The Effects of Photo, Physical and Mechanical (PPM) Pre-treatments in Controlling Certain Pests and Diseases in Carrots, *Daucus carota* L. var. Sativus cv. Carson

by

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Submitted in partial fulfillment of the requirements
for the degree of Master of Science

at
Dalhousie University
Halifax, Nova Scotia

and

Nova Scotia Agricultural College
Truro, Nova Scotia
October 2009

DALHOUSIE UNIVERSITY

NOVA SCOTIA AGRICULTURAL COLLEGE

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Dated: October 16th, 2009

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NOVA SCOTIA AGRICULTURAL COLLEGE

DATE: October 16th, 2009

AUTHOR: Kathryn Ruth Pickle

TITLE: The Effects Of Photo, Physical And Mechanical (PPM) Pre-treatments In Controlling Certain Pests and Diseases In Carrots, Daucus carota L. var. Sativus cv. Carson

DEPARTMENT OR SCHOOL: Department of Plant and Animal Science

DEGREE: MSc CONVOCATION: May YEAR: 2010

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Abstract

Pests and diseases have a significant economic impact on yield, quality and sale of processing carrots. In an effort to combat certain pests and diseases, the effects of photo, physical and mechanical (PPM) treatments on carrot (*Daucus carota* L. var. *Sativus* cv. Carson) were investigated. Generally, the incidence of certain pests and diseases was reduced through a variety of PPM treatments during two separate field experiments. However, not all of the reductions were statistically significant owing in large part to the low level of incidence of certain pests and diseases during the growth trials. To counteract this difficulty, experiments were conducted to determine the conditions necessary to induce blight in a controlled laboratory environment, with little success. In addition, HPLC-based detection of the defence hormone, salicylic acid, in carrot seedlings was established to help characterize the effects of PPM treatments from a biochemical perspective in future experiments.

List of Abbreviations and Symbols Used

13-HPLA - 13(S)-hydroperoxylinolenic acid

4-HBA - 4-hydroxy-benzoic acid

6-MM - phytoalexin, 6-methoxymellein

ABA - abscisic acid

ATCC - American Type Culture Center

CRD - complete randomized design

DHBA - 3,4-dihydroxybenzylamine

GAP - Good Agricultural Practices

GC-MS - gas chromatography – mass spectrophotometry

HPLC - high performance liquid chromatography

HR - hypersensitive reaction

IQF - Individually Quick Frozen

IR - induced resistance

JA - jasmonic acid

K - potassium

LA - linolenic acid

LapA - leucine aminopeptidase

LC-MS - liquid chromatography – mass spectrophotometry

MeJA - methyl jasmonic acid

MeSA - methyl salicylic acid

N - nitrogen

P - phosphorous

PAGE - polyacrylamide gel electrophoresis

PAL - phenylalanine ammonia-lyase

PCD - programmed cellular death

PCRP - Processing Carrot Research Program

pin - proteinase inhibitor

PIs - protease inhibitors

PPM - Photo, Physical and Mechanical

PR - pathogenesis related

ROS - reactive oxygen species

SA - salicylic acid

SAR - systemic acquired resistance

TCA - trichloroacetic acid

UV - ultraviolet

Acknowledgements

I sincerely thank my supervisor Dr. Raj Lada, and committee members Drs. S.K, Asiedu, C.D. Cladwell, J. Hoyle, and J. Norrie, for their supervision, guidance, patience and encouragement over the years. I would like to thank Anna Fitzgerald and Dr. A. Strurz for teaching me pathology techniques, Dr. R. McDonald from the University of Guelph for sending me research material for my experiments and Angus and Amanda from Oxford Frozen Foods for guiding me through the NS carrot industry.

I would also like to thank past and present members of ERG (formally PCRP), Azure, Phillip, Aru, Mason, Jim and Paul for being more than just co-workers, but for being friends as well. A special thanks is extended to my officemates Limin and Shannon, who shared with me all the ups and downs of being a graduate student.

Finally, I would like to thank my family and friends for providing never ending support and encouragement, especially my Aunts Norma and Arlene and husband Tom. Without them I would never have completed my education, thank you.

Chapter I: Introduction

1.1 Introduction

As more consumers are demanding chemically free and safe food, farmers have become increasingly dependent on alternative disease and pest control strategies, especially for vegetable crops. A desire to maintain healthy farmland and the increasing costs of crop inputs, have led to research to establish Good Agricultural Practices (GAP). GAP utilizes science to develop management practices that maintain and improve upon the health and quality of both our foods and our environment. One subset of GAP goals is to understand the plant defence mechanisms involved in carrot pests and diseases and subsequently develop eco-friendly technologies aimed at decreasing the dependency on agrochemicals while maintaining existing productivity.

Carrots (*Daucus carota* L. var Sativus) are the second largest horticultural crop in Nova Scotia, Canada, contributing nearly 12 million dollars annually to the economy (Ells, A., personal communication). By developing alternatives to conventional pest and disease management strategies and technologies, we can reduce agrochemical use and may increase carrot quality. Carrot blights, both early and late, are serious diseases in carrot fields of Atlantic Canada. In traditional management, the chemical fungicides chlorothalanil (Benlate) (Du Pont Canada (Mississauga, ON) and benomyl (Benlate) (ISK-Biotech, Mentor, OH, USA), are sprayed once every two weeks after blight symptoms have reached 25% row coverage (Bragg Lumber, 1999). While the application of fungicides is based on scouting information, the intent of the carrot industry is to reduce or eliminate fungicide use, if possible. This would not only reduce costs of production but also help in production of niche “green” or “eco-friendly” carrots. The two main fungal pathogens involved in carrot blight are *Cercospora carotae* (Pass.) Solh (early blight) and *Alternaria dauci* (Kühn) Groves & Skolko (late blight). Leaf blights not only compromise the plant"s ability to photosynthesize by reducing functional leaf area but also weaken the petioles making harvesting difficult. Mechanical harvesters clasp carrot leaves and pull the root into the harvester. Blighted carrot petioles and leaves can easily snap off, leaving the edible roots in the ground, thereby reducing harvest recovery (Rubatzky *et al.*,

1999 and Ben-Noon *et al.*, 2001). The unrecovered carrots then become a source of infection for the succeeding carrot crop as blights can over-winter in crop debris. Carrot blight is a continuous problem for carrot farmers, which affects the crop all through the season, reducing the yield in whole or in part, depending on its severity.

When *Cercospora* or *Alternaria* enter the carrot, a series of biochemical reactions take place, starting with recognition of the pathogen and triggering certain plant defences. Plants and/or pathogens release elicitors and certain unknown chemical messages, which initiate plant defence reactions (Prell and Day, 2000). The chain of defence reactions involves many different plant molecules, including plant growth regulators (phenols, phytoalexins, etc.) and plant growth regulators, such as the jasmonates and salicylates, which are known to be part of a complex network of plant defence mechanisms (Prell and Day, 2000). These hormones can also be induced after insect or herbivore attack (Baldwin *et al.,* 1997; Baldwin, 1998)**,** leading to the idea that mimicking this damage may result in an induced plant resistance. In order to imitate these defence reactions, the idea of photo, physical and mechanical (PPM) technologies was developed at the Nova Scotia Agricultural College (Lada, 2004a). It is hypothesized that certain PPM factors trigger plant defences, prior to infection, thereby conferring plant disease and/or pest resistance. If this works, PPM technology(ies) holds promise for increasing plant resistance in an eco-friendly way and preventing economic loss of carrots..

The model of plant defence reaction is quite complex. A plant must first recognize that it is under attack and then it must quickly respond and defend itself from the attack. This model becomes more complicated when all the possible modes of recognition, responses and defences are taken into consideration. This study investigates the potential uses of PPM technology in controlling carrot blights and other diseases and examines the role of salicylic acid (SA) as a possible mechanism through which plant defences are triggered due to PPM treatments: it also evaluates the techniques to confirm the response in field trials.

This information will help carrot growers reduce their dependency on fungicides and pesticides and encourage better GAP for carrots.

1.1.1 Hypotheses

A. *Alternaria* and/or *Cercospora* symptoms can be induced *in vitro*;

B. Photo (UV-C), physical (clipping), mechanical (brushing) (PPM) treatments inhibit certain pests and diseases in field-grown carrots;

C. PPM treatments do not have any negative effects on yield and quality of carrots.

D. PPM treatments induce resistance through an increase in endogenous levels of salicylic acid (SA).

1.1.2 Objectives

The intent of this project was to assess various photo, physical and mechanical (PPM) methods in reducing disease, and their effects on yield and carrot quality. The putative benefits of possible defence compounds and signals, such as salicylic acid (SA), were also examined to determine if they play any role in PPM defences.

