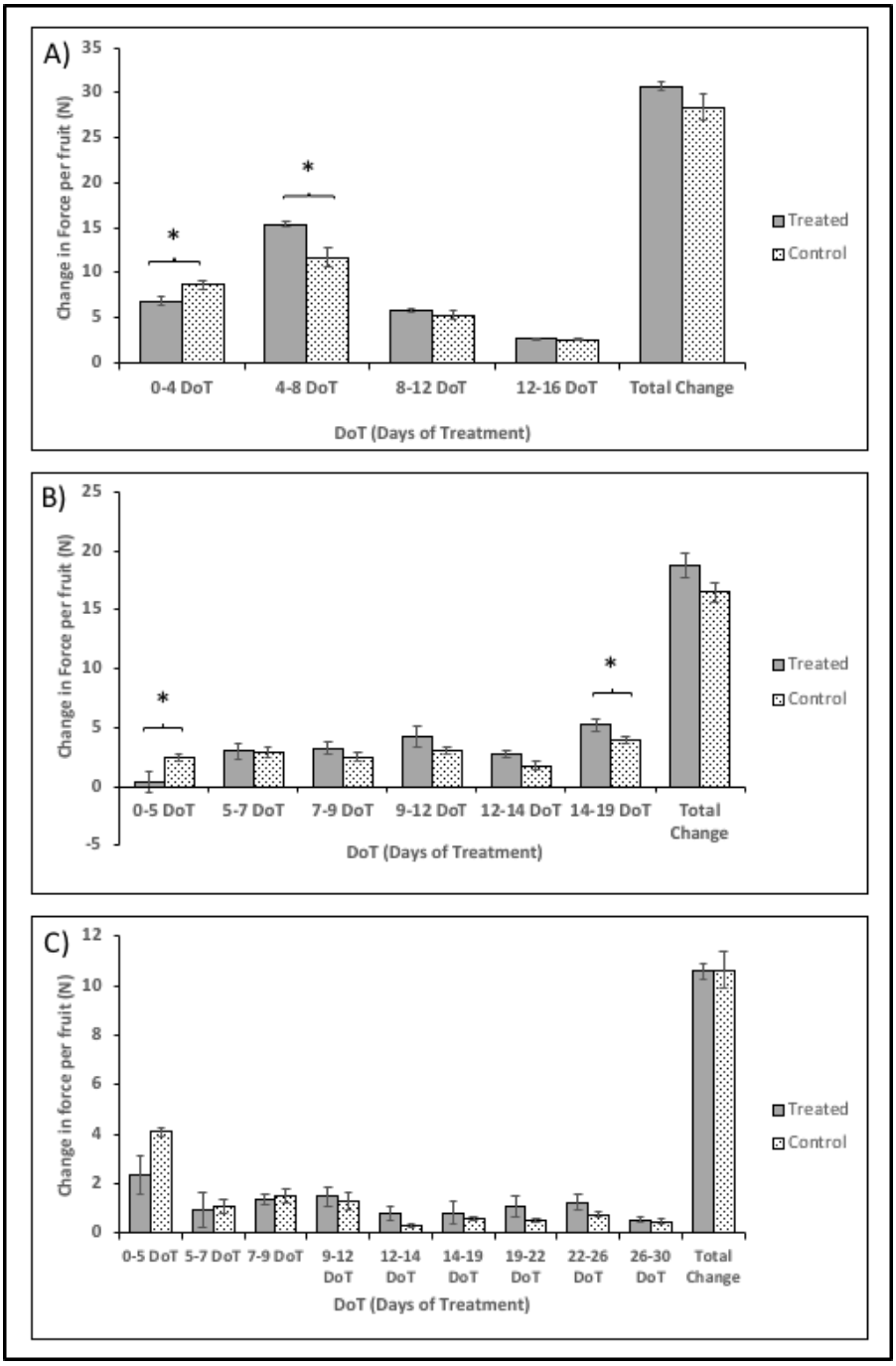


Figure 4.8. Mean change in force (N) required to compress mature green tomato varieties the specified amount (section 4.2.2) during a period of daily, Monday to Friday 10 ml ethylene injections into both storage chambers. Total change represents the total change force required from the first day of storage to the last recorded day. Error bars represent SEM. An asterisk (*) represents a significant difference ($p < 0.05$) between results in the given time period. A) Shirley variety tomato results. B) Meccano variety tomato results. C) Brioso variety tomato results.

For Shirley and Meccano variety fruit, the mean change in force required to compress the tomato 4 mm was significantly lower than the change in force required for control chamber fruits. For the Brioso variety tomatoes, although the result is not significant, it shows the same trend as seen with Shirley and Meccano. These results indicate that the control fruits are initially softening at a quicker rate and therefore the UV photocatalytic ethylene scrubbing is delaying fruit ripening.

However, following this initial period of ripening delay, results show that the treated tomato fruits soften at a quicker rate than the control fruit to end up with a very similar total change in firmness. This effect can be observed clearly in Figure 4.8(a) for Shirley tomatoes where the initial significantly greater softening of control fruit is followed by a significantly greater softening of treated fruit. These ethylene injection experiments were performed during a period of high RH maintenance within the treatment chamber, therefore the increased rate of fruit softening following the initial delay will not be related to humidity reductions within the treatment chamber. This means that the increased rate of softening after the initial delay is likely to be due to natural ripening once the fruits hit their climacteric peak within the treatment chamber.

Results presented here with fruit softening match the results observed with colour evolution where we see an initial delay in fruit ripening, quickly followed by fruit ripening at a similar rate to control fruits. The delay observed here supports previous research by Maneerat *et al.* (2003) which shows TiO_2 photocatalytic reaction delaying the softening of fruit. However, in this previous research, the ripening delay of mature green fruit observed lasted for a much longer period than what is observed here (Maneerat *et al.*, 2003). This could potentially be due to the method of treatment used here compared with the

one used by Maneerat *et al.* (2003). They stored mature green tomatoes directly next to a TiO_2 solution irradiated with UV light meaning ethylene was removed instantly at the location of the tomatoes. Furthermore, no external ethylene was applied to the tomatoes meaning it took longer to get to their climacteric peak. Our photocatalytic system meant that ethylene, both produced and injected, had to move around the system to the ethylene scrubber in order to be removed meaning ethylene at decreasing quantities is present around the tomatoes longer, as can be seen in Figure 3.4. A future experiment could involve only applying exogenous ethylene to both chambers prior to fruit storage. This way, treated fruits wouldn't be subjected to daily ethylene. This could potentially increase the time taken for the climacteric peak of treated fruits to be reached, further delaying fruit ripening.

These results do however suggest that the UV photocatalytic ethylene scrubber used in this research has potential for delaying the rate of ripening and extending the shelf/storage life of fresh produce. The results from the ethylene injection experiments demonstrate this especially, as ripening delays are induced even though treated fruits are still subject to daily ethylene treatment for the short period as the ethylene is being oxidised. Comparing the results presented here with those from Maneerat *et al.* (2003) suggests that the treatments may work more effectively in delaying fruit ripening if the ethylene scrubber was closer to the fruit samples. This potentially means that should the UV photocatalytic ethylene scrubber be used in fresh produce storage industrially, a method of implementing the equipment close to the fresh produce, without the heat from the UV lamps causing determent should be considered.

4.6 EFFECTS OF UV PHOTOCATALYTIC ETHYLENE SCRUBBING ON FRUIT WEIGHT

The effects of the ethylene scrubber on fruit weight loss was examined. Fruits naturally lose weight during the ripening process (Moneruzzaman *et al.*, 2008). Here, the change in weight of fruit between chambers was analysed to determine whether the action of ethylene scrubbing treatment had an effect on the rate of weight loss in fruit.

4.6.1 Rate of weight loss between chambers

It was identified that ethylene scrubbing treatment increased the rate of weight loss in tomato fruits. This result was consistent across all tomato varieties, as well as on tomatoes that began storage in the chambers at mature green and red ripe stages (Figure 4.9 and Figure 4.10). The consistency of treatment induced increases in weight reduction suggests a significant effect of UV photocatalytic ethylene scrubbing treatment on the development, weight and quality of fruit.

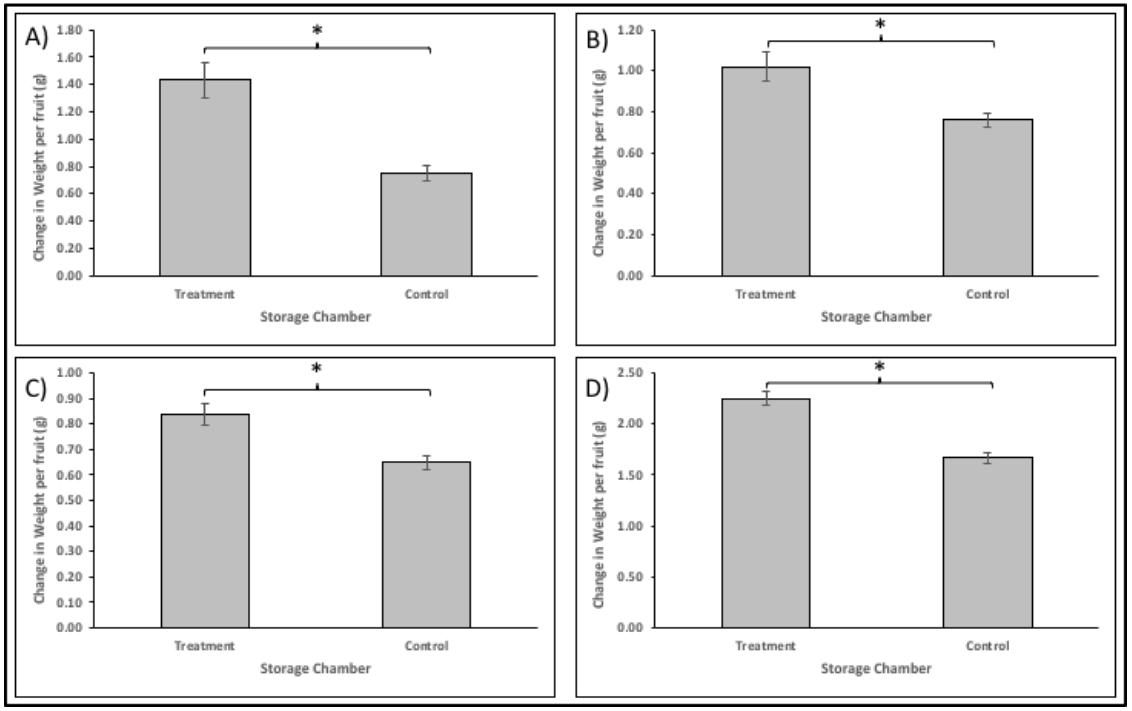


Figure 4.9. The effects of UV photocatalytic ethylene scrubbing treatment on the mean change in weight of mature green tomato fruits of different varieties. Error bars represent SEM. An asterisk (*) represents a significant difference ($p < 0.05$) between results. A) Mean total change in Shirley variety tomato weights taken from three replicate experiments after 16 days of storage. B) Mean total change in Moneymaker variety tomato weights taken from 2 replicate experiments at 16 days of storage. C) Mean change in Brioso variety tomato weights after 30 days of storage. D) Mean change in Meccano variety tomato weights taken from two replicate experiments after 26 days of storage.

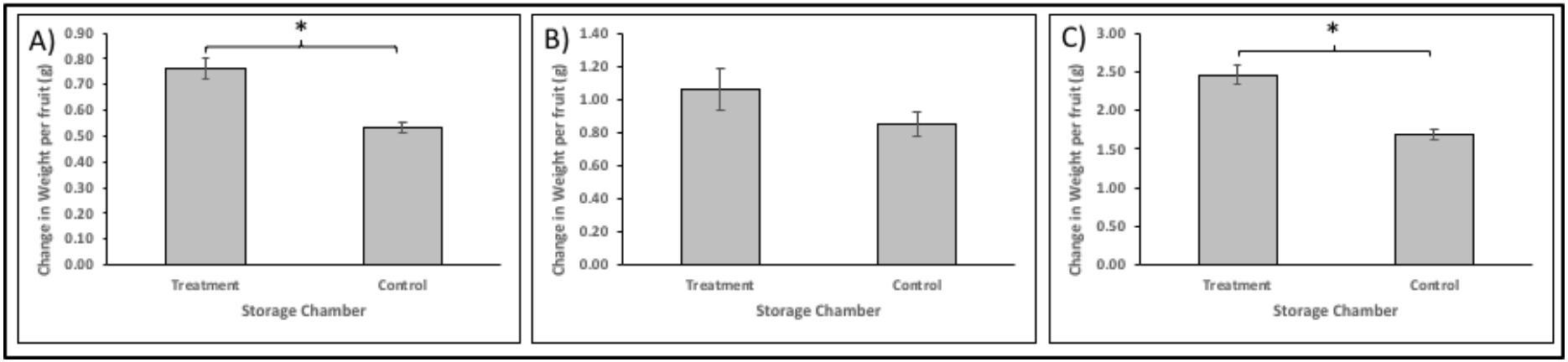


Figure 4.10. The effects of UV photocatalytic ethylene scrubbing treatment on the mean change in weight of red ripe tomato fruits of different varieties. Error bars represent SEM. An asterisk represents a significant difference ($p < 0.05$) between results. A) Mean change in Shirley tomato variety weights taken from two replicate experiments after 16 days of storage. B) Mean change in Brioso tomato variety weights after 30 days of storage. C) Mean change in Meccano variety tomato weights taken from two replicate experiments at 26 days of storage.

Results presented here clearly demonstrate the effects of the ethylene scrubber causing significant increases in the rate of weight reduction in tomato fruit. The consistency of this result across multiple tomato varieties, as well as in tomatoes stored at both mature green and red ripe stages suggests that the ethylene scrubbers' action will potentially produce similar outcomes in other fleshy fruit crops.

Due to the ethylene scrubbing induced ethylene removal most likely being able to cause reductions in the ripening and development rate in fruit produce, the increased rate of weight loss with ethylene scrubbing treatment could be deemed somewhat surprising. However, the effects of the scrubber on the storage environments relative humidity (RH) (section 3.5.2) is most likely the reason for this increasing weight loss. Low RH in the storage environment is associated with the drying out of fruits resulting in weight loss (Hardenburg *et al.*, 1986; Shin *et al.*, 2008; Keller *et al.*, 2013). Due to the action of the ethylene scrubber decreasing the RH within the chamber storage environment, it is perhaps unsurprising to see the increased rate of weight loss observed here.

To confirm that decreasing RH induced by ethylene scrubbing was the primary cause of hastened weight loss, change in weight results from experiments performed during periods of high RH (≥ 87%) in the treatment chamber where compared to experiments performed where ethylene scrubbing treatment induced decreasing RH levels (Table 4.1).

Table 4.1. The mean change in weights of mature green Shirley and Moneymaker tomatoes across 16 days of storage during experiments with varying RH levels (%) in the ethylene scrubbing treatment chamber. A) Experiments where the action of the ethylene scrubber was allowed to naturally effect chamber RH. B) Experiments where RH % in the treatment chamber remained ≥ 87%.

	Shirley Tomatoes			*Moneymaker Tomatoes*		
Relative Humidity Levels	*Treated*	*Control*	***Difference in Change of Weight (g) between chambers***	*Treated*	*Control*	***Difference in Change of Weight (g) between chambers***
A	1.32	0.57	**0.75**	1.14	0.70	**0.44**
B	0.98	0.85	**0.13**	0.91	0.81	**0.10**

The results presented in Table 4.1 demonstrate the effects of RH on the rate of fruit weight loss. The difference in the change of weight between chambers is greatly reduced when the RH is higher in the treatment chamber, indicating that it is in fact the decreasing RH induced by the action of the UV photocatalytic ethylene scrubber which is causing a significant increase in the rate of weight removal.

The small differences in change of weight seen in the high RH experiment can be attributed to RH levels still fluctuating between 99% and 87% where in the control chamber humidity would still have remained at 99%.

The results presented here demonstrate the issues associated with the ethylene scrubbers' effects on the RH of the storage environment. The

increased rate of weight reductions observed here represents a decrease in overall fruit quality. These results further demonstrate the requirement for postharvest produce storage units to identify a method of utilising the benefits of ethylene scrubbing induced ethylene removal whilst maintaining high humidity within the storage environment. Whilst the effects of humidity reductions may prove beneficial in decay development, microbial contamination and the onset of pathogen attack (Hardenburg *et al.*, 1986; Shirazi & Cameron, 1992; Estrada *et al.*, 2000), the negative effects associated with fruit weight loss and quality parameters would be a real issue in the postharvest market for the consumer. Therefore it is important that storage units are able to identify a way of applying the UV photocatalytic ethylene scrubber to a storage environment whilst maintaining RH levels at around 85-95% for optimum fruit storage (Hardenburg *et al.*, 1986). The absence of any real increase in weight reduction when RH levels in the treatment chamber remained high (Table 4.1) is a promising result for the potential application of the UV photocatalytic ethylene scrubbers in fresh produce storage environments.

4.7 OVERALL CONCLUSIONS

Results presented here demonstrate potential positive and negative effects of postharvest UV photocatalytic ethylene scrubbing on fruit physiology. They show the potential for the UV photocatalytic ethylene scrubber to cause slight delays in the onset of ripening and therefore cause increases in fruit shelf/storage life. This was confirmed by assessing the colour development and softening rate of fruits stored in either chamber. These results were only observed however during periods of ethylene injections into either chamber, and without this source of external ethylene, it was seen that the ethylene

scrubber was having no effect at reducing the rate of ripening. However, in the storage of fruit and fresh produce, ethylene levels are likely to vary, and potentially accumulate higher than what is observed in this research without the use of external ethylene injections (Figure 3.2) (Lawton, 1991; Wills *et al.*, 2000). The results presented with ethylene injections demonstrate the ability of the UV photocatalytic technology to remove high concentrations of applied ethylene efficiently enough to stimulate a delay in the onset of ripening. Had it not been for the daily subjection of treated fruits to ethylene prior to its removal, it is highly likely that the delay in the onset of ripening would have been extended. This is a scope for future research. Therefore, it can be concluded from the research presented here, in combination with previous research demonstrating ripening delays as a result of photocatalytic ethylene degradation (Maneerat *et al.*, 2003; de Chiara *et al.*, 2015) that the UV photocatalytic ethylene scrubber could be applied to fruit and fresh produce storage environments to delay ripening and extend shelf/storage life.

However, the method of ethylene scrubbing application used here appears to have some negative effects on fruit physiology in storage. The effects of the ethylene scrubbing treatment on chamber humidity are observed to be causing significant increases in fruit weight loss as well as potentially reducing fruit turgor pressure which in turn increases the rate of softening. As previously stated, it is important that in postharvest fresh produce storage that a method of UV photocatalytic ethylene scrubber application can be utilised without it causing a reducing effect on RH.

Furthermore, it was occasionally observed that the effects of ethylene scrubbing were effecting the ripening uniformity. However, due to the rarity

that this result was observed, it can be concluded that it is not too much of a concern for the postharvest market.

CHAPTER FIVE

5 THE EFFECTS OF UV PHOTOCATALYTIC ETHYLENE SCRUBBING ON POSTHARVEST DISEASE PREVENTION

THE EFFECTS OF UV PHOTOCATALYTIC ETHYLENE SCRUBBING ON POSTHARVEST DISEASE PREVENTION

5.1 INTRODUCTION

Postharvest decay of fruit and vegetable crops due to fungal pathogens is a major global problem affecting the quality and marketing value of fresh produce. In industrialised countries, 25% of total production can be lost postharvest (Nunes, 2012), whilst in developing countries the figure can reach as high as 50% (Eckert & Ogawa, 1985; Janisiewicz & Korsten, 2002). In fruits and vegetables, attack by fungal pathogens contributes to much of these losses due to the high nutrient content of the produce, and reductions in intrinsic resistance that protects the fruits whilst they are unripe and attached to the plant (Droby *et al.*, 1992; Alkan & Fortes, 2015).

Currently, fungicide application is the most common disease control method used postharvest. However, growing concern about the effects of chemical treatments on both environmental and human health has led to an increased need to search for alternative disease control strategies. Ethylene removal has been explored as a strategy to reduce disease incidence postharvest. The phytohormone which stimulates the ripening of climacteric fruits brings about positive fruit characteristics for human consumption including changes in colour, flavour and texture. However, it has been reported that ethylene action postharvest can result in many negative effects on the quality of fruits and vegetables, including increased pathogen susceptibility. Because of the negative effects associated with ethylene action postharvest, much research has been focussed on assessing various methods of ethylene control

(Blankenship & Dole, 2003; Palou & Crisosto, 2003; Martínez-Romero *et al.*, 2007; Zhang *et al.*, 2017).

Although ethylene is well known to play an important role in defence signalling against many pathogens (Thomma *et al.*, 2001; Bari & Jones, 2009; Yu *et al.*, 2009; Jia *et al.*, 2013; Broekgaarden *et al.*, 2015), there are numerous examples of ethylene being detrimental in prevention of the onset of various diseases. Ethylene has been observed to aid infections by *Diplodia natalensis* (Porat *et al.*, 1999) and *C. gloeosporioides* (Cooper *et al.*, 1998), whilst ethylene removal and perception inhibition treatments including use of the ethylene perception inhibitor 1-MCP have been observed to reduce postharvest decay severity on a variety of crops, from pathogens such as *B. cinerea*, *Penicillium* spp., and *C. gloeosporioides* (Saftner *et al.*, 2003; Seglie *et al.*, 2009; Scariot *et al.*, 2014; Li *et al.*, 2017; Xu *et al.*, 2017).

The aim of these studies was to investigate whether the effects of a novel method of ethylene removal, UV photocatalytic ethylene scrubbing, was able to prevent/reduce disease incidence of *B. cinerea*, *P. expansum* and *C. gloeosporioides* on tomato fruits within a fruit storage environment. The effects of the treatment on the pathogens *in vitro* as well as *in planta* were investigated. Additionally, work was undertaken looking at ethylene scrubbing treatment in combination with UV-C light treatments.

5.2 UV PHOTOCATALYTIC SCRUBBING ON *IN VITRO* PATHOGEN GROWTH

As well as fruits, plant pathogenic fungi are able to produce ethylene. Whilst plants produce ethylene via the 1-aminocyclopropane-1-carboxylic acid (ACC)

pathway (Yang & Hoffman, 1984), in ethylenogenic microorganisms, ethylene can be biosynthesised by two known pathways (Fukuda *et al.*, 1993). One pathway uses 2-oxoglutarate as a precursor catalysed by ethylene forming enzyme (EFE) (Fukuda *et al.*, 1992). Most ethylene producing fungi and bacteria however, utilise an α-keto-methylthiobutyric acid (KMBA) pathway, where ethylene is derived via methionine with KMBA as the immediate precursor (Fukuda *et al.*, 1993; Chagué *et al.*, 2002; Cristescu *et al.*, 2002; Daundasekera *et al.*, 2003; Qadir *et al.*, 2011). Increasing numbers of plant pathogenic fungi are known to utilise methionine as a precursor of ethylene including *C. musae* (Daundasekera *et al.*, 2003) and *B. cinerea* (Qadir *et al.*, 1997; Chagué *et al.*, 2002; Cristescu *et al.*, 2002).

Ethylene is an important hormone in defence against pathogens as its signalling during biotic interaction with pathogens modulates defence signalling pathways, including those regulated by salicylic acid and jasmonic acid (Broekgaarden *et al.*, 2015). However, in postharvest produce, ethylene induces ripening, which is known to bring about increased susceptibility to disease and pathogens (Alkan & Fortes, 2015). Therefore, it could be hypothesised that certain postharvest pathogens produce ethylene locally during infection in order to stimulate a ripening response, and increase fruit susceptibility to their attack. However, the role of ethylene production by pathogens still remains unclear. For example, with *C. musae*, it was identified that although the fungus was able to produce ethylene *in vitro* (Daundasekera *et al.*, 2003), during infection into bananas, the source of ethylene was of host origin alone; indicating that the ability of *C. musae* to produce ethylene *in vitro* plays no role in its pathogenicity *in planta* (Daundasekera *et al.*, 2008). Similar results with *B. cinerea* were observed in work performed by Chagué *et al.*

(2006) where it was determined that the fungus does not produce significant amounts of ethylene during plant colonization.

Perhaps it could be hypothesised that the production of ethylene by pathogens *in vitro* is to stimulate autocatalytic growth and development as if the pathogens were actually developing on an ethylene producing host. Cristescu *et al.* (2002) identified that on unamended PDA absent of L-methionine supplementation, *B. cinerea* was still able to produce small amounts of ethylene. PDA supplementation with L-methionine was able to increase ethylene production by the pathogen due to its role in the KMBA pathway. However, even without L-methionine, *B. cinerea* was still able to produce ethylene. This therefore could indicate that some pathogens naturally produce ethylene in order to promote self-growth.

Here, the effects of UV photocatalytic ethylene removal on *B. cinerea* and *C. gloeosporioides* growth rates *in vitro* were analysed to determine whether the removal of external ethylene could affect growth rates. Pathogens were grown in culture with and without methionine supplementation to determine whether increasing ethylene production by the pathogens could affect fungal growth. It was hypothesised that ethylene removal from the pathogens growth environment would reduce the pathogens rate of growth.

5.2.1 *In vitro* growth experimental methods.

The same experimental procedures were performed for both pathogens (*B. cinerea* and *C. gloeosporioides*) subject to *in vitro* experimentation.

5.2.1.1 In vitro fungal growth

On the day the experiments began, 4 mm plugs of specified fungal material (section 2.8) were sub-cultured from plates of an equal age and placed into the centre of 9-cm-diameter Petri dishes containing 22.5 ml of solidified PDA. Cultures were either grown on unamended PDA or PDA supplemented with L-methionine. Methionine amended PDA was supplemented with a 5 mM concentration of L-methionine as results from work performed by Cristescu *et al.* (2002) showed that this concentration of L-methionine caused *B. cinerea* to produce the greatest quantities of ethylene. Cristescu *et al.* (2002) also adjusted the pH of methionine amended PDA to 3.8; however, for the experiments presented here, pH was adjusted to 4.5 prior to autoclaving due to issues with media solidity at pH values lower than 4.5.

5.2.1.2 Experiments without ethylene injections

In vitro cultures were stored within either the ethylene scrubbing treatment chamber or control chamber. Each chamber contained 60 culture plates: 40 replicate plates of methionine amended PDA and 20 replicate plates of unamended PDA.

The growth room was maintained at 16^{o}C and lights were left on throughout the duration of the experiment. Lights were left on as light is required to photo-oxidise KMBA secreted into the media by the pathogen in order for ethylene to be released (Chagué *et al.*, 2002).

5.2.1.3 Experiments with ethylene injections

In vitro cultures were stored within either the ethylene scrubbing treatment chamber or the control chamber. Each chamber contained 60 plates, all amended with 5 mM L-methionine; however, in these experiments, the pH of the media was left unamended.

Daily 5 ml ethylene injections into both treatment and control chambers occurred throughout the duration of experimentation. The growth room was maintained at 16°C throughout, and the lights were left off. Lights were only switched on during the scoring of the plates. In darkness, KMBA accumulates in the medium, and light stimulates photo-oxidation (Chagué *et al.*, 2002); therefore, in the ethylene injection experiments performed after those performed without ethylene injections, it was interesting to see if the lights off storage method potentially had an effect on pathogen growth.

5.2.1.4 Monitoring of culture growth

Culture growth was recorded daily from 24 h – 48 h post storage until the pathogens mycelia reached the edge of the Petri dish, or plate contamination occurred. Culture growth was recorded by measuring the diameter (mm) of the mycelia at 2 perpendicular points and taking an average of the two measurements for each individual plate.

5.2.2 *In vitro* culture growth results and discussion

For both *B. cinerea* and *C. gloeosporioides*, UV photocatalytic ethylene scrubbing treatment resulted in slight increases in the rate of mycelial growth.

It was observed that on both types of PDA used, and in experiments with or without ethylene injections, the growth of the fungal culture occurred quicker in the treatment chamber (Figure 5.1 and Figure 5.2).

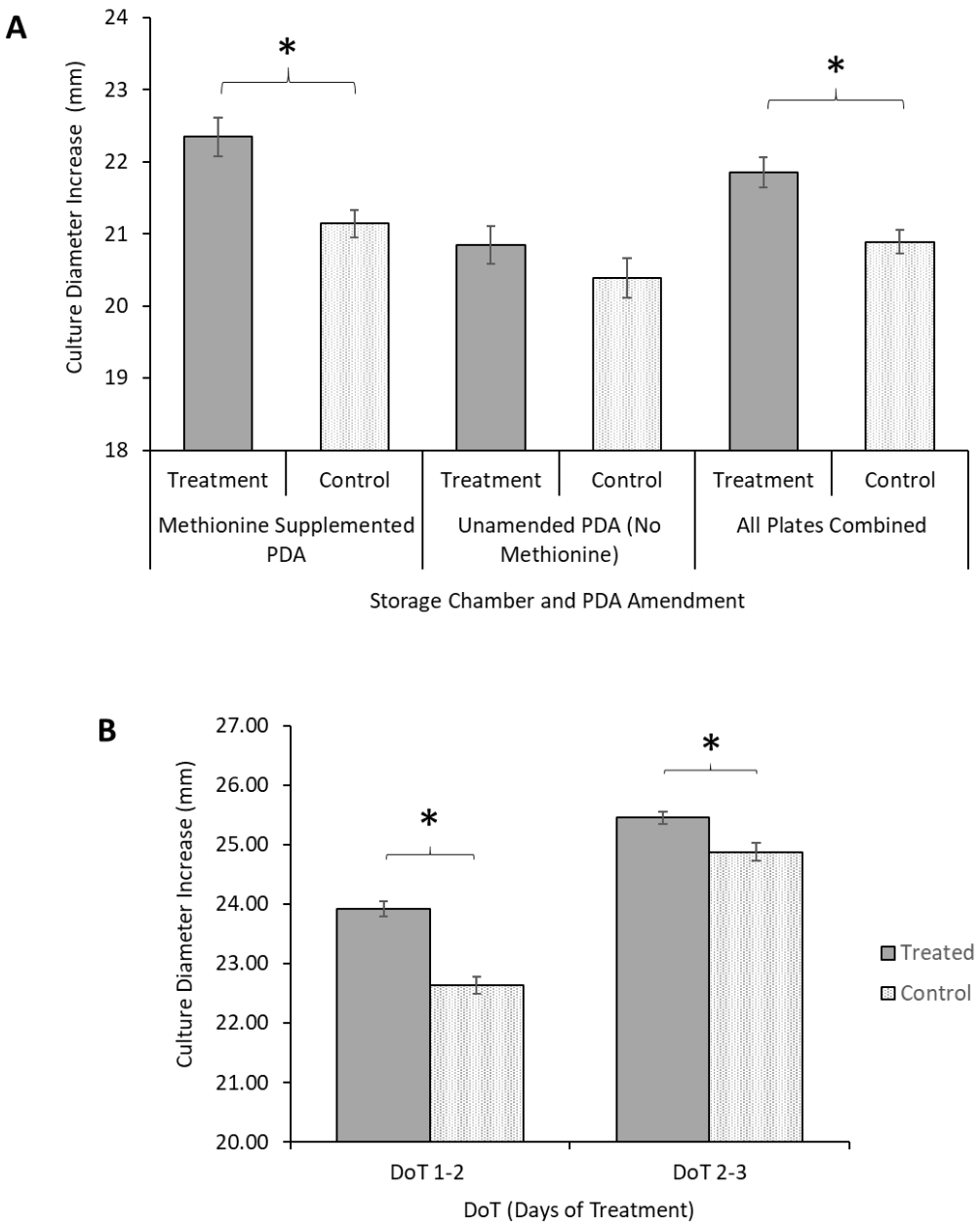


Figure 5.1. Mean *in vitro* growth increase of *Botrytis cinerea* stored in either the ethylene scrubbing treatment chamber or control chamber. Error bars represent SEM. Asterisks (*) represent a significant difference ($p < 0.05$) in results between the two chambers. A) Compiled results from two repeat experiments performed without ethylene injections. Results show the mean growth increase of the cultures between 1 and 2 days post storage. B) Results from ethylene injection experiments.

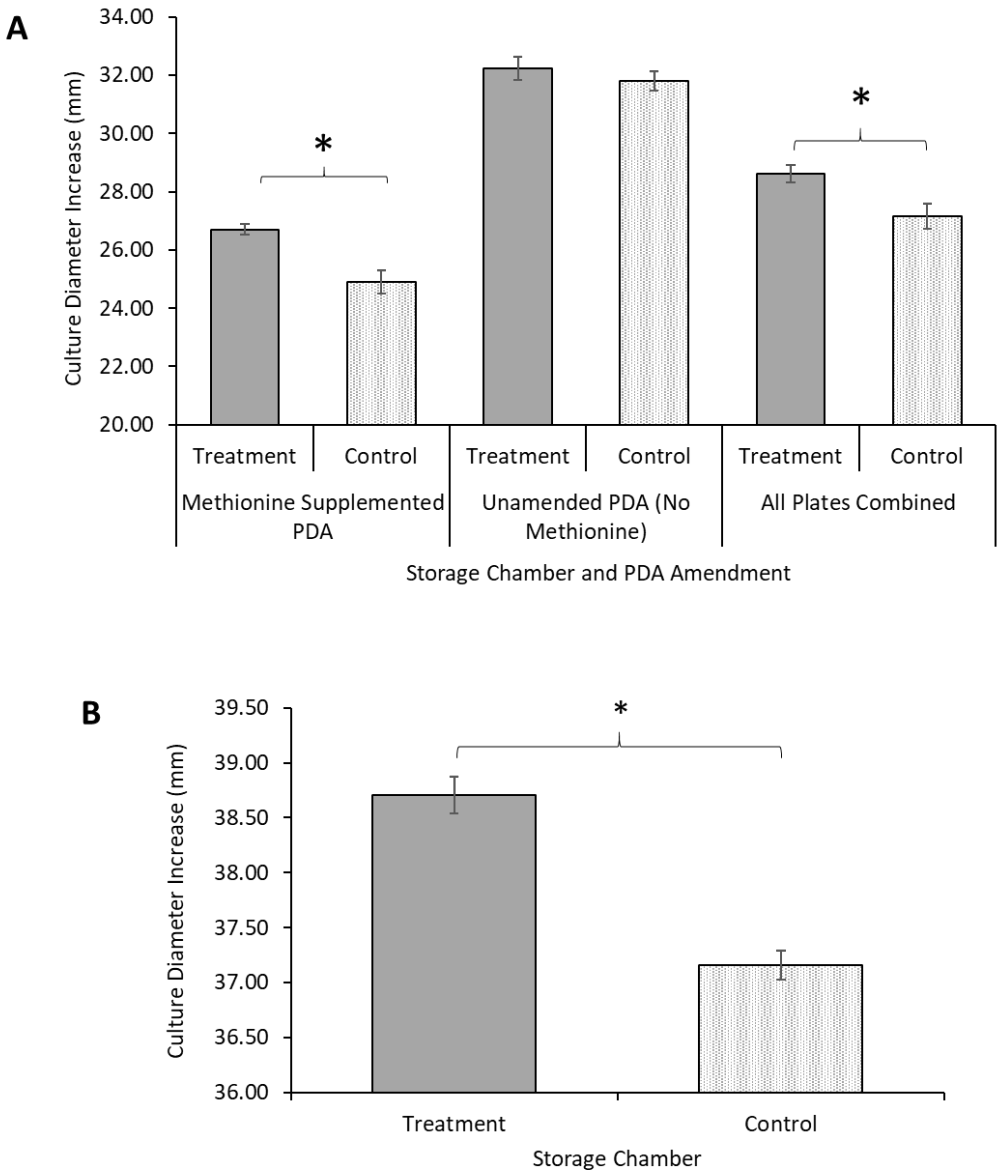


Figure 5.2. Mean *in vitro* growth increase of *Colletotrichum gloeosporioides* stored in either the ethylene scrubbing treatment chamber or control chamber. Error bars represent SEM. Asterisks (*) represent a significant difference ($p < 0.05$) in results between the two chambers. A) Compiled results from two repeat experiments performed without ethylene injections. Results show the mean growth increase of the cultures between 1 and 5 days post storage. B) Results from ethylene injection experiments. Results represent the mean growth increase between 1 – 6 days post storage.

Results clearly demonstrate an increase in mycelial growth as a result of ethylene removal. The results support what was previously seen in work performed by Chagué *et al.* (2006) who observed that exposure of *B. cinerea* to ethylene *in vitro* caused an inhibition of mycelial growth. It would appear that a similar effect has happened with *C. gloeosporioides*. Although the growth inhibition is only small, it was consistent across all experiments and results between chambers in most cases were significantly different.

The ethylene sensitivity of *B. cinerea and C. gloeosporioides* observed here demonstrates a role for ethylene in plant defence. As previously stated, ethylene is known to modulate the regulation of other defence signalling pathways in plant responses to pathogens (Broekgaarden *et al.*, 2015). However, work performed here along with previous research (Chagué *et al.*, 2006) suggests that ethylene itself may play a role in reducing the development of plant pathogens.

The ability of pathogens themselves to produce ethylene *in vitro* seems an odd trait if ethylene is able to reduce the rate of pathogen growth. However, the results presented here show some interesting effects of methionine on each pathogen. In Figure 5.1a, it can be seen that methionine supplemented PDA is triggering an increased growth rate in both chambers when compared to unamended PDA. This results implies that the action of *B. cinerea* producing ethylene can stimulate increased growth rates; however, the response to ethylene is able to slow the growth as seen by differences between chambers. With *C. gloeosporioides* however, it is observed that methionine supplemented plates reduce pathogen growth rates in comparison to unamended PDA plates (Figure 5.2a). Although it is unknown as to whether *C. gloeosporioides* is able to produce ethylene, a member of the same genus, *C. musae*, is able to

produce ethylene via the KMBA pathway (Daundasekera *et al.*, 2003) in the same way as *B. cinerea*. It is interesting to see that the potential effects of ethylene production *in vitro* by *C. gloeosporioides* have a slight inhibitory effect on *C. gloeosporioides* mycelial growth, especially when the necessity for ethylene in *C. gloeosporioides* conidial germination and appressorium formation is considered (Flaishman & Kolattukudy, 1994; Kolattukudy *et al.*, 1995). There is of course the potential that the difference in growth rates between the two types of PDA are as a result of the pH differences in the PDA. If the experiment was to be repeated, unamended PDA would also have its pH reduced to 4.5 in order to confirm that the results described here are definitely as a result of methionine supplementation.

Interestingly, 1-MCP application to *in vitro C. gloeosporioides* plates has been shown to inhibit mycelial colony growth (Xu *et al.*, 2017). However, this is more as a result of the antifungal activity of 1-MCP inducing ROS generation in the pathogen, damaging the mitochondria and destroying spore plasma membrane integrity. Therefore, this is not as a result of the ethylene perception blocking effects of 1-MCP treatment.

These experiments were performed prior to any fruit experiments with the UV photocatalytic ethylene analyser. It was therefore important to see how the results presented here would transfer to *in planta* experiments. Results would suggest that ethylene scrubbing treatment could increase disease on fruits as it has *in vitro*. These results were observed with *B. cinerea* on *Nicotiana bethamiana* leaves where plant ethylene production was prevented due to aminoethoxyvinylglycine (AVG), resulting in increased necrosis compared to ethylene producing controls (Chagué *et al.*, 2006). Work following this would

look to examine effects of UV photocatalytic ethylene removal on postharvest *in planta* disease prevention.

5.3 UV PHOTOCATALYTIC ETHYLENE SCRUBBING ON *BOTRYTIS CINEREA*

Botrytis cinerea is a plant pathogenic fungus that causes grey mould disease on many ornamental, fruit and vegetable crops. *Botrytis cinerea* infestation can occur at any point from the seedling stage all the way through to product ripening, with the costs of *Botrytis* damage difficult to measure because of the pathogens broad host range (Dean *et al.*, 2012). Fungal hyphae spread from previously colonized dead tissues into healthy ones by penetrating through wound sites and/or natural openings. Although considered a typical necrotroph, the fungus is able to adopt a quiescent lifestyle after entering a host, before rapidly rotting tissues when the environment becomes conducive, and host physiology changes (Williamson *et al.*, 2007), for example, during the process of fruit ripening. Therefore, *B. cinerea* causes serious damage of fruit and fresh produce following the harvest of seemingly healthy crops during transport and storage, which incurs large yield and economic losses.

Much research shows that ethylene plays an important role in defence against *B. cinerea* as well as many plant pathogens (Diaz *et al.*, 2002; Chagué *et al.*, 2006; Bari & Jones, 2009; Jia *et al.*, 2013; Broekgaarden *et al.*, 2015). However, *B. cinerea* has been reported to have sensitivity to ethylene with research suggesting that ethylene is able to induce the germination of conidia (Kępczyńska, 1989), as well as stimulating germ tube elongation and appressorial formation (Prusky, 1996). High ethylene levels have also been observed to increase necrosis caused by *B. cinerea*. Furthermore, work on cut

flowers has demonstrated that the use of the ethylene inhibitor, 1-MCP, resulted in reductions of *B. cinerea* damage (Seglie *et al.*, 2009; Scariot *et al.*, 2014) with similar results being observed on tomato fruit in work performed by Su & Gubler (2012).

This study investigated the role of UV photocatalytic ethylene scrubbing in reducing *B. cinerea* damage in fruit and fresh produce storage. The specific objective was to determine whether ethylene scrubbing treatment could be used to slow *B. cinerea* disease on various tomato fruit varieties as well as lettuce leaves within fruit storage chambers.

5.3.1 Results and Discussion

Ethylene scrubbing treatment appeared to have no effect on grey mould disease prevention in mature green tomato fruits inoculated at harvest/on arrival. This result was consistent across all varieties tested. In fact, results suggested that external ethylene removal in this manner may cause increasing levels of disease (Figure 5.3).

The same results were observed in fruits inoculated at a red ripe stage and can be seen in Figure 5.4. A slight increase in disease severity was observed on red ripe fruits whether they were inoculated on arrival or after a period of storage either in the ethylene scrubbing chamber or alternative storage facilities. Whatever the experimental parameters were, *B. cinerea* disease was consistently worse in red ripe fruits due to ethylene scrubbing treatment.

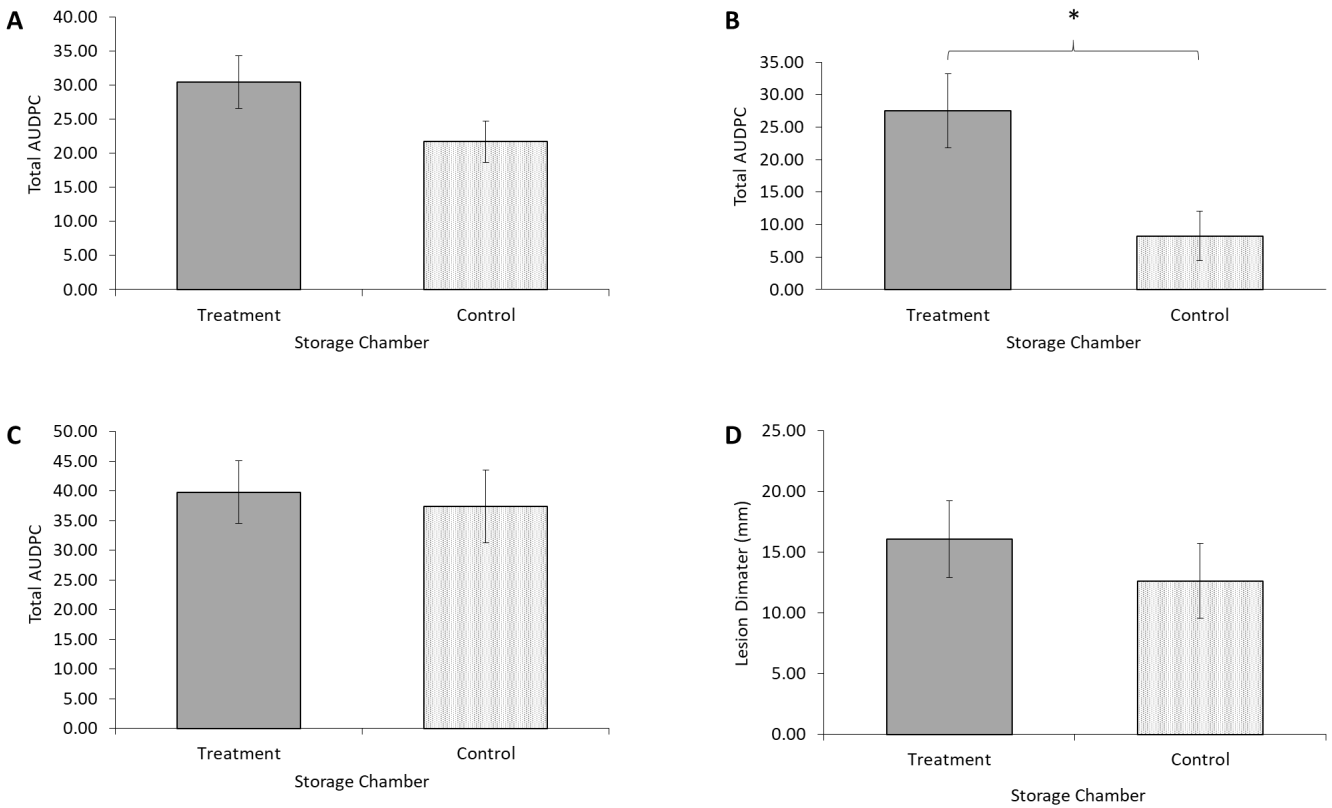


Figure 5.3. *Botrytis cinerea* disease development on mature green tomato fruit varieties inoculated at harvest/on arrival and stored in either the ethylene scrubbing treatment chamber or control chamber. Error bars represent SEM. Asterisks (*) represent a significant difference ($p < 0.05$) between chambers. A) Brioso variety. B) Shirley variety. C) Moneymaker variety. D) Ailsa Craig variety showing mean *B. cinerea* lesion size on sample fruits.

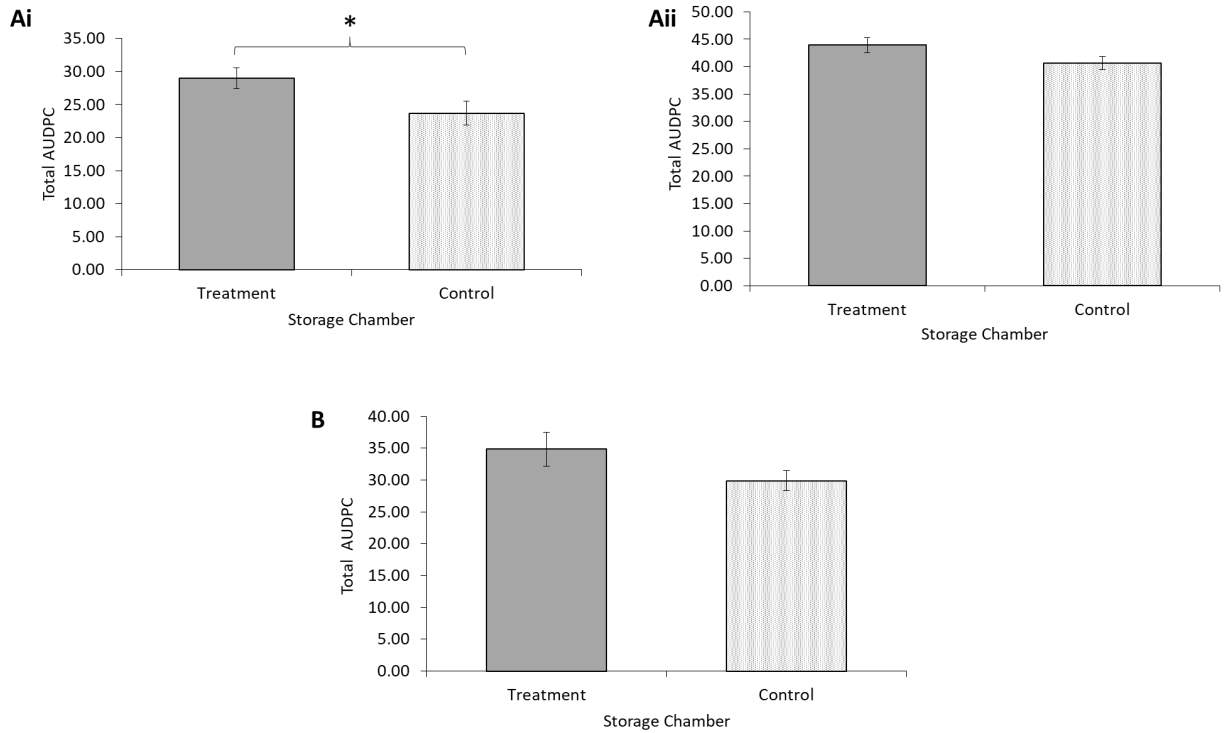


Figure 5.4. *Botrytis cinerea* disease development on red ripe tomatoes stored in either the ethylene scrubbing treatment chamber or control chamber. Error bars represent SEM. Asterisks (*) represent a significant difference ($p < 0.05$) between chambers. Ai) Brioso variety inoculated on arrival. Aii) Brioso variety inoculated after a period of 7 d storage with either specified chamber. B) Meccano variety inoculated after a period of 10 d storage within a 13^{o}C incubator before placement into specified chambers.

The results presented here match what was observed with pathogen growth *in vitro* (section 5.2) where the development of *B. cinerea* occurs at a slightly increased rate as a result of ethylene scrubbing treatment. It would therefore suggest that the presence of ethylene has an inhibitory effect on *B. cinerea* growth and that the pathogen is able to sense and respond to ethylene. Alternatively, the greater concentrations of ethylene present within the control chamber could be acting to stimulate increasing defence responses from control chamber fruit samples. As previously discussed, ethylene can modulate defence signalling pathways (Broekgaarden *et al.*, 2015) and therefore, the absence of ethylene in the treatment chamber could be reducing the modulation of defence signalling pathways such as JA and SA in comparison to the control chamber.

The results observed here are in contrast to results in work performed by Su & Gubler (2012) looking at the effects of the ethylene antagonist 1-MCP on disease resistance of tomatoes to *B. cinerea.* They identified that application of ethylene action inhibitor 1-MCP significantly reduced disease incidence and severity compared to control fruit. Furthermore, 1-MCP treatments have proved effective against *B. cinerea* in cut flowers (Seglie *et al.*, 2009, 2012; Scariot *et al.*, 2014).

Interestingly, storage of mature green tomato fruit varieties within the ethylene scrubbing treatment chamber prior to inoculations with *B. cinerea* appeared to have a disease reducing effect. Fruits left to ripen for a period of time from a mature green stage in the ethylene scrubbing chamber incurred a slightly increased level of resistance to *B. cinerea* to fruit stored within the control chamber (Figure 5.5). These results were the opposite of what was seen when fruits were inoculated at harvest.

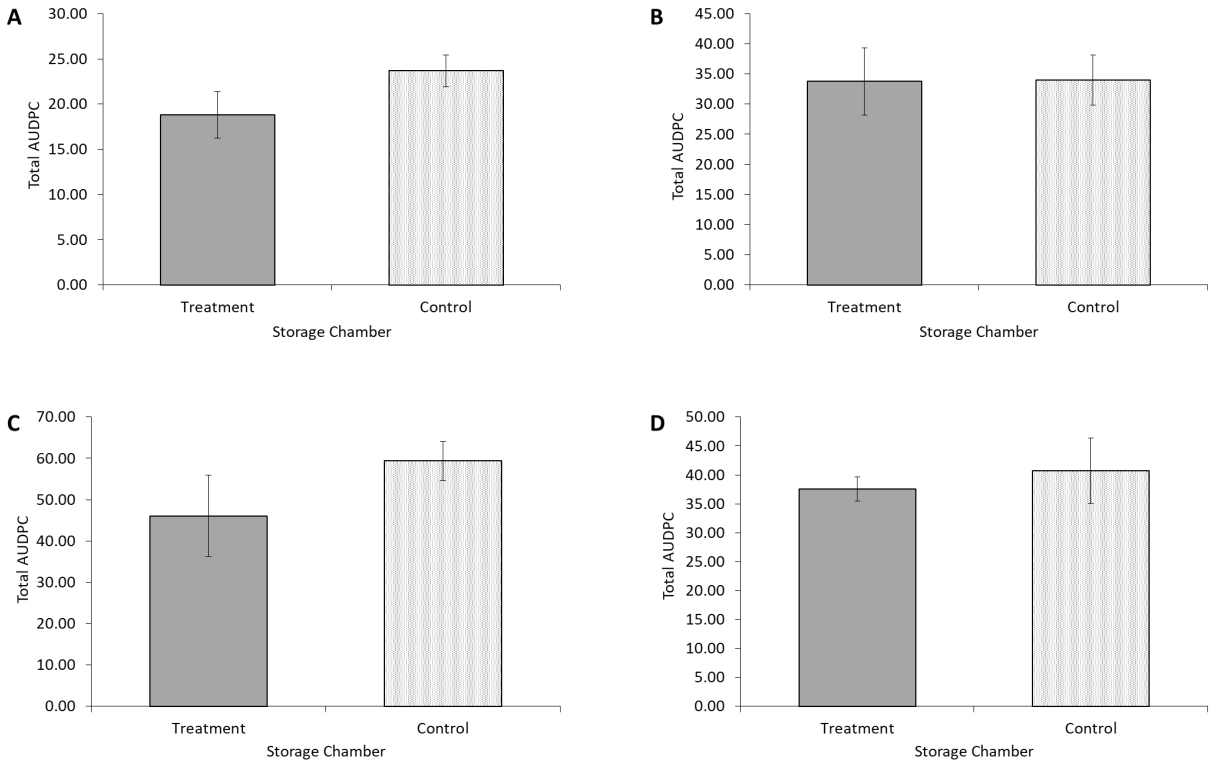


Figure 5.5. *Botrytis cinerea* development on originally mature green tomato fruits inoculated after a period of storage within specified chambers. Error bars represent SEM. A) Brioso variety fruits inoculated after 7 d storage. B) Shirley variety fruits inoculated after 14 d storage. C) Moneymaker variety after a period of 14 d storage. D) Ailsa Craig variety after a period of 14 d storage. For figures B and C, results exclude infection sites were no disease was observed.

As can be seen in Figure 5.5, for each variety, disease levels are either the same between chambers or slightly greater in the control chamber. These results contradict what was seen when the fruits were inoculated at the start of storage. Results here are likely due to ripening delays caused by ethylene scrubbing treatment. It is well known that fruits have increased susceptibility to pathogens as they ripen (Alkan & Fortes, 2015). Although in the previous chapter (chapter 4) it was observed that no obvious visible reductions in ripening rate were observed without the use of ethylene injections, it would appear that the treatments may still have an effect on reducing the rate of ripening related susceptibility to pathogens. Furthermore, the varieties here where the greatest differences in disease severity was observed, Brioso and Moneymaker, demonstrated the greatest physiological differences between chambers at the point of inoculations as shown in Table 5.1.

Table 5.1. The mean tomato colour index (TCI) of originally mature green tomatoes at the point of inoculation with *B. cinerea* spores. The table corresponds directly with the results presented in Figure 5.5.

Tomato Variety	***Corresponding Figure 5.5 Graph***	***Storage Chamber***	***Mean TCI at Point of Inoculation***	***Visual Colour According to the López Camelo & Gómez (2004) Ripening Index Values***
Brioso	A	Treatment	-14.51	Mature Green
		Control	-10.70	Breaker
Shirley	B	Treatment	31.70	Red
		Control	31.14	Red
Moneymaker	C	Treatment	7.09	Pink
		Control	21.61	Red
Ailsa Craig	D	Treatment	20.37	Red
		Control	20.88	Red

As can be seen in Table 5.1, Brioso and Moneymaker tomato colour index results at the point of inoculation show differences in the physiological stages

of fruit development. This helps explain why the greater differences in *B. cinerea* disease susceptibilities are observed in Figure 5.5 with these varieties.

Future work could look into repeating the experiments performed for Figure 5.5, but using ethylene injections into either chamber (as described in section 3.4.1.1) in order to determine whether further induced differences in ripening rate are able to increase the difference in disease observed between chambers.

5.3.2 Effects of UV photocatalytic ethylene removal on *B cinerea* resistance in lettuce plants

Botrytis cinerea is also a problem in lettuce plants and can cause necrosis on leaves (De Meyer *et al.*, 1998; Card, 2005). Furthermore, ethylene has previously been reported to effect the storage life of lettuce, with increasing ethylene levels reducing the shelf life of the crop (Kim & Wills, 1995). Here, the effects of ethylene scrubbing on *B. cinerea* disease incidence and severity on lettuce leaves was examined.

5.3.2.1 Methods

Temira variety lettuce seeds were sowed into Rockwool and watered daily for 2 wks prior to being transferred to a NCP hydroponic system for the continuation of growth. Lettuce plants were harvested after 2 wks growth in the hydroponic system and subjected to experimentation at harvest.

Lettuce plants were randomized between ethylene scrubbing treatment and control groups. From each plant, a leaf from the inner ring of lettuce leaves

and a leaf from the outer ring of leaves were extracted and placed onto Petri dishes containing 30 ml of 0.7% agar (Figure 5.6a). Inner ring leaves were physiologically shorter and wider whereas outer ring leaves were longer and thinner. On each plate there were 4 leaves from two plants. *Botrytis cinerea* spore solutions were created and adjusted to 1 x 10^6 spores per ml using a 50% PDB solution in order to increase to likelihood of infection. A 5 µl droplet of spore solution was placed just to the side of the spines of the leaf (Figure 5.6b). Per chamber, one agar plate was used for leaves mock inoculated with a 50% PDB solution.

Plates were left lidless and placed into the assigned chambers. Growth conditions within the chambers remained at 16^{o}C with the lights off. Humidity boxes were placed at the bottom of each chamber. Disease scores were recorded 4 DPI by photographing the agar plates from a height of 634 mm using a lens length of 55 mm. ImageJ was then used to analyse the necrotic area of each lesion. Results were obtained from 3 repeat experiments, each experiment examining disease on leaves extracted from 10 lettuce plants per chamber.

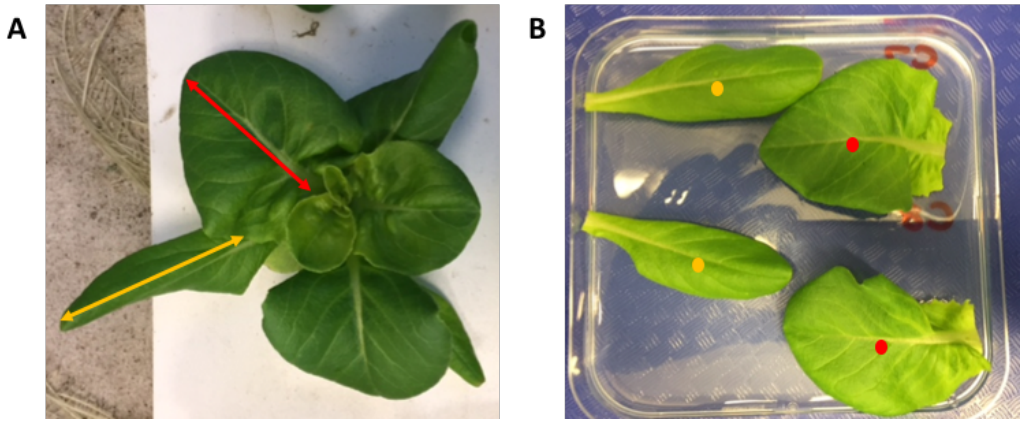


Figure 5.6. A) Representation of leaves extracted from lettuce plants inner and out rings. The red arrow highlights an example of leaf taken from the inner ring of the plant and the yellow arrow highlights an example of a leaf taken from the outer ring of the plant. B) Leaf set up on the agar plates. Yellow and red dots indicate the inoculation points used on the leaves. Colours are coordinated to A.

5.3.2.2 Results and discussion

Results from all three experimental repeats demonstrated that ethylene scrubbing treatment was able to reduce disease caused by *B. cinerea* infection. These results were observed on leaves from both the inner and outer leaf rings (Figure 5.7 and Figure 5.8).

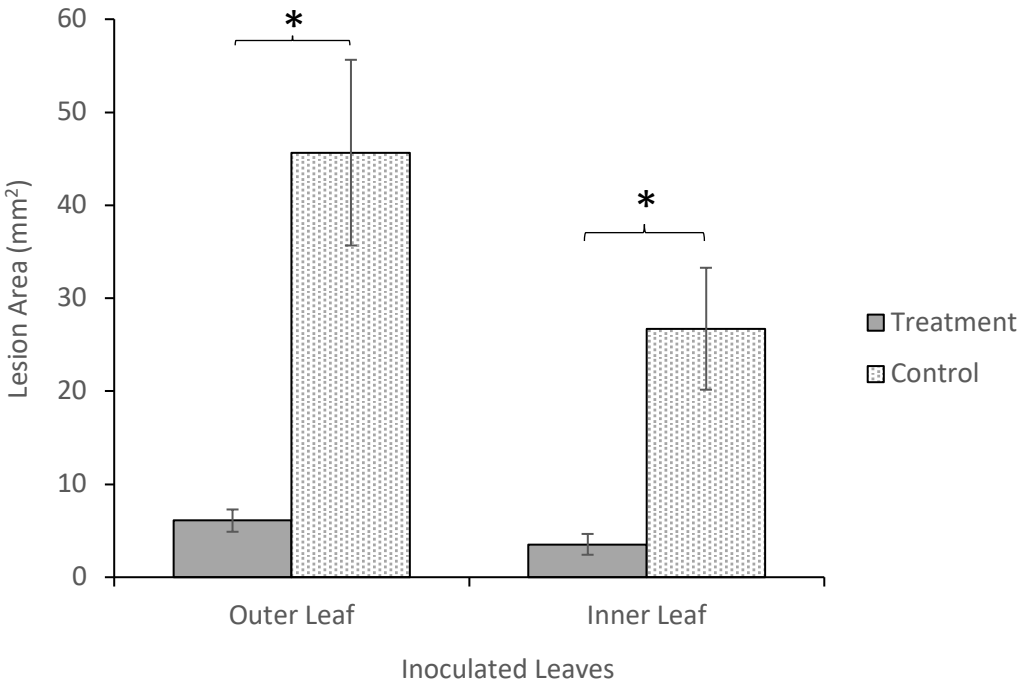


Figure 5.7. Mean lesion area recorded at 4 DPI on leaves stored in either the ethylene scrubbing chamber or control chamber. Error bars represent SEM. An asterisk (*) represents a significant difference ($p < 0.05$) between chambers.

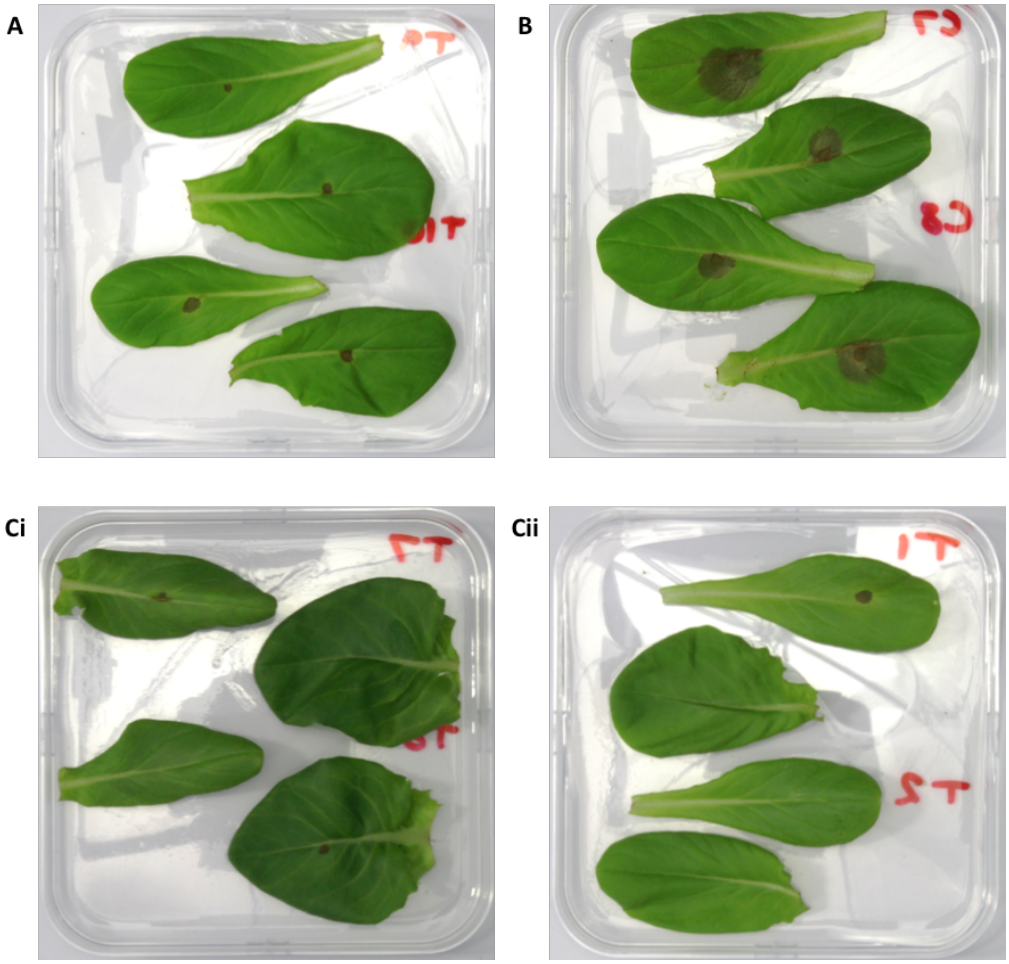


Figure 5.8. Examples of necrotic lesions observed on leaves after 4 DPI. A) Disease observed on leaves stored within the ethylene scrubbing chamber. B) Disease observed on leaves in the control chamber. Ci and Cii) Leaves stored within the treatment chamber which show a prevention of *Botrytis cinerea* disease.

The significant differences between necrotic lesions on lettuce leaves stored in the treatment chamber compared to the control chamber can be clearly observed when comparing images in Figure 5.8a and Figure 5.8b. These results suggest that ethylene scrubbing is inhibiting the disease development of *B. cinerea*.

Figure 5.7ci and Figure 5.7cii show examples demonstrating that on some leaf samples, disease development was completely prevented on the lettuce leaves resulting in no lesions. This was observed far more regularly in the treatment chamber than the control chamber, as highlighted by Table 5.2.

Table 5.2. The percentage of leaves per chamber where necrotic lesions from *Botrytis cinerea* infection were observed.

Percentage of leaves showing disease symptoms (%)

Storage Chamber	***Outer Leaf***	***Inner Leaf***
Treatment	63.33	36.67
Control	96.67	73.33

Results presented in Table 5.2 suggest that ethylene scrubbing treatment is causing disease prevention as well as disease inhibition. A larger quantity of lettuce samples in the control chamber were showing symptoms of disease compared with treatment chamber samples. This is a promising result for the postharvest market as complete disease prevention would increase the

amount of lettuce plants making it to the shelves. Even when lesions did develop on the treatment chamber leaves, they were consistently smaller than on the control chamber leaves, as highlighted by Figure 5.8a and Figure 5.8b.

As well as ethylene removal, results could have been in part due to the humidity reductions caused by ethylene scrubbing treatment. On the treatment chamber plates, some drying out of the agar was noticeable. Future work in this area would look into identifying how these reductions in humidity affect lettuce physiology during storage. The benefits of disease reductions due to treatment would be redundant if the physiological characteristics of the vegetable were altered so that no consumers would want to purchase it. Treatment times would also be analysed to determine whether a treatment duration could be met to prevent/inhibit disease without having an adverse effect on physiology. Furthermore, the experiments would be repeated with humidity levels in the treatment chamber being maintained as high as possible. This would be to determine the main cause of the disease inhibition: ethylene removal or humidity reductions.