1.2 Literature Review

1.2.1 The Carrot Industry

Nova Scotia is the largest producer of Individually Quick Frozen (IQF) processing carrots in Canada, with nearly 1000 ha of farmland planted with carrots every year. Carrot growers encounter many problems including poor stand establishment due to cool planting temperatures, drought stresses, physiological disorders such as greenshoulder, and insect and disease outbreaks. The two main foliar diseases of carrots, *Cercospora carotae* and *Alternaria dauci,* have been shown to cause as much as 15% to 74% reduction in total yield and can also reduce carrot quality (Bragg Lumber, 1999; Ben-Noon *et al.*, 2001).

1.3 Carrot Blight

Two fungi, *Alternaria dauci* and *Cercospora carotae* cause carrot blights. These two disease-causing organisms belong to Ascomycetes and are similar in their symptomology but differ in the timing of pathogenic development.

Cercospora infects younger carrots, while *Alternaria* is more commonly found on older leaves and plants. They are non-obligate parasites with the ability to complete their life cycle on living and/or dead materials. *Cercospora* sp. and *Alternaria* sp. are eukaryotic spore-bearing organisms that lack chlorophyll and have chitin and glucans embedded in their cell walls (Agrios, 2004). Their vegetative bodies are called mycelia (haploid with cross walls) and are comprised of hyphae that can enter through stomata or between surface cell walls. These serve as normal points of entry for both pathogens (Rotem, 1994; Takaichi and Oeda, 2000).

Alternaria and *Cercospora* both reproduce by spores. Ascomycetes produce both asexual conidia and sexual ascospores but it is uncommon to find them in a sexual stage (teleomorph), unless it is at the end of the season or their food supply has run out (Agrios, 2004). Both conidia and ascospores can over-winter in carrot debris remaining after harvest.

Carrot blights occur throughout the growing season. Damp weather and high humidity create an ideal environment for blight sporulation. The spores of *Cercospora* and *Alternaria* are easily carried by the wind or in water droplets. A combination of humidity and wind can disperse carrot blight spores throughout the field allowing them to adhere to the bottom of the carrots leaves and germinate.

1.3.1 *Cercospora carotae*

C. carotae is the most common and destructive carrot disease in Quebec (Brodeur *at el.*, 1998) and is found in all carrot production areas. *Alternaria* may be the most common carrot blight worldwide, but *Cercospora* is the most common in Canada (Davis and Raid, 2002). *C. carotae* affects younger carrots

(van Delden and Carisse, 1993) and therefore, can be found earlier in the growing season than *Alternaria* leaf blight, which infects in mid- to late summer.

Cercospora mycelia are light brown and may appear as a light coating on the underside of carrot leaflets (Davis and Raid, 2002). Their spores are dark in colour, multiseptate and cylindrical. The spores generally develop in long rows and may bunch together. *C. carotae* may produce cercosporin; a perylenequinone toxin, that is light-activated and produces oxygen species that attack plant cell walls (Daub and Ehrenshaft, 2000). This toxin may facilitate the entry of the pathogen through the plant"s stomata (Brodeur *et al.,* 1998) allowing more of the fungus to infect and invade the carrot leaves.

1.3.1.1 *Cercospora* Symptoms

Cercospora lesions are almost circular in shape and can easily be identified as *Cercospora* leaf blight. The blight is a grey brown colour and can appear on the leaflet"s surface or petioles (Figure 1.1). The center of the infected tissue becomes necrotic with a surrounding yellow ring and no clear borders. As the lesions develop and merge together, the necrotic centers may fall out. Petioles and stems can be completely encompassed, causing the entire leaf to starve and die, however roots are not infected.

Figure 1.1 Early carrot blight, *Cercospora carotae*, depicting the yellow halo often seen surrounding blight necrosis.

1.3.1.2 *Cercospora* Disease Cycle

Cercospora require the leaf surface to be damp in order to successfully infect the leaf. As such, long periods of high humidity, rain or dew aid in the spread and rate of *C. carotae* infection (Carisee and Kushalappa, 1989). In addition, the light spores are easily carried by the wind or splashed off the leaf surfaces. Lesions are first seen on the younger leaves within ten days of infection if dampness and optimal temperatures are present (above 20ºC). After infection, *C. carotae* is able to over-winter in soil and crop residues to continue the cycle the following growing season (Brodeur *et al.,* 1998).

1.3.2 *Alternaria dauci*

Alternaria is the most common foliar disease in carrots and has been found on carrots all over the world (Davis and Raid, 2002). The conidia are formed individually and have a filamentous beak that is three times the length of the main body. As with most *Alternaria* species, the conidia are obclavate (club shaped), pointed and have both horizontal and vertical cross walls and they appear light olive in colour, as seen in Figure 1.2 (Davis and Raid, 2002; Rotem, 1994).

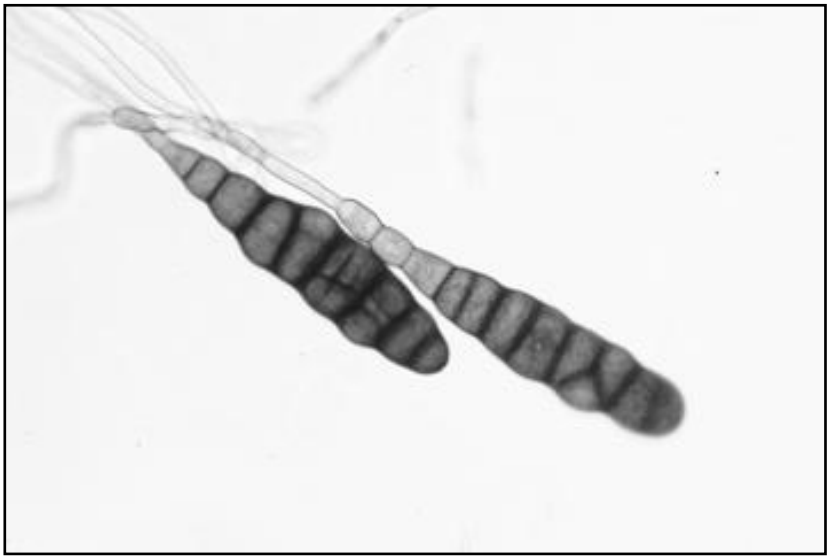


Figure 1.2 Conidia of *Alternaria dauci*.
(Source:http://ag.arizona.edu/PLP/alternaria/online/alternaria_species/pages/Alternaria_Dauci.htm, 2005).

Alternaria is a diurnal sporulator and has both a light and a dark sporulation phase. Sunlight increases the production of conidiophores and conidia are formed in the dark (Rotem, 1994). *Alternaria dauci* will not sporulate in culture unless supplied with UV-C light (Rotem, 1994).

Alternaria spores are parasitic and enter the host plant *via* stomata (Rotem, 1994) or directly though the epidermal cell walls of the leaf (Takaichi and Oeda, 2000). High humidity and leaf surface dampness are necessary for infection to take place (Standberd, 1988a). *Alternaria* weakens the host by absorbing various plant metabolites. High humidity and dampness increase *Alternaria's* ability to derive nutrition from the plant (Rotem, 1994) and therefore, they have a higher infection and invasion rate when the climate is warm and damp. As disease spreads over the leaf surface, the carrot"s ability to photosynthesize is diminished leading to limitation in carbohydrate synthesis and translocation to its storage organ, the root, potentially reducing yield.

Uptake of nutrients by the fungi is accomplished by the release of cell wall degrading enzymes, such as polygalacturonase, pectin lyase, pectin methylesterase (Rotem, 1994), and non-enzymatic compounds including alternariol (Rotem, 1994; Dugdale *et al.*, 2000) and/or AL-toxin (Vesonder *et al.,* 1992). As the fungal hyphae spread into the leaf"s mesophyll, pigment cells and the plasma lamellae are digested and symptoms such as a yellow halo can be seen around the site of infection.

1.3.2.1 *Alternaria* Symptoms

The fungus attacks leaves and petioles of carrots, causing leaf spot and blight. Figure 1.3 depicts a typical carrot plant infected with *Alternaria* leaf blight. The lesions are irregular dark brown/black with yellow margins, and are usually found at the edge of leaflets. As the disease progresses, the entire tip of the leaflet may turn yellow then shrivel up. Petioles can show elongated lesions without infection being detected on the leaves.