Results presented here also suggest that the inner ring of lettuce leaves confer a higher level of disease resistance to the outer ring of lettuce leaves (Figure 5.7). The reason for this is unknown. However, it would be interesting to perform a study looking into defence related genes within the lettuce plants to determine whether there are differing levels of defence gene expression though the different layers or lettuce leaves.

Here, UV photocatalytic ethylene removal treatment has demonstrated the ability to inhibit *B. cinerea* on lettuce leaves during storage. This supports work by Elad (1990) who observed that ethylene supplied to infection sites

increased necrosis, whereas ethylene inhibitors reduced the development of grey mould. However, it contradicts work performed by Chagué *et al.* (2006) who observed that exogenous ethylene application to ethylene-nonproducing *Nicotiana bethamiana* leaves was able to restrict the spread of *B. cinerea* on leaves. It would be interesting to see if ethylene injections into each chamber could affect the continued necrotic spread of the fungus on the lettuce leaves.

It would be interesting to examine the use of UV photocatalytic ethylene removal on other non-climacteric fruit crops, such as strawberries. The results presented here with lettuce contradict what was observed with climacteric tomato fruit. A study could be conducted into whether the external ethylene removal caused by ethylene scrubbing can confer differing disease resistance levels between climacteric and non-climacteric fruits. This work could look firstly into disease reductions on the fruit and potentially view the molecular mechanisms within the different fruit types to analyse potential differences in defence gene regulation. A study of this nature could further elucidate the most beneficial applications of the ethylene scrubber. It could potentially show that it would be more beneficially applied to the storage and transport units of non-climacteric produce.

As previously stated, the results presented here with lettuce contradict what was seen with both mature green and red ripe tomato fruits inoculated prior to storage. This could potentially be due to the type of inoculation used. For the lettuce inoculations, a *B. cinerea* spore solution was applied as a droplet onto the leaf surface. For the tomato inoculations however, spore solutions were applied directly into a wound (as described in section 2.9.1). This means that the spores in the droplet inoculations on the lettuce leaves were more open to sense the lack of ethylene presence and also more vulnerable to reductions in

humidity. The wound inoculations placed spores directly into a moist environment with all the sugars required to continue development. Moreover, there would have been a high internal ethylene concentration (Saltveit, 1999), meaning the pathogen would have had less trouble contending with ethylene removal as it did when externally applied to non-climacteric lettuce.

5.4 UV PHOTOCATALYTIC ETHYLENE SCRUBBING ON *PENICILLIUM EXPANSUM*

Penicillium expansum is a fungal plant pathogen regarded as one of the most important postharvest phytopathogens (Jurick *et al.*, 2011). *Penicillium expansum* operates by producing organic acids to lower the ambient pH of the host environment in order to provide optimum conditions for its cell-wall-degrading enzymes to operate (Prusky *et al.*, 2004; Jurick *et al.*, 2011). Able to enter through wound sites and natural openings, *P. expansum* causes browning, sunken lesions and blue mould symptoms.

Previously, ethylene removal treatments have been attributed to suppression of *P. expansum* both *in vivo* and *in vitro*. The effects of 1-MCP have been observed to decrease disease severity of *P. expansum* on apple fruits, as well as suppress the mycelial growth and spore germination of the fungus *in vitro* (Li *et al.*, 2017). Furthermore, the TiO_2 photocatalytic reaction has demonstrated antifungal activity against the pathogen as it reduced conidial germination *in vitro* and was able to reduce/prevent fruit rot across a range of different fruits (Maneerat & Hayata, 2006).

Here the effects of UV photocatalytic ethylene removal on *P. expansum* disease during fruit storage was investigated. The objective of these

experiments was to determine whether ethylene scrubbing could be used as an effective treatment to reduce fruit losses to blue mould rot in fruit storage and transport environments.

5.4.1 Results and Discussion

The AUDPC of *P. expansum* infection sites on fruit inoculated at a red ripe stage were greater in the ethylene scrubbing treatment chamber than in the control chamber. This result was consistent across various tomato varieties as well as in fruit inoculated at harvest, or after a period of storage (Figure 5.9). Although in most cases, the difference was not large or significant, the results do show a trend towards ethylene playing a role in defence against *P. expansum*.

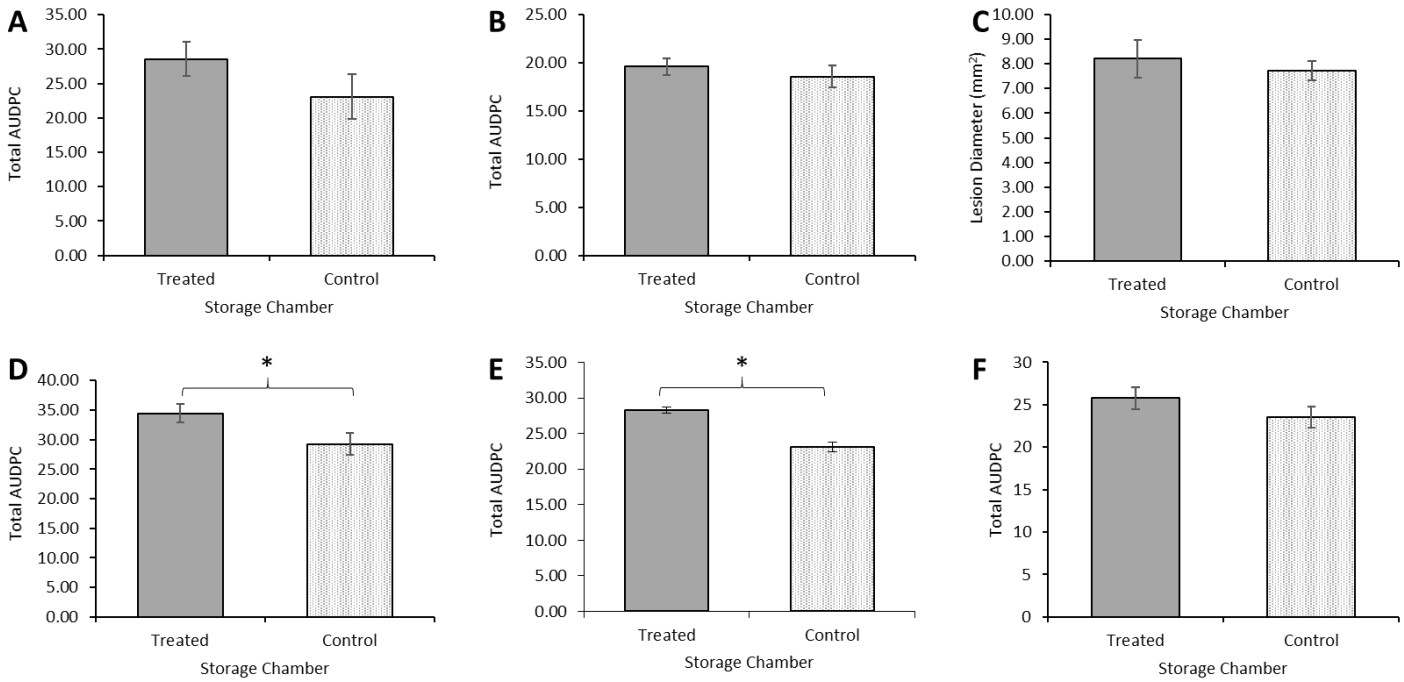


Figure 5.9. Mean *Penicillium expansum* disease present on red ripe tomatoes at the beginning of experimentation. Error bars represent SEM. Asterisks (*) represent a significant difference ($p < 0.05$) between chambers. All figures created without including results from disease sites where no disease was recorded. A) Shirley variety inoculated at harvest. B) Brioso variety inoculated at harvest. C) Lesion diameter at 3 DPI on Ailsa Craig variety inoculated at harvest. Results compiled from two repeat experiments. D) Shirley variety inoculated after 8 d storage. Results compiled from two repeat experiments. E) Brioso variety inoculated after 7 d storage. F) Brioso variety inoculated after 30 d storage.

Results presented in Figure 5.9 are similar to the results observed with *B. cinerea* infections on red ripe tomato fruits in Figure 5.4. This suggests that the ethylene is inhibiting the development of *P. expansum* on fruit interactions. This is likely again as a result of ethylene gas modulating defence signalling pathways in control chamber fruit resulting in increased disease resistance.

Interestingly, the results in Figure 5.9 contradict what has previously been observed with ethylene removal methods on *P. expansum* control in fruit crops (Maneerat & Hayata, 2006; Li *et al.*, 2017) where both 1-MCP and TiO_2 photocatalytic reaction methods were able to reduce disease severity. However, these studies both discuss the effects of their respective treatments on the pathogen itself. Both studies discuss reactive oxygen species (ROS) in their work and how it could be attributed to causing blue mould disease reductions. Whilst Maneerat & Hayata (2006) discussed the generation of ROS on the irradiated TiO_2 surface as the potential cause of *P. expansum* inhibition, Li *et al.* (2017) observed increased ROS within 1-MCP treated *P. expansum* spores leading to enhanced oxidative damage and reduced integrity of spore plasma membranes, resulting in significant decreases in disease severity. Future work could therefore look to observe ROS levels within both *P. expansum* spores and tomato fruits subject to storage with either treatment or control chambers. If no differences in ROS were observed between the two chambers, it would help explain why the method of ethylene removal tested here is producing different results to previous studies. A further explanation to the differences between these results could be due to the close proximity of the 1-MCP and TiO_2 applications performed by Li *et al.* (2017) and Maneerat & Hayata (2006) respectively. In the discussed publications, treatment solutions were applied directly to the spores, or within close proximity to the fruits. The method utilised in the present study however

examines external ethylene removal treatment situated far away from the samples. This could potentially reduce the effects which could be seen by the ethylene scrubber. The lack of any contact between the *P. expansum* and the FRESH+ proprietary formulation photocatalyst in the ethylene scrubber will mean that any antifungal properties the catalyst may have are not coming into contact with the pathogen as was seen with TiO_2 in the work by Maneerat & Hayata (2006).

The effects of storing mature green fruit for a period of time prior to *P. expansum* inoculations was also examined. Results of these experiments can be observed in Figure 5.10.

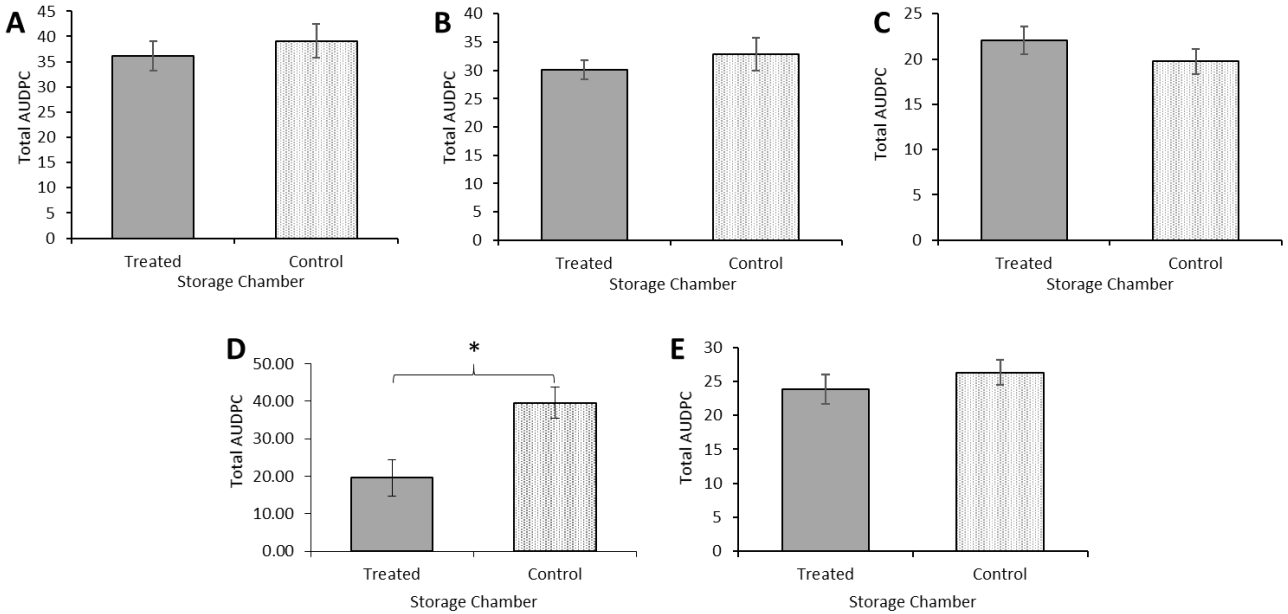


Figure 5.10. Mean *Penicillium expansum* disease present tomato fruit varieties which began storage in either chamber at a mature green stage prior to inoculation at the specified day and placed back into the chambers. Error bars represent SEM. Asterisks (*) represent a significant difference ($p < 0.05$) between chambers. A) Shirley variety tomatoes inoculated after 16 d storage. Results compiled from two repeat experiments. B) Moneymaker variety tomatoes inoculated after 16 d storage. Results compiled from two repeat experiments. C) Meccano variety tomatoes inoculated after 30 d storage and subject to daily ethylene injections into each chamber throughout. D) Shirley variety tomatoes inoculated at 16 d of storage at 13ºC. E) Brioso variety tomatoes inoculated after 30 d storage and subject to daily ethylene injections into each chamber throughout.

The results observed through these experiments were relatively inconsistent. This is demonstrated in Figure 5.10 where some disease inhibition can be seen on varieties as a result of ethylene scrubbing treatment, but no disease inhibition due to treatments in other varieties. Even in the experiments where daily ethylene injections were performed throughout (c and e for Meccano and Brioso tomatoes respectively), no real differences in disease suppression between chambers are observed regardless of the known effects these ethylene injections had on the rate of fruit ripening and changes in fruit physiology (chapter 4). However, in those experiments at the point of inoculation, tomatoes from both chambers were already at a red ripe stage. The only time significance was observed from tomatoes beginning storage at a mature green stage was on Shirley variety tomatoes stored at 13ºC inoculated after 16 d storage (Figure 5.10d). Physiological analysis showed that at the point of inoculation, treatment chamber samples averaged a TCI of 10.8 and control chamber samples averaged a TCI of 32.7 equating to visual colours of pink and red respectively according to the López Camelo & Gómez (2004) ripening index values. This information in combination with the other graphs presented in Figure 5.9 suggest that ethylene scrubbing can only prevent *P. expansum* disease by potentially reducing the rate of ripening within the treatment chamber, which would therefore reduce the rate of the fruits turning from highly resistant to susceptible to pathogen attack (Alkan & Fortes, 2015). *Penicillium expansum* could not cause disease on mature green fruits as identified in preliminary work and can induce increasing disease severity on fruits the riper they are.

The ethylene scrubbing treatments examined here are therefore more likely to be effective in *P. expansum* disease prevention over shorter storage periods where there may be increased levels of ethylene within the storage of fresh

produce to be removed compared to what was seen throughout this research without the use of ethylene injections. This is a scope for future work.

5.4.1.1 Effects of treatment on Penicillium expansum *disease symptoms.*

Throughout the research conducted with *P. expansum*, differences in disease symptoms were observed in numerous experiments between the treatment and control chamber. Whilst increased disease severity, by way of larger sunken lesion diameters, was observed in the treatment chamber, the white mycelial leakage from the wound sites was remaining white for longer than in the control chamber. Within the control chamber, infection sites were producing more characteristic blue-green mould symptoms early from infection, whereas within the treatment chamber, mycelial growth from the wound sites was observed to be taking longer to develop the characteristic blue mould symptoms. Examples of the symptoms observed from each chamber can be seen in Figure 5.11.



Figure 5.11. Examples of the differences in disease symptoms sometimes observed on infected tomato fruits from within the ethylene scrubbing treatment chamber and control chamber.

As can be seen in Figure 5.11, in the control chamber fruit samples, classic blue-green mould symptoms appear to be developing at the infection site. However, within the treatment chamber, pathogen growing out from the infection site appears to be remaining white.

This effect was seen on not all, but a high number of experiments. Therefore, the day in which blue-green colouration was observed at the infection sites was recorded for certain experiments. An example from one experiment can be seen in Table 5.3.

Table 5.3. Percentage of *Penicillium expansum* infection sites showing blue-green colouration in disease symptoms on red ripe Shirley tomatoes.

5 DPI Percentage of infection sites showing blue-green colouration	
Treated tomatoes	5
Control tomatoes	70
6 DPI Percentage of infection sites showing blue-green colouration	
Treated tomatoes	30
Control tomatoes	100

Table 5.3 clearly demonstrates the delay in symptom colouration observed in some of the experiments performed. The change in colour from white mycelia to blue-green occurs as the pathogens asexual spores mature.

Due to the results observed on fruits indicating reductions in the rate of asexual spore maturation as a result of ethylene scrubbing treatment, *in vitro* experiments were performed to see if these results occurred on culture media.

5.4.1.2 Methodology of in vitro Penicillium expansum *asexual spore maturation experiment*

Throughout experimentation, the growth room housing the experiment chambers remained at 22°C with the lights off. Temperature was maintained at 22°C to be closer to room temperature and closer to the temperature used by Li *et al.* (2017) who performed a similar experiment. Throughout this experiment, there were no humidity boxes in the treatment or control chamber. Furthermore, samples in both chambers were subjected to external ethylene as daily 10 ml ethylene injections were being delivered into both chambers.

There were 29 sample plates to be used per chamber. Five cm Petri dishes were used and filled with 10 ml of PDA amended with streptomycin and penicillin. *Penicillium expansum* spores were extracted from a 5 – 10 d old culture (as described in section 2.8.4) and corrected to 1 x 10^5 spores per ml in SDW using a haemocytometer. A 5 µl droplet of spore solution was placed into the centre of each PDA plate and placed lidless into either the ethylene scrubbing treatment chamber or control chamber.

Images were taken of spore plates from 3 DPI onwards to assess the development of pathogen cultures within either chamber.

5.4.1.3 In vitro *results and discussion*

On *in vitro* cultures of *P. expansum*, clear differences were observed in the development of the pathogen between chambers. Within the control chamber, cultures of *P. expansum* were displaying the characteristic blue-green colour associated with the disease at 3 DPI. Within the treatment chamber however,

cultures were remaining white indicating that the asexual spores of the pathogen were being inhibited from maturing. Only at 7 DPI did plates within the treatment chamber show the onset of asexual spore maturation around the outer edge of the developing mycelia. These results can be observed closely on individual plates in Figure 5.12, with the consistency of the results across almost all the sample plates demonstrated in Figure 5.13.

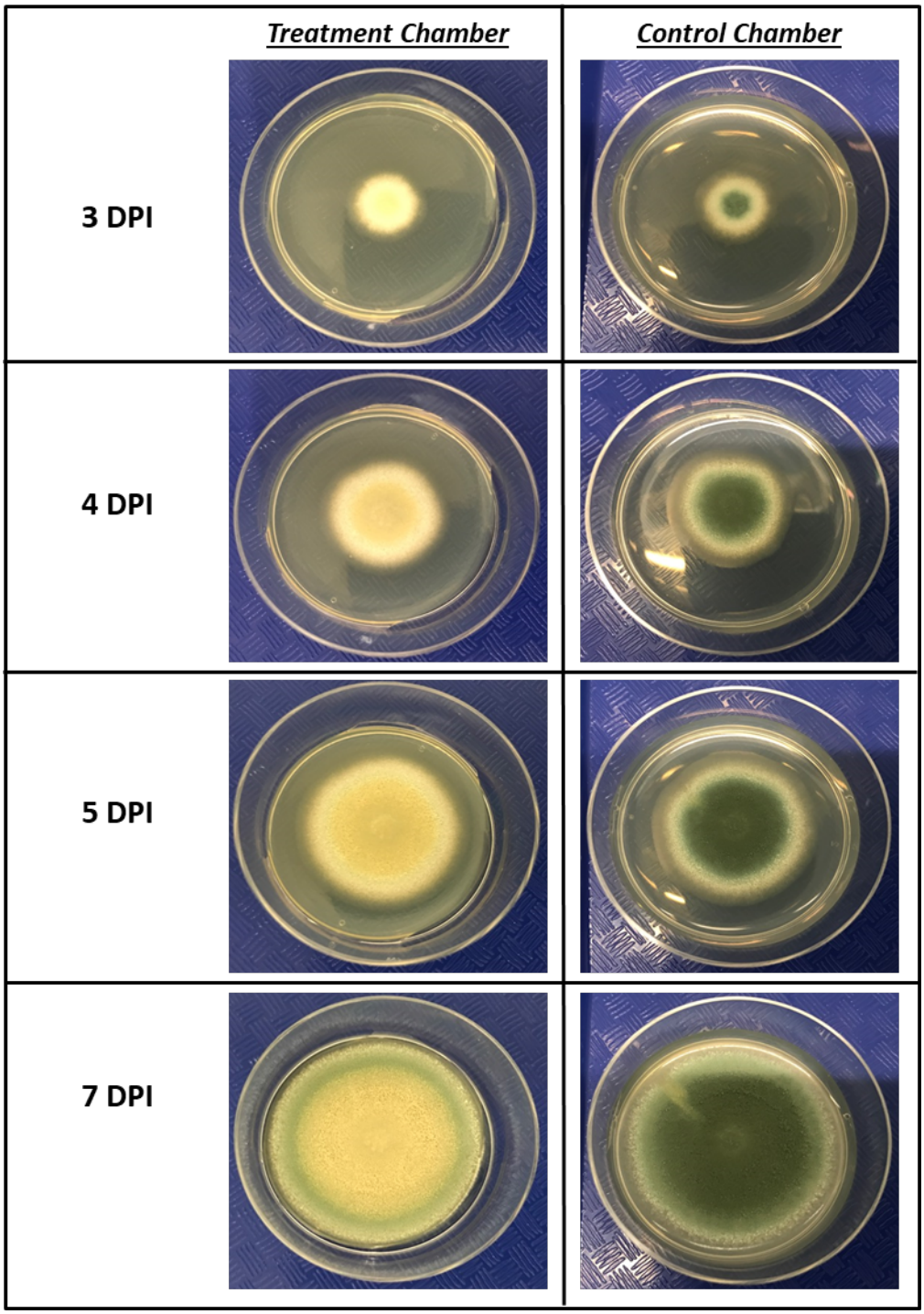


Figure 5.12. Daily comparison of the *Penicillium expansum* cultures observed in either chamber

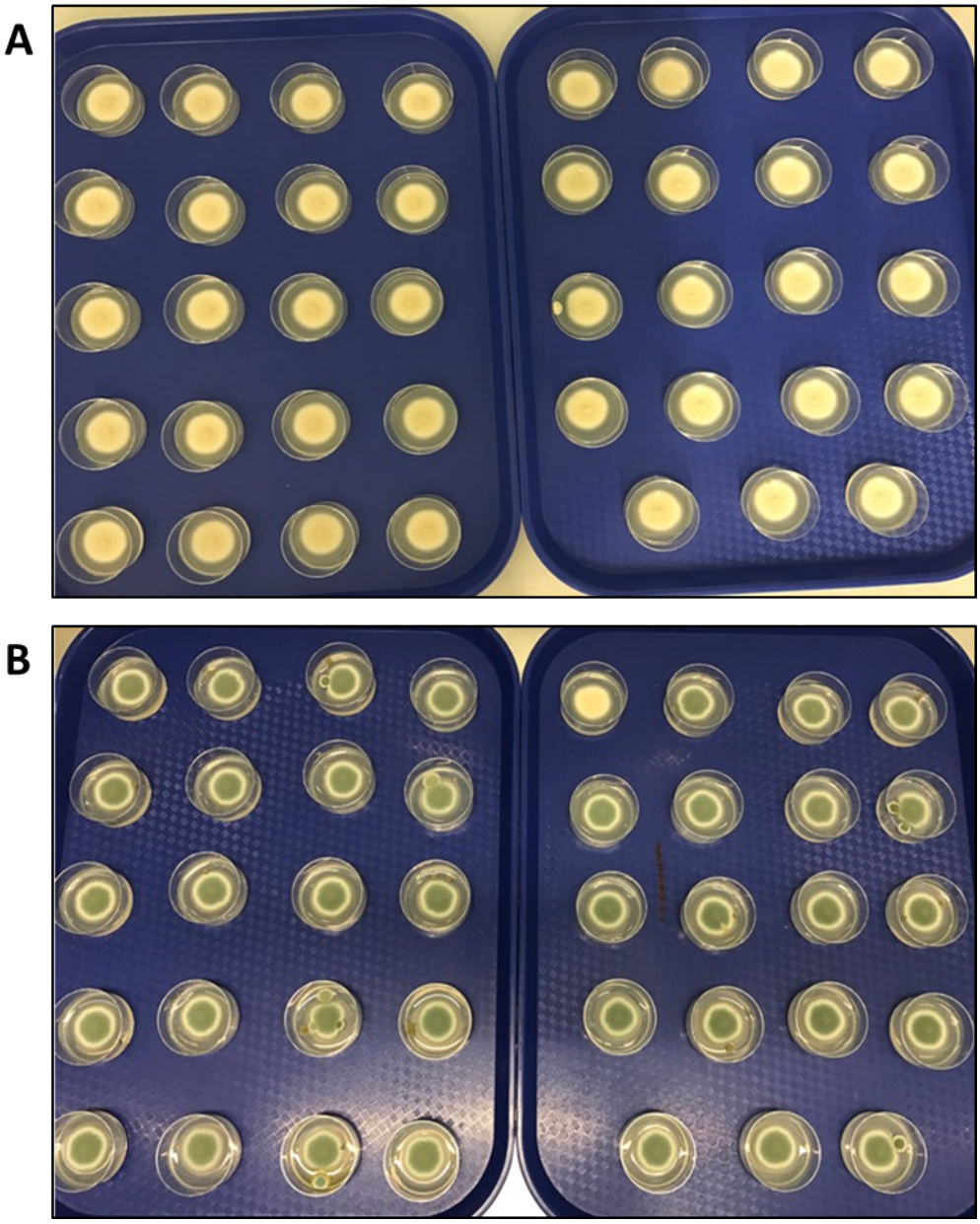


Figure 5.13. *Penicillium expansum* cultures within A) the treatment chamber and B) the control chamber at 5 DPI. This figure demonstrates the consistency of samples observed in Figure 5.12 across almost all samples within either chamber.

The results presented in Figure 5.12 and Figure 5.13 in combination with results observed in Figure 5.11 and Table 5.3 demonstrate a delay/prevention of asexual spore maturation of *P. expansum* as a result of ethylene scrubbing treatment. Similar results have been observed by Li *et al.* (2017) *in vitro* where 1-MCP treated cultures had showed delays in turning green or around 48 h compared to control cultures. These results combined therefore suggest that ethylene may be required for the maturation of asexual *P. expansum* spores.

These results, however, did not repeat in a follow-up experiment, where all plates in the treatment chamber resulted in the maturation and blue colouration of asexual spores at the same rate as in the control chamber. Furthermore, the effects observed in Figure 5.11 and Table 5.3 were not consistent across every experiment. This demonstrates a level of inconsistency with the effects of the treatment on the pathogen. The reasons for these inconsistencies are unknown. Future work should look to identify why this inconsistency is observed and figure out what particular aspect of the treatment is preventing asexual spore maturation. If there is a specific reason behind the inhibitions, then future ethylene removal treatments could look to target and upregulate factors which may stimulate the prevention of spore maturation. These results could be significant in the storage and transport of fruit and vegetable crops.

5.4.1.4 Effects of ethylene scrubbing treatment on tomato vines

A colleague, Dr. Hassan Moeiniyan Bagheri, who was also working on UV photocatalytic ethylene scrubbing analysing the physiology of vine tomatoes, identified mould growing on the vines of Piccolo variety control tomatoes which was not present on his ethylene scrubbed vines (Figure 5.14). He provided me

with sample vines from treated tomatoes and control tomatoes to conduct DNA analysis to identify the pathogen.

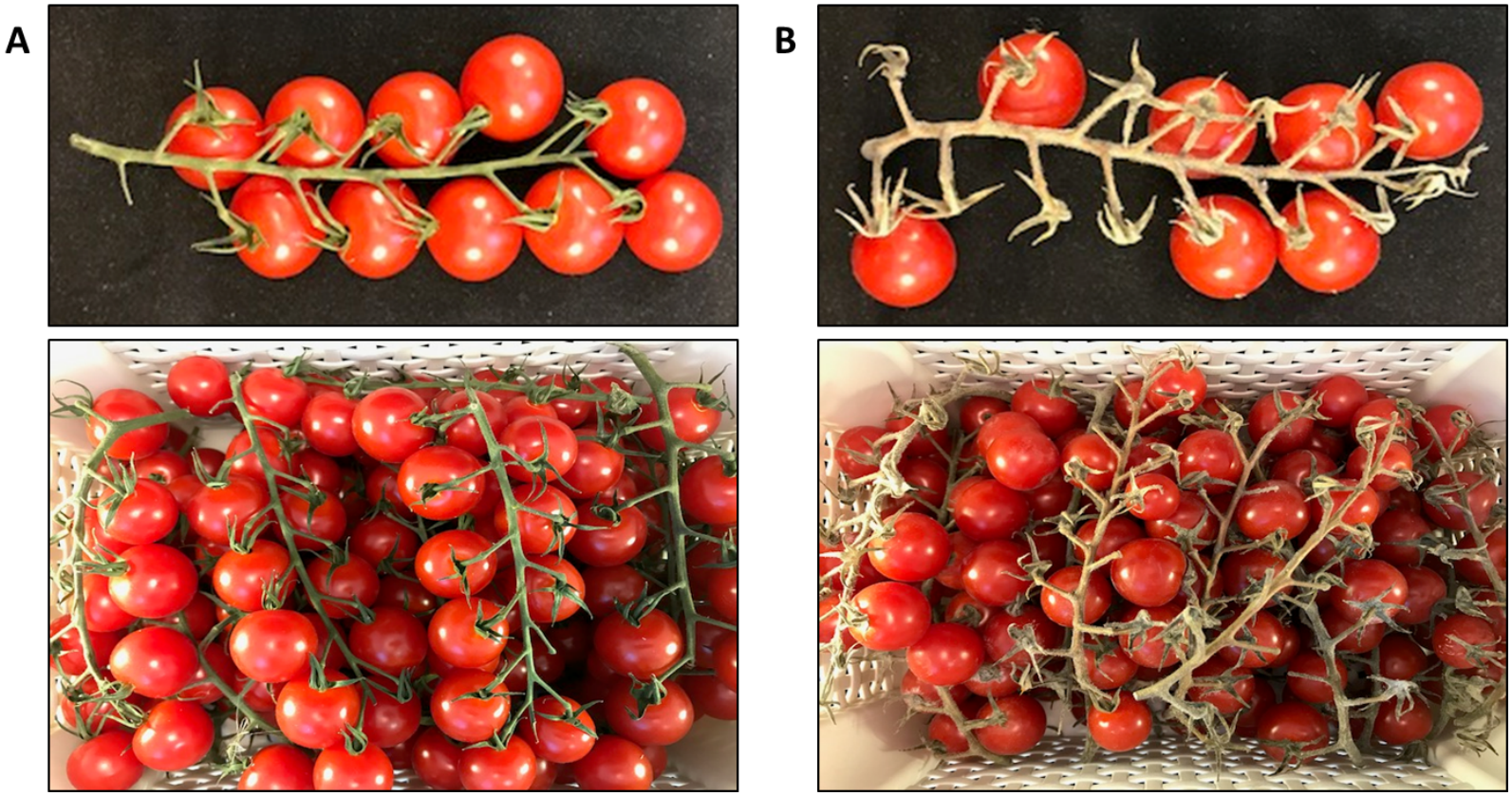


Figure 5.14. Images provided by Dr. Hassan Moeiniyan Bagheri of vine tomatoes from his experiment. A) Vine tomatoes subject to ethylene scrubbing treatment. B) Control Vine tomatoes.

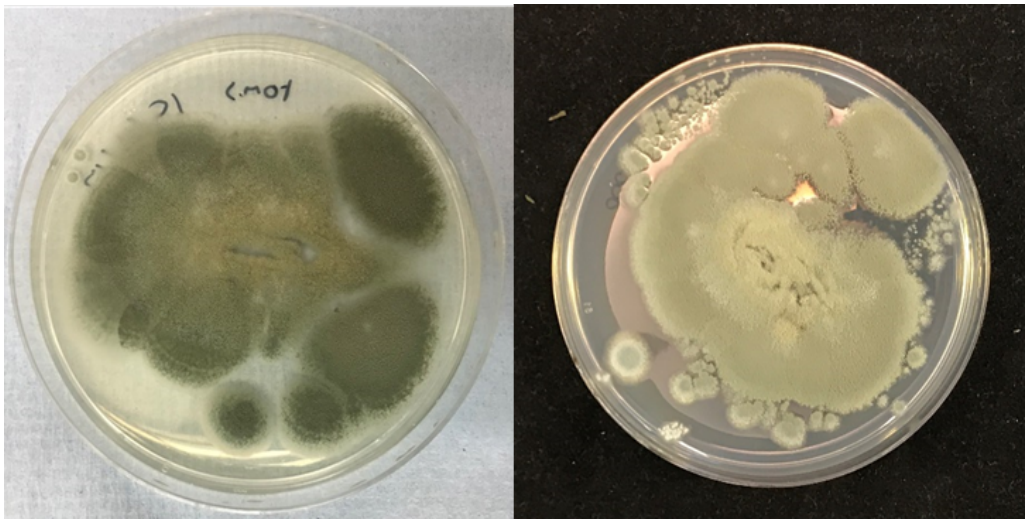


Figure 5.15. Images of the pathogen present on tomato vines cultured onto PDA growth media.

As can be seen in Figure 5.14, the treated vines appear healthy displaying a good green colour, whereas the control chamber vines are covered in mould. Due to this, the mould from the control chamber tomato vines was plated on PDA, by swabbing some of the spores off the vine, and left to grow prior to DNA analysis. The pathogen grown on PDA can be observed in Figure 5.15. Whatever pathogen was identified on the control chamber vine tomatoes was clearly being inhibited due to UV photocatalytic ethylene scrubbing treatment. Once the pathogen had developed for approximately 1 wk on the PDA, DNA sequencing of the pathogen was performed from six of the plates using the same methods as described in section 2.8.2. Disease symptoms present on the tomato vines (Figure 5.14), and the growth of the pathogen on PDA (Figure 5.15) indicate that the pathogen was probably from the *Penicillium* genus. However, it was still important to confirm this by performing DNA analysis.

DNA analysis from the plated pathogens confirmed that the species was from the *Penicillium* genus. BLAST analysis in the majority of cases identified the species as *P. olsonii*. *Penicillium olsonii* has previously been observed in the storage of vine tomatoes in British Columbia with swab samples of the pathogen taken from fruit demonstrating similar *in vitro* characteristics to what has been observed here (Chatterton *et al.*, 2012). However, in the same research conducted by Chatterton *et al.* (2012), there was no report of any *P. olsonii* disease presence on the vines of the tomato, only the surface of the fruit. Furthermore, it was shown here that other species of *Penicillium* including *P. brevicompactum, P. astrolabium* and *P. volgaense* also gave 100% identification matches to the sequences extracted from the fungal plates, probably due to inaccurate annotation / identification of the species

from which sequences had been submitted to GenBank. Therefore, it is not entirely clear which specific species of *Penicllium* was present on the vines.

Future work should look to identify the exact species being inhibited as identifying what pathogen the ethylene scrubbing treatments are effective against would be beneficial in the storage of fresh produce commonly affected by the specified pathogen. Identification of the exact pathogen infected would lead to appropriate application of the treatment against its commonly affected host crop species.

The work presented here does however suggest that UV photocatalytic ethylene removal treatment is effective in inhibiting *Penicillium* disease or at least the disease symptoms the pathogen causes. This could prove to be important for the postharvest market as conidia from *Penicillium* species are known to be ubiquitous within the atmosphere of packinghouses (Barkai-Golan, 1966). Potential reductions in symptoms produced by the pathogen could lead to reductions in the presence of conidia in storage environments and therefore reductions in the spread of disease. It would also be interesting to perform an experiment where spores present within the storage atmosphere were affected by being drafted through the fans of the ethylene scrubber. The movement of air through the scrubber would bring conidia with it. It would be hypothesised that the UV light within the ethylene scrubber could potentially affect the spores for when they come out the other side and perhaps inhibit disease.

Further future work could also look into other species of *Penicillium* disease prevention such as *P. digitatum* or *P. italicum* diseases on citrus fruits. If the method of treatment used here is preventing both asexual spore maturation of

P. expansum and the onset of another species of *Penicillium* on tomato vines in storage, then there could potentially be an application for it against *P. digitatum* and *P. italicum,* which are prevalent on citrus fruits (Holmes & Eckert, 1999; Macarisin *et al.*, 2007). This would be particularly interesting as previous research has suggested that ethylene perception is important for increased resistance to these pathogens, and furthermore, that 1-MCP ethylene perception blocking can increase disease susceptibility (Porat *et al.*, 1999; Marcos *et al.*, 2005). Because UV photocatalytic ethylene scrubbing removes ethylene without effecting fruit ethylene perception, the treatments may be able to beneficially inhibit de-greening as 1-MCP has been observed to do (Porat *et al.*, 1999), but also inhibit *Penicillium* species as seen here, therefore not increasing fruit disease susceptibility. Future work here could also be carried out against further species of *Penicillium* to determine how effective treatments are against many species from the genus.

5.5 UV PHOTOCATALYTIC ETHYLENE SCRUBBING ON *COLLEOTOTRICHUM GLOEOSPORIOIDES*

Colletotrichum gloeosporioides is a hemibiotrophic plant pathogen that causes postharvest anthracnose disease on fruit crops. It is regarded as one of the most important postharvest pathogens on a wide variety of tropical and subtropical fruits (Coates *et al.*, 1993; Wattad *et al.*, 1997).

In order to promote colonization on its host, the fungal pathogen exhibits sequential quiescent and active necrotrophic infection strategies (Prusky *et al.*, 2013). The time taken for *C. gloeosporioides* to switch from a quiescent, biotrophic stage of infection to an aggressive necrotrophic stage is dependent on the physiological stage of the infected fruit cuticle. On unripe fruit, the

pathogen can remain dormant in the quiescent stage for weeks until the fruit ripens, at which point the pathogen switches to necrotrophic growth (Prusky, 1996; Alkan *et al.*, 2015). If *C. gloeosporioides* conidia were to land on ripe, susceptible fruit however, the biotrophic quiescent phase of infection is very short and the switch from quiescent structures to necrotrophic growth is rapid (Alkan & Fortes, 2015).

Ethylene's role in the ripening of climacteric fruit brings about the necessary physiological changes required for the onset of *C. gloeosporioides* necrotrophic infection. Furthermore, ethylene has been observed to be crucially important for *C. gloeosporioides* infection (Cooper, 1993; Cooper *et al.*, 1998) due to the importance of the phytohormone in stimulating conidial germination, germ tube elongation and appressorial formation (Brown, 1975; Flaishman & Kolattukudy, 1994; Flaishman *et al.*, 1995). Moreover, ethylene perception inhibitor, 1-MCP, has been observed to inhibit *C. gloeosporioides* development on avocado and mango fruit (Wang *et al.*, 2006; Xu *et al.*, 2017). These studies demonstrate a potential for ethylene removal treatments in the prevention of postharvest anthracnose disease caused by *C. gloeosporioides*.

Here, the effects of UV photocatalytic ethylene scrubbing treatments were examined to determine whether treatments could prevent anthracnose disease severity and occurrence. Although not a typical host of *C. gloeosporioides*, tomatoes were used as a model for more naturally infected crop species such as mango, avocado and dragonfruit.

5.5.1 Results and discussion

UV photocatalytic ethylene scrubbing proved highly effective in causing anthracnose disease reductions caused by *C. gloeosporioides* on tomato fruits. Across all varieties tested, whether tomatoes were harvested and inoculated at a red ripe or mature green physiological stage, disease severity and occurrence was always consistently worse within the control chamber. These results can be seen in Figure 5.16 and Figure 5.17.

What was particularly promising about the results was that as well as significant levels of disease inhibition and reductions, the majority of samples within the ethylene scrubbing treatment chamber were coming out with no anthracnose disease symptoms present whatsoever. This can be seen in Figure 5.17a where the samples photographed in the treatment chamber are showing no symptoms of disease after 27 DPI. This suggests that ethylene scrubbing could potentially be applied to storage units to prevent anthracnose on fruit crops.

Previous research would suggest that the results observed here are due to ethylene absence resulting in the inhibition of conidial germination, germ tube elongation and appressorial formation of *C. gloeosporioides* spores (Brown, 1975; Flaishman & Kolattukudy, 1994; Flaishman *et al*, 1995).

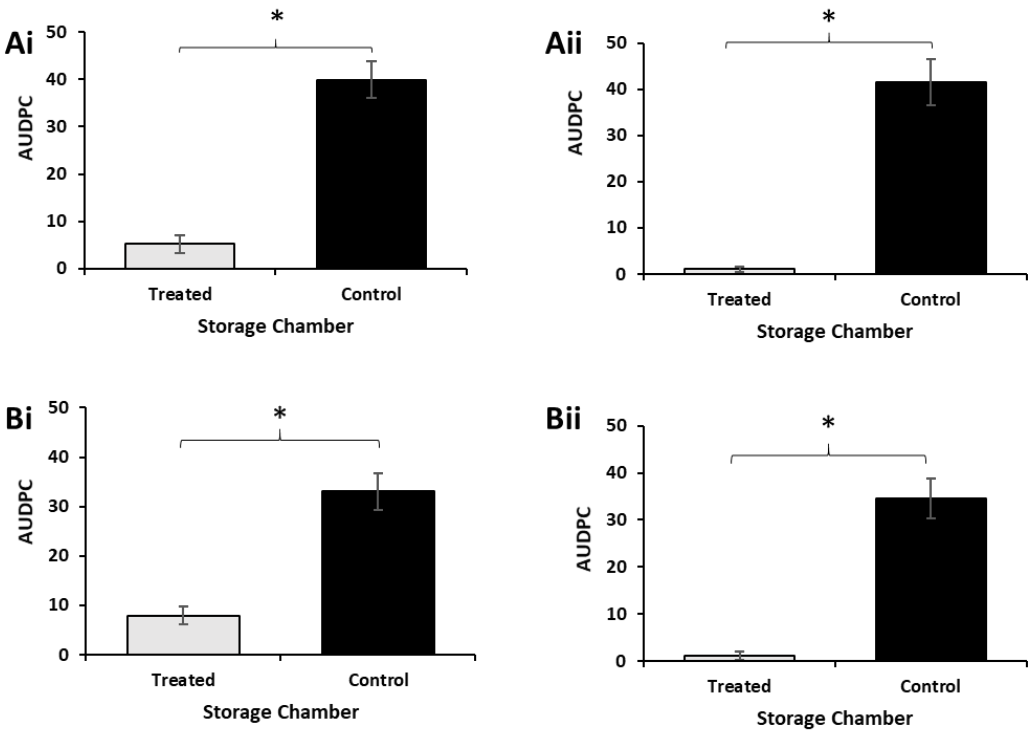


Figure 5.16. Area under the disease progression curve (AUDPC) of anthracnose disease caused by *Colletotrichum gloeosporioides* on tomato fruits stored in either the ethylene scrubbing treatment chamber or control chamber. Error bars represent SEM. Asterisks (*) represent a significant difference ($p < 0.05$) in results between chambers. A graphs represent tomatoes harvested and inoculated at mature green stage. B graphs represent tomatoes harvested and inoculated at a red ripe stage. Ai) Shirley tomato variety tomatoes results compiled from three repeat experiments. Aii) Moneymaker variety tomato results compiled from two repeat experiments. Bi) Shirley variety tomato results compiled from 4 repeat experiments. Bii) Ailsa Craig variety tomato results compiled from two repeat experiments,

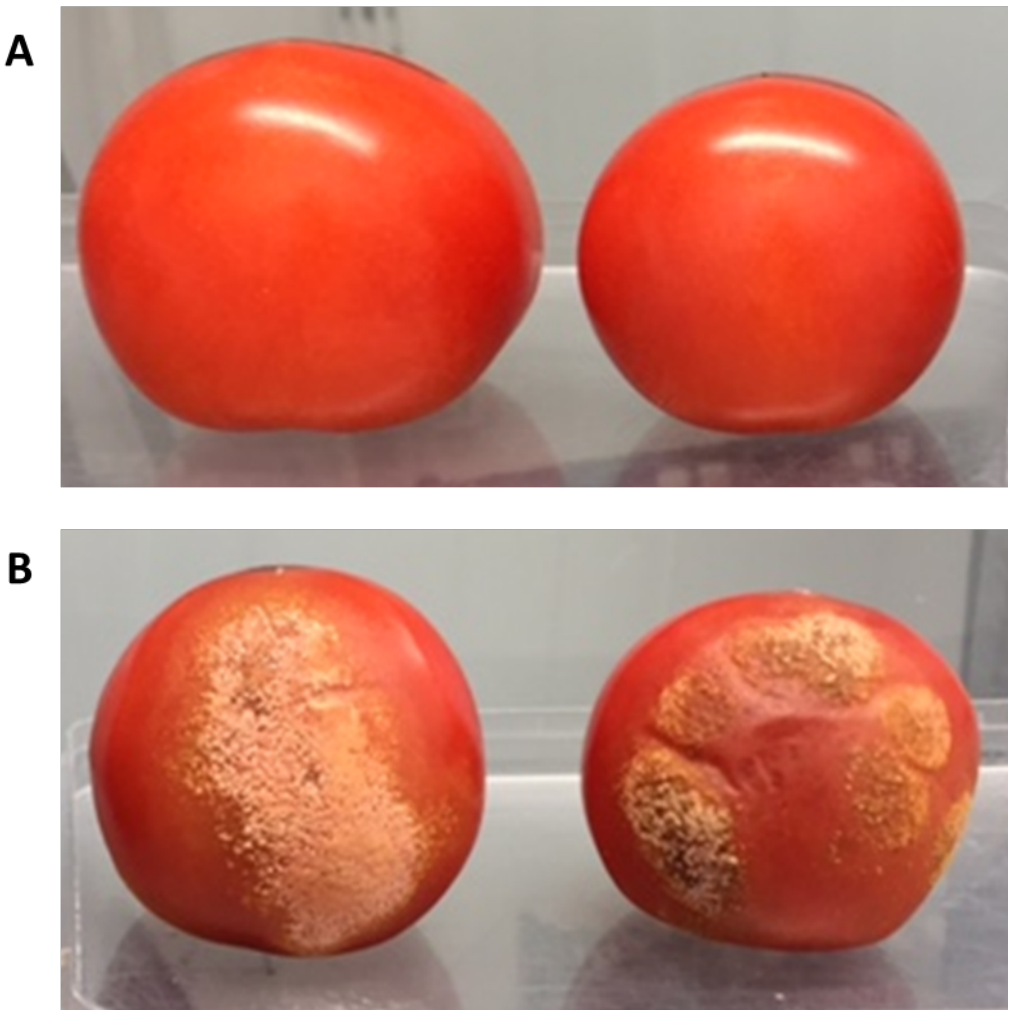


Figure 5.17. Images to represent the absence and presence of anthracnose disease present on tomato fruits stored after inoculation in the treatment and control chamber respectively. A) Treatment chamber tomato samples. B) Control chamber tomato samples. Tomatoes pictured were Moneymaker tomatoes harvested and inoculated at a mature green stage. Here, they are pictured 27 DPI.

The red ripe physiological stages of fruits within both chambers demonstrated by Figure 5.17 suggests that *C. gloeosporioides* should have been able to infect the treated fruits in the same manner as the control due to the increased ripening related susceptibility of fruits within both chambers (Alkan & Fortes, 2015; Alkan *et al.*, 2015). This could have come at a slight delay if the ethylene scrubbing treatment delayed the rate of fruit ripening in the treatment chamber. However, the prolonged ripening of the treated fruit should have still induced increased disease susceptibility, so it is unlikely ripening rate could be the reason for disease prevention. It could be hypothesised that delays in the pathogen switching to its aggressive necrotrophic phase of infection due to ethylene absence have allowed more host response time to detect and respond to *C. gloeosporioides* infection, perhaps meaning that the defence response to *C. gloeosporioides* is stronger in the treatment chamber resulting in disease reductions. In the following chapter, the fruit molecular mechanisms between chambers in response to pathogen attack have been investigated to test this hypothesis.

Previous work with ethylene perception inhibitor 1-MCP has also demonstrated *C. gloeosporioides* disease reductions in fruits. Mango fruits inoculated and stored in the presence of 1-MCP demonstrated clear disease reductions in comparison to control fruit (Xu *et al.*, 2017). However, it is clear from images of the fruit within the publication that the 1-MCP has delayed the ripening of the mango fruits, which consequently would have resulted in smaller lesion diameters of treated fruits at the point of disease scoring. Furthermore, it is suggested that 1-MCP may have bound to the ethylene receptors of *C. gloeosporioides*, inhibiting lesion development by inducing ROS accumulation in *C. gloeosporioides* spores and causing oxidative damage. Here, no real ripening delays were observed and a completely

external ethylene removal method was used. Therefore, disease prevention observed here is unlikely to have been induced in a similar manner to the research performed by Xu *et al.* (2017). In other research performed by Wang *et al.* (2006), 1-MCP treatment affected the accumulation of antifungal dienes in avocado fruit. Treatments proved beneficial or not depending on the harvesting time of the fruits. Here, UV photocatalytic ethylene scrubbing has proved to be effective against anthracnose regardless of fruit harvesting times. This suggests a positive effect of the treatment over 1-MCP.

5.5.1.1 **Colletotrichum gloeosporioides *on ripening mutant tomatoes***

Studies using tomato fruit ripening mutants have been beneficial in promoting our understanding of fruit development and ripening (Klee & Giovannoni, 2011; Osorio *et al.*, 2013; Kumar *et al.*, 2016). Ripening mutant tomatoes are characterised by deficiencies in ripening associated characteristics observed in normally ripening fruit.

Mutant *rin* (*ripening inhibitor*) tomatoes have previously been observed to be resistant to *C. gloeosporioides* infection, except when external ethylene is applied to the fruits (Cooper, 1993; Cooper *et al.*, 1998). Here, work was performed inoculating the Ailsa Craig variety ripening mutant tomatoes *rin*, *nor* (*non-ripening*), *Nr* (*Never-ripe*) and *Cnr* (*colourless nonripening*). The transcription factors *rin*, *nor* and *Cnr* all act to regulate ripening, with mutations in the genes for these transcription factors resulting in fruit which fail to complete normal ripening (Vrebalov *et al.*, 2002; Manning *et al.*, 2006; Cantu *et al.*, 2009). Perception of ethylene occurs in His-kinase ethylene receptors (Chang & Shockey, 1999). The *Nr* mutation occurs within these receptors and

interferes with ethylene perception, consequently delaying fruit ripening (Wilkinson *et al.*, 1995).

Experiments were performed to determine firstly whether the specified mutations would affect the onset of anthracnose disease between the treatment and control chamber, and secondly, whether the pathogens ability to cause disease would be effected as a result of each ripening mutation. The results of *C. gloeosporioides* inoculations on each mutant tomato variety can be observed in Table 5.4.

Table 5.4. Anthracnose disease scores (as demonstrated in **Error! Reference source not found.** and section 2.10.2) present on Ailsa Craig variety ripening mutant tomatoes stored within either the ethylene scrubbing treatment chamber of control chamber. *Nr* mutation results compiled from two repeat experiments at 49 DPI. *Cnr* mutation results compiled from two repeat experiments on the last day of result taking. *Nor* mutation results compiled from two repeat experiments at 49 DPI. *rin* mutation results at 49 DPI.

	Nr Mutation		***Cnr Mutation***		***nor Mutation***		***rin Mutation***	
Disease Score	*Treated*	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*	*Control*
0 (0%)	14	0	14	0	14	2	8	0
1 (1%)	0	6	0	2	0	3	0	0
2 (2%)	0	9	0	3	0	9	0	4
3 (5%)	0	0	0	0	0	0	0	2
4 (10%)	0	0	0	0	0	0	0	0
5 (20%)	0	0	0	2	0	0	0	1
6 (40%)	0	0	0	2	0	0	0	0
7 (60%)	0	0	0	5	0	0	0	1
8 (≤ 80%)	0	0	0	1	0	0	0	0

As can be seen in Table 5.4, for each ripening mutation, within the treatment chamber there was no disease symptoms present across multiple experiments. Within the control chamber however, there are disease symptoms present on all ripening mutants of varying severities dependant on the ripening mutant inoculated. These results support results observed by Cooper (1993) and Cooper *et al.* (1998) who showed that *C. gloeosporioides* inoculated *rin* tomato fruits only showed visible infection with the pathogen in the presence of ethylene. Here, we see this effect across other ripening mutations as well as *rin* fruit. Future work should look to repeat these experiments utilising external ethylene injections. It would be interesting to determine whether increased ethylene levels within the control chamber could act to increase disease severity in *Nr* and *nor* mutation tomatoes as seen with *Cnr* and *rin* mutation tomatoes. Furthermore, it would be interesting to leave the experiment running for a longer period of time. Here, experiments had to be stopped due to the requirement for space within the chambers for further experiments.

The consistency across the different ripening mutations here as well as in wild type fruit varieties would suggest that disease resistance is observed due to the ethylene scrubbing treatments effects on the pathogen itself. What is interesting about the results presented in Table 5.4 is the difference in anthracnose disease severity in control chamber fruit between ripening mutations. It suggests increased baseline disease resistance of *Nr* and *nor* mutations over *Cnr* and *rin* mutations. With *Nr* and *nor*, disease symptoms were not able to go above 2%, whereas with *Cnr* and *rin*, disease was able to progress to much more severe symptoms. Previous research by Cantu *et al.* (2009) has shown that ripening related susceptibility of tomato fruit to *B. cinerea* requires *nor* but not *rin,* as demonstrated by increased disease

incidence and severity on *rin* over *nor* tomatoes. Additionally, *Nr* mutant tomatoes showed reduced grey mould disease symptoms in comparison to *rin*. This demonstrates further similarly to what has been observed here with differences in anthracnose disease severity symptoms.

Further future work in this area should look to determine why different levels of anthracnose disease severity have been observed between the ripening related mutants. Research should be conducted into identifying why *C. gloeosporioides* was able to cause severe disease symptoms in *Cnr* and *rin* mutation tomatoes, but progress no further than forming small black lesions in *Nr* and *nor* mutant tomatoes. It would be of interest to find out what aspect of the *Nr* and *nor* fruits is preventing the development of anthracnose disease. Identifying the reasons behind this disease inhibition could lead to the development of future methods of anthracnose disease prevention, such as breeding for specific host defence genes and responses from *Nr* and *nor* plants.

5.5.1.2 Chamber swap anthracnose disease experiment

An experiment was conducted to determine whether the effects of ethylene scrubbing treatment could be maintained if half the treated tomatoes were swapped from the treatment chamber to the control chamber. Conversely, half the tomatoes beginning storage within the control chamber were to be swapped to the treatment chamber to determine whether the ethylene scrubbing treatment could play a role in reducing the severity of disease once the onset of pathogen virulence had begun. The experiment consisted of inoculating 40 mature green Shirley tomatoes at harvest and storing 20 in each chamber. At the first sign of disease, which turned out to be 16 DPI, 10

tomatoes from each chamber were swapped around and disease was recorded from 17 DPI onwards to 29 DPI. Results can be observed in Figure 5.18.

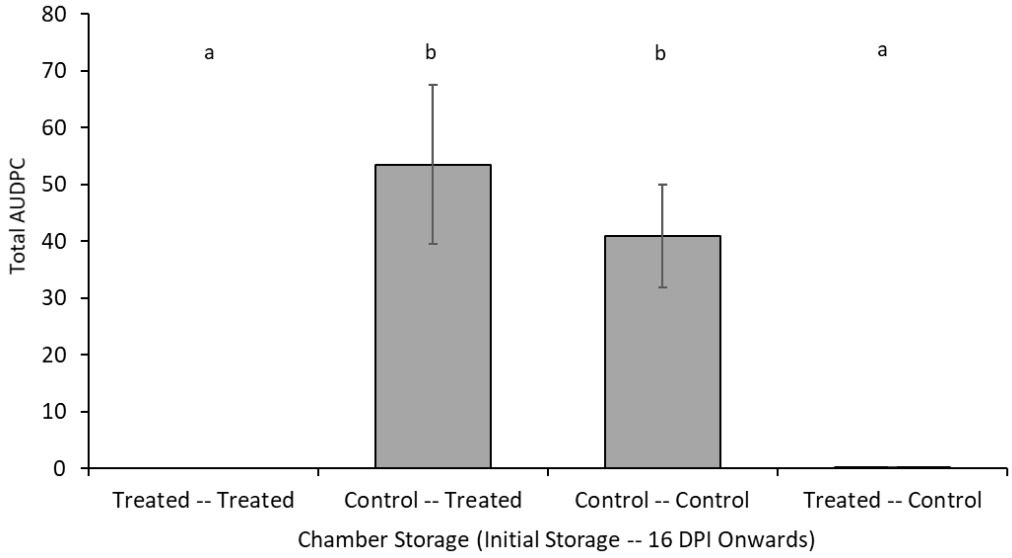


Figure 5.18. Mean AUDPC of mature green Shirley tomatoes subjected to varied storage chamber regimes. Tomatoes had an initial period of storage in either chamber before half of the samples from each chamber were swapped at 16 DPI. Error bars represent SEM. Labelling indicates statistical significance ($p < 0.05$) where groups sharing the same labels are not significantly different.

As can be seen in Figure 5.18, there were significant differences between tomatoes that began storage in the treatment chamber and tomatoes that began storage within the control chamber. For tomatoes that began storage within the treatment chamber, only one showed any disease symptoms and this was a disease score of only 1. However, 84% of the tomatoes that began storage within the control chamber developed varying levels of disease.

This experiment suggests firstly, that UV photocatalytic ethylene scrubbing treatment is able to confer lasting disease inhibition against *C.*

gloeosporioides. This is due to tomatoes swapped from the treatment chamber to the control chamber exhibiting no disease after a period of storage within the control chamber. Furthermore it suggests that the treatment should be used as a preventative treatment for anthracnose disease as opposed to a curative one. Tomatoes which began storage in the control chamber and were swapped to the treatment chamber continued disease development within the treatment chamber.

In order to confirm these results, it is important that this experiment is repeated. Unfortunately, due to time constraints and chamber space, a repeat experiment could not be performed. Furthermore, it would be interesting to run this experiment for a longer time to further confirm that anthracnose disease prevention due to treatments can be maintained for extended storage in other storage units without an ethylene scrubber.

5.5.1.3 Experiments conducted with ethylene injections and maintained high relative humidity within the treatment chamber

Research was also conducted in order to examine how the effects of ethylene injections, and maintenance of higher RH within the treatment chamber, may affect disease incidence. Ethylene injections occurred (as described in section 3.4.1.1) and RH was maintained high as seen in Figure 3.7(a). It was hypothesised that disease within the treatment chamber may increase slightly due to higher maintained levels of humidity, and the initial perceptions of increased ethylene levels due to daily ethylene injections. Although it was observed that ethylene from the injections is quickly removed from the treatment chamber (section 3.4.1.1), perhaps the small period of ethylene

detection by the fruits and pathogen could stimulate increased disease on treated fruit.

If disease during these experiments was maintained at the incredibly low levels seen in previous results (Figure 5.16 and Figure 5.18), it would indicate that the ethylene scrubbing treatment would show strong disease preventative qualities against *C. gloeosporioides* in a variety of different storage environment conditions. Results from these experiments can be observed in Figure 5.19.

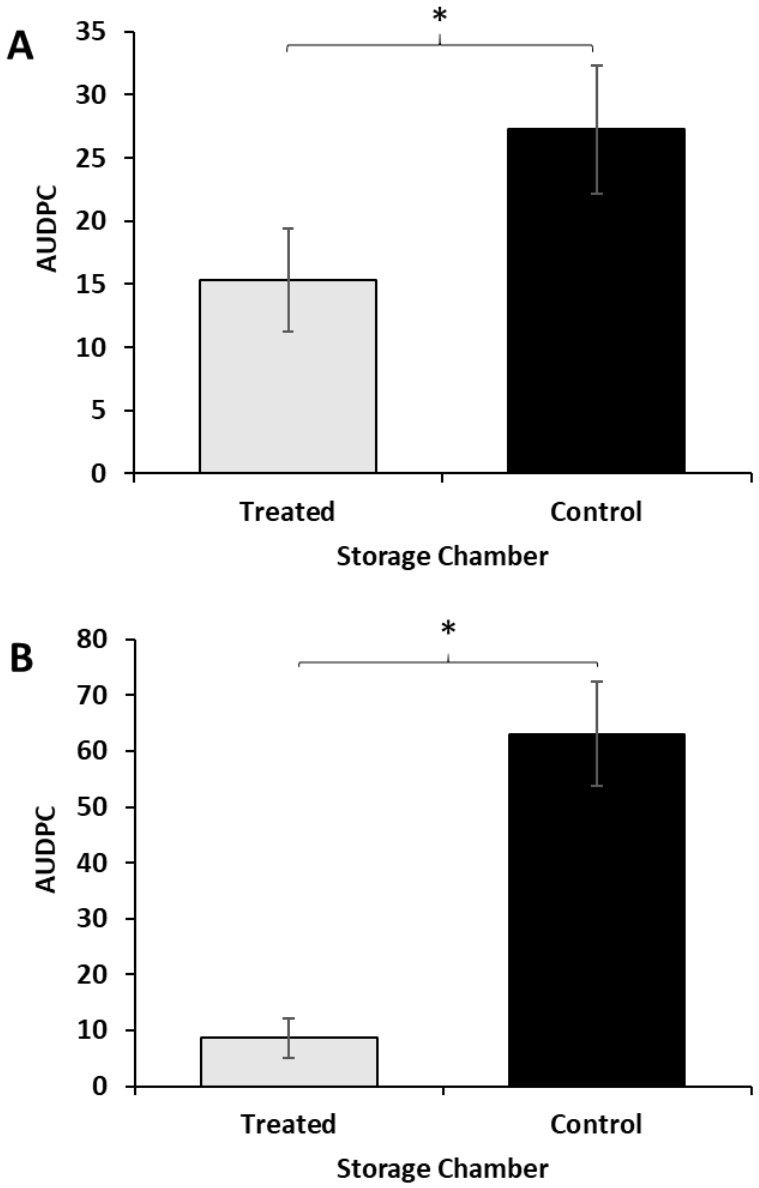


Figure 5.19. Mean AUDPC of Shirley variety tomato fruit following inoculation with *Colletotrichum gloeosporioides* and storage within either the ethylene scrubbing treatment chamber or control chamber compiled from 3 experimental repeats. Throughout experiments, RH was maintained as high as possible within the treatment chamber and both chambers were subjected to daily Monday – Friday 10 ml ethylene injections. Error bars represent SEM. Asterisks (*) represent a significant difference ($p < 0.05$) between the results in each chamber as determined by a Mann Whitney U test. A) Tomatoes harvested and inoculated at a mature green stage. B) Tomatoes harvested and inoculated at a red ripe stage.

Results from these experiments demonstrated that increasing humidity levels in combination with short periods of external ethylene detection results in slightly higher levels of anthracnose disease incidence within the treatment chamber. A breakdown of disease scores recorded on the final day of disease scoring from these experiments (26 DPI) can be observed in Table 5.5.

Table 5.5. The distribution of anthracnose disease scores present on tomatoes on the final day of disease recordings (26 DPI). Results were used as part of the calculation to generate the AUDPC's observed in Figure 5.19. Tomatoes were of Shirley variety.

	Mature Green Fruit		***Red Ripe Fruit***	
Disease Score	**Treated**	**Control**	**Treated**	**Control**
0 (0%)	10	4	6	0
1 (1%)	4	0	2	0
2 (2%)	3	0	2	0
3 (5%)	1	2	2	0
4 (10%)	0	2	0	1
5 (20%)	2	6	1	2
6 (40%)	4	1	0	2
7 (60%)	0	2	0	0
8 (≥80%)	0	6	0	6

The disease incidence of anthracnose in these three experiments was greater than previously seen in the other experiments. Increasing humidity and ethylene injections have clearly increased *C. gloeosporioides* aggressiveness within the treatment chamber. As previously discussed (section 5.5.1.1), external ethylene has been observed to induce *C. gloeosporioides* development on *rin* ripening mutant tomatoes (Cooper, 1993; Cooper *et al.*, 1998). Potentially, the addition of external ethylene into the treatment chamber may have induced increasing incidence of conidial germination and appressorial formation, resulting in greater disease incidence amongst treated samples. Alternatively, nullifying the effects of treatment chamber humidity reductions by the ethylene scrubber, by maintaining high RH within the treatment chamber, could have incurred greater levels of conidial germination, as *C. gloeosporioides* conidial germination is far more frequent at greater humidity levels (Estrada *et al.*, 2000).

However, regardless of the slight increases in disease incidence and severity observed in the treatment chamber as a result of higher humidity levels and ethylene injections, disease still proved to be significantly more severe and consistent in the control chamber. Furthermore, across the three repeat experiments, within the treatment chamber, there were still numerous samples displaying no symptoms of disease (Table 5.5). For fruits harvested and inoculated at a mature green stage, within the treatment chamber, 58% of samples showed disease varying from 1% - 40% severity. In the control chamber however, 82% of the samples were diseased, varying from 5% - ≥80%. Similarly with fruit harvested and inoculated at a red ripe stage, only 64% of treated samples showed disease symptoms varying from 1% - 20% severity, whereas 100% of control samples produced disease symptoms varying from 10% - ≥80% severity. These figures clearly demonstrate that

even with the effects of ethylene injections and increased treatment chamber RH, UV photocatalytic ethylene scrubbing is effective in reducing and preventing anthracnose disease severity and occurrence. Images of fruit samples from these experiments days after the final disease recordings can be seen in Figure 5.20.

Figure 5.20 demonstrates that within the treatment chamber, although there are overall increases in disease incidence as a result of ethylene and humidity supplementation, there is still anthracnose disease prevention brought about by UV photocatalytic ethylene scrubbing treatment.

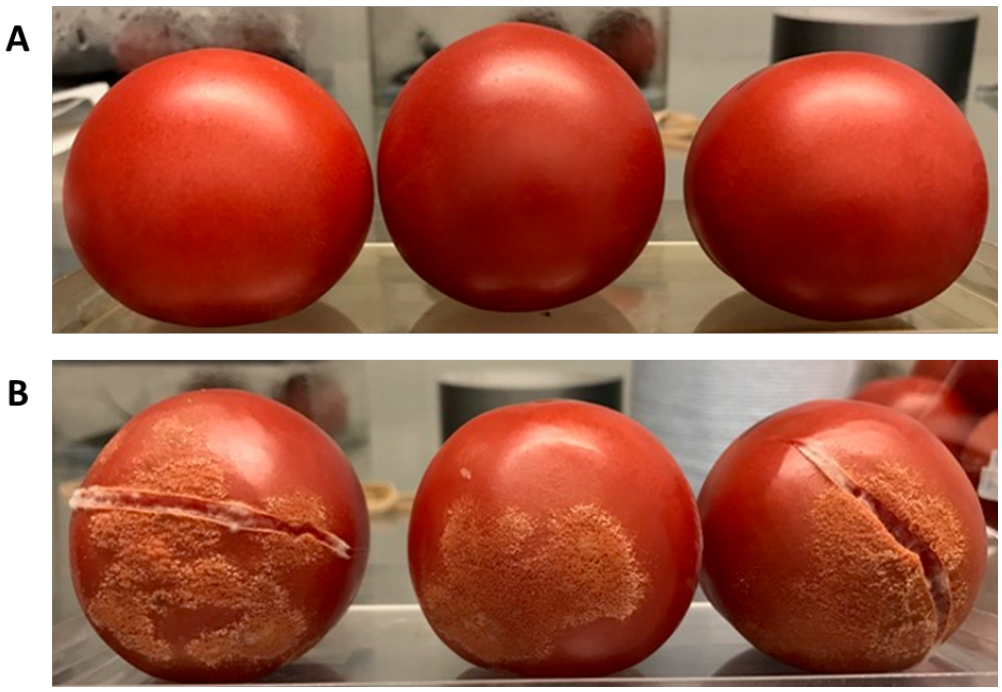


Figure 5.20. Sample fruit stored within either A) the ethylene scrubbing treatment chamber or B) the control chamber. Images were taken at 31 DPI. Fruit samples presented here were stored during daily Monday - Friday 10 ml ethylene injections and during periods of maintain high humidity. Shirley tomatoes were originally harvested and inoculated at a mature green physiological stage.