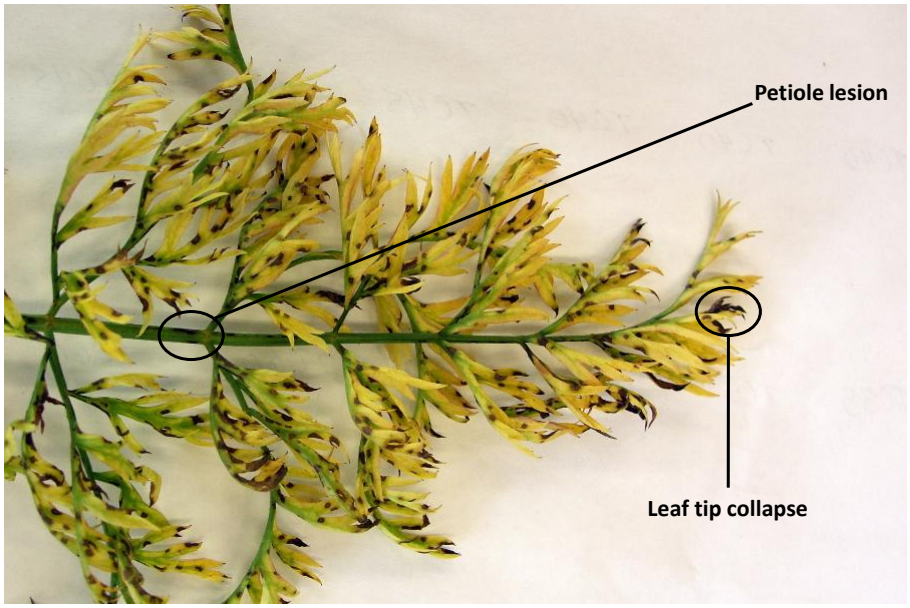


Figure 1.3 Carrot blight, *Alternaria dauci*, depicting petiole lesions and necrotic leaf-top collapse.

Alternaria dauci causes damping-off in carrot seedlings, but this is uncommon. The resistance of young plants to blight, combined with the normal environmental conditions, can lead to late emergence of this blight, usually in August in Nova Scotia. Carrots are susceptible to most *Alternaria* species; they are attacked for a short period of time in their seedling stage and then they have a long period of latent infection until they mature, after which they are vulnerable for a longer time during maturation (Rotem, 1994).

1.3.2.2 *Alternaria* Disease Cycle

Spores over-winter within and on carrot seeds, on weeds surrounding the field and on carrot leaf debris that could not be harvested because of their weakened petioles. In Israel, *Alternaria dauci* could retain its ability to sporulate on carrot material for longer during dry periods than wet periods (Rotem, 1994). Spores can be windborne or carried by water droplet movement. When environmental conditions are met, spores begin to grow and infect new carrot material. The optimum environmental conditions for sporulation and infection are when the day temperature is between 16 to 24ºC, and the night temperatures are greater than 12ºC.

1.3.2.3 Blight and Its Response to Fertilizer

Vintal *et al.* (1999) reported that the relationship between *A. dauci* and the degree of infection is more related to carrot health than to fertilizer application. When carrots have optimum nutrition, they are stronger and more capable of resisting attack. When fertilizer was halved to 50 ppm nitrogen (N), 10 ppm phosphorous (P) and 37 ppm potassium (K), carrot plants become nutritionally stressed and were therefore, more susceptible to blight. If phosphorous and potassium are in excess, there is little effect on blight. Carrots do not take up nutrients past their needs; even increasing the fertilizer application three fold did not increase the levels of NPK in the carrot leaf tissue (Vintal *et al.*, 1999; Pettipas *et al*., 2006).

Increased nitrogen has a differential effect on blight. When a carrot has ample or mildly excessive nitrogen, its maturity is delayed, and younger plants are less susceptible to *A. dauci* (Vintal *et al.*, 1999). Vintal"s recommendation is not to alter fertilizer application patterns in response to blight, as the optimal rate of fertilization for carrots and disease resistance is too great for carrots to be grown economically.

1.3.3 The Response of Carrots to Blights

Carrots have evolved many strategies to defend themselves from biotic and abiotic disturbance. Increasing defensive compounds is one of the mechanisms that carrots have to defend against invading pathogens. Kurosaki and Nishi (1983) identified a phenolic compound known as phytoalexin, 6-methoxymellein (6-MM), in carrot root while Dugdale *et al.* (2000) identified the same compound in carrot cell suspensions. The 6-MM production increases within the tissues after infection with *A. dauci*. Mercier and Kuć (1997) reported that petiole 6-MM concentrations were quadruple the concentration in the leaf. The 6-MM concentration peaked at the same time as lesions began to appear, and this peak was maintained for several days. Mercier and Kuć (1997) reported that *C. carotae* does not produce 6-MM, therefore, the carrot may synthesize this phytoalexin at first infection in an attempt to reduce the severity of lesion

formation. The concentration remains high in order to actively reduce fungal growth and lesion development (Mercier and Kuć, 1997).

Research on *Cercospora* resistance has been carried out with induced systemic resistance as both the induction for resistance (challenge) and as a measure of induced resistance. Mercier and Kuć (1996) determined that a concentration of 3 X 10^4 conidia per mL of this species is the optimum inoculation to induce resistance. The experiment involved newly emerged carrot leaves that were challenged with the pathogen 23 days later. As the lesions were forming, the 6-MM phytoalexin concentrations in the leaf increased. When carrots were treated with 6-MM and again with *C. carotae,* the disease severity was not as pronounced as in the control group (Mercier and Kuć, 1996) indicating that carrots produce natural blight defence compounds after the plant senses an attack. If biotic factors such as blight can trigger plant defences and protect from further attack, mimicking the fungal infection through abiotic treatments, PPM factors may also trigger these essential protective compounds and confer resistance to blight.

Takaichi and Oeda (2000), using carrot cell culture *in vitro,* demonstrated a decrease in chlorophyll and total soluble polyphenols content compared to control plants that were not treated with fungi. They measured these parameters six days after inoculation with *A. dauci,* several days before symptoms usually appeared. As previously mentioned, *A. dauci* degrades pigment organelles, but its effect on phenols, a group of compounds involved in plant defence, is not well understood.

1.4 Plant-fungal Interactions

When a spore lands on a plant, both organisms react. This developing relationship begins with a series of chemical and physical reactions within both organisms (Prell and Day, 2000). Elicitation starts when the plant or pathogen releases an elicitor after they recognize each other; this starts the plant defence process (Prell and Day, 2000). The plant must have an elicitor receptor and then this combination sets off an effector. The effector, a low molecular weight

compound, initiates a chain of defence reactions. Defence reactions are categorized into two levels; the first level of plant defence mechanisms are the physical barriers, such as waxy cuticle, cell walls, membranes and spines and the second level is metabolic and involves a variety of messengers, proteins, signals and pathways.

1.5 Defence Mechanisms

One of the first noticeable reactions of plant defence is the hypersensitive reaction (HR). This involves rapid cell death and necrosis of adjacent cells when injured by invading pathogens or other external force (Prell and Day, 2000). When a cell is penetrated and undergoes HR and cell death, it sends protein messengers to the surrounding cells (Prell and Day, 2000) for them to die or starve/inhibit the invading pathogen. This may contribute to the yellow halo often seen around a developing fungal lesion (Figure 1.1). The strength of the HR translocation may explain why the closest tissue to the site of infection is killed, but more distal cells only form defence compounds (Prell and Day, 2000). The progression of HR can be examined by monitoring the progression of phenols and reactive oxygen species (ROS) with UV light (Prell and Day, 2000).

Oxygen bursts are the development of ROS, which aid in both HR and message translocation. One of the results of the oxygen burst is the synthesis of hydrolytic enzymes that destroy fungal walls (Prell and Day, 2000). Free radical oxygen, also known as superoxidized oxygen may also play a role in cell wall strengthening. Its production stimulates proline-rich proteins to form cross walls in cells near the site of fungal infection (Prell and Day, 2000).

Systemic acquired resistance (SAR) is the spreading of resistance to other cells and tissues after a plant senses an invasion (Prell and Day, 2000). These can include the signals for the formation of structural proteins to strengthen cell walls, the production of digestive enzymes that will attack the fungal cell walls, and defence toxin production (Prell and Day, 2000).

Pathogenesis related (PR) proteins are a sub-group of defence-related proteins. These proteins are involved in most defence reactions that are not

purely physical, but also serve as developmental proteins. Their developmental role is not well understood, but they are expressed after an invasion has been perceived by the plant (Prell and Day, 2000). This indicates that a defence gene must be activated by the interaction between plant and fungi. Five different families of PR proteins have been identified by polyacrylamide gel electrophoresis (PAGE), including; ß-1,3-glucanases (PR-2), chintinases (PR-3) and osmotin (PR-5) (Sticher *et al.*, 1997; Prell and Day, 2000).

1.5.1 Salicylic Acid (SA)

SA is a simple phenol (2-hydroxybenzoic acid, Figure 1.4) that is ideal for long-distance transport within phloem, making it a prime candidate as a SAR messenger (Raskin, 1992). SA is involved in several defence functions as well as normal developmental processes such as plant thermogenesis, flower development and allelopathy (Raskin, 1992).

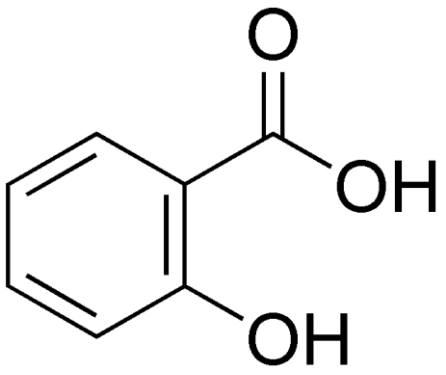


Figure 1.4 The structure of salicylic acid, $C_7H_6O_3$.