5.5.1.4 Location of treatment chamber disease symptoms

Throughout experimentation, it was observed that within the treatment chamber, the majority of samples that developed any disease symptoms were only able to produce these symptoms around the calyx scar of the fruit. Examples of this can be seen in Figure 5.21.



Figure 5.21. Examples of anthracnose disease development in the majority of disease cases within the treatment chamber. Disease tended to develop around the calyx scar with no disease symptoms present on the tomato surface.

The reasons behind the location treatment chamber disease symptoms are not completely clear. Successful *C. gloeosporioides* infection of control chamber fruit samples can be clearly observed on the area of inoculation as demonstrated in Figure 5.17b and Figure 5.20b. However, successful *C. gloeosporioides* infection within the treatment chamber was only really seen around the fruit calyx scar when inoculation droplets had ran spores down to the area. Only very rarely could disease symptoms be observed at the exact inoculation site as was seen within the control chamber.

It could be hypothesised that the fruit tissue around the calyx scar is more susceptible to disease and therefore *C. gloeosporioides* spores were more easily able to cause infection. Furthermore, another theory could be that the calyx scar of the tomato fruit releases greater quantities of ethylene so spores in that specific area of the fruit are able to detect ethylene before it is removed, promoting conidial germination and appressorial formation, consequently resulting in successful infection. Previous research has shown that in response to certain stresses, increased ethylene production in persimmon calyxs has been induced (Nakano *et al.*, 2002; Besada *et al.*, 2016). Water stress due to low RH was actually observed to increase ethylene production in the persimmon calyx (Nakano *et al.*, 2002). It could be possible here that decreasing RH due to the action of the ethylene scrubber could be increasing ethylene production in the calyx scar of the fruit, resulting in increased disease around this area. Future work could look to test this hypothesis to determine whether ethylene scrubbing treatment is inducing greater ethylene production at the calyx scar. This would help identify why disease in the treatment chamber occurs predominantly at this area.

A further hypothesis could be that there could be higher RH in the microenvironment around the calyx scar as a result of inoculation droplets falling to that are of the tissue, increasing the moisture of the area. The potential increase in RH at that specific area would likely cause increases in *C. gloeosporioides* conidial germination.

5.5.1.5 Effects of humidity on Colletotrichum gloeosporioides *infection*

Due to observations that increasing humidity within the treatment chamber results in increased anthracnose disease incidence (section 5.5.1.3), it was important to determine how much of an affect humidity reductions caused by the ethylene scrubbing treatment were having on anthracnose disease. Experiments were performed to access varying humidity levels on the ability of the pathogen to produce disease on tomato fruits.

Three experiments (using the same growth room conditions as described in section 2.4) were performed each containing 12 red ripe store-bought tomatoes per high or low humidity groups. In the first experiment, tomato samples were all stored in boxes. Samples subjected to higher humidity levels had saturated tissue within the box whereas samples subjected to ‘low humidity’ had dry tissue within the box. In this first experiment, humidity levels were recorded as being 99% within each type of box used. However, conditions were clearly much drier in the ‘low humidity’ boxes due to no condensation present within the boxes. In the ‘high humidity’ boxes, there was condensation present within the box coating the sides.

In the second experiment, boxes with saturated towels were again used for the ‘high humidity’ tomatoes as previously described. For the ‘low humidity’

tomatoes, RH was maintained at 60% - 65% in order to test the pathogen in really low RH.

In the third experiment, ‘high humidity’ tomatoes were again stored in the same way as the previously described. ‘Low humidity’ tomatoes were stored on trays with saturated towels similarly to the high humidity boxes. However the boxes remained lidless to allow airflow and reductions in humidity. Following an initial day at 99% RH, RH was maintained between 74% and 89% therefore finding a good medium between the first two experiments.

The results from the three experiments can be observed in Figure 5.22, where a, b and c, represent experiment 1, 2, and 3 respectively.

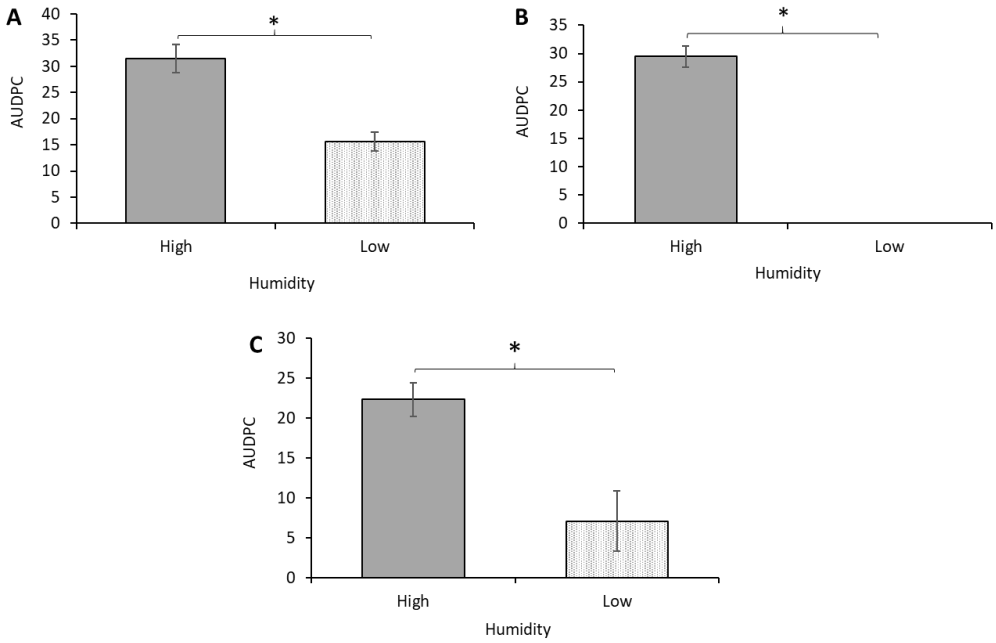


Figure 5.22. The effects of varying humidity levels on the prevalence of *C. gloeosporioides* infection. Error bars represents SEM. Asterisks (*) represent a significant difference ($p < 0.05$) between chambers. A) Experiment one where low humidity was 99% RH but noticeably dryer than high humidity. B) Low humidity storage was maintained between 60-65% RH. C) Low humidity was maintained between 74-89% RH.

Figure 5.22 shows that in each experiment, high humidity induces significantly greater anthracnose disease than lower humidity conditions. These results are supported by Estrada *et al.* (2000), who showed that the percentage of *C. gloeosporioides* conidia that germinate increase as RH increases to 100%. These results further support the work performed by Estrada *et al.* (2000) as the mean AUDPC for 'low humidity' stored samples is greatest in experiment one, with the highest recorded RH, and lowest in experiment two, where RH is maintained at the lowest of the three experiments. Experiment three's 'low humidity' storage mean AUDPC score sits between the two other experiments.

Results presented here show that at RH as low as 60- 65%, there is a complete inhibition and prevention of disease as no disease symptoms were observed. However, there is disease of varying levels present when tomatoes were stored at ≥74% RH and above. These results, in combination with those observed in section 5.5.1.3 show that throughout experimentation, disease prevention observed within the ethylene scrubbing chamber isn't completely due to humidity reductions caused by the action of the UV photocatalytic ethylene scrubber. However, because of the observed disease reductions caused by decreased humidity levels observed in Figure 5.22, as well as results from previous research (Estrada *et al.*, 2000), we can infer that the decreasing RH caused by the ethylene scrubber is playing a role in disease reductions.

Further to these varied humidity experiments, initial preliminary experimentation using mature green Brioso variety tomatoes as the host fruits, demonstrated anthracnose disease on treatment chamber fruits when RH never got above 82% throughout the entire period of storage within the treatment chamber. The resulting disease caused by *C. gloeosporioides* during this period of storage can be observed in Figure 5.23.

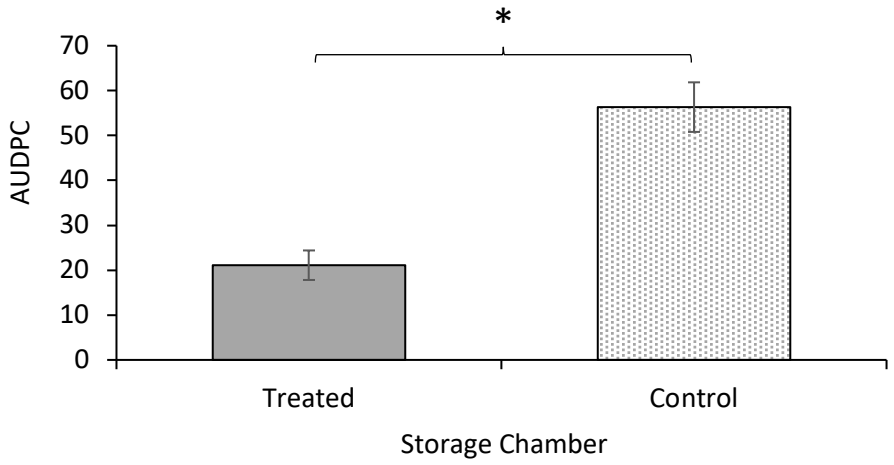


Figure 5.23. Anthracnose disease on originally mature green Brioso variety tomato fruits at inoculation stored within either the ethylene scrubbing treatment chamber or control chamber during preliminary work. Mean AUDPC on samples with either chamber. Error bars represent SEM. The asterisk (*) represents a significant difference ($p < 0.05$) between chambers.

Table 5.6. Representation of the disease scores recorded from tomatoes stored within either the treatment or control chamber at 35 DPI during the experiment performed and represented as AUDPC in Figure 5.23.

Disease Score	***Treated***	***Control***
0 (0%)	0	0
1 (1%)	3	1
2 (2%)	10	1
3 (5%)	1	1
4 (10%)	0	1
5 (20%)	0	2
6 (40%)	1	2
7 (60%)	0	3
8 (+80%)	0	3

The results presented in Figure 5.23 and Table 5.6 further support the suggestion that decreasing humidity levels caused by UV photocatalytic ethylene scrubbing can't completely prevent disease as even at RH as low as 82%, 100% of samples produced anthracnose disease symptoms following *C. gloeosporioides* inoculations.

Results presented in Figure 5.22, Figure 5.23 and Table 5.6 therefore suggest that throughout this research, the observed reductions in anthracnose disease severity and occurrence as a result of UV photocatalytic ethylene scrubbing treatment is as a result of a combination of ethylene removal and humidity reductions caused by the scrubber.

Overall however, we have confirmed that the application of UV photocatalytic ethylene scrubbing to storage chambers is an effective method of preventing postharvest anthracnose disease on tomato fruits. Results proved to be consistent across all tomato varieties tested, and at all physiological stages the tomato fruits were inoculated.

CHAPTER SIX

6 UV-C HORMETIC TREATMENT ON DISEASE PREVENTION AND ITS EFFECTS IN COMBINATION WITH UV PHOTOCATALYTIC ETHYLENE SCRUBBING

6.1 INTRODUCTION

UV-C describes a wavelength of light that falls between 100 – 280 nm. UV-C light demonstrates germicidal properties and has commonly been used in high doses in decontamination processes due to its ability to inactivate a range of microbes. Hormesis is a phenomenon where low doses of a potentially damaging agent result in the organism being treated having a beneficial response. A mechanism was proposed for hormesis by (Luckey, 1980) where it was suggested that low doses of UV could inflict repairable damage to DNA, and that the slight trauma would activate repair mechanisms for radiation-induced DNA damage. This suggested a positive change in plant homeostasis as a result of sub-lethal radiation stimulating vital intracellular processes (Shama & Alderson, 2005).

UV-C hormesis has demonstrated numerous beneficial effects on a variety of fruits and fresh produce including ripening delays, increased nutritional content and pathogen resistance (Shama & Alderson, 2005; Civello *et al.*, 2007; Ribeiro *et al.*, 2012; Turtoi, 2013). The effects of UV-C hormesis on disease resistance are well documented with much research demonstrating the effects of treatments in reducing postharvest decays. UV-C hormesis induced disease resistance is achieved through defence response upregulations in combination with alterations to physiology and metabolism (Scott *et al.*, 2018). The production of phytoalexins and pathogenesis-related (PR) proteins, delayed ripening and senescence, and the establishment of physical barriers which inhibit pathogen progression are all responses to UV-C hormetic treatment which increase disease resistance (Mercier *et al.*, 2000; Romanazzi *et al.*, 2006; Charles *et al.*, 2008a, 2009; Scott *et al.*, 2018).

Conventional low-pressure mercury sources which emit UV light at a peak emission of 254 nm at relatively low intensities have been used to conduct UV-C hormetic treatments in the majority of previous research and been referred to as low intensity UV-C (LIUV). Publications recently however have demonstrated the potential of high-intensity, pulsed polychromatic light (HIPPL) to deliver hormetic treatments, as they have been shown to induce similar hormetic benefits to LIUV treatments (Koyyalamudi *et al.*, 2011; Rodov *et al.*, 2012; Pataro *et al.*, 2015; Scott *et al.*, 2017). In a previous study, it was observed that a 16 pulse HIPPL treatment at 4.6 kJ/m^2/pulse induced disease resistance in tomato fruits against *B. cinerea* at a level comparable to LIUV treatments at a 3.7 kJ/m^2 dose (Scott *et al.*, 2017). The treatment times of LIUV required to apply the necessary UV-C doses to fruit crops (3.7 kJ/m^2 for tomatoes (Maharaj *et al.*, 1999)) are long and one of the reasons why the horticultural sector have shown disinclination to adopt UV-C hormetic treatments. However, 97.3% reduction times from 370 s to 10 s were observed by using HIPPL in comparison to LIUV (Scott *et al.*, 2017).

The aims of this this study were to investigate the effects of UV-C hormetic light treatments, from both LIUV and HIPPL sources, in combination with ethylene scrubbing treatment against disease caused by *B. cinerea* and *C. gloeosporioides*. If the combination of treatments proved successful in postharvest disease prevention against these pathogens, it could suggest a model for postharvest treatment regimens to reduce/prevent decay caused by *B. cinerea* and *C. gloeosporioides*. Furthermore, work performed with the HIPPL source against *C. gloeosporioides* would be the first of its kind. Therefore, experiments were also performed to investigate whether the HIPPL source was able to induce resistance against anthracnose disease caused by *C. gloeosporioides*.

6.2 MATERIALS AND METHODS

6.2.1 UVC light treatment

Meccano variety tomato fruits were treated with specified UV light treatments on the day of arrival within glasshouses at the University of Nottingham, Sutton Bonington Campus (UK).

UV light treatments were performed with the help of Dr George Scott. Treatments with the LIUV source were performed using a U-shaped amalgam UV source (UVI 12OU2G11 CP15/469) obtained from Dr Hönle AG, Gräfelfing, Germany with a 254 nm peak emission and mounted within an anodised aluminium parabolic reflector. Specific doses of 3.7 kJ/m^2 at an intensity of 20 W m^{-2} were delivered following the procedures of (Charles *et al.*, 2008b) and (Scott *et al.*, 2017). A portable radiometer (Model UVX, UVP Instruments, Cambridge) fitted with a 254 nm sensor was used to measure intensity.

Treatments with the HIPPL source were performed using a XENON LH-840 16” ozone-free B lamp powered by an RT-847 cabinet and RC-802 controller, supplied by Lambda Photometrics (Harpenden, Herts). Per pulse, 505 J of energy were produced by the source with a pulse width of 360 µs at 3.2 pulses per second. Source spectral emission lay between 240 nm and 1050 nm. Fruit were treated at a distance of 10 cm from the window of the lamp housing as described in (Scott *et al.*, 2017). An estimated 4.6 kJ/m^2/pulse was delivered at fruit level through extrapolation of the manufacturer’s data.

For either treatment source, fruit received exposure on two opposing sides through 180^o axial rotation. A control group was taken out to the glasshouses

and remained untreated with either light source as a comparison to the two sources. Following treatments, fruits were immediately stored in the dark until surface sterilisation (as described in section 2.7). Following sterilisation, fruits were stored in the dark and moved into storage within either the ethylene scrubbing treatment chamber or control chamber, dependent on what treatment regime they were assigned. Fruits were stored in the dark to prevent the effects of photoreversal – where subsequent exposure to visible light can negate the effects of UV-C hormesis (Kelner, 1949). Fruits were stored for the 10 d within either chamber prior to inoculation with a specified pathogen.

Results from each pathogen, and each physiological stage that the tomato fruits were light treated at were collected from two independent replicate experiments.

6.3 RESULTS

6.3.1 UVC light treatments in combination with ethylene scrubbing against *Botrytis cinerea*

There were clear and significant differences observed in disease severity as a result of both light treatments and ethylene scrubbing against *B. cinerea* disease. With regards to light treatments, the effects of both HIPPL and LIUV sources proved to be far more effective in disease prevention when applied to fruits at a mature green physiological stage. For both mature green and red ripe fruits, *B. cinerea* disease in the treatment chamber was more severe than on fruits stored in the control chamber. This result was more severe in red ripe fruit which therefore shows strong similarity to the results seen previously with *B. cinerea* in section 5.3.1. These results can be seen in Figure 6.1.

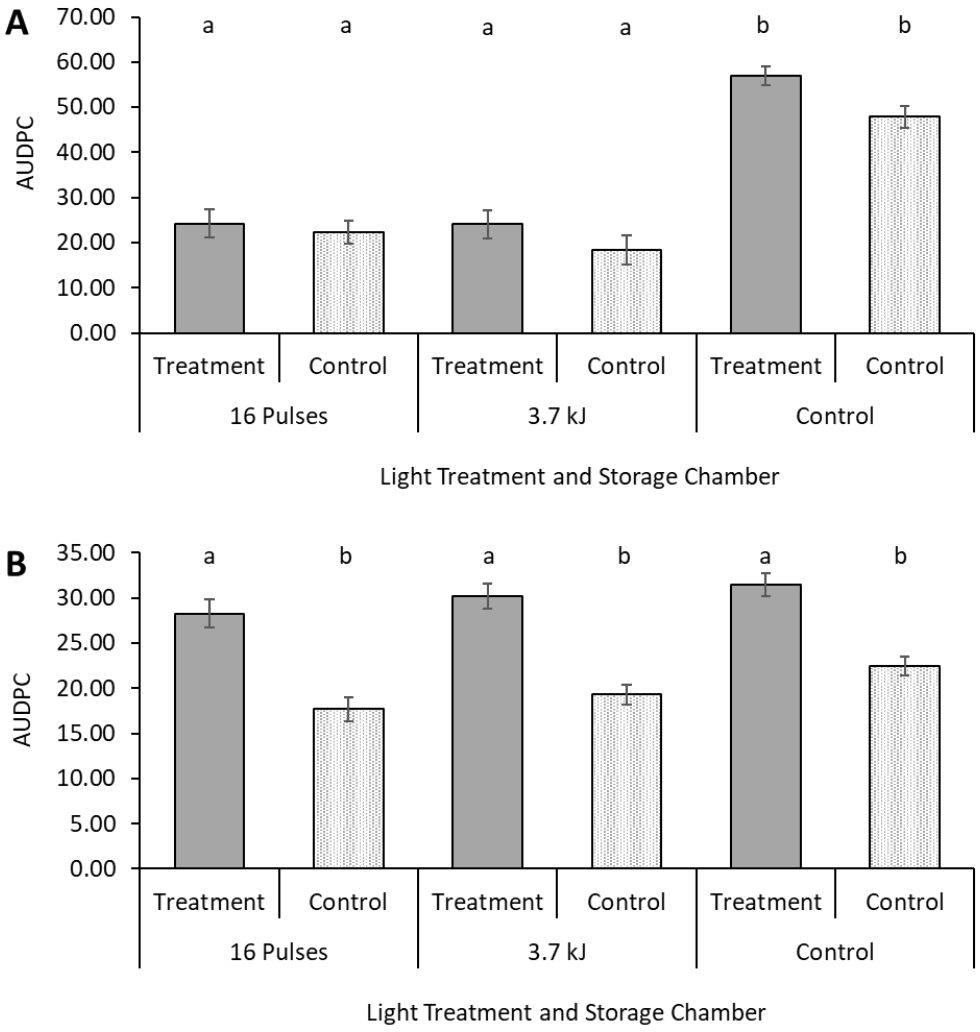


Figure 6.1. The effects of UV-C hormetic light treatments in combination with either storage in the ethylene scrubbing treatment chamber or control chamber on the mean AUDPC caused by *Botrytis cinerea*. Error bars represent SEM. Labelling indicates statistical significance ($p < 0.05$). Means sharing the same label are not significantly different from each other. A) Fruits light treated and stored prior to inoculation at a mature green stage. B) Fruits light treated and stored prior to inoculation at a red ripe stage.

The results presented in Figure 6.1 suggest that there are no benefits in combining the UV-C hormetic treatments with ethylene scrubbing treatments to prevent *B. cinerea* disease on tomato fruits. As seen in section 5.3.1, disease caused by *B. cinerea* is made more severe by storage within the treatment chamber than in the control chamber. Here, this effect has been seen in both red ripe and mature green fruits. Disease severity differences between chambers are greater in red ripe fruit (Figure 6.1b). In mature green fruits (Figure 6.1a), although disease is greater in the treatment chamber, the results are not significant as they are with red ripe fruit. Perhaps this is as a result of ripening delays, further to those caused by light treatments, induced during the 10 d storage in the absence of ethylene. Ripening delays would mean that at the point of inoculation, treatment chamber fruits would be slightly less susceptible to disease than control chamber fruits due to the higher intrinsic resistance associated with unripe fruit (Droby *et al.*, 1992; Alkan & Fortes, 2015). However, even with these slightly higher levels of disease resistance, *B. cinerea* disease severity appears slightly worse as a result of ethylene scrubbing treatment.

In mature green fruit, both 16 pulse HIPPL and 3.7 kJ LIUV treatments induced significant disease resistance against *B. cinerea*. This result was observed in both storage chambers. This result supports the results from our previous study (Scott *et al.*, 2017) as well as numerous other publications demonstrating UV-C hormetic treatments reducing *B. cinerea* disease severity (Liu *et al.*, 1993; Mercier *et al.*, 2000; Marquenie *et al.*, 2003; Romanazzi *et al.*, 2006; Charles *et al.*, 2008b; Scott *et al.*, 2018). Induced disease resistance to *B. cinerea* can be attributed to the stimulation of defence responses which are mediated by salicylic acid, jasmonic acid and ethylene as a result of treatment from either source (Scott *et al.*, 2018).

With red ripe fruits, although there was a slight decrease in disease severity as a result of UV-C hormetic treatments, the results were not as clear as seen with mature green fruits, nor were they significant. These results both support and contradict what was previously seen in our research (Scott *et al.*, 2017). Furthermore, the failure of the LIUV treatment to significantly induce disease resistance is supported by Obande *et al.* (2011) who demonstrated that 3 kJ/m^2 treatments pre-harvest could not inhibit *P. digitatum* disease progression on ripe Meccano tomatoes. Previously, it was shown that LIUV treatment on red ripe fruits resulted in no significant *B. cinerea* reductions; however, the use of a 16 pulse HIPPL treatment did result in a significant drop in disease severity compared to control fruit. Here, disease in both chambers was lower as a result of 16 pulse treatment in comparison to 3.7 kJ/m^2 LIUV treatment and the control group although the result was not significant across two experiments.

The differences in disease reductions as a result of light treatments in fruits treated at a mature green or red ripe physiological stage could be attributed to the light treatments ability to delay fruit ripening. As previously discussed, fruit ripening brings about increased disease susceptibility (Alkan & Fortes, 2015). The ability of UV-C hormetic light treatments to reduce the ripening rate of mature green tomato fruits will no doubt contribute to the observed reductions in disease incidence and severity compared with control fruits. Due to red ripe fruits already being at a highly susceptible stage for pathogen attack, the induction of defence responses by UV-C hormetic light treatments are always likely to be less effective than on mature green fruits were the induction of defence gene pathways by treatments can supplement induced ripening delays to produce much more resistant host fruits.

6.3.2 UVC light treatments in combination with ethylene scrubbing against *Colletotrichum gloeosporioides*

As expected from the results in section 5.5.1, regardless of light treatments the fruits were previously subjected to, disease severity was consistently worse on fruits stored within the control chamber in comparison to the treatment chamber. This was seen on fruits harvested at both red ripe and mature green physiological stages (Figure 6.2 and Figure 6.3).

The effects of light treatments on fruits produced some interesting results. For mature green light treated fruits within the control chamber, disease was significantly less severe than on control fruits (Figure 6.2). However, in red ripe fruits, the opposite effects were observed and light treatments appeared to increase fruit disease susceptibility to anthracnose (Figure 6.3).

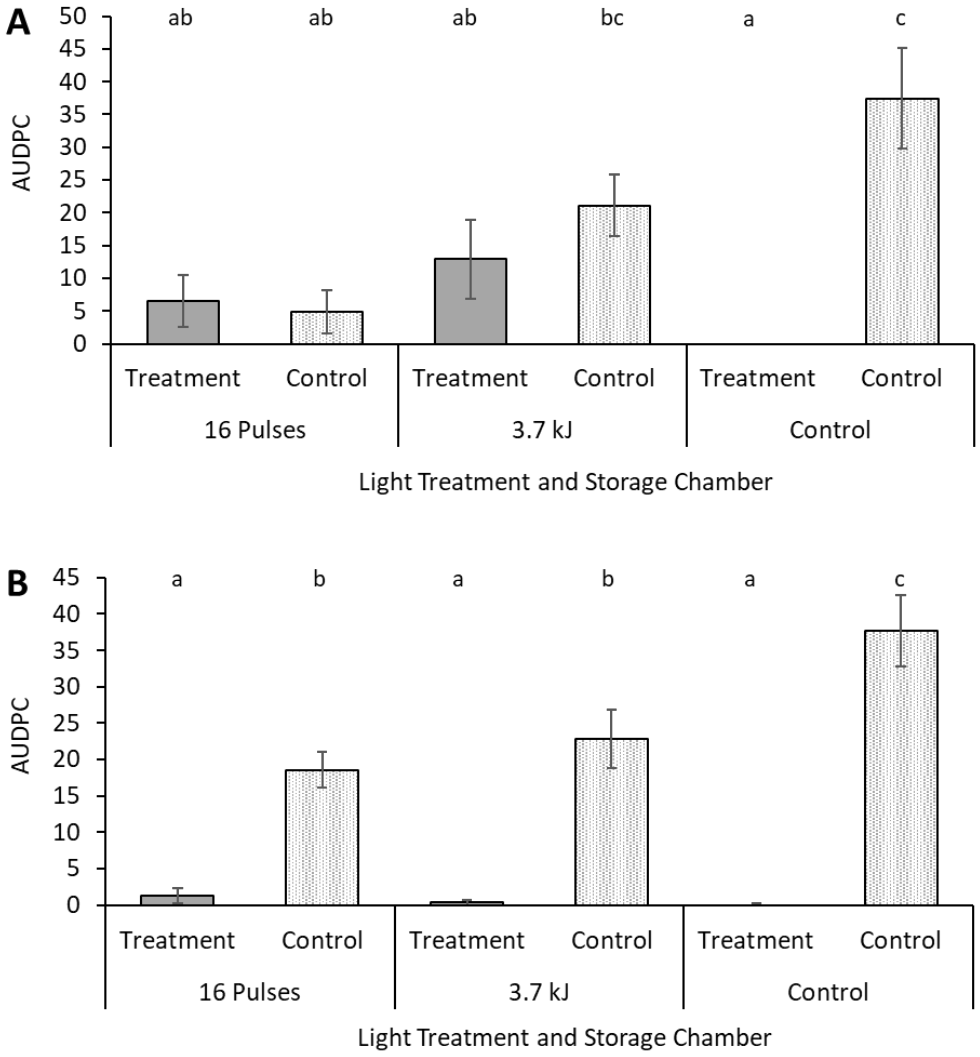


Figure 6.2. The effects of UV-C hormetic light treatments on mature green tomatoes in combination with either storage in the ethylene scrubbing treatment chamber or control chamber on the mean AUDPC caused by *Colletotrichum gloeosporioides*. Error bars represent SEM. Labelling indicates statistical significance ($p < 0.05$). Means sharing the same label are not significantly different from each other. A) Results from the original experiment. B) Results from the repeat experiment. Results could not be compiled due to disease being scored on different DPI to calculate AUDPC. Different disease scoring days were used as a result of more rapid pathogen progression in the repeat experiment.

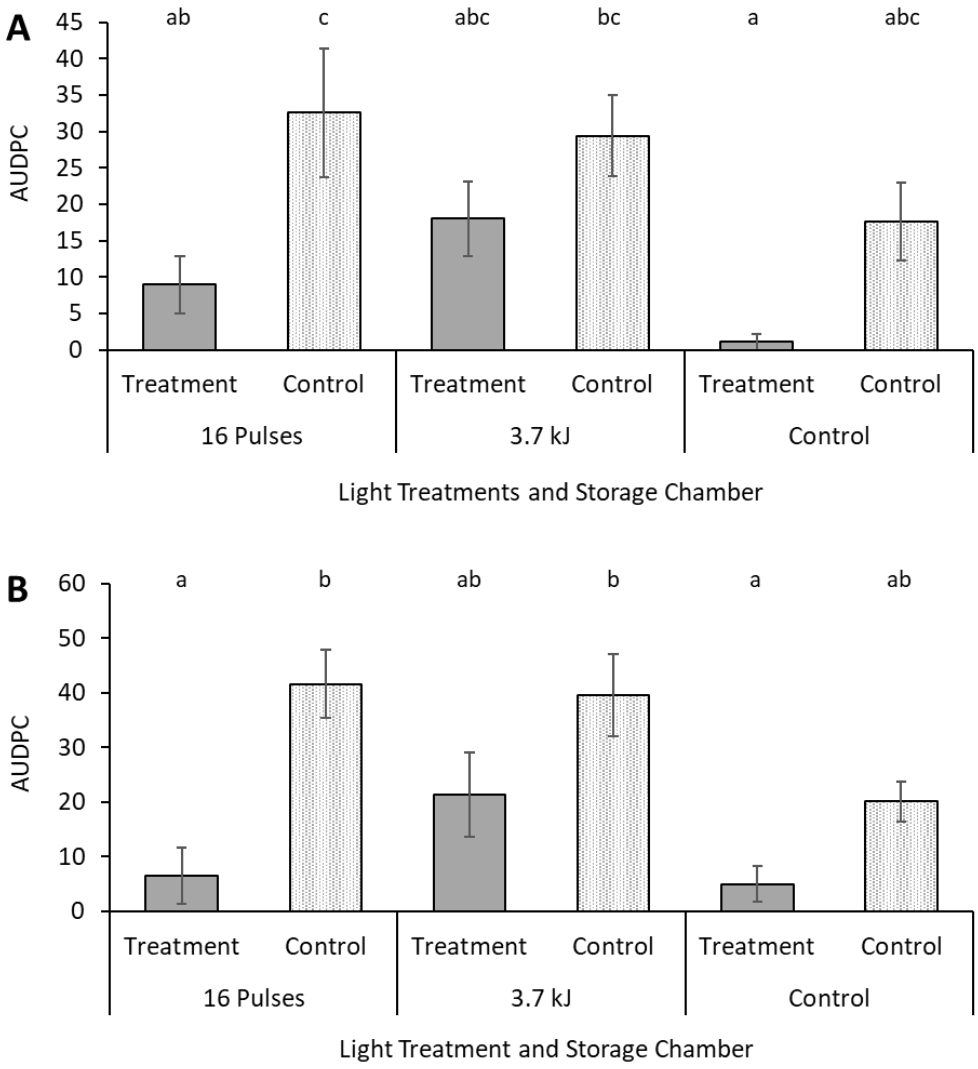


Figure 6.3. The effects of UV-C hormetic light treatments on red ripe tomatoes in combination with either storage in the ethylene scrubbing treatment chamber or control chamber on the mean AUDPC caused by *Colletotrichum gloeosporioides*. Error bars represent SEM. Labelling indicates statistical significance ($p < 0.05$). Means sharing the same label are not significantly different from each other. A) Results from the original experiment. B) Results from the repeat experiment. Results could not be compiled due to disease being scored on different DPI to calculate AUDPC. Different disease scoring days were used as a result of more rapid pathogen progression in the repeat experiment.

Results presented here demonstrate positive and negative effects of UV-C hormetic treatments in postharvest *C. gloeosporioides* prevention. In mature green fruits, it is clear that UV-C light treatments from both LIUV and HIPPL sources result in anthracnose disease reductions within both storage chambers (Figure 6.2). However, the effect of light treatments on red ripe fruits is induction of increased disease severity in comparison to control fruits (Figure 6.3).

Previously in the literature, UV-C treatment has demonstrated positive and negative effects against *C. gloeosporioides*. As well as anthracnose, the pathogen is known to cause bitter rot in apples. It has been observed that the application of UV-C has reduced bitter rot decay caused by *C. gloeosporioides* on apple fruit (Stevens *et al.*, 1996). Furthermore, *in vitro* UV-C treatment of the strain of *C. gloeosporioides* which causes papaya anthracnose resulted in mycelial growth inhibitions and reductions in fungal sporulation (Cia *et al.*, 2007). However, the same study reported that there was no effect of UV-C doses on anthracnose control and fungal sporulation in fruit lesions (Cia *et al.*, 2007).

Here, the effects of both LIUV and HIPPL source treatments on mature green tomato fruits have been demonstrated to induce significant reductions in anthracnose disease (Figure 6.2). These are the first reported effects of HIPPL sources on *C. gloeosporioides* disease. Moreover, they are the first reports of UV-C radiation inducing disease reductions on *in planta* postharvest anthracnose disease caused by *C. gloeosporioides*. It is likely that disease resistance is stimulated in the same way as observed against *B. cinerea* and other pathogens by inducing the up-regulation of defence related pathways,

and delaying the ripening of fruits. These results could prove significant to the postharvest market.

CHAPTER SEVEN

7 MOLECULAR BASIS OF SCRUBBER INDUCED *COLLETOTRICHUM GLOEOSPORIOIDES* RESITANCE

MOLECULAR BASIS OF SCRUBBING INDUCED *COLLETOTRICHUM GLOEOSPORIOIDES* RESISTANCE

7.1 INTRODUCTION

Consistent and significant reductions in anthracnose disease as a result of ethylene scrubbing led to questions regarding why disease prevention was observed. Here, the aim was to elucidate the molecular mechanisms induced, within both fruit and pathogen, to determine how the effects of ethylene scrubbing consistently prevents *C. gloeosporioides* infection.

Upregulation of defence responses in combination with alterations in metabolism and physiology stimulate disease resistance in host plants. The production of pathogenesis-related (PR) proteins and phytoalexins, delays in ripening and senescence, and inhibition of pathogen progression due to establishment of physical barriers, are all changes that give rise to disease resistance (Spoel *et al.*, 2007; Spoel & Dong, 2008; Charles *et al.*, 2009; Alkan & Fortes, 2015; Scott *et al.*, 2018). When confronted by a pathogen such as *C.* gloeosporioides, which can utilise both biotrophic and necrotrophic lifestyles, cross talk must be regulated within the host in order to fight off the pathogen at different stages of its attack (Spoel *et al.*, 2007; Spoel & Dong, 2008; Bari & Jones, 2009; Alkan & Fortes, 2015). This cross talk has been shown to optimize the response of a host against a single attacker (Spoel *et al.*, 2007).

In this study, the aim was to use genes associated with defence against both biotrophic and nectrotrophic pathogens to develop an understanding of how fruits alter gene regulation in order to defend against the *C. gloeosporioides*

hemibiotrophic lifestyle. Results between both chambers could then be compared to identify at what stage of infection the treatment caused disease resistance. This investigation aimed to identify which of the main signalling pathways, jasmonic acid (JA), salicylic acid (SA) and ethylene, if any, were induced by the ethylene scrubbing treatment.

Regulation of genes associated with fruit ripening was to be observed to determine whether any differences in expression between chambers could delay the development of *C. gloeosporioides* infection and ultimately prevent disease. Tomato genes examined for regulation during pathogen attack were also to be monitored on mock inoculated fruit. This would determine if there were any underlying effects on gene regulation prior to pathogen attack as a result of ethylene scrubbing treatment.

Further to understanding the processes that occur within tomato defence responses, it is important to understand the distinct fungal arms strategy that is associated with *C. gloeosporioides* attack (Alkan *et al.*, 2015). Many interactions between pathogen and host occur during plant infection (Jones & Dangl, 2006), so it is important to identify whether disease resistance could actually be due to treatment effecting the pathogen as opposed to upregulating defence response. Pathogens utilise many different methods in order to overcome plant defence mechanisms. *Colletotrichum gloeosporioides* has many different components of its genome required for successful infection of a host. Upregulation of specific genes at specific times during infection is crucial for successful colonization (Stephenson *et al.*, 2000, 2005; Kim *et al.*, 2002; Chagué *et al.*, 2009; Nesher *et al.*, 2011; Alkan *et al.*, 2013; Shnaiderman *et al.*, 2013; Sharma & Kulshrestha, 2015).

Regulation of pathogenicity genes from *C. gloeosporioides* was analysed to identify whether resistance was caused due to inhibition of pathogenicity genes. With this information we could identify whether resistance is therefore a result of: up-regulation of tomato defence genes, inhibition of pathogenicity genes, or a combination of the two. Specific pathogenicity associated gene primers were selected for experimentation in order to examine any differences in gene regulation between chambers associated with spore germination through to aggressive necrotrophic tissue maceration.

7.2 MATERIALS AND METHODS

7.2.1 Tomato gene primers

The same primers, primer concentrations and annealing temperatures were used for experimentation as seen in Scott *et al.* (2018) (see Table 7.1). Primer concentrations were tested on C. gloeosporioides infected tissue and healthy uninfected tissue from Shirley tomato fruit samples prior to this investigation by performing a 5-point 5-fold dilution series using a pooled cDNA sample to calculate primer efficiency (see Equation 7.1). Calculated amplification efficiency (AE) was used to confirm appropriate use of specified primer concentrations (Table 7.1). Furthermore, this prior testing was performed to confirm qPCR machine specifications used for these primers (i.e. appropriate annealing temperatures) as well as to confirm primers worked with Shirley variety tomatoes.

$$AE = D\left(\frac{-1}{V}\right) \qquad \text{Equation 7.1}$$

Amplification efficiency (AE). Equation shows fold dilution (D) and gradient of the logarithmically plotted dilution curve (V) (Pfaffl, 2004).

Table 7.1 Details of tomato primers used in qPCR. All primers were used following work performed by Scott *et al.* (2018).

Target Gene	*Reference*	*Accession No.*	*Product Tm (°C)*	*Concentration (nm)*	*Anneal (°C)*	*Efficiency (%)*	*Sequence 5'-3'*	*Primer Source*
ACTIN	Aimé *et al.* (2008)	U60480	74.4	100	60	103.5	F:AGGCACACAGGTGTTATGGT R:AGCAACTCGAAGCTCATTGT	Eurofins Genomics
ACO1	Van de Poel *et al.* (2012)	X04792	72.3	500	60	106.7	F:ACAAACAGACGGGACACGAA R:CCTCTGCCTCTTTTTCAACC	Eurofins Genomics
CHI9	Aimé *et al.* (2008)	Z15140	77.8	50	58	105.4	F:GAAATTGCTGCTTTCCTTGC R:CTCCAATGGCTCTTCCACAT	Eurofins Genomics
CRTR-B1	Tiecher *et al.* (2013)	SGN-U58606	78.2	500	60	101.3	F:TTGGGCGAGATGGGCACAC R:TGGCGAAAACGTCGTTCAGC	Sigma-Aldrich
FLS	Tiecher *et al.* (2013)	GI 225321931	71.8	250	60	90.7	F:ATGGAGGCAGCTGGTGGTGAA R:CAGGCCTTGGACATGGTGGATA	Sigma-Aldrich
GLUB	Aimé *et al.* (2008)	M80608	75.2	100	60	89.9	F:TCTTGCCCCATTTCAAGTTC R:TGCACGTGTATCCCTCAAAA	Eurofins Genomics
OPR3	Blanco-Ulate *et al.* (2013)	Solyc07g007870	76.1	300	60	94.0	F:TGGGTTTCCTCATGTGCCAG R:GCAGCTCCAGCAGGTTGATA	Eurofins Genomics
PAL	Bovy *et al.* (2002)	M83314.1	74.0	500	60	97.5	F:ATTGGGAAATGGCTGCTGATT R:TCAACATTTGCAATGGATGCA	Eurofins Genomics
PG	Xie *et al.* (2014)	X05656.1	74.3	250	58	94.9	F:ATACAACAGTTTTCAGCAGTTCAAGT R:GGTTTTCCACTTTCCCCTACTAA	Eurofins Genomics
PR1a	Aimé *et al.* (2008)	AJ011520	80.6	250	58	100.8	F:TCTTGTGAGGCCCAAAATTC R:ATAGTCTGGCCTCTCGGACA	Eurofins Genomics

7.2.2 *Colletotrichum gloeosporioides* gene primers

Primers and primer conditions used for experimentation are listed in Table 7.2. Prior to this investigation, cDNA from Shirley tomato fruit samples infected with *C. gloeosporioides* was pooled and used to test various primer concentrations by running a 3-point 5-fold dilution series to calculate primer AE (Equation 7.1). Optimum primer concentration was selected for whereby sample serial dilutions within a specified primer concentration gave a primer AE percentage as close to 100% as possible. Primers for the target genes Chip6, Cap20 and CgRac1 were all designed through online software package Primer3.

7.2.3 Specificity of primers

Product specificity from each primer pair was confirmed by sequencing products from the 5-point 5-fold serial dilution ran on each primer qPCR plate (section 7.2.9). Products were prepared using the QIAquick® PCR purification Kit to purify cDNA following manufacturers guidelines. The purified products were then sent to Eurofins Genomics (Europe) for sequencing. Specificity of product was confirmed using NCBI BLAST analysis.

Table 7.2 Details of *Colletotrichum gloeosporioides* primers used in qPCR

Target Gene	*Reference*	*Accession No.*	*Product Tm (°C)*	*Concentration (nm)*	*Anneal (°C)*	*Efficiency (%)*	*Sequence 5'-3'*	*Primer Source*
ACTIN	Alkan *et al.* (2013)	* Unknown*	80.1	100	60	97.2	F: AGCGGAAAGCCTCGCAGT R: TGTCGTTACCATCTCGACCCA	Sigma-Aldrich
PEL-B	Kramer-Haimovich *et al.* (2006)	U32942	76.2	100	60	94.4	F: CACCAAGCCCGACTACAGCT R: AGCCTTACCTTGGAGGAGCC	Sigma-Aldrich
CHIP6	Designed with Primer 3	AF001285.3	80.9	100	60	91.5	F: TGCATGGTGAGAGGTCAGAG R: GCACCCTGGAAGTCCTGATA	Sigma-Aldrich
MEPB	Shnaiderman *et al.* (2013)	JN660156.1	81.7	100	60	91.5	F: ATGGTCTTCGCCTTCATCTGG R: AGGAAATGTGGACGGGAGTA	Sigma-Aldrich
Cap20	Designed with Primer 3	U18061	80.5	100	60	89.8	F: GGCCACTGCTCAGGTAAGAG R: CGAGAGGGTTGTTCCTGAAG	Sigma-Aldrich
CgRac1	Designed with Primer 3	EU348861.1	81.1	100	60	90.2	F: ACATCCCCATCATCCTCGTC R: CTTGATTTCCTTGGCGCAGA	Sigma-Aldrich

7.2.4 Plant Material

Tomato fruit, cv. Shirley were harvested from glasshouses at the University of Nottingham (UK) at a mature green developmental stage (see section 2.5.1).

7.2.5 Experimental Design

Data was collected from three independent replicate experiments. For each experiment, three fruit per treatment group per time point were analysed. All fruits were either pathogen inoculated or mock inoculated on the day of harvest before being placed into either the ethylene scrubbing or control chamber. Fruits were sampled pre-treatment and pre-inoculation following harvest (baseline expression), and at 14 d and 24 d post inoculation (DPI) and of treatment (DoT) from pathogen inoculated and mock inoculated fruit respectively. Fourteen DPI was selected as the first tissue sampling time point as it was when the first early symptoms (disease score 1) could be detected on some of the tomatoes from previous experiments. Furthermore, preliminary work during *C. gloeosporioides* primer testing showed no detection of *C. gloeosporioides* target gene expression prior to this time post inoculation. Twenty-four DPI was selected as the second sample point as by this stage post inoculation disease would be severe on the fruit. Therefore, it would be possible to analyse gene expression within fruit and pathogen at what would be a necrotrophic, very aggressive stage of infection. Five further fruit were inoculated with *C. gloeosporioides* per group and disease scores were recorded at 24 DPI.

7.2.6 Tissue sampling

A scalpel was sterilised and used to take a 50 to 150 mg sample of epidermis and underlying pericarp from tissue directly pathogen or mock inoculated. A scalpel was used to cut approximately a 1 by 2 cm rectangle from the tissue. Samples were placed into a micro-centrifuge tube containing a 4 mm steel bead (Qiagen) and immediately frozen in liquid nitrogen. Samples were stored at -80°C until required for RNA extraction.

7.2.7 RNA extraction

Samples were taken from the -80°C and homogenised using a Tissuelyser II (Qiagen). Samples were homogenised across two runs at 30 Hz for 30 s whereby tissue lyser blocks were rotated in between runs. The RNeasy Plant Mini Kit (Qiagen) was used for RNA extraction by following manufacturer's guidelines. An RNase-free DNase kit (Qiagen) was used following manufacturer's guidelines as an extra on-column DNase treatment. RNA purity and yield were assessed using a Nanodrop (Thermo Scientific) following extraction. Sample RNA's were then diluted to <250 ng/µl where required. Samples where RNA was undetected or showed impurity were discarded and replaced by extra samples taken from each treatment group.

7.2.8 Reverse transcription

Prior to qPCR, RNA required converting to cDNA. A High-Capacity RNA to cDNA kit (Applied Biosystems) was used to perform a 20 µl reverse transcription reaction following manufacturer's protocol. A Flexigene (Techne)

PCR machine was used to run the reverse transcription reaction. The resulting cDNA was stored in -20°C until required.

7.2.9 qPCR

Each 10 µl reaction contained 2 µl of sample template cDNA and 5 µl of 2 x Fast SYBR® Green master mix (Applied Biosystems). Annealing temperatures and primer concentrations were as stated in Table 7.1 and Table 7.2. Reactions were run on a LightCycler 480c (Roche). A two-step amplification cycle was run as follows: pre-incubation at 95°C for 10 min followed by 50 cycles of 95°C for 5 s and the anneal step for 45 s. The second derivative maximum method was used to calculate Cycle threshold (Ct) values. Products were checked for specificity by running a melting curve between 90°C and 60°C following amplification.

Each gene of interest was run on its own 384 well plate (Roche). Three technical replicates were performed for each sample as well three technical replicates of a non-template control (NTC). Plates contained a 5-point 5-fold dilution series used to calculate efficiency of amplification (Equation 7.1). Application efficiency percentages calculated from serial dilutions on each qPCR primer plate are listed in Table 7.1 and Table 7.2.

Melt curve analysis was used to test for non-specific products within each sample well. Correct product Tm values are also listed in Table 7.1 and Table 7.2. Samples were considered non-specific if sample wells produced a Tm value ≥ ±1.5°C the listed figure. Samples were also considered non-specific if the resulting Ct value was ≥ 45. Sample wells exhibiting characteristics of non-specific products were considered unfit for further analysis.

Application efficiency calculated from the serial dilutions of each plate were used to correct Ct values (see Equation 7.2). Results from technical repeats were then averaged prior to further analysis.

$$CtE = Ct \times \frac{Log10(AE)}{Log10(2)} \quad \text{Equation 7.2}$$

Efficiency correction of cycle threshold (Ct) values. CtE represents the efficiency corrected Ct value and AE is the efficiency of application (Scott *et al.*, 2018).

Two different types of actin genes were used as reference genes depending on genes being tested. For target genes in tomato fruits, the actin gene previously used in Scott *et al.* (2018) was used. For *C. gloeosporioides* related target genes, the actin gene previously used in Alkan *et al.* (2013) was used. Following efficiency correction, the specified actin genes were used to normalise the data giving ∆Ct (Equation 7.3).

$$\Delta Ct = CtE(goi) - CtE(ref) \quad \text{Equation 7.3}$$

Gene of interest normalisation using the reference gene. CtE(goi) represents the efficiency corrected Ct value for the gene of interest and CtE(ref) represents the efficiency corrected Ct value for the reference gene.

Data collected was normalised to baseline level (pre-treatment) gene expression. Fold change between treatment groups was calculated following Equation 7.4.

$$Foldchange = 2^{-(\Delta CtET - \Delta CtEC)}$$ Equation 7.4

Calculating fold change. ΔCtET is normalised and efficiency corrected mean Ct value for the treatment group and ΔCtEC is the normalised and efficiency corrected mean Ct value for the control group (Livak & Schmittgen, 2001).

7.2.10 Data analysis

Statistical analysis of results was performed on the normalized and efficiency corrected Ct values (ΔCt). Method of statistical analysis is as described in section 2.11.

7.3 RESULTS AND DISCUSSION

As stated in section 7.2.5, per experiment, five extra fruit per chamber were inoculated with *C. gloeosporioides*. This was to confirm that results from these three experiments matched what was seen in section 5.5. It would also confirm that these particular pathogen inoculations worked. Results compiled from all three experiments can be seen in Figure 7.1 whilst examples of the disease symptoms produced on sample fruits from both chambers can be seen in Figure 7.2.

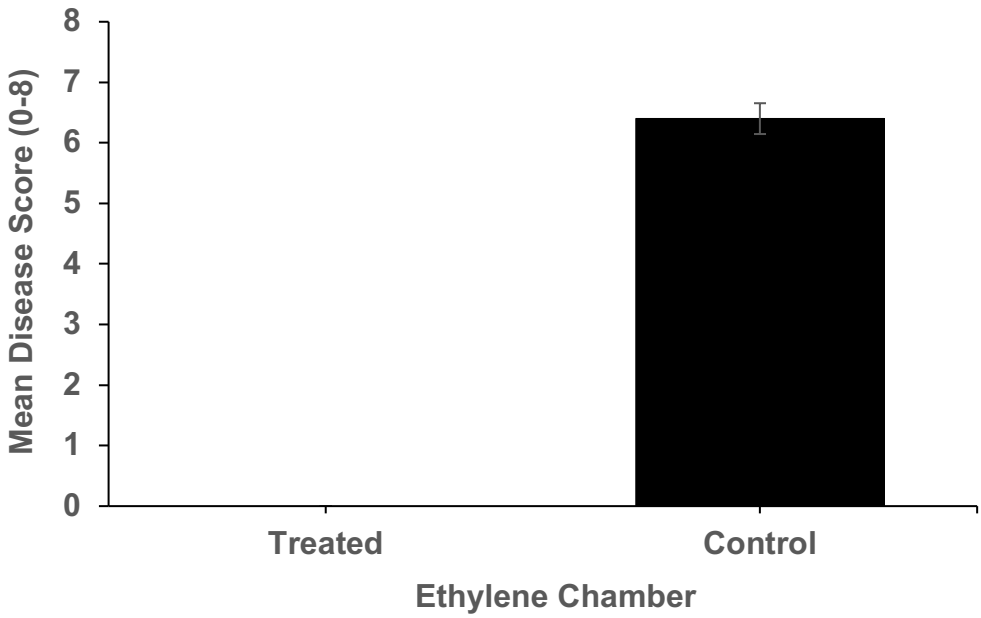


Figure 7.1. Mean of the combined disease scores from both chambers at 24 DPI. Results presented here represent compiled data from all three molecular experiments. Error bars represent standard error of the mean (SEM).



Figure 7.2. Images of diseased un-sampled tomatoes from the second molecular experiment at 24 DPI. A) Tomatoes stored in the treatment chamber. B) Tomatoes stored in the control chamber.

As can be seen in Figure 7.1 and Figure 7.2, the results from the three molecular experiments clearly follow on from the results seen in chapter 5. The ethylene scrubbing chamber tomatoes are showing no signs of disease whereas all the control chamber tomatoes have varying levels of pathogen leakage from the epidermis with internal softening covering almost the entirety of the inoculated area.

7.4 TOMATO GENE RESULTS AND DISCUSSION

Gene expression profiles were analysed and compared between ethylene scrubbing and control chambers. Analysis of genes involved in plant defence, secondary metabolism and ripening was made at 14 DPI/DoT and 24 DPI/DoT. Baseline expression pre-treatment was used to calculate relative changes in gene expression at each time point.

7.4.1 *ACO1* gene

As well as playing a primary role in many fruit physiological processes, ethylene can affect the expression of plant defence response genes (Ecker & Davis, 1987; Van Loon *et al.*, 2006) . Pathways regulated by jasmonic acid and salicylic acid, are often modulated by ethylene (Broekgaarden *et al.*, 2015). Ethylene is known to play a major role in regulating plant defence responses against necrotrophic pathogens (Bari & Jones, 2009) and has been reported to play a significant roles susceptibility to *B. cinerea* on tomato fruits and plants (Diaz *et al.*, 2002; Cantu *et al.*, 2009). ACO1 is one of five identified ACO enzymes in tomato which are involved in ethylene biosynthesis (Barry *et al.*, 1996; Sell & Hehl, 2005). *ACO1* is the main gene, out of *ACO1, ACO2* and

ACO3, which is expressed during *C. gloeosporioides* infection on tomato fruit (Cooper *et al.*, 1998).

ACO1 expression results can be seen in Figure 7.3. The results show that there were no real differences in *ACO1* expression between chambers at either 14 or 24 DPI for mock inoculated fruit. This result suggests that ethylene scrubbing treatment did not affect the production of ethylene by the treated fruit. It infers that ethylene is produced at the same rate as in untreated fruit. This result supports the hypothesis that the ethylene scrubber acts as an external ethylene removal system and is effectively removing produced ethylene as opposed to preventing ethylene production.

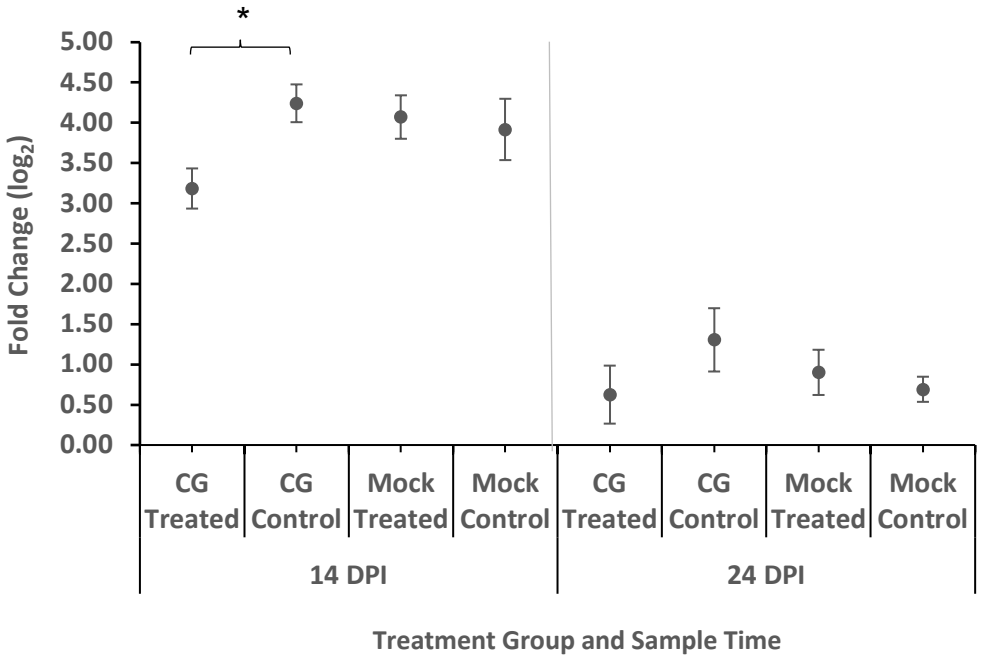


Figure 7.3. Relative expression of *ACO1* following ethylene scrubbing treatment (Treated) or storage within a control chamber (Control). Samples were inoculated with either a *Colletotrichum gloeosporioides* pathogen inoculum (CG) or a water inoculum (Mock). Samples were taken before inoculation and storage (baseline expression), at 14 days post inoculation (DPI) and 24 DPI. Fold changes (log_2) are relative to baseline expression. An asterisk (*) indicates statistical significance ($p < 0.05$) between groups recorded by independent T-test ($p \leq 0.05$). Error bars represent ± 1 S.E.M.

For the fruit inoculated with *C. gloeosporioides*, there is 2.08-fold and 1.60-fold less *ACO1* expression in the treatment chamber compared to control chamber at 14 DPI and 24 DPI respectively. At 14 DPI this difference between chambers is significant. Furthermore, at 14 DPI within the treatment chamber, pathogen inoculated fruit are producing significantly less (1.85 fold) *ACO1* expression than the mock inoculated fruit. This anomaly in results is difficult to explain. It would be expected that *C. gloeosporioides* inoculated fruits within the control chamber would have greater expression levels than those in the treatment chamber at 14 DPI as a response to the onset of pathogen virulence. However, the pathogen inoculated sample expression in the control chamber is in line with the mock inoculated samples. Therefore, the result that the *C. gloeosporioides* inoculated treated fruits *ACO1* expression is significantly less than what is seen in the control chamber is an apparently unexplainable anomaly.

7.4.2 *OPR3* gene

The phytohormone jasmonic acid (JA) plays a major role in defence against necrotrophic plant pathogens (Bari & Jones, 2009). 12-Oxophytodienoate reductase 3 (OPR3) acts as a catalyst in the penultimate step in JA biosynthesis. OPR3 reduces 9*S*, 13*S*-12-oxophytodienoate to the corresponding cyclopentanone, which is the precursor to JA (Schaller *et al.*, 2000; Breithaupt *et al.*, 2006). Expression of *OPR3* determines the expression of major genes in JA biosynthesis and furthermore, silencing of the *OPR3* gene has been shown to reduce callose deposition in tomatoes which leads to increased susceptibility to *B. cinerea* (Scalschi *et al.*, 2015).

Results presented here (Figure 7.4) show that at 14 DPI, samples from both chambers using either pathogen or mock inoculations, all expressed similar levels of *OPR3* at around 11-fold less than what is expressed at a mature green stage. This result is consistent with high levels of *OPR* genes expressed in immature and mature green fruit which decrease in expression during ripening (Kumar *et al.*, 2014).

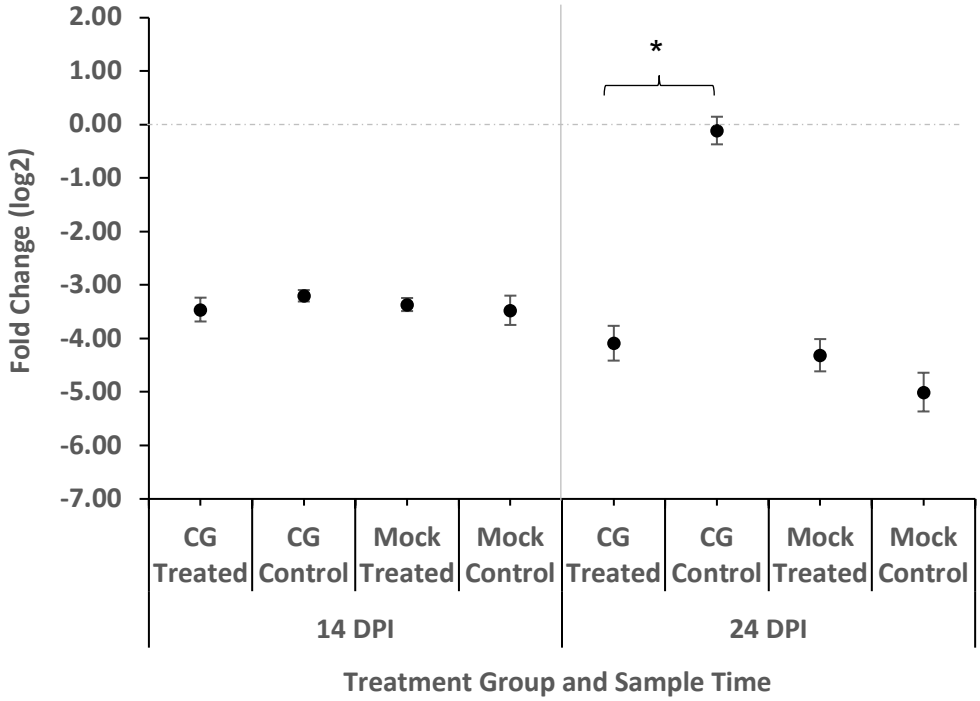


Figure 7.4. Relative expression of *OPR3* following ethylene scrubbing treatment (Treated) or storage within a control chamber (Control). Samples were inoculated with either a *Colletotrichum gloeosporioides* pathogen inoculum (CG) or a water inoculum (Mock). Samples were taken before inoculation and storage (baseline expression), at 14 days post inoculation (DPI) and 24 DPI. Fold changes (log_2) are relative to baseline expression (dashed line). An asterisk (*) indicates statistical significance ($p < 0.05$) between groups recorded by independent T-test ($p \leq 0.05$). Error bars represent ± 1 S.E.M.

At 24 DPI, *OPR3* expression in *C. gloeosporioides* inoculated samples in the control chamber is 15.76-fold greater than the corresponding samples in the treatment chamber. Expression of *OPR3* of control chamber inoculated samples is almost at the same detected level as was seen in the mature green fruits sampled at harvest. This indicates that the fruits are producing greater amounts of JA in order to combat the attacking *C. gloeosporioides*. This result makes sense as at 24 DPI in the control chamber, *C. gloeosporioides* is clearly in its necrotrophic stage based on symptoms observed on the fruit (Figure 7.2). This advanced necrotrophy of the pathogen will result in increased JA levels in order to activate JA-inducible plant defences involved in resistance against necrotrophic pathogens (Glazebrook, 2005).

At 24 DPI within the treatment chamber, very little difference in *OPR3* expression is detected between pathogen and mock inoculated fruit. This infers that the pathogen has not needed to increase its defence responses to fight off an invading pathogen. Instead it suggests that *C. gloeosporioides* has been prevented from entering its necrotrophic stage and remains inactive on the surface of inoculated fruit. This result suggests that possibly the ethylene scrubbing treatment is preventing the *C. gloeosporioides* functioning properly as opposed to stimulating increased defence responses within fruit samples.

7.4.3 *PR1a* gene

P4 (PR1a) is a salicylic acid-inducible PR protein monitored to infer changes in salicylic acid (SA) biosynthesis due to there being at least two SA biosynthesis pathways (Lee *et al.*, 1995; Scott *et al.*, 2018). Defence responses against biotrophic pathogens are regulated by SA (Glazebrook, 2005; Spoel & Dong, 2008; Bari & Jones, 2009). Salicylic acid activity has been seen to decrease

susceptibility to *C. gloeosporioides* early in infection by inhibiting the fungal conidia (Zhang *et al.*, 2016). The phytohormone also plays a role in establishing systemic acquired resistance (SAR). Defence pathways induced by SA often lead to programmed cell death which is why the phytohormone is so effective against biotrophic pathogens and not so beneficial against necrotrophs (Glazebrook, 2005; Spo*el et al*., 2007; Spoel & Dong, 2008).

Figure 7.5 shows that at both 14 DPI and 24 DPI, there are no significant differences in *PR1a* expression in mock inoculated fruits between the chambers. Furthermore, there is no significant difference between *C. gloeosporioides* inoculated fruit at 14 DPI. However, similarly to *OPR3* expression, at 24 DPI, expression of *PR1a* in control chamber samples is 12.83-fold greater than in treated samples. The fact that a similar result is not seen in the mock inoculated fruits at 24 DPI indicates that this increased expression is as a result of pathogen attack as opposed to the presence of ethylene within the control chamber.

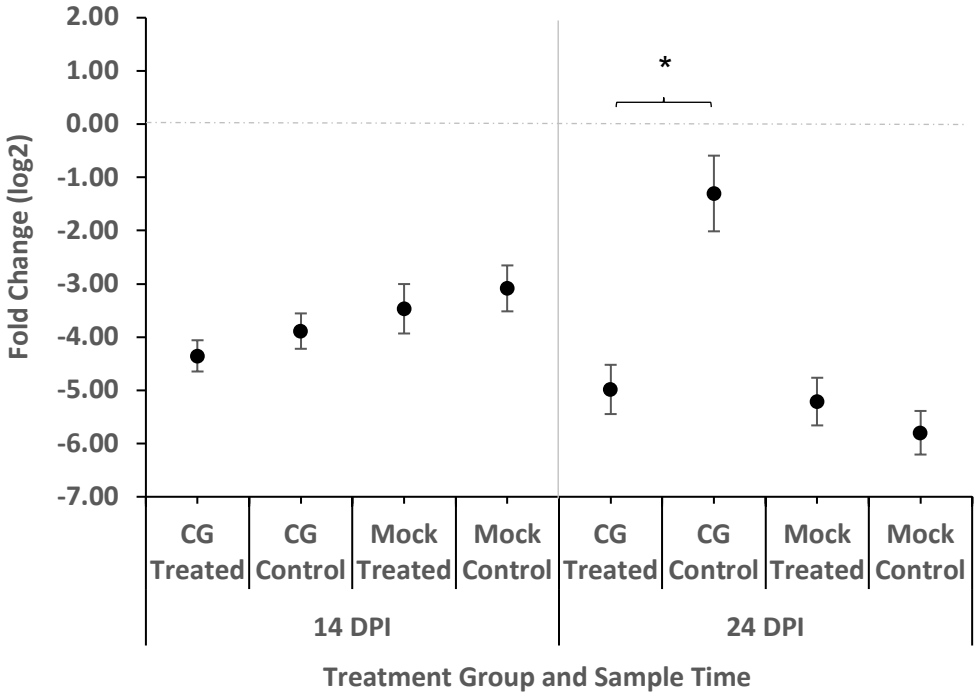


Figure 7.5. Relative expression of *PR1a* following ethylene scrubbing treatment (Treated) or storage within a control chamber (Control). Samples were inoculated with either a *Colletotrichum gloeosporioides* pathogen inoculum (CG) or a water inoculum (Mock). Samples were taken before inoculation and storage (baseline expression), at 14 days post inoculation (DPI) and 24 DPI. Fold changes (log_2) are relative to baseline expression (dashed line). An asterisk (*) indicates statistical significance ($p < 0.05$) between groups recorded by independent T-test ($p \leq 0.05$). Error bars represent ± 1 S.E.M.