The exact biosynthetic pathway of SA is not well understood, but it is believed that SA is a degradation of cinnamic acids, and therefore, is formed in conjunction with the shikimic acid pathway (Raskin, 1992; Yalpani *et al.*, 1994; Wildermuth *et al.*, 2001). UV radiation increases levels of phenols and cinnamic ester (Jansen *et al.,* 2001) and SA (Yalpani *et al*., 1994). As UV radiation induces SA, a plant previously stimulated by UV-C radiation may have already

triggered an SAR response, which may induce defence against later pathogen attack.

Interaction between JA and SA defence pathways may bridge differences between short term and long-term plant defences, respectively (Doares *et al.,* 1995; Chao *et al.,* 1999). The relationship between SA and JA in defensive pathways is not well understood. For example, these compounds have been shown to hinder each other (SA inhibits the octadecanoid pathway), yet it is not clear whether they are involved in the same pathways or produce similar reactions through very different pathways (Doares *et al.,* 1995). Also, both JA and SA increase after pathogen attack in *Arabidopsis thaliana* (L.) Heynh*,* but activate different genes (Thomma *et al.,* 1998). PR-1, 2 and 5 all require SA signals, but PR-3 and 4 are SA-independent and require JA (Thomma *et al.,* 1998). This work was carried out on various JA and SA deficient mutants. PR-4 is induced independently of the defence genes *LapA* and *pin*2, but ethylene and SA seem to be the necessary signals involved, though they are independent of each other (van Kan *et al.,* 1995).

Aspirin (acetyl salicylic acid) prevents wound-induced gene expression by inhibiting the 13-HPLA to 12-oxo-phytodienoic hydroxyperoxide-dehydrase within the octadecanoid pathway (Peňa-Cortés *et al.*, 1993). SA also inhibits the important *LapA* gene that systemin and abscissic acid (ABA) induces (Chao *et al.*, 1999). *LapA* proteins are abundant after pathogen invasion in tomatoes (*Lycopersicon esculentum* L.), leading to the idea that they are important in defence processes (Pautot *et al.*, 1991). Chao *et al.* (1999) used tomato mutants to demonstrate that there are at least four different, possibly overlapping, signalling pathways utilized in defence responses.

SA has been identified as a lesion inhibitor in tobacco (*Nicotiana tabacum* L.) and other plant species (Raskin, 1992). This may be due to its HR activity. Plants will sacrifice a few cells in order to isolate a pathogen and thereby reduce the ability of the disease to spread. Ethylene is involved in programmed cellular death (PCD) and SA is involved as a messenger for a possible next step in plant

defence, SAR. SA can induce the production of PR genes, a necessary precursor to SAR.

1.5.2 Jasmonates

Jasmonates are derived from a polyunsaturated fatty acid called linolenic acid (LA) within lipid membranes (Figure 1.5). This class of hormones is involved in many biological activities, including the synthesis of volatiles, vegetative storage proteins, air-borne signaling, growth inhibition and defence signaling and pathways (Srivastva, 2001).

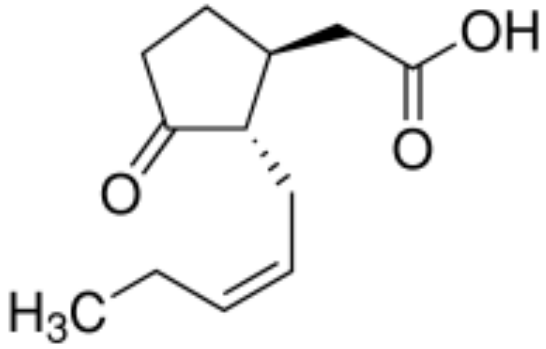


Figure 1.5 The structure of jasmonic acid, $C_{12}H_{18}O_3$.

The defensive properties of JA are activated by several substances, and possibly through different pathways. In members of the Solanaceae family, JA and methyl jasmonate (MeJA) have been found to be induced by cleaving of prosystemin into systemin, an 18 amino acid polypeptide (Srivastva, 2001). Systemin can induce JA synthesis both locally and systemically (Narváez-Vásquez *et al.,* 1995). Baldwin (1998) reported that JA accumulates in various wild plant leaves after an herbivore attack. Wild plants that were treated with MeJA prior to grasshopper attack had a significantly higher fitness level than the control plants (Baldwin, 1998) indicating the importance of jasmonates in defence. Baldwin *et al.* (1997) also found that wounding flowering tobacco"s (*Nicotiana sylvestris* Speg. & Comes) leaves resulted in a 10-fold increase of JA leaf concentration within 90 minutes and a 3.5-fold increase in root concentration after three hours. Their results suggest that JA is a major component in both defence signaling and pathways (Baldwin *et al.,* 1997).

Wounding of cell walls by a fungal attack releases elicitors and oligosaccharide fragments from both the fungal cell walls and the injured plant cell walls. Doares *et al.* (1995) reported that elicitors like oligogalacturonides (plant-derived), chitosan oligosaccharides (fungus-derived) and systemin all increase the levels of JA in tomatoes. The work of Gundlach *et al.* (1992) supports the hypothesis that an elicitor-receptor complex activates a lipase that releases LA, a precursor to JA, and also allows for intercellular communication (Farmer and Ryan, 1992).

Several octadecanoid precursors of JA have also been analyzed for their ability to induce wound signaling and intracellular communication. Farmer and Ryan (1992) believe that JA amplifies the wounding signal and may saturate proteinase inhibitor (*pin*) receptors, switching on their genes. Exogenously applied LA, 13(S)-hydroperoxylinolenic acid (13-HPLA) and phytodienoic acid all induce *pin*1 and 2 synthesi*s*, which occur naturally in wounded plants (Farmer and Ryan, 1992). 13-HPLA may also be cleaved to produce volatile aldehydes and 12-oxo-acids, which are defensive compounds found in wounded tissue (Srivastva, 2001). Eleven phenolic compounds are identified in orange carrots, along with 35 volatile compounds (Alasalvar *et al.,* 2001), some of which are known to come from 13-HPLA precursors.

Fungal elicitors like chitosan, are also known to induce *pin* synthesis (Farmer and Ryan, 1992). Creelman *et al.* (1992) measured the increase of JA and MeJA in wounded soybean hypocotyls and determined that they are part of the signaling for *pin* genes. Other defence genes encode structural proteins that aid in the prevention of the spread of fungal hyphae. These are glycine and proline-rich proteins; perhaps similar to the proteins that the oxygen burst is known to induce.

Leucine aminopeptidase (*LapA)* is modulated by some of the same molecules as the *pin*2 gene; MeJA, systemin, and octadecanoid pathway intermediates (Chao *et al.,* 1999). Peňa-Cortés *et al.* (1993) have concluded that ABA is essential for *pin*2 gene expression and acts early in the octadecanoid pathway (Chao *et al.,* 1999) but there is no consensus among researchers.

Hildmann *et al.* (1992) and Wasternack and Parthier (1997) proposed the idea that ABA responds to stress and may operate upstream to the JA pathway.

Jasmonates play an integral role in plant defence but their place in carrot blight defence is unknown. This review suggests that this hormone could play one or several roles in defence, which can overlap and associate with fungal-derived elicitors, plant signals and other hormones.

1.5.3 Other Defence Hormones

JA alone does not always induce defence pathways; other hormones and compounds are necessary in initiating plant defence signals. Peña-Cortés *et al.* (1993) reported that ABA could also trigger the activation of wound-induced genes involved in the octadecanoid pathway. Xu *et al.* (1994) also reported that ethylene and MeJA work synergistically to induce PR-1 and PR-5 in tobacco seedlings. Ethylene blockers do not prevent PR-5 genes from producing mRNA, therefore these two PR genes are induced, at least partially, by separate pathways (Xu *et al.,* 1994).

There are many types of plant defence genes and they are activated by different pathways and signals. Ethylene is involved with the development of HR and necrotic lesions that develop after a pathogen invasion (Chao *et al.*, 1999). Ethylene produced after wounding increases the gene expression of chitinase (PR-3) and ß-glucanase (PR-2) (Creelman *et al.,* 1992), which encodes fungal cell wall hydrolysis enzymes.

Phytoalexins are low-molecular-weight antimicrobial secondary metabolites often associated with plant-pathogen defence (Hammerschmidt, 1999). They are a group of chemically diverse compounds that are derived from many separate pathways. They are important compounds in plant defence, and it is believed that phytoalexins are induced after the plant recognizes a pathogen elicitor, like N-acetylchitoheptaose (Nojiri *et al.*, 1996). There are specific cell wall receptors for N-acetylchitoheptaose and rapid membrane depolarization occurs shortly after recognition (Kuchitu *et al.,* 1993).