The increasing *PR1a* expression as a result of pathogen attack is somewhat surprising. At 24 DPI *C. gloeosporioides* is very much in its necrotrophic lifecycle and therefore it is far more likely to observe increasing expression of JA regulated products to induce resistance (Glazebrook, 2005). However, SA biosynthetic and signalling pathways have been observed before in tomatoes as a response to *C. gloeosporioides* infections (Alkan & Fortes, 2015; Alkan *et al.*, 2015). Although this SA signalling has been shown to have an important role in the necrotrophic colonization stage of *C. gloeosporioides* on tomato, SA treatment to mango fruits inoculated with *C. gloeosporioides* has been shown to decrease lesion severity and occurrence. SA treatment was observed to increase chitinase, *β*-1,3-glucanase (GLUB), phenylalanine ammonia-lyase (PAL), polyphenoloxidase and the content of lignin and total phenolic compounds in mango fruit to control anthracnose decay and maintain fruit firmness (He *et al.*, 2017). Therefore, it is feasible that the fruit could be upregulating SA production in order to increase the activity of various other compounds listed above in order to fight off *C. gloeosporioides*.

It would have been good if fruit tissue had been sampled earlier than 14 DPI to examine the expression of this SA inducible protein during the biotrophic colonization phase of *C. gloeosporioides*.

7.4.4 *CHI9* gene

Chitinases are a group of enzymes that act to hydrolyse chitin within the fungal cell wall. Many chitinases are involved in defence against plant pathogenic fungi with SA treatments also said to increase chitinase activities (He *et al.*, 2017). Here, the activity of an ethylene, JA and wound inducible chitinase: *CHI9* (chitinase 1) (Diaz *et al.*, 2002; Wu & Bradford, 2003; Scott *et al.*, 2018)

was monitored. This gene has been reported to be upregulated during attack by *B. cinerea* (Levy *et al.*, 2015) indicating that it plays a role in defence against necrotrophic pathogens.

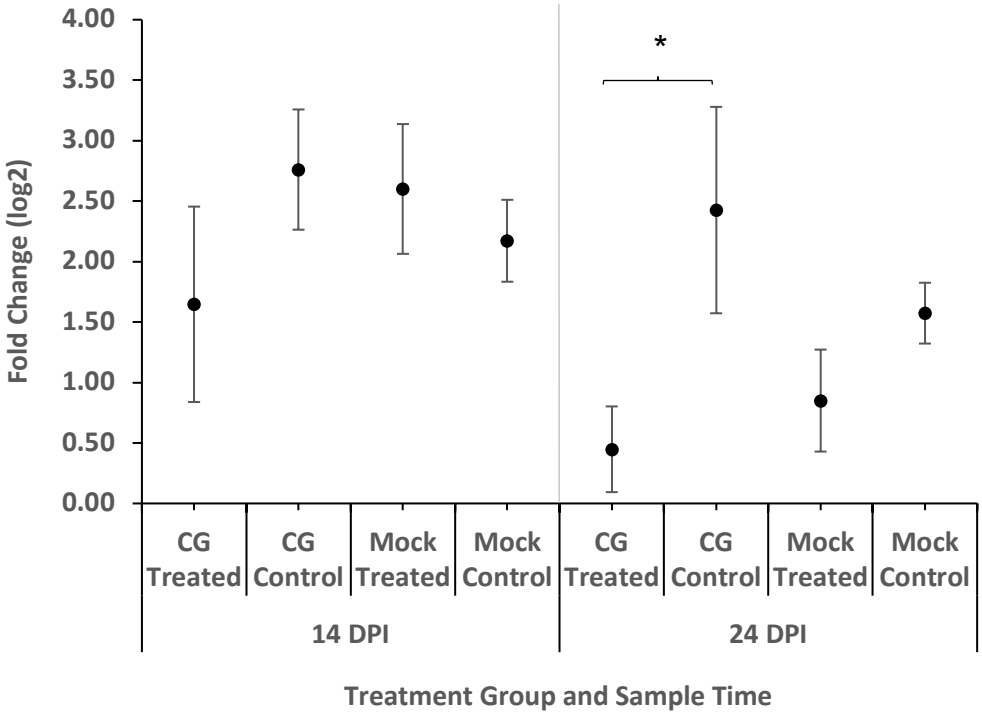


Figure 7.6. Relative expression of *CHI9* following ethylene scrubbing treatment (Treated) or storage within a control chamber (Control). Samples were inoculated with either a *Colletotrichum gloeosporioides* pathogen inoculum (CG) or a water inoculum (Mock). Samples were taken before inoculation and storage (baseline expression), at 14 days post inoculation (DPI) and 24 DPI. Fold changes (log_2) are relative to baseline expression. An asterisk (*) indicates statistical significance ($p < 0.05$) between groups recorded by independent T test ($p \leq 0.05$). Error bars represent ± 1 S.E.M.

The results show that at 24 DPI, *C. gloeosporioides* inoculated fruits in the control chamber produced significantly greater amounts of *CHI9* than in the control chamber (Figure 7.6). Expression of *CHI9* from pathogen inoculated fruit was 3.94-fold greater in the control chamber at 24 DPI. This suggests that the onset of virulence from *C. gloeosporioides* in the control chamber has stimulated the fruit to produce chitinase to fight off the pathogen, a result that correlates with what was seen with *OPR3* and *PR1a*. The observed correlation with *PR1a* is unsurprising as the increase in the SA inducible PR product indicates increasing SA levels; treatment with SA has been show to increase chitinase production in mango fruits to help fight off *C. gloeosporioides* infection (He *et al.*, 2017). At 24 DPI, mock inoculated control chamber fruit were also observed to express 1.65-fold more *CHI9* than samples in the treatment chamber, although this result was not significant. However, the higher detected levels of *CHI9* expression could be as a result of ethylene levels within the control chamber. *CHI9* is an ethylene inducible chitinase (Diaz *et al.*, 2002; Wu & Bradford, 2003; Scott *et al.*, 2018); therefore, the greater levels of ethylene present within the control chamber appear to be having an effect on expression levels. This same result however was not seen at 14 DPI.

At 14 DPI for *C. gloeosporioides* inoculated fruits, *CHI9* expression within the treatment chamber was 2.16 -fold less than what was observed within the control chamber and 1.94 fold less than mock inoculated treated fruits. Although these results are not significant, they are anomalies similar to what was seen with *ACO1*. However, with *CHI9* being an ethylene inducible gene, it makes sense that *CHI9* expression would correlate well the *ACO1* expression witnessed in section 7.4.1.

7.4.5 *GLUB* gene

Here we look at expression of an ethylene inducible and PR basic intracellular β-1,3-glucanase (*GLUB*) (Wu & Bradford, 2003; Aimé *et al.*, 2008; Scott *et al.*, 2018). β-1,3-glucanases are involved in many plant processes including seed germination and defence against pathogen attack. β-1,3-glucanases are endoglucanases that act primarily in fungal cell walls on fungal glucans by catalysing the hydrolytic cleavage of (1,3)- β-D-glucosidic linkages in (1,3)- β-glucans (Gupta *et al.*, 2013).

At 14 DPI, results show that *GLUB* expression was significantly higher in the control chamber with a 3.18-fold and 3.11-fold greater expression detected for *C. gloeosporioides* inoculated and mock inoculated samples respectively (Figure 7.7). This could be as a result of the greater levels of ethylene within the control chamber. Wu & Bradford (2003) observed that external ethylene strongly induced *GLUB* in leaves. Therefore, it is possible that the ethylene levels in the control chamber were great enough to induce increasing levels of *GLUB* expression in comparison to the treatment chamber where there was little to no ethylene. At 14 DPI, results also suggest some effect of the pathogen inoculation on *GLUB* expression. *Colletotrichum gloeosporioides* inoculated fruit showed 1.56-fold and 1.59-fold greater *GLUB* expression than mock inoculated fruit for the treatment chamber and control chamber respectively. This could be in response to pathogen activity on the fruit if *C. gloeosporioides* is just exiting its dormant stage and entering its necrotrophic stage.

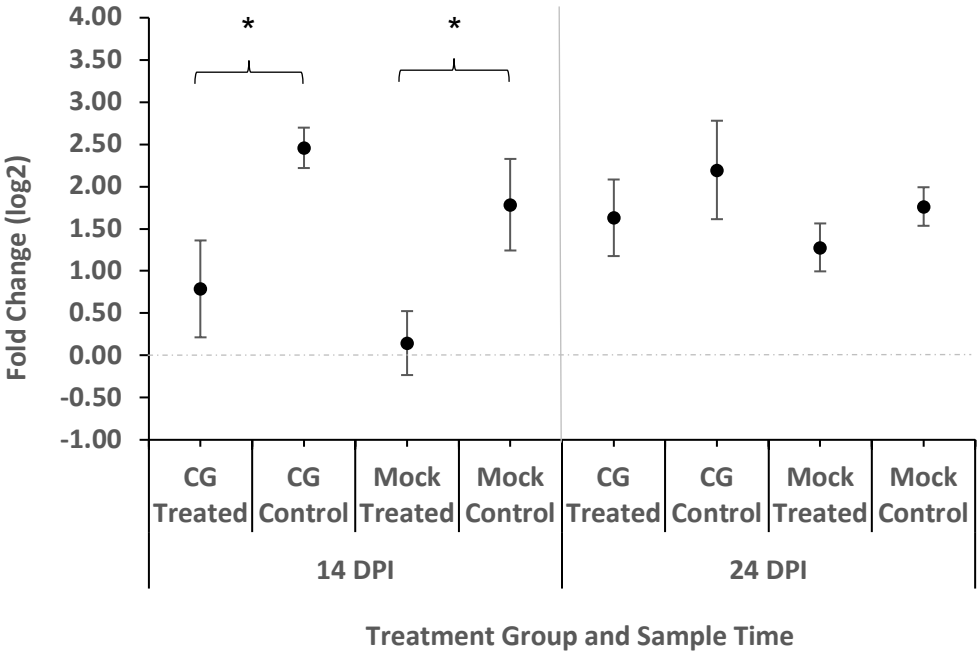


Figure 7.7. Relative expression of *GLUB* following ethylene scrubbing treatment (Treated) or storage within a control chamber (Control). Samples were inoculated with either a *Colletotrichum gloeosporioides* pathogen inoculum (CG) or a water inoculum (Mock). Samples were taken before inoculation and storage (baseline expression), at 14 days post inoculation (DPI) and 24 DPI. Fold changes (log_2) are relative to baseline expression (dashed line). An asterisk (*) indicates statistical significance ($p < 0.05$) between groups recorded by independent T-test ($p \leq 0.05$). Error bars represent ± 1 S.E.M.

At 24 DPI, no differences between groups were observed. The same pattern of expression between groups at 14 DPI however shows expression between groups to be much closer together. The result suggests again that external ethylene is playing a role in increased *GLUB* expression and therefore, that the action of the ethylene scrubber decreases *GLUB* expression. Results presented here suggest that although pathogen inoculation appears to slightly increase *GLUB* expression, the effect of external ethylene appears to have a greater impact on the expression of the gene. Furthermore, increased *GLUB* expression observed in the control chamber appears to have no real impact on *C. gloeosporioides* infection as fruits within the chamber became heavily diseased anyway (Figure 7.2).

7.4.6 *PG* gene

During the ripening process, pectin within the cell wall of fruits is broken down and softening is initiated. Polygalacturonase (PG) is a primary hydrolase which acts to breakdown pectin and therefore a key enzyme in controlling fruit softening (King & O'Donoghue, 1995). PG is also produced by *C. gloeosporioides* (Prusky *et al.*, 1989; Yakoby *et al.*, 2000) and is reported to play a role in the pathogen as a cell wall degrading enzyme during tissue maceration in the necrotrophic stages of the pathogens lifecycle (Cooper *et al.*, 1998).

The results show a massive increase of *PG* expression across all groups at 14 DPI in mature green fruit (Figure 7.8). This result is expected due to the high abundance and rapid synthesis of PG which acts to break down the long-chain pectin polymers during tomato fruit ripening (King & O'Donoghue, 1995).

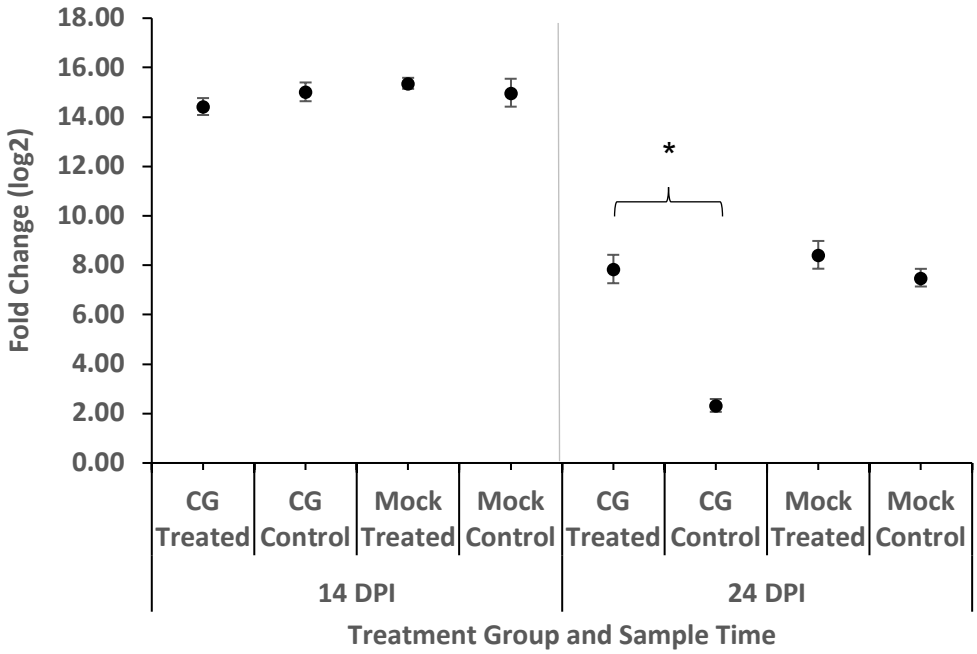


Figure 7.8. Relative expression of *PG* following ethylene scrubbing treatment (Treated) or storage within a control chamber (Control). Samples were inoculated with either a *Colletotrichum gloeosporioides* pathogen inoculum (CG) or a water inoculum (Mock). Samples were taken before inoculation and storage (baseline expression), at 14 days post inoculation (DPI) and 24 DPI. Fold changes (log_2) are relative to baseline expression. An asterisk (*) indicates statistical significance ($p < 0.05$) between groups recorded by independent T-test ($p \leq 0.05$). Error bars represent ± 1 S.E.M.

At 24 DPI, in the control chamber *C. gloeosporioides* inoculated tomatoes, there is 45.89 fold less *PG* expressed than in the corresponding treatment chamber tomatoes. This indicates that there is a decrease in *PG* expression within the tomato in response to the onset of pathogen attack, a result supported by work performed by Scott *et al.* (2018) who observed a decrease in *PG* expression in response to *B. cinerea* inoculation. This result makes sense as a response to attack as the fruit could be trying to prevent tissue softening to prevent pathogen tissue maceration. The fact that there was no significant differences between *C. gloeosporioides* inoculated fruits and mock inoculated fruits within the treatment chamber indicates that there has been no pathogen attack detected on the treatment chamber fruits. This suggests that the scrubbing chamber is affecting the pathogens ability to enter the necrotrophic phase and macerate fruit tissue.

There appears to be no significant effects of the ethylene scrubbing treatment on the regulation of *PG* expression. However, at 24 DPI, there is a 1.90-fold greater expression level detected in treatment chamber samples over the control chamber samples for mock inoculated fruits. Although this result is not significant; this slight increase in *PG* expression as a result of ethylene scrubbing could explain why some slight increase in levels of softening is seen in treatment chamber fruit compared to control chamber fruit in many experiments in section 4.5.

7.4.7 *PAL* gene

Phenylalinine ammonia-lyase (PAL) is directly involved in phenolics, phytoalexins and lignin biosynthesis. The enzyme is the first in the resistance related phenylpropanoid pathway and it is involved in SA biosynthesis and

localized resistance processes (Prusky *et al.*, 1994; Chen *et al.*, 2014; He *et al.*, 2017; Scott *et al.*, 2018).

The results show that at 24 DPI, *C. gloeosporioides* inoculated fruit in the control chamber expressed 19.44-fold greater *PAL* than in the corresponding treatment chamber fruit (Figure 7.9). This result correlates with what was seen with the expression of SA protein: PR1a (Figure 7.5). This makes sense as PAL expression is related to SA biosynthesis (Scott et al., 2018). Furthermore, SA treatment has been seen to increase PAL enzyme activity which is known to correlate with the onset of resistance induction (He *et al.*, 2017). Therefore, increasing levels of *PAL* expression would be expected with increasing levels of disease in order for the fruit to induce resistance. Moreover, enhanced PAL activity increases the flavonoid epicatechin concentrations which inhibit the decline of natural antifungal diene effecting avocado resistance to *C. gloeosporioides* attack (Prusky *et al.*, 1994; Yakoby *et al.*, 2001; Guetsky *et al.*, 2005). Again therefore, the increase in *PAL* observed here in response to *C. gloeosporioides* attack observed here is unsurprising. At 24 DPI, all other groups produce *PAL* expression at less than what was observed at harvest in mature green fruit. This indicates that the significant increase in *PAL* expression is as a direct result of the onset of *C. gloeosporioides* virulence.

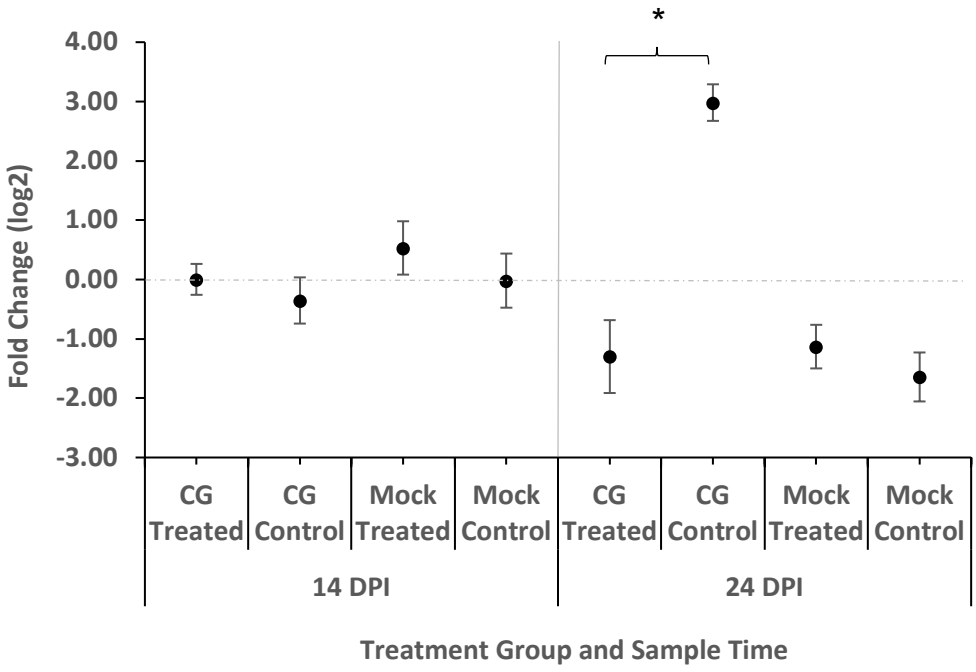


Figure 7.9. Relative expression of *PAL* following ethylene scrubbing treatment (Treated) or storage within a control chamber (Control). Samples were inoculated with either a *Colletotrichum gloeosporioides* pathogen inoculum (CG) or a water inoculum (Mock). Samples were taken before inoculation and storage (baseline expression), at 14 days post inoculation (DPI) and 24 DPI. Fold changes (log_2) are relative to baseline expression (dashed line). An asterisk (*) indicates statistical significance ($p < 0.05$) between groups recorded by independent T-test ($p \leq 0.05$). Error bars represent ± 1 S.E.M.

For groups where *PAL* expression has clearly not been affected by pathogen virulence, there is a consistency in results between chambers. At 14 DPI for *C. gloeosporioides* inoculations and mock inoculations, and at 24 DPI for mock inoculations, there is a 1.28-fold, 1.47-fold and 1.43-fold greater level of *PAL* expression respectively observed in the treatment chamber in comparison to the control chamber. Although these results are not significant, they suggest an effect of the ethylene removal on the *PAL* expression. It would be interesting to see if the difference between these groups is greater if there was a higher concentration of ethylene within the control chamber. The result described suggests that potentially the ethylene scrubbing treated fruit are better prepared for pathogen attack with regards to *PAL* expression due to PALs involvement in defence against pathogens.

7.4.8 *CRTR-B1* gene

Tomato fruit colour change from green to red is a clear indicator of ripening. The change is associated with chlorophyll degradation and a shift in carotenoid composition from leaf-like xanothophylls to carotenes (Fraser *et al.*, 1994; Su *et al.*, 2015). Carotenoids are organic molecules responsible for the induction of the vivid yellow, orange and red colours found on fruits and flowers. As well as providing horticultural crops with their visual appeal, carotenoids are known to enhance fruit nutritional value and health benefits for humans (Yuan *et al.*, 2015). Orange pigmentation in tomato fruit is down to the carotenoid, β-carotene (Tadmor *et al.*, 2005; Su *et al.*, 2015; Yuan *et al.*, 2015). β-carotenes are synthesised by β-cylases from lycopene (the major carotenoid in tomato fruit giving rise to red pigmentation) before being transformed into lutein and zeaxanthin by heme and non-heme β-carotenes hydroxylases (CYP97 and CRTR-b) (Giuliano, 2014; Su *et al.*, 2015). The

gene *CRTR-B1* encodes a β-carotene hydroxylase enzyme involved in β-carotene modification (Scott *et al.*, 2018) and its expression was monitored here.

The results suggest a delay in fruit ripening as a result of the ethylene scrubbing treatment. These results fall in line with some of the results seen in section 4.4.1.1 when exogenous ethylene was applied. At 14 DPI, although the difference observed in *CRTR-B1* expression between chambers is not significant, there is a 1.57-fold and 1.47-fold difference in *C. gloeosporioides* inoculated fruit and mock inoculated fruit respectively (Figure 7.10). Further to this, at 24 DPI, there is a significant difference observed between chambers in the mock inoculated fruit. In the treatment chamber fruit samples, *CRTR-B1* expression is 1.77-fold greater than in the control chamber which indicates that the fruits are still going through colour evolution. This is in comparison to control chamber fruits which are showing a similar level of *CRTR-B1* expression to what was seen at a mature green stage at harvest indicating that they have completed their colour evolution and are no longer expressing β-carotene. These results indicate that the ethylene removal caused by ethylene scrubbing is causing a slight delay in the colour evolution of the fruits due to changes in carotenoid regulation. This result is supported by work performed by An *et al.* (1990) who identified that exogenously applied ethylene was able to hasten carotenoid synthesis and development in papaya fruits indicating that external ethylene levels can effect carotenoid levels. On the other hand however, it was found in tomatoes by Watada *et al.* (1976) that β-carotene levels were not affected by supplementary ethylene (Saltveit, 1999). Furthermore, it was observed by Marty *et al.* (2005) that 1-MCP ethylene removal treatment had only a small effect on carotenoid pigment accumulation in apricots (Watkins, 2006) This contradicts the results produced by Tiecher *et*

al. (2013) in tomato fruits. Tiecher *et al.* (2013) found that ethylene prevention caused by 1-MCP treatment retarded carotenoid accumulation and consequently delayed colour development. The results in the current study demonstrate that the ethylene scrubbing method of ethylene removal has a similar effect to what was seen by Tiecher *et al.* (2013) whereby ethylene removal has effected carotenoid regulation and delayed ripening meaning it took longer for the fruits to reach full colour maturity (Figure 7.10) (section 4.4.1.1).

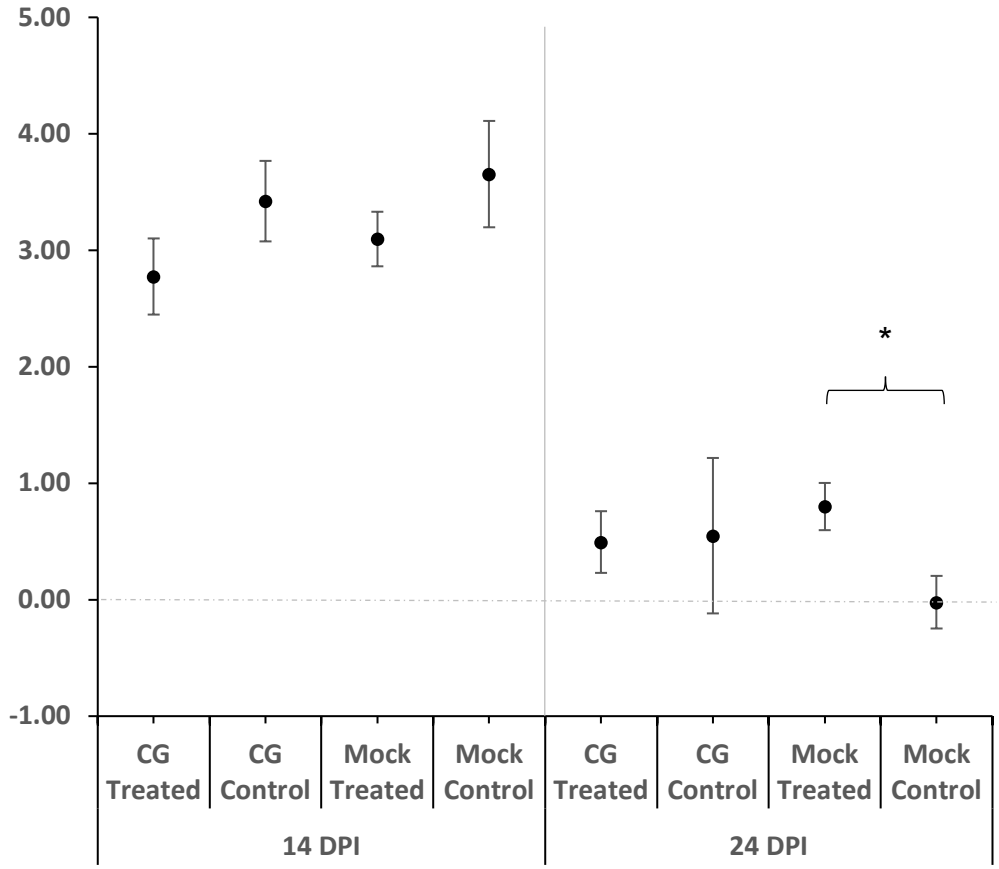


Figure 7.10. Relative expression of *CRTR-B1* following ethylene scrubbing treatment (Treated) or storage within a control chamber (Control). Samples were inoculated with either a *Colletotrichum gloeosporioides* pathogen inoculum (CG) or a water inoculum (Mock). Samples were taken before inoculation and storage (baseline expression), at 14 days post inoculation (DPI) and 24 DPI. Fold changes (log_2) are relative to baseline expression (dashed line). An asterisk (*) indicates statistical significance ($p < 0.05$) between groups recorded by independent T-test ($p \leq 0.05$). Error bars represent ± 1 S.E.M.

7.4.9 *FLS* gene

Often targeted for their health promoting benefits, flavanols are a group of naturally occurring phenolic flavonoid antioxidants found in plants (Schroeter *et al*., 2010; Choudhary *et al.,* 2016b). Furthermore, flavonols have an important role in plant pathogen interactions due to their antioxidant properties (Scott *et al.*, 2018). Here the expression of an enzyme directly involved in flavonol biosynthesis: flavonol synthase (*FLS*) has been monitored. Results from this work can be seen in Figure 7.11.

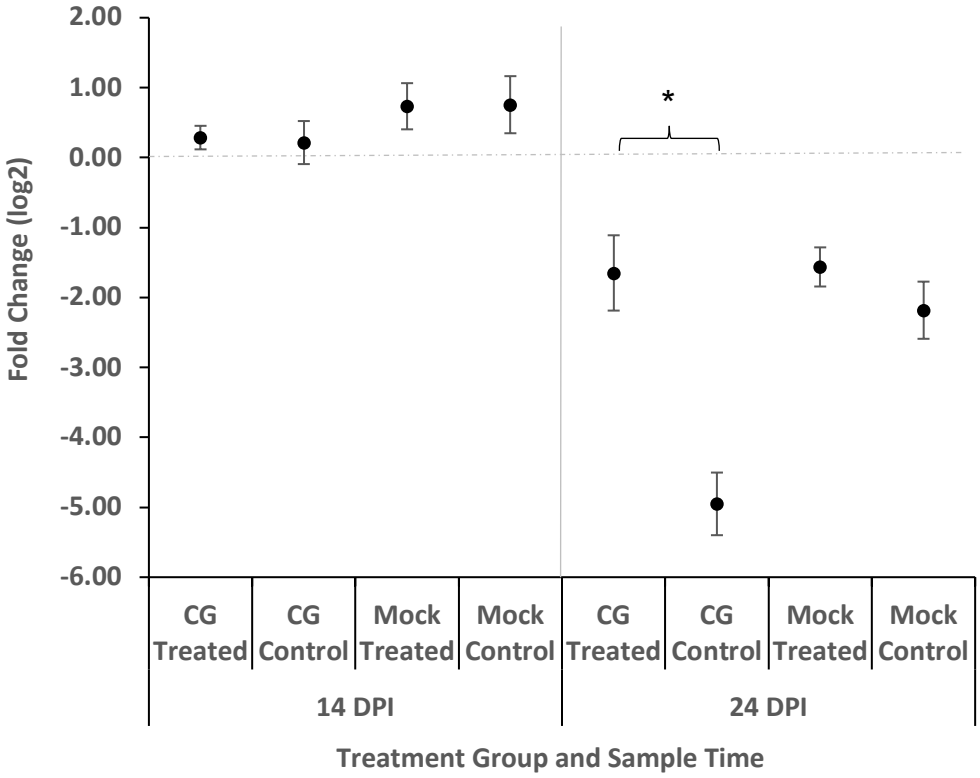


Figure 7.11. Relative expression of *FLS* following ethylene scrubbing treatment (Treated) or storage within a control chamber (Control). Samples were inoculated with either a *Colletotrichum gloeosporioides* pathogen inoculum (CG) or a water inoculum (Mock). Samples were taken before inoculation and storage (baseline expression), at 14 days post inoculation (DPI) and 24 DPI. Fold changes (log_2) are relative to baseline expression (dashed line). An asterisk (*) indicates statistical significance ($p < 0.05$) between groups recorded by independent T-test ($p \leq 0.05$). Error bars represent ± 1 S.E.M.

The results show that the only significant difference in results is at 24 DPI for the *C. gloeosporioides* inoculated fruit between the treatment and control chamber. Results show that there is 9.86-fold less *FLS* expression within the control chamber than there is within the treatment chamber samples. Due to this being the only real difference seen between chambers across the two time points, it suggests that the difference is as a direct result of *C. gloeosporioides* disease virulence as opposed to the action of the ethylene scrubber.

7.4.10 Tomato gene conclusions

Due to the majority of significant differences observed between results being only as a direct result of the onset of *C. gloeosporioides* attack on control chamber fruit and not a result of ethylene scrubbing treatment, it can be inferred that the ethylene scrubbing treatment is affecting the pathogen itself instead of altering defence gene regulation pathways in tomatoes. The genes *OPR3, PR1a, CHI9, PG, PAL* and *FLS* all showed significantly different expression levels between treatment and control chambers in response to pathogen inoculation, whereas the corresponding mock inoculated fruit demonstrated no real differences in gene expression between chambers. This clearly demonstrates that the results are pathogen related and not treatment related. Furthermore, it suggests that anthracnose disease prevention is as a result of ethylene scrubbing treatment effecting the *C. gloeosporioides* fungus directly.

The gene regulation observed with *CRTR-B1* and *PG* correlate well with what was seen in chapter 4. Results seen with *CRTR-B1* indicate a delay in colour evolution as a result of ethylene scrubbing treatment whilst results seen with *PG* demonstrate slightly higher PG activity as a result of scrubbing treatment.

In section 4.5.1, the slight increase in softening rates observed across some experiments as a result of ethylene scrubbing was difficult to explain. Although this is still likely in some part down to dehydration related softening as a result of decreasing chamber humidity in the treatment chamber, the slight increase in *PG* activity helps to further explain the decrease in softening rates in some of the treated fruits.

7.5 *COLLETOTRICHUM GLOEOSPORIOIDES* PATHOGENICITY GENES

Due to results showing no real variations in tomato gene regulation as a result of ethylene scrubbing treatment, it could be assumed that disease was prevented as a consequence of the treatment effecting the pathogen itself. Expression profiles of genes involved in *C. gloeosporioides* pathogenicity were therefore analysed and compared between ethylene scrubbing and control chambers. A description of the function of each gene analysed, underlining their reason for selection in these experiments is detailed below.

Actin was used as the reference gene in order to normalise the Ct values of each sample for all the other target genes.

PELB encodes for pectate lyase (PL) and is a *C. gloeosporioides* virulence gene (Yakoby *et al.*, 2001). The PL enzyme from *C. gloeosporioides* acts to degrade the plant cell wall and becomes active and easily seen during the pathogens necrotrophic phase of infection (Sharma & Kulshrestha, 2015). The naturally occurring alkalinisation of fruit during the ripening process strongly effects pectate lyase expression. During fruit ripening, as the pericarp pH rises from 4.0 to 6.0, extracellular proteins from *C. gloeosporioides*, including PL, are secreted and accumulated (Drori *et al.*, 2003). Furthermore, the pathogen

itself is able to secrete ammonia to increase the amount of ammonia accumulated by the host (Prusky *et al.*, 2001) in order to create an alkaline environment necessary for the activation of extracellular lyase enzymes including PL (Wattad *et al.*, 1997; Kramer-Haimovich *et al.*, 2006; Miyara *et al.*, 2008). This fruit tissue alkalisation during *C. gloeosporioides* infection is important in stimulating the transition from a biotrophic stage to a necrotrophic stage during fungal colonization so to stimulate the secretion of the cell wall degrading such as PL (Kramer-Haimovich *et al.*, 2006).