Carrot cultures accumulate the phytoalexin 4-hydroxy-benzoic acid (4-HBA) in response to fungal elicitors. 4-HBA is synthesized from the phenylpropanoid pathway, and is preceded by a rapid increase of Ca^{2+} and efflux of K^{+} ions (Bach *et al.,* 1993). An elicitor that stimulates 4-HBA also triggers programmed cell death in carrots (Veit *et al.,* 2001). Phytoalexins have only been studied in nature or field environments to a limited degree. It is not well known if they are produced only after an attack or wounding, as seen in controlled greenhouse experiments or if natural stresses that plants encounter keep phytoalexin production turned on continuously (Hammerschmidt, 1999). Hammerschmidt (1999) asked the question whether phytoalexins are truly defence compounds or a means through which plants "shunt" carbon stores to antimicrobial stores.

1.5.4 Interactions Between Various Defence Compounds

In rice (*Oryza sativa* L.) cell cultures, JA was a signal in the production of momilactone A, a phytoalexin that is induced by N-acetychitoheptaose (Nojiri *et al.*, 1996). Another phytoalexin, camalexin, which may be required for the signaling of JA synthesis (Saskia *et al.*, 2003) was identified in JA-deficient carrot mutants. These mutants are highly susceptible to *Alternaria brassicicola*, however the ethylene and salicylic acid deficient mutants are not (Saskia *et al.*, 2003). Saskia *et al.* (2003) also reported that normal plants accumulated JA in response to pathogen attack and that JA synthesis requires camalexin, but that JA does not increase the camalexin concentration. This indicates that camalexin production is independent from JA.

Studies on resistant and susceptible mutants of Arabidopsis to *A. brassicicola* (Schwein.) Wiltshire, indicate that camalexin and JA are necessary for resistance to this disease (van Wees *et al.*, 2003). van Wees *et al.* (2003) goes on to report that JA signaling-deficient mutants (*pad1* and *coi1*) were more vulnerable to *A. brassicicola* attack than both wild-type and SA and ethylene deficient, suggesting the *A. brassicicola* resistance is depend on JA signaling and not SA or ethylene. The effect of *Pseudomonas syringae* Van Hall. on

Arabidopsis is well mapped, and resistance to this diseases is dependent on SA and ethylene pathways and the activation of *COI1* gene (van Wees *et al.*, 2003), supporting the idea that complex cross-talk between signaling compounds are key to understanding the various resistance between diseases.

1.5.5 Induced Defences

Wounding can trigger the synthesis of JA and SA, as well as other defence compounds (Baldwin, 1998). As these hormones are produced, they may then activate the transcription of other defence compounds and genes (Ryan, 1990). Plant-fungal interactions may also induce these same pathways (Farmer and Ryan, 1992), possibly inducing defence compounds like phytoalexins and phenols as well (Bach *et al.,* 1993). Figure 1.6 outlines a proposed model, for plant defence (Lada, R., personal communication). The intent of this research was to establish connections between known inductions of plant defence mechanisms by biotic factors to the unknown induction of possible plant defences by abiotic means, (e.g. PPM technologies). Some abiotic stresses are known to cause the accumulation of phenolic compounds in plants, like UV-B (Jansen *et al.,* 2001) and wounding (Birkenmeier and Ryan, 1998). If abiotic factors also induce the production of plant growth regulators such as SA, ABA, JA and ethylene as in other biotic stresses, then using an abiotic factor could help to prepare the plants to defend against potential pathogen attack.

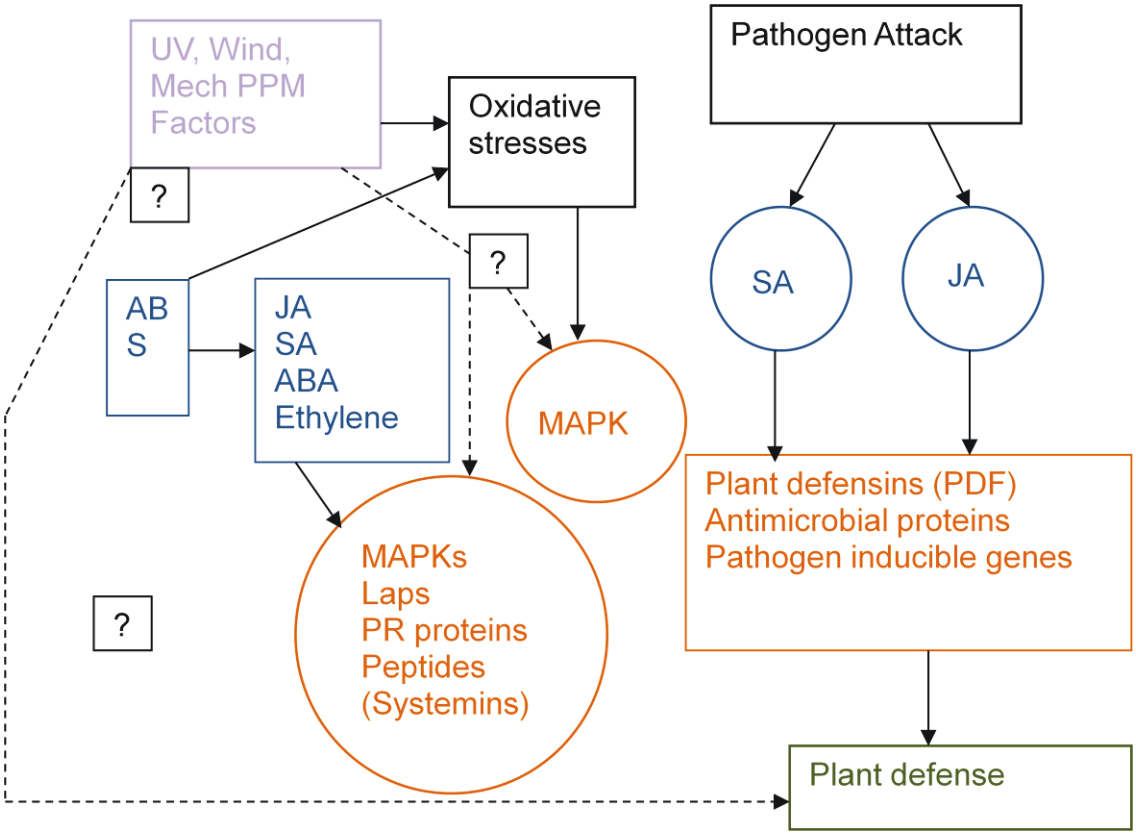


Figure 1.6 Proposed plant defence model (Lada, R., personal communication).

1.6 Photo, Physical and Mechanical Factors

Photo factors involve the application of UV radiation to the surface of plants. Physical (brushing) factors are those that disturb the plant while not necessarily damaging the cell walls, while mechanical (trimming) factors involve the destruction and removal of sections of the plant.

UV-C radiation, leaf brushing and clipping were examined in 2004 field trials (Truro, NS), where the preliminary results suggested that UV-C at 4 weeks or brushing at 8 weeks (40 strokes) can significantly reduce the severity of carrot blight, Figure 1.7 (Lada, 2004b).

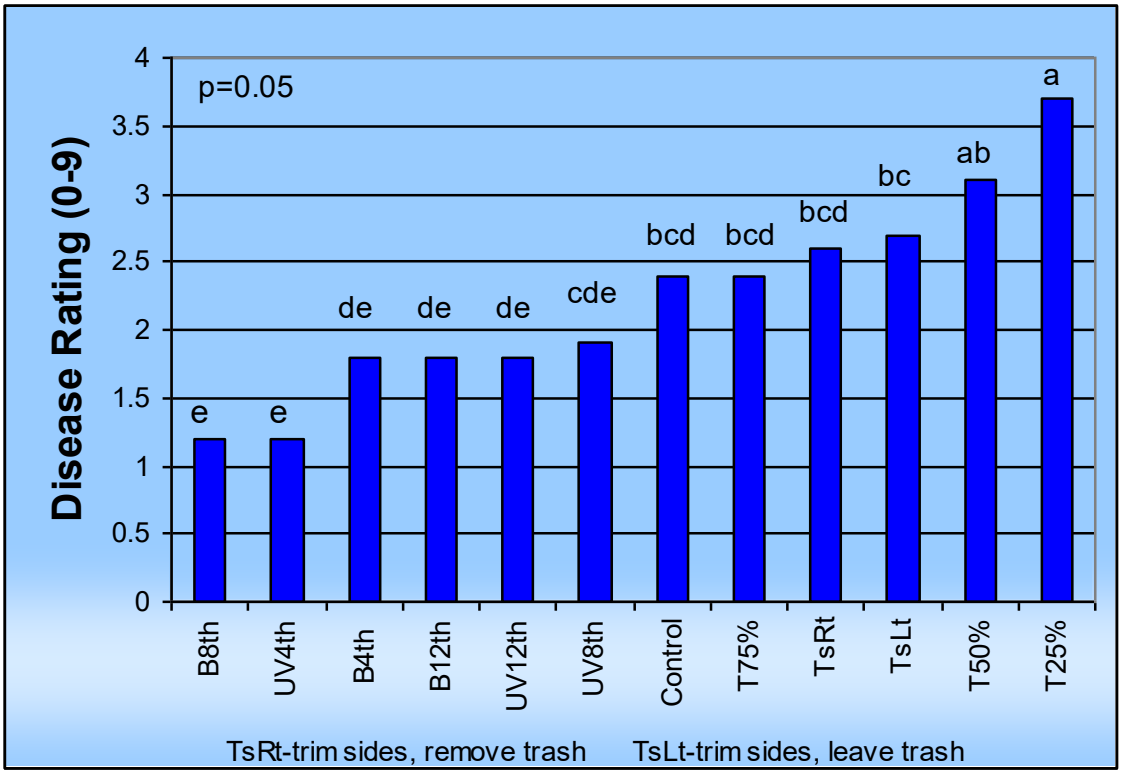


Figure 1.7 Rating (0-9) of severity of *Alternaria* and *Cercospora* blight infection at harvest, following brushing (B), trimming (T) and UV-C radiation (UV) on „Carson" carrots (Lada, 2004b).

1.6.1 UV Radiation

Ultraviolet (UV) radiation is light of certain wavelengths that is longer than x-ray and shorter than human visible light. It is sub-divided into three groups: UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (100-280 nm). UV-C is of little physiological importance to plants because it seldom passes through the earth"s stratosphere to reach the plants" surfaces (Danon and Gallois, 1998). When UV-C radiation does reach the earth"s surface it can affect plants in a similar fashion to UV-B radiation and stimulates changes within the plant (Danon and Galllois, 1998). A low level of UV-B radiation has been shown to increase phenol concentrations in cucumber (*Cucumis sativus* L.) leaves, while still maintaining plant health (Teklemariam and Blake, 2003). Phenols can act as defensive compounds and scavenge free radicals. This reduces the damaging

effect these free radicals have on cell membranes. UV radiation has increased the levels of phenolics (like flavonoids), cinnamate esters, lignin and tannin (Jansen *et al.,* 2001), all of which may be involved in plant defence. SA is a simple phenol and exposure to UV light increases phenol production. Therefore, SA may also increase after exposure to UV. Danon and Gallois (1998) report that UV-C radiation may physically induce PCD in plant nuclei. If UV-C radiation can induce PCD, this would begin a cascade of other plant defence reactions similar to those induced by HR and SAR cell death protecting plants against pests and diseases.

Ultraviolet radiation protection is essential as more UV-B rays can pass through the depleting ozone layer that surrounds the earth. The negative effects of UV-B radiation have been well documented and include; reduced photosynthesis, biomass reduction, damage to chloroplasts, DNA, RNA and proteins and ROS production (Yannarelli *et al.*, 2005).

Hydrogen peroxide, an ROS, is also a signaling compound that has been identified in both abiotic and biotic stress defences (Mackerness *et al.*, 2001). UV-B radiation stimulates ROS, which in turn stimulates ROS defences; antioxidants, phenols and ROS absorbing pigments. The phenylpropanoid pathway may produce many of these ROS defence compounds, such as anthocyanins, flavanoids, phenols and phytoalexins which then can induce further defences like lignin formation (Teklemariam and Blake, 2004). Phenylalanine ammonia-lyase (PAL) is an enzyme upstream of the phenylpropanoid pathway and Teklemariam and Blake (2004) have shown that PAL increases after mild UV-B stress. Flavonoids and phenolics (sinapic acid esters) in the epidermis of plants are necessary to reduce damage caused by increasing levels of UV-B radiation (Li *et al.*, 1993). Costa *et al.* (2002) reported that a dose of 15.0 and 30.0 kJ/m of UV-B light is enough to induce antioxidant defences (CAT, GDH) and protect plant cells from ROS. Ascorbic acid, peroxidases and glutathione reductase have all been reported to increase under UV-B stress in various plants (Jain *et al.*, 2003). The line between where UV-B radiation can induce defences without causing too much harm to the plant and

excess radiation damage is not well defined. If mild doses of UV-B or UV-C radiation can induce these protective compounds, like flavonoids and phenols (Li *et al.*, 1993) and protect against ROS (Mackerness *et al.*, 2001), they may also be utilized for other protective functions, like biotic defence.

1.6.2 Wounding: Brushing and Clipping

Wounding, like brushing and exposure to high water and air pressures, have also been shown to increase defensive compounds, hormones, phenols, antioxidants and toxins (Birkenmeier and Ryan, 1998; Seljasen *et al.,* 2001; Reyes and Cisneros-Zevallos, 2003). There is an increase of 6-MM, ethylene and ethanol, and a decrease of total terpenes and sugars in carrots after they have been harvested and wounded (Seljasen *et al.,* 2001).

The interest in wounding as a PPM treatment comes from observations from nature. Plants that have been previously damaged by biotic stresses, like herbivory, exhibited higher fitness after a second attack than plants that were not previously attacked. Artificial wounding is not an exact replica of herbivory wounding, as demonstrated by De Moraes *et al.* (1998). In De Moraes *et al.* (1998), plant defences are similar between artificial wounding and caterpillar damage but the volatile profiles are different (Constabel, 1999). Protease inhibitors (PIs) bind proteolytic enzymes (from insects) and block their activity. Some PIs have also been shown to be wound and pathogen induced and may therefore play a critical role in disease resistance as well (Constabel, 1999).

Clipping, a simulated mechanical injury, may also stimulate JA and/or SA pathways and increase compounds like PR proteins and phytoalexins that are known to confer defence to biotic stress like fungal infection (Prell and Day, 2000). It is not known whether these PPM factors would trigger plant defence. Similarly, the hormones and compounds involved in carrot blight defence are not known.

1.7 Conclusion

Although there is considerable information about plant-pathogen interactions, plant defences and metabolic pathways, there are gaps in our knowledge of how these plant defence concepts work together. As seen in the model plants *Arabidopsis* and tomato, not all plants use the same pathways in defensive strategies or the same defence compounds. The purpose of this series of experiments is to determine if PPM technologies can be used to trigger plant defences and if SA is involved in PPM-induced carrot defence.

Chapter II: Disease symptom index for *Cercospora carotae* and *Alternaria dauci* on *Daucus carota* var. Sativus cv. Carson

2.1 Introduction

Carrot leaf blight, whose causal agents are *Cercospora carotae* (Pass.) Solh and *Alternaria dauci* (Kühn) Groves & Skolko, is a destructive and economically damaging disease that carrot producers worldwide must control. Leaf blight causes species-specific lesions on the petioles and surfaces of both young and mature carrot (*Daucus carota* L.) leaves. The carrots affected by leaf blight are adversely affected in three ways; (1) energy and resources are diverted from root bulking to defence mechanisms, (2) functional leaf area is reduced which inhibits photosynthesis and (3) harvest losses increase due to weakened carrot petioles breaking before the carrot is pulled from the ground. Section 1.3 explains leaf blight diseases and their effects in greater detail.

The most common and widely used method of blight control is a combination of conventional farming practices and chemical suppression. Conventional farming practices such as soil cultivation and crop rotations can reduce the spore load within the environment, as blight spores remain in crop debris and weeds from previous years (Pryor *et al.*, 2002). Foliar application of chemical fungicides may also be employed to reduce the growth and spread of the pathogen. Despite such practices, pressure for higher yields and continuous year-round production has forced carrot producers to apply additional inputs to their systems in order to control diseases and produce healthy, higher-yielding crops.

In addition to farming practices and chemical suppression of diseases, carrot-breeding programs have developed strains of carrots which exhibit an increased resistance to blight; however, this effect is often only effective for a short period of time as leaf blight has exhibited a concomitant increase in pathogenicity (Ma and Michailides, 2004; Madison and Stevenson, 2008). Thus, new methods are required to control this disease. In order to evaluate the effectiveness of new plant protection protocols for carrot blight, models must be created to determine the effect of blight in controlled environmental conditions before the methods can be further tested in natural systems (i.e. field trials).

Several studies performed by Carisse (Strandberg, 1988; Carisse and Kushalappa, 1989; Carisse *et al.*, 1993; van Delden and Carisse, 1993; Mercier and Kuć, 1996) demonstrated that carrot seedlings could be successfully inoculated with *C. carotae* and *A. dauci,* respectively, in controlled environmental conditions. In the present study, we attempted to determine the optimum spore-containing concentration and seedling age needed to induce visual symptoms of blight. The information gathered from these experiments was used in subsequent experiments aimed at evaluating the effectiveness of PPM technologies in reducing carrot blight symptoms.

2.2 Hypothesis

Carrot blight causal agents, *C. carotae* and *A. dauci* can be used to induce blight symptoms in carrot seedlings grown in a controlled environment.

2.3 Objectives

1. To determine the optimum spore concentration of *C. carotae* and *A. dauci* to produce disease symptoms on three week old *D. carota* seedlings.
2. To determine the ideal *D. carota* seedling age for successful inoculation with *C. carotae* and *A. dauci*.