The *chip6* gene is induced in the conidia of *C. gloeosporioides* within a few hours of hard surface contact with a host. It encodes a protein with homology to sterol glycosyl transferase which is important for conidial germination and appressorial formation. It has been observed that *chip6* gene disrupted *C. gloeosporioides* mutants demonstrate a marked decrease in virulence on avocado fruits which shows that the transferase encoded by *chip6* plays an important role in pathogenesis (Kim *et al.*, 2002).

As alluded to previously, *C. gloeosporioides* is an alkalinizing fungus which is able to secrete ammonia to modulate the pH of a hosts' environment in order to modulate its own fungal pathogenicity (Prusky *et al.*, 2001; Miyara *et al.*, 2010). *MEPB* is the most highly expressed gene of a family of *MEP* genes expressed during pathogenicity which encode ammonium transportation (Miyara *et al.*, 2012; Shnaiderman *et al.*, 2013). Disruption of the *MEPB* gene has been observed to result in overexpression of *MEPA* and *MEPC* but reduced colonization; which suggests *MEPB*'s contribution to pathogenicity (Shnaiderman *et al.*, 2013). As well modulating ammonia secretion, *MEPB* also contributes to ammonia accumulation within germinating spores and mycelia. This accumulation activates cAMP pathways which contribute to *C.*

gloeosporioides pathogenicity. As well as *MEPB* contributing to the pathogens necrotrophic stages, this ammonia accumulation by germinating spores regulates appressorium formation determining the initiation of the biotrophic stages in avocado-fruit colonization (Miyara *et al.*, 2010).

Abundant in conidia and hyphal tips, *CgRAC1* encodes a CgRAC1 protein which is the major regulator of asymmetric development in *C. gloeosporioides* (Nesher *et al.*, 2011; Sharma & Kulshrestha, 2015). Moreover, the gene is involved in the regulation of both nuclear division and morphogenesis. *CgRAC1* activity has also been identified to play a big role in pathogenicity. This was confirmed as transgenic strains expressing constitutively active *CgRAC1* proved to be completely non-pathogenic. This modification of *CgRAC1* caused various abnormalities including reduced conidial and hyphal adhesion, difficulties in establishing and maintaining cell polarity and the formation of immature appressoria, consequently preventing fungal infection (Nesher *et al.*, 2011).

The *cap20* gene is induced by hard surface contact with a host and expressed uniquely in *C. gloeosporioides* during appressorium formation (Hwang *et al.*, 1995; Kolattukudy *et al.*, 1995). The *cap20* gene encodes a 20-kDa protein which is localised within the appressorial wall (Hwang *et al.*, 1995; Perfect *et al.*, 1999). The *cap20* gene is reported to play a significant role in host infection. Evidence of this comes from a *cap20* gene disrupted mutant which produced appressoria with normal morphology that was however unable to develop lesions on tomato and avocado fruits (Hwang *et al.*, 1995).

7.5.1 *Colletotrichum gloeosporioides* pathogenicity gene results and discussion

As previously stated, *Actin* was used as the reference gene in order to normalise the Ct values of each sample for all the other target genes. However, upon analysis of the actin qPCR run, it was identified that actin expression was only detected in the samples stored in the control chamber, presumably because of the slow rate of growth of the fungus in the treated chambers. Only 7% of ethylene scrubbing chamber sample wells had actin detected by the qPCR machine across both time points (14 and 24 DPI). However, in the control chamber, actin was detected in 56% and 100% of the wells at 14 DPI and 24 DPI respectively (Table 7.3).

Table 7.3. *Colletotrichum gloeosporioides actin* detection by the Lightcyler 480®. Figures represent Ct values. The smaller the Ct value, the greater the expression of the gene. Asterisks (*) represent no detection of the target gene.

***Colletotrichum gloeosporioides Actin* Detection**			
14 DPI		24 DPI	
Treatment Chamber	*Control Chamber*	*Treatment Chamber*	*Control Chamber*
*	*	*	22.55
*	35.79	*	22.46
*	*	*	22.55
*	36.32	*	22.31
*	*	*	22.17
*	35.6	*	22.11
*	*	*	23.25
*	*	*	22.85
*	35.81	*	23.27
35.05	34.76	38.24	22.75
*	*	*	22.37
*	*	*	22.6
*	*	*	22.34
*	36.17	*	21.88
*	*	*	21.94
*	33.14	*	22.21
*	33.89	41.89	22.23
*	34.54	*	22.28
*	34.86	*	23.85
*	*	*	23.55
*	42.92	*	23.67
*	*	*	25.12
*	36.14	*	24.72
*	*	*	25.01
*	32	35.26	26.47
*	31.62	*	25.97
*	32.49	*	26.32

These results indicate firstly that there is almost a complete lack of activity by *C. gloeosporioides* within the treatment chamber. The fact that actin cannot be detected at either time point within the study shows that treatment has prevented all disease progression. This result alone would explain why there has been little to no anthracnose disease present on the tomatoes within the ethylene scrubbing chamber throughout the entirety of this study. The results could indicate that either the treatment has managed to kill off the pathogen completely or that the ethylene scrubbing treatment has maintained the pathogen in a state of dormancy. It is possible that this ethylene deprivation could lead to dormancy and long term cell cycle arrest, and due to the complete lack of ethylene within the environment this could happen before spore or appressorial germination therefore halting the whole disease process if ethylene is the required germination triggering factor (Prusky, 1996). It has previously been reported that exogenous ethylene has been required to induce spore germination as well as multiple appressorial and lesion formation of *C. gloeosporioides* on transgenic tomatoes and oranges unable to produce ethylene (Flaishman & Kolattukudy, 1994). Therefore, it is feasible that ethylene removal from the environment is enough to prevent spore germination, multiple appressorial production and consequently lesion formation in this manner. Further to this, some biocontrol agents actually work by maintaining the dormancy of *C. gloeosporioides* in order to control disease (Korsten & Jeffries, 2000; Janisiewicz & Korsten, 2002). It was reported by Korsten *et al.* (1995) that using *B. subtilis* as a biocontrol agent was effective against *C. gloeosporioides* on avocado by effecting spore and appressorium germination. It is possible that the ethylene scrubbing treatment has acted in a similar manner to this by maintaining pathogen dormancy and therefore preventing the onset of disease symptoms.

Alternatively to ethylene deprivation being the causing factor to the absence of pathogen activity, decreasing RH brought about by the action of the ethylene scrubber could be playing a significant role. As demonstrated in section 3.5.2, the action of UV photocatalytic ethylene scrubbing causes constant reductions in RH within the treatment chamber. It has previously been observed that RH levels below 95% can prevent *C. gloeosporioides* conidial and appressorial germination (Estrada *et al.*, 2000). Therefore, it is possible that the decreasing RH levels induced by the ethylene scrubber are responsible for preventing *C. gloeosporioides* germination and development, and the reason why no *actin* expression in the ethylene scrubbing chamber is observed throughout these experiments.

These issues clearly haven't arisen for *C. gloeosporioides* in the control chamber as the tomatoes are exhibiting severe disease symptoms by 24 DPI (Figure 7.2). The fact that there is an increase in actin detection from 14 to 24 DPI in the control chamber suggests that the pathogen is coming out of its quiescent phase and into a necrotrophic one. This is backed up by symptoms observed on the fruit. At 14 DPI, disease scores ranged from 0-1 on the fruit, whereas by 24 DPI, within the control chamber, disease scores ranged from 4-7. This indicates that at 14 DPI the pathogen was either still dormant and exhibiting its quiescent lifestyle or just beginning its necrotrophic lifestyle where genes are being expressed again; hence 56% of sample wells showing actin expression. This is compared to 24 DPI where there are disease symptoms across all control chamber tomatoes which is why we see 100% actin detection.

The complete inactivity of *actin* within the treatment chamber indicates a complete inactivity of all the other *C. gloeosporioides* pathogenicity genes

analysed throughout these experiments. Analysis of the other pathogenicity genes confirmed that this was the case. Similarly to *actin*, there was no expression detected in either all, or the vast majority of the treatment chamber wells from each pathogenicity genes (*PELB*, *chip6*, *MEPB*, *CgRAC1* and *cap20*) qPCR plates at both 14 and 24 DPI. These results were highly unsurprising following the run of the *actin* plate however.

The absence of any gene expression within the treatment chamber for any of the examined pathogenicity genes explains why throughout this research little to no anthracnose disease has been observed on any tomatoes subjected to ethylene scrubbing treatment. Furthermore, the absence of consistent actin expression from the same samples indicates that within the ethylene scrubbing chamber, *C. gloeosporioides* loses all function. The indicated inactivity of the pathogens 'housekeeping gene' suggests that the action of the ethylene scrubber has completely prevented fungal development. It would therefore be concluded the scrubbers' primary resistance effect is on the pathogen and not the fruit.

7.6 OVERALL CONCLUSIONS

Results presented from the gene expression of both tomato defence genes as well as *C. gloeosporioides* pathogenicity genes indicate that anthracnose disease prevention is as a result of ethylene scrubbing treatment inhibiting *C. gloeosporioides*. Results from both *C. gloeosporioides* inoculated fruits and mock inoculated fruits in section 7.4 suggest that UV photocatalytic ethylene removal has no real effects on tomato defence gene regulation. Results presented in section 7.5 however, demonstrate that the treatment causes almost a complete loss of function of *C. gloeosporioides*. These results

confirm therefore that the action of the ethylene scrubber is to prevent successful *C. gloeosporioides* colonization and infection on tomato fruits.

CHAPTER EIGHT

8 FUTURE WORK

FUTURE WORK

8.1 FUTURE WORK

In order for our understanding of the potential uses of the UV photocatalytic ethylene removal technology to develop, it is important that research continues into the way the postharvest treatment can be utilised to benefit fruit storage and transport systems. The conclusions drawn from this work can now be used as a framework to conduct further research. Research must continue in both the lab environment and in real -world postharvest storage and transport systems in order to really investigate if and how the technology can help in the prevention of food waste.

Here, a breakdown of potential future experiments that could be conducted, based around the work performed and conclusions drawn from each chapter, will be listed. Finally, there will be a brief overview of the key areas of the research to be developed and taken forward.

8.1.1 Ethylene removal, and changes in temperature and humidity.

During this work, there were issues with identifying the exact ethylene levels present within the control chamber due to problems with the equipment used to detect ethylene concentrations (section 3.3). In future work, determining the exact concentration of ethylene present is important because ethylene at any level is reported to have deleterious effect on produce, with decreases in postharvest life linearly related to increasing $\log_{10}$ ethylene concentrations (Wills *et al.*, 2001; Keller *et al.*, 2013). Within the treatment chamber this would not be such an issue, as according to the two current methods of ethylene

analysis tested, the ethylene scrubber is continuously removing ethylene from the environment. However, in the control chamber, the accurate representation of ethylene concentrations would enable us to reliably correlate observed changes in fruit physiology and disease resistance with changes in ethylene levels present in the chamber. Potential alternative ethylene detection technologies including laser-based sensors (photoacoustic detection), electro-catalytic sensor technology and non-dispersive infrared spectroscopy, are well described by Cristescu *et al.* (2013) and Janssen *et al.* (2014) and could be utilised in future work.

Unfortunately for the work produced here, the temperature differences observed between chambers (Figure 3.6) were present throughout all research performed. Therefore, they could have had an effect on the outcomes of fruit physiology and disease resistance described in this research. Future work would therefore look to perform similar experiments to those in this research, but with modifications to the treatment chamber. This could potentially involve a longer piping system to help dissipate more of the heat. Results would determine whether the viewed temperature differences had any detrimental effects on fruit physiology and disease resistance.

Throughout the research conducted, constant humidity reductions were observed within the treatment chamber as a result of the action of the ethylene scrubber (section 3.5.2). Humidity levels effect fruit physiology and also the ability of pathogens to cause infection (Hardenburg *et al.*, 1986; Estrada *et al.*, 2000; Keller *et al.*, 2013). Therefore, moving forward, within storage environments where the ethylene scrubber is applied, efforts would be required to maintain high humidity levels for the optimum storage conditions of fruit and fresh produce. Furthermore, the equipment will need to be tested in

real-world storage and transport environments to see how it effects RH and consequently, fruit quality within the store environment.

8.1.2 Effects of treatment on fruit physiology

It was observed in chapter 4 that external ethylene injections were able to induce differences in ethylene concentrations between chambers which resulted in initial delays in the ripening of treated fruit compared to control fruit.

Future work in this area should involve around optimising and maximising the positive effects of treatment. This would involve identifying methods of applying the UV photocatalytic ethylene scrubber within a fresh produce storage environment to further delay fruit ripening and extend the shelf/storage lives of fruits. Maneerat *et al.* (2003) reported delays in ripening for up to 4 weeks storage at 25^{o}C, possibly due to the close proximity of the TiO_2 photocatalytic activity to the fruit samples. Perhaps applying the ethylene scrubbing treatment used in this research to fruit storage units at a closer proximity to the fruits would stimulate increased delays in fruit ripening. However, a method of application would need to be identified so that the heat generated by the UV lamps would not cause detrimental effects on the quality of fresh produce.

Furthermore, it would be interesting to see how pre-storage ethylene injections only were able to trigger differences in the rate of ripening between chambers. The daily ethylene injections utilised in these experiments could have been sufficient in stimulating ripening within the treatment chamber, all be it at a slightly delayed point to within the control chamber. However, if a large, given quantity of external ethylene (for example 100 ppm), was placed into both

chambers a couple of days prior to sample storage, with no further ethylene injections to occur, theoretically, the complete removal of ethylene within the ethylene scrubbing chamber could alter the time of the onset of ripening. This would be a good test for the application of the UV photocatalytic ethylene scrubber in fruit storage, to determine whether scrubbers applied to storage units with large concentrations of accumulated ethylene could have instant benefits of extending the shelf/storage lives of fruits stored following application.

8.1.3 Effects of treatment on disease prevention

8.1.3.1 Botrytis cinerea

Work performed *in vitro* with *B. cinerea* and *C. gloeosporioides* cultures demonstrated that ethylene removal treatment stimulated an increase in the growth rate of both pathogens in culture. It has previously been suggested that *B. cinerea* can sense and respond to ethylene, and a gene, *Bcg1*, in the Gα subunit of the G protein signalling pathway (Schulze Gronover *et al*., 2001) is responsible for ethylene response (Chagué *et al.*, 2006). Future work in this area could look to test the *B. cinerea* Δ*bcg1* mutants response to the method of ethylene scrubbing performed here to see if the mutant cultures grow at the same rate in both chambers to further support the previous work performed by Chagué *et al.* (2006). Furthermore, future work could look at identifying ethylene responsive genes within *C. gloeosporioides* and performing gene knockouts of these genes to see if the mutants are then affected by ethylene removal similarly to the *B. cinerea* mutants.

Furthermore, future work in this area should look to identify whether *C. gloeosporioides* is capable of producing ethylene at all, and if so, using the same KMBA pathway as *C. musae* (Daundasekera *et al.*, 2003) and *B. cinerea* (Cristescu *et al.*, 2002). If it is shown that it does produce ethylene *in vitro*, it would be interesting to identify whether this ethylene production is utilised during fruit infection.

With *B. cinerea* infections performed pre-storage, it was observed that disease symptoms were worsened when tomatoes were then subject to ethylene scrubbing. It was concluded that this was due to the absence of ethylene preventing the activation of various defence related pathways, based on ethylene's role in co-ordinating defence related pathways (Bari & Jones, 2009; Broekgaarden *et al.*, 2015). Future work here should therefore look to analyse the molecular differences of fruit samples between chambers to determine whether there are differences in the expression of defence signalling genes and pathways in response to *B. cinerea* infection. This could help determine whether increased *B. cinerea* susceptibility caused by ethylene scrubbing treatment is as a result of ethylene regulating tomato defence genes or *B. cinerea*'s ability to sense and respond to ethylene. Moreover, experiments could be performed *in planta* by performing inoculations using the ethylene insensitive Δ*bcg1* mutant strain of *B. cinerea* (described above). If the pathogen was able to cause the same levels of disease between chambers, then it would suggest that the lower levels of disease observed here in the control chamber are as a result of the pathogens ability to sense and react to ethylene. If that was the case, it could lead to further understanding of *B. cinerea* control in fruit storage and transport.

The results presented were the opposite to what was observed using the 1-MCP ethylene perception blocking treatment in a study performed by Su & Gubler (2012). Future work should look to compare 1-MCP and ethylene scrubbing treatments under the same conditions. The reasons behind the observed differences between the two methods of ethylene action inhibition are currently unknown. A study which compares the treatments under the same conditions would begin to help identify why these differences are observed. Perhaps 1-MCP treatments are having direct effects on the pathogen itself by interfering with its metabolism for example. Comparative studies of this nature could help identify which storage treatment would provide the most benefit to postharvest produce. It could also help identify whether one treatment is more beneficial to certain crops against specific pathogens, and maybe the other is more beneficial in the storage and protection of different fruits against different diseases.

Furthermore with *B. cinerea*, it would be good to develop and external inoculation method. Prior to experimentation, in preliminary work, methods of external *B. cinerea* spore solution application were tested. Unfortunately, it was not possible to get one to work consistently or without risking excess pathogen growth and potential contamination of other samples. Future work with tomato inoculations will look to develop a method of effectively applying *B. cinerea* spore solutions to the tomatoes externally. This would mean that the pathogen is more exposed to the ethylene removal as well as further consequences of UV photocatalytic ethylene removal treatment such as RH reductions. Furthermore, it could more effectively resemble the conditions of fruit and fresh produce within storage environments where the hope would be that there are minimal wound sites prior to sale.

8.1.3.2 Penicillium expansum

During this research, it was observed that UV photocatalytic ethylene scrubbing treatment could affect *P. expansum* asexual spore maturation (section 5.4.1.1). If ethylene scrubbing is able to cause consistent inhibition of *P. expansum* spore maturation, then it could potentially be preventing the viability of a secondary inoculum. This could prevent the spread of disease within storage environments and therefore reduce disease occurrence. This effect would be particularly beneficial in the storage of pome fruits where disease is most prevalent (Jurick *et al.*, 2011; Ballester *et al.*, 2015). In order to test this hypothesis, an attempt was made to perform a cross contamination experiment, which was unfortunately unsuccessful. However, future work should look to build on this and develop an experiment where disease produced from initial inoculations on fruits in either chamber could be used to potentially infect wounded fruit within the vicinity. It would be interesting to observe whether disease symptoms produced on treatment chamber infections were able to infect other fruits wound sites in the same way you would expect the spores from control fruit to be able to do. An experiment of this kind could elucidate whether these ethylene removal treatments have the potential to reduce the spread and occurrence of *P. expansum* disease postharvest.

In further future work, it would be good to repeat the *in vitro* spore maturation experiment (section 5.4.1.2), however this time, daily spore extraction and counting would occur from 3 plates per chamber to determine whether the amount of spores produced on the plates was affected. Furthermore, it would be interesting to perform an inoculation experiment with this secondary inoculum to help determine whether the resulting treatment chamber spores

from experimentation were as pathogenic as spores produced from within the control chamber. It would also be interesting to measure colony growth as was measured with *B. cinerea* and *C. gloeosporioides* in section 5.2. It has previously been observed that 1-MCP treatment can significantly decrease *P. expansum* colony growth (Li *et al.*, 2017), so it would be interesting to determine whether the UV photocatalytic ethylene scrubbing method of ethylene removal could inhibit colony growth in the same manner; or whether it increases pathogen colony growth as was observed in section 5.2.

Recently, work performed by Yang *et al.* (2017) showed that *P. expansum* was able to produce ethylene via the KMBA pathway, as described in section 5.2.1. However, the pathogen could only produce the hormone in the presence of KMBA. A further interesting *in vitro* study would be to repeat the *P. expansum in vitro* experiment (section 5.4.1.1) but, supplement the PDA growth media with KMBA or L-methionine. It would be interesting to see if ethylene production by the pathogen in the treatment chamber was capable of inducing spore maturation. It was suggested that ethylene production by *P. expansum* was not involved in conidiation, but in fungal metabolism (Yang *et al.*, 2017). However, it would be interesting to observe whether the production of ethylene by the pathogen was capable of inducing conidial maturation within the ethylene scrubbing chamber.

Furthermore, an interesting experiment would be to repeat the *in vitro* plate experiment, and after 5 DPI, swap half of the treatment chamber plates into the control chamber. If the plates that moved over into the control chamber began to produce the characteristic blue-green colour associated with *P.*

expansum disease, then it would suggest further that ethylene is crucial for *P. expansum* asexual spore maturation.

8.1.3.3 Colletotrichum gloeosporioides

With all the work performed using *C. gloeosporioides* as the host pathogen, it was concluded that the disease prevention was likely as a result of the effects of treatment on both ethylene and RH levels. Whilst it is know that ethylene plays a key role in triggering *C. gloeosporioides* infection, RH levels are also known to play a significant role (Estrada *et al.*, 2000). Future work would revolve around elucidating the main cause of disease prevention from the treatment: ethylene removal or humidity reductions. This is because if humidity reductions are the primary cause, then in future work it would be important to identify an appropriate treatment time for the storage of the fruits. Reductions in humidity in fruit storage can be detrimental to fruit physiology (Hardenburg *et al.*, 1986). Therefore, future research would need to determine a storage treatment time to prevent anthracnose disease by rendering *C. gloeosporioides* conidia permanently unviable, without becoming detrimental to fruit physiological characteristics.

There is however further interesting work that can be taken forward using *C. gloeosporioides* as the infecting pathogen.

Future work on *C. gloeosporioides* could involve performing microscopy experiments looking at incidence of conidial spore germination *in vitro* between the treatment and control chambers. A study of this kind could help to further explain why significant differences are seen in disease incidence between chambers. It would be hypothesised that due to ethylene removal

and reductions in humidity, there would be a smaller percentage of *in vitro* conidial germination within the treatment chamber.

It would also be interesting to look at disease incidence between chambers following *C. gloeosporioides* wound inoculations. In this research, spray inoculations were selected to portray a more realistic imitation of natural conidial dispersal. However, it would have been interesting to look at wound inoculations to see if the results replicated what was observed with spray inoculations. The wound inoculation means that the conidial spores would not be subjected as much to ethylene absence and humidity reductions due to the high internal ethylene levels and moisture content within the tomato fruits. Therefore, if disease reductions were still observed in the treatment chamber, it would suggest that treatments were having an effect on increasing the defence regulation of the fruits themselves against the pathogen. If no differences were observed however, it would suggest that the observed disease prevention in the treatment chamber from spray inoculations are from the effects of treatment on the conidial spores.

In further future work, it would be important to test the treatments against more common hosts of *C. gloeosporioides* such as mangos (Tucho *et al.*, 2014) and avocados (Beno-Moualem & Prusky, 2000). *Colletotrichum gloeosporioides* is not a common pathogen of tomato plants due to its requirement for tropical and subtropical environments to spread and cause successful infections. Therefore, it is important to confirm that the results observed on tomatoes transfer to more common hosts so that the UV photocatalytic ethylene removal treatments can be beneficially applied to storage of these host crops to prevent anthracnose disease. Additionally, *C. coccodes* is an important anthracnose disease causing pathogen on tomato fruits in regions with regular

rainfall, warm temperate climates and high humidity (Dillard & Cobb, 1998). Although this pathogen tends to not be a problem in glasshouse grown tomatoes in northern Europe, it may serve as a better model for testing postharvest anthracnose disease prevention using UV photocatalytic ethylene removal.

Furthermore, interesting and important studies would involve performing pre-harvest inoculations of immature developing fruits growing in a glasshouse for a period of time prior to harvest and storage. *Colletotrichum gloeosporioides* infects immature fruit crops pre-harvest and causes disease symptoms much later following harvest and a period of storage. It would be good to perform an experiment looking at the more natural infection time of the pathogen. This would also help confirm whether the treatments are effective over this increased period of time and whether they can prevent severe disease symptoms after the pathogen has been set into its quiescent stage of infection for a long period of time. A study of this nature could help determine how useful the treatments may be against anthracnose disease for crop storage and transport in the postharvest market.

8.1.3.4 UVC hormetic light treatments

During this research, the effects of both LIUV and HIPPL source treatments on mature green tomato fruits have been demonstrated to induce significant reductions in anthracnose disease (Figure 6.2) and disease caused by *B. cinerea* (Figure 6.1). These are the first reported effects of HIPPL sources on disease caused by *C. gloeosporioides* and the first reported effects of UVC radiation against disease caused by *C. gloeosporioides in planta*.

Future work must now look to investigate whether these treatments could induce the same levels of disease reductions on tropical fruits which are more at risk to postharvest anthracnose disease. These would include fruits such as mangoes. Previous research has demonstrated the benefits of UV-C irradiation in preventing postharvest mango decay (González-Aguilar *et al.*, 2001); therefore, the results here show added potential for the tropical fruit postharvest market with these effective new treatments for anthracnose disease reductions.

The production of increased anthracnose disease levels on red ripe tomatoes (Figure 6.3) could be a dose related response. Perhaps the doses of UV-C used to induce disease resistance in mature green fruit has caused more of a damaging effect to the fruit tissues in red ripe fruit, therefore allowing the onset of increased disease incidence and severity. Perhaps future work should look to identify whether alternative UV-C doses from both LIUV and HIPPL sources are capable of inducing increased or decreased disease resistance. If higher doses induce greater disease severity it would infer that the treatments are too damaging and going beyond what is a 'hormetic dose'. Whereas lower doses may be able to produce a hormetic dose and induce disease resistance. Future work should look into various potential dose responses.

The ability of the ethylene scrubber to prevent anthracnose disease postharvest meant that no real effects of light treatment in combination with ethylene scrubbing treatment could be seen. However, an interesting study would be to perform *C. gloeosporioides* inoculations pre-harvest as well as conduct UV-C hormetic treatments pre-harvest. This could really benefit the postharvest market of tropical fruits. The known germicidal effects of UV light

could potentially act to reduce disease further to what has been observed here. If a high proportion of *C. gloeosporioides* spores were killed off prior to storage, then disease reductions will be seen prior to ethylene scrubbing treatment. UV photocatalysis in postharvest storage and transport could then act to prevent disease of any surviving spores on the produce. Furthermore, the induction of increased defence levels as a result of UV-C hormetic treatment could help fruits prevent disease onset against already treated and weakened conidia.

In further future work, it would be good to look at the effects of UV-C light treatment in combination with ethylene scrubbing on ripening rate and fruit physiology. Much previous research demonstrates the use of UV-C light treatments in ripening delays and extension of shelf life in fruit crop (Obande *et al.*, 2011; Tiecher *et al.*, 2013; Severo *et al.*, 2015; Scott *et al.*, 2017, 2018). Furthermore, previously, UV-C irradiation in combination with modified atmosphere packaging (MAP), which decreases ethylene synthesis in fruits, resulted in colour change delays of cherry tomatoes (Choi *et al.*, 2015). In this research, work was performed looking at the combination of light treatments and ethylene scrubbing on fruit ripening; however, results were inconsistent and contained too small sample size. Unfortunately, there was neither the time nor resources to perform repeat experiments. Therefore, future work should look to perform experiments of a similar nature on a larger scale to determine whether the treatments in combination could act to further prolong the shelf life of fruit crops.

8.1.3.5 Molecular studies

The results from the molecular experimentation in chapter 7 confirm that UV photocatalytic ethylene scrubbing treatment prevents the onset of anthracnose disease by completely inactivating *C. gloeosporioides*. With fruits inoculated with *C. gloeosporioides*, there were no real differences observed in tomato defence gene expression between chambers until disease really started to take hold of control chamber fruits by 24 DPI (section 7.4). Furthermore, in mock inoculated fruits, there were no real differences observed in defence gene expression between chambers at either 14 or 24 DoT. These results indicated that disease prevention of treated fruits was likely as a result of the effects of treatment on the pathogen itself. This hypothesis was confirmed as at both 14 and 24 DPI, there was no gene expression of *Actin* or any *C. gloeosporioides* pathogenicity genes.

The molecular work presented here allowed for the ethylene scrubber to effect chamber environment with minimal human interaction to determine the overall effects of the treatment on tomato gene expression as well as pathogen gene expression. Now it can be concluded that the ethylene scrubbing treatment prevents anthracnose disease by affecting the pathogen alone, efforts would be made to determine by what cause the pathogen was affected: ethylene deprivation, decreasing RH levels or a combination of the two. The above experiments could be repeated, this time however, with greater efforts made to keep treatment chamber RH at 99%. This could involve the continual topping up of humidity boxes within the treatment chamber as well as adding greater numbers of humidity boxes. If 99% RH was achieved within the treatment chamber and results remained the same as observed here, it would determine that *C. gloeosporioides* prevention is as a result of ethylene removal.

However, if at 99% RH, *C. gloeosporioides* was able to cause infection on the tomatoes and *C. gloeosporioides* gene expression was detected from the samples, it would demonstrate that the disease resistance observed here is actually as a result of decreasing humidity levels.

If the experiment were to be repeated, it would be good to analyse the genetic regulation of fruit tissue at specific points between 2-24 hours post inoculation and hours of storage. This way, the early responses of fruits between chambers to *C. gloeosporioides* inoculation could be analysed. This would give a fuller understanding of fruit responses to *C. gloeosporioides* spore germination, quiescence and the early biotrophic stage of infection and furthermore, it would inform as to whether the ethylene scrubbing treatment had an effect on defence response pathways at this early stage of infection and treatment. Moreover, it would inform us at what stage in the *C. gloeosporioides* life cycle the pathogen was inhibited. It would allow us to identify where changes in *C. gloeosporioides* gene expression begin to differ between treated and control samples; therefore enabling us to further understand how treatments might prevent anthracnose disease. In repeating these experiments, greater spore concentrations would be used in order to increase the likelihood of detection as preliminary work was unable to detect *C. gloeosporioides* expression as early as is being suggested.

A further interesting study would be to look into identifying ethylene responsive genes within the *C. gloeosporioides* genome and knocking those genes out to create mutants. It would be interesting to see if it was possible to create *C. gloeosporioides* mutants which were able to show virulence on fruits in conditions absent of ethylene. Although this type of experiment would be intricate and challenging, it may help clarify how ethylene is able to trigger

aggressive infections. Understanding *C. gloeosporioides'* mode of action with regards to ethylene response could lead to the development of potential methods of preventing anthracnose disease on fruits.

8.1.4 The bigger picture

Moving forward, it is important that the UV photocatalytic ethylene scrubber is tested in real-world postharvest storage and transport settings to determine how effective it can be in extending fruit shelf life and preventing postharvest rot.

There is little data comparing ethylene removal methods in storage and transport settings (Keller *et al.*, 2013). The testing of this equipment in postharvest storage and transport will be crucially important to see the effects of treatment on actual fruit yields. Although laboratory experimentation provides us with an understanding of how the equipment might benefit the postharvest market, the way to really understand its effects would be by applying it in the real-world. By doing this, it is possible to also then test the equipment's effects on a much wider range of fruit species as well as against a much wider range of potential pathogens.

Application of the equipment would first be tested in countries where anthracnose disease caused by *C. gloeosporioides* is a major issue. The results observed throughout this research suggest that this is where the ethylene scrubbing equipment may prove to be most beneficially utilised. By using the equipment in these real-world settings, it would enable us to see just how effective the treatments could be in anthracnose control and prevention.

It would also be crucial to apply the equipment to real-world settings to determine how it effects storage atmosphere. Throughout this research, we saw continual reductions in humidity levels as a result of the treatment. Postharvest produce requires specific RH levels during storage and transport so to maintain optimum physiological condition (Hardenburg *et al.*, 1986). Therefore, it is important to see the effects of treatment on this parameter to determine if the treatment could prove detrimental to fruit physiology for the consumer.

Studies to determine how effective the treatments could be in postharvest storage and transport environments should be conducted over an extended time period, of at least 3 years, across several different storage and transport units, similarly to the methodology in the study performed by Wills *et al.* (2000). A study of this nature would allow for a well-rounded assessment of UV photocatalytic ethylene scrubbing treatment on a range of different fruit crops, against a range of different pathogens, and in a variety of different postharvest environmental conditions around the world.

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PIPS Reflective Statement Template

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.
The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken

PIPS Reflective Statement

I undertook my placement with Naturally Scientific Technologies Ltd (NST), a Nottingham based privately owned company focused on the production of high value vegetable oils using a unique biotech process. NST has spent the last six years researching, patenting and proving the scalability of its scientific processes.

During my placement, NST were transitioning from a research and development organisation towards commercial production, and were in the process of constructing a new production facility in Nottingham. Through the placement, I worked as part of a multi-disciplinary team, which included plant and production personnel, lab personnel and other technical and commercial professionals.

During my time with NST, I undertook several roles and responsibilities. I lead a team whose primary objective was to investigate methods optimising product efficiency, and wrote a report on behalf of that team to demonstrate our research findings. Being employed within the company as it was just starting production meant that I was fortunate enough to experience many different elements of work in science industry. I paid several visits to their new production site and was involved in the process of developing the site under construction. Furthermore, I gained experience in business product marketing and attended a global conference in London to advertise their products with a team of specialists.

My placement allowed me to build on many skills whilst also learning new ones. As the main objective of my placement was to undertake a laboratory based project, I spent a large proportion of my time with NST designing and undertaking an experiment to identify whether production efficiency could be optimised. As a PhD student, I was seen fit to be team lead of an undergraduate placement student and lab technician. Together, we outlined an appropriate experimental design to perform within the time period in order to produce results which may help the company in increasing oil yields, whilst reducing microbial contamination. Our experiments involved looking at varying concentrations of confidential media on the rate of oil production, as well as how sugar levels in the mixtures effected contamination by fungi and bacteria. I was then tasked to produce a report of our findings for the use of the company moving forward. This experience developed my skills in team and project management