2.4 Materials and Methods

Methods were derived and modified from the protocols of Strandberg (1988); Carisse *et al.* (1993); van Delden and Carisse (1993); Pryor *et al.* (2002). They examined the relationship between seedling age, spore concentrations and visible disease symptoms in several *D. carota* cultivars. According to their research, three to five week old seedlings inoculated with 5 to 10 conidia mL^{-1} of *C. carotae* or *A. dauci* produced easy to identify and count symptoms.

2.4.1 Plant Culture

Daucus carota var. Sativus cv. Carson seeds (20-25 seeds) were sown into 5 cm cone pots in Promix BX (Premier Horticulture Inc., Red Hill, PA, USA) and thinned to ten plants per pot one week after emergence. The pots were kept

in a growth chamber at 24ºC/12ºC day/night temperature, with 94+/-2% relative humidity and a 16/8 h light/dark cycle. The pots were rotated counter clockwise every three days to reduce localized chamber fluctuations. In order to maintain high humidity, plants were watered (50 mL) every day. This volume was low enough to prevent over saturation.

2.4.2.1 General Fungal Procedures

All procedures involving fungal cultures were performed in a sterile environment, within a Laminar flow hood or within 30 cm of a burning flame. Tools were dipped in 80% ethanol and flamed to insure their sterility.

2.4.2.2 Fungal Cultures

C. carotae and *A. dauci* lesions were identified and removed, along with some healthy tissue surrounding the lesion, from carrot leaves from the Processing Carrot Research Program (PCRP) fields in Truro, NS. Cultures were also ordered from the American Type Culture Center (ATCC), Manassas, VA, USA. Work was done within 30 cm of a Bunsen burner flame within the laminar flow chamber. The lesions were then surface sterilized by dipping in 70% ethanol for 5 to 15 s, then placed in 10% bleach for up to 3 min. The diseased tissue was then rinsed twice in distilled water, blotted and placed in a Petri dish with V8 agar (Fitzgerald, 2000). The tools used (scalpel and forceps) were rinsed in ethanol and flamed between each transfer. The plates were kept at 18°C in the dark.

2.4.2.2.1 Sub-culturing

After the specimens were allowed to grow for 3 to 7 days, sub-culturing was necessary to prevent bacterial contamination. Aseptic transfer with a flamed fungal hook (allowed to cool) was used to slice a section of agar containing the desired fungi cultures, which then was placed on a new plate containing V8 agar (V8 agar: sterilize 150mL V8 juice, 3g $CaCO_3$, 15g Agar in 850 ml H_2O). This was done at least three times to ensure at least one plate was free of

contaminants. To ensure healthy colonies, cultures were again sub-cultured to prevent the fungi from reaching the outer edges of the agar.

2.4.2.2.2 Single Spore Culturing

To ensure a pure isolate, a sample of fungal culture was added to a sterile slide with a drop of sterile water. A serial bacteria loop was dipped into the suspension and gently streaked over a new agar plate. Four lines were made, all intersecting except the last, at approximately 90-degree angles. After the cultures were allowed to develop at 18°C in the dark for 24 h, individual spores were removed from the agar under a dissecting microscope with a sterile modified fungi hook and placed on a new agar plate (Sturz, A.V., personal communication). Each plate was developed from one spore or hyphal tip, and therefore represented only one species.

2.4.3 Inoculum Preparation

Spores were collected from the 7 day old fungal cultures by adding 10 mL of sterile water with 0.01% Tween 80 in the Petri dish and gently streaked the culture with a glass rod, so as not to disturb the agar. The resulting suspension was then filtered through cheesecloth. This suspension was reused with more fungal plates to increase the spore population. The average number of spores was measured by counting the number of spores on a haemocytometer four times under a microscope in a 0.25 mL droplet. Once the spore population was determined, serial dilution was used to dilute the solution to the desired concentrations.

2.4.4 Inoculation on Carrot Leaves

Carrots were placed in a large clean plastic bag and sprayed with the suspended spores onto the undersides of leaves until they were dripping. This inoculation occurred within 2 h after the lights came on in the growth chamber to ensure that the stomata were open, thereby allowing the fungus to enter the carrot. The age of the plants for inoculation is indicated in the experiments. The

carrot plants remained sealed in their plastic bags for 48 h, and unsealed for an additional 48 h before being removed from the plastic bags.

2.4.5 Disease Measurements

Plants were monitored for three weeks post-inoculation, time to lesion formation and total number of lesions at the end of the three weeks were recorded.

2.5 Experiments

2.5.1 Experiment One, First ATCC Culture Screen

One of five spore load concentrations was applied to 30 three week-old carrot seedlings (three replicates of ten seedlings). The concentrations were: 0, 10,000, 15,000, 20,000 and 25,000 spores mL^{-1}. This was done for both *C. carotae* and *A. dauci* ATCC cultures.

2.5.2 Experiment Two, Second ATCC Culture Screen

One of five spore load concentrations was applied to 30, three week-old carrot seedlings (three replicates of ten seedlings). The concentrations were: 0, 30,000, 40,000, 50,000 and 100,000 spores mL^{-1}. This was done for both *C. carotae* and *A. dauci* ATCC cultures.

2.5.3 Experiment Three, Second Generation ATCC Culture Screen

One of five spore load concentrations was applied to 30 three and four week-old carrot seedlings (three replicates of ten seedlings). The concentrations were: 0, 40,000, 50,000, 60,000 and 80,000 spores mL^{-1}. This was done for both *C. carotae* and *A. dauci* ATCC cultures.

2.5.4 Experiment Four, Second Generation ATCC Culture Screen Number Two

One of six spore load concentrations was applied to three, four, five, six, seven and eight week-old carrot seedlings. A total of 144 seedlings were treated

(two replicates of 72). The concentrations were: 0, 30,000, 50,000, 60,000, 80,000 and 90,000 spores mL^{-1}. This was done for both *C. carotae* and *A. dauci* ATCC cultures.

2.5.5 Experiment Five, "Valley" Culture Screen

One of five spore load concentrations was applied to 30 three and four week-old carrot seedlings (three replicates of ten). The concentrations were: 0, 15,000, 30,000, 45,000 and 90,000 spores mL^{-1}. This was done for three unknown cultures derived from carrot leaf tissue from PCRP fields in the Annapolis Valley, NS in August 2005 as described in section 2.4.2.2.

2.5.6 Experiment Six, University of Guelph Culture Screening

One of five spore load concentrations of *C. carotae* was applied to 30 three and four week-old carrot seedlings (three replicates of ten each). The concentrations were: 0, 15,000, 30,000, 45,000 and 90,000 spores mL^{-1}. This culture was a gift from the lab of Mary Ruth McDonald, University of Guelph, Ontario.

2.6 Results

Of the six experiments, only *A. dauci* from ATCC at 50,000 spores mL^{-1} (Experiment Two) produced any blight symptoms. These lesions were cultured (Section 2.4.2.2) and used to prepare an inoculation for experiments three and four. These cultures did not produce disease symptoms on any carrot seedlings, regardless of age or spore load concentration. Neither the cultures derived from blight symptoms isolated from carrot leaves from the Annapolis Valley, NS, nor the cultures sent from the University of Guelph, produced blight symptoms on carrot seedlings.

2.7 Discussion

Of the six separate experiments undertaken, five involved disease from culture plates. The sixth (Experiment Five) used fresh disease tissue from the

field as was used previously by Strandberg (1988); Carisse and Kushalappa (1989); Carisse *et al.* (1993); van Delden and Carisse (1993); Mercier and Kuć (1996). This diseased material was isolated, identified, monocultured and inoculated onto lab carrot plants to insure continuous freshly diseased material. This approach was duplicated in our experiment, but without success. Although disease symptoms did appear and were successfully cultured in Experiment Two, this new spore-containing inoculum did not produce visible disease symptoms.

According to the literature and protocols, having high humidity and leaf moisture is paramount for infection to take place (Carisse *et al.*, 1993). These conditions were met in the growth chamber by placing carrot seedlings in clear plastic garbage bags after inoculation. Temperature was consistent over time, but in retrospect, increasing the night temperature to above 12˚C may help produce a more conducive environment for fungal infection.

Cultures that lose or have reduced pathogenic strains or isolates are common in cultures that have been repeatedly subcultured without being re-introduced to their host plant (Fitzgerald, A. and Strurz, A.V. personal communication). This may have also contributed to the poor pathogenicity of our fungal cultures. In addition, the lack of blight in our research fields in 2005 made it difficult to isolate and culture fresh carrot blight organisms (Chapter 3). In future, obtaining fresh diseased material, with strong symptoms, would likely be an appropriate method to obtain pathogenic material.

2.8 Conclusion

The pathogenicity of cultures is paramount for induced blight symptoms. Without cultures that can produce pathogenic inoculums it is difficult to simulate carrot blight in controlled environments. The development of this protocol will have to be modified in order to determine the effectiveness of alternative disease control treatments.

Chapter III: The effect of PPM technologies on pest and disease incidence, growth and harvest quality of *Daucus carota* L.

3.1 Introduction

Carrot (*Daucus carota* L.) production contributes over 12 million dollars to the Nova Scotia economy annually (Ells, A., personal communication). Carrot producers, as a result of carrot leaf blight, incur significant costs and yield losses. Fungicides including Bravo, (ISK-Biotech; Mentor, OH, USA) and Benlate, (Du Pont Canada; Mississauga, ON) are applied frequently in order to reduce yield losses, as a severe infection of blight can reduce yield by as much as 15% to 74% (Bragg Lumber, 1999; Ben-Noon *et al.*, 2001). However, fungicides are not always a solution. For example *Alternaria* fungicidal resistance to iprodine (Bayer, Toronto, ON) has been well documented (Fancelli and Kimati, 1991; Solel *et al.*, 1996; Pryor *et al.*, 2002; Ma and Michailides, 2004). Ma and Michailides (2004) also reported a resistance to the fungicide benomyl, which had been a common fungicide used in Canada until it was removed from the market in 2001. Other alternative methods need to be developed not only to prevent blight from becoming a serious constraint to Atlantic Canadian carrot producers, but also to promote ecologically sustainable carrot production. The rising costs of agrochemicals, both in terms of financial input for the producer and detrimental effects on the environment, are putting great constraint on the carrot industry.

There are two fungal organisms that cause carrot leaf blight: (1) *Cercospora carotae* (Pass.) Solh and (2) *Alternaria dauci* (Kühn) Groves & Skolko, also known as early and late blight, respectively. Both blight species cause leaf chlorosis, appearing as lesions generally found at the bottom and edges of carrot leaves. Infection can occur throughout the growing season, however, the symptoms and severity of the disease are greatly affected by the environment. High humidity coupled with moderate temperatures can create an ideal growing and reproductive environment for blight, increasing the potential spore load in a field (Agrios, 2004). In addition to blight, environment plays a key role in the development and intensity of a number of other crop diseases, disorders and pest infestations. Diseases induced by *Sclerotinia sclerotiorum* (Lib.) De Bary (white mold), *Botrytis cinerea* (De Bary) Whetzel (bacterial soft rot) and aster yellows (a mycoplasmal disease caused by leaf hoppers, *Macrosteles*

quadrilineatus (Forbes)), physiological disorders like green shoulders, and insects such as *Psila rosae* (Fab.) (carrot rust fly) and *Listronotus oregonensis* (Le Conte) (carrot weevil) can cause considerable damage and yield lost (MacNab *et al,,* 1983).

Blight reduces carrot yield by three modes; (1) leaf chlorosis destroys functional leaf area, thereby reducing the plants photosynthetic capacity, (2) energy is diverted from storage development (root bulking) to defence mechanisms, and (3) damaged petioles are more susceptible to breakage when mechanical harvesters clasp them to pull the roots from the ground. Unrecovered carrots can also lead to further crop loss in subsequent seasons as the decaying plant material can be a host to blight spores, providing a putative source for future infection.

Due to their sessile nature, plants are naturally equipped with unique defence mechanisms. Many plant defences are morphological in nature, such as waxy cuticles, trichomes, hair, spines and thick epidermal cell walls. Chemical defences, like pathogenesis related (PR) proteins, reactive oxygen species (ROS) (Prell and Day, 2000), phenolics and phytoalexins (Kurosaki and Nishi, 1983) also exist and have increasingly become the subject of research (Karban and Kuć, 1999; Durrant and Dong, 2004; Korthari and Patel, 2004; Molloy *et al.*, 2004; Bais *et al.,* 2005). Both physical and chemical defences can either be constitutive or inducible, and the latter has received more attention by agrologists.

One of the first reactions that plants undergo after perceiving a stress is the oxygen burst, which may lead to the hypersensitive reaction (HR). HR involves rapid cell death via ROS of a tissue closest to the site of infection or stress (Prell and Day, 2000). The yellow halo that often surrounds the blight lesion is a result of ROS and the HR (Figure 1.1). HR is one method the plant can utilize to isolate the infected cells so that the growing fungal hyphae cannot infect the adjacent cells. ROS and HR lead to many defence pathways, including induced resistance (IR) or systematic acquired resistance (SAR). IR or SAR against pathogens and insects has been known since the early 1900s and 1970s,

respectively (Karban and Kuć, 1999); however, the application of these protective mechanisms to agriculture is in its infancy. IR and SAR have the potential to form a foundation for the next generation of crop protection.

The effect of wounding on plant defences has been well documented (Baldwin *et al.,* 1997; Hara-Nishimura and Matsushima, 2003; Li *et al.*, 2002; Reyes and Cisneros-Zevallos, 2003). After a plant perceives a disturbance or stress, messages are sent throughout the plant to produce defence compounds, such as proteinase inhibitors (PIs) (Arimura *et al.*, 2005), defence genes (Birkenmeier and Ryan, 1998) and secondary metabolites including terpenes (Seljasen *et al.*, 2001) and phenolics (Reyes and Cisneros-Zevallos, 2003). Thus, it is possible that any physical disturbance that mimics an insect or pathogen attack may stimulate a series of defence reactions and thereby confer protection against diseases and pests. In theory, brushing and trimming the leaves of plants is expected to induce common defence compounds, since both actions can cause physical and mechanical damage. Brushing on leaves is expected to physically damage and induce defence reactions without destroying plant cells, whereas trimming would mimic insect damage by physically wounding the leaves. However, artificial wounding is not an exact replica of herbivory wounding, as demonstrated by De Moraes *et al.* (1998) and Constabel, (1999). Physiologically, their studies revealed that plant defence mechanisms are similar between artificial wounding and caterpillar damage despite the biochemical differences observed (Constabel, 1999).

The effect of UV-C radiation on plants is not as well understood as an inducer of wounding. The pathways and modes of action are still under much observation and experimentation. Danon and Gallois (1998) reported that UV-C radiation may physically induce programmed cell death (PCD) in plant nuclei. If UV-C radiation can induce PCD, this would begin a cascade of other plant reactions similar to those induced by HR and SAR cell death, likely through ROS. UV-B radiation stimulates the production of signaling compounds, such as hydrogen peroxide (a ROS), which has been identified in both abiotic and biotic defence pathways (Yannarelli *et al.*, 2005). Teklemariam and Blake (2004)

reported that UV-B radiation activates ROS and phenylalanine ammonia lyase (PAL), which is an enzyme associated with the phenylpropanoid pathway. Both ROS and PAL can activate this pathway, which in turn produces many different defence compounds, including flavanoids and other phenolics (Teklemariam and Blake, 2004), which have been hypothesized to act as "sun-screen" for the epidermis, absorbing free radicals formed by the solar radiation damaging plant cell tissue (Li *et al.*, 1993).

UV-A and UV-B radiation have both been applied to carrots (or carrot cell cultures) to measure their effect on phenolics (Gleitz and Seitz, 1989; Glabgen *et al.*, 1998; Hirner *et al.,* 2001; Takeda *et al.*, 2002). Both UV-A and UV-B seem to stimulate anthocyanin biosynthesis and increase the activity of PAL. UV-C has also been investigated as both a post-harvest treatment for the reduction of rots (Mercier and Arul, 1993) and for its successful stimulation of the defence pathways (phytoalexin) in carrots (Mercier and Arul, 1993).

The line between beneficial UV-B radiation and excess radiation damage is not well defined. If mild doses of UV-B or UV-C radiation can induce protective compounds such as flavanoids, other phenolics (Li *et al.*, 1993) and ROS (Mackerness *et al.*, 2001), these compounds may also be utilized for other protective functions, such as biotic defence. Inducing plant defences may be a novel way to protect crops from both abiotic and biotic stresses that are inevitable in a natural system. Natural field studies are therefore required in order to properly identify and quantify both the benefits and unforeseen consequences of PPM treatments.

3.2 Hypothesis

Photo (UV-C), physical (brushing), mechanical (clipping/trimming) (PPM) factors induce resistance to certain carrot pests and diseases, and reduce physiological disorders in field-grown carrots.

3.3 Objective

The intent of this field study was to determine the effects of selected PPM pre-treatments on certain pests and disease incidences, physiological disorders, growth and harvest quality of *D. carota* var. Sativus cv. Carson.

3.4 Materials and Methods

3.4.1 Plant Selection and Growth and Site Location

Carson (Bejo Seeds Inc., California, USA), a dicer cultivar of carrots, was seeded on June 2 in field 201 (2005 field experiment) and on May 31 in field 200 (2006 field experiment) on the Nova Scotia Agricultural College farm grounds at Brookside, NS (44° 32′ 57.8″ N, 63° 42′ 59.3″ W). Carrots were grown according to standard Oxford Frozen Food guidelines (Bragg Lumber, 1999). The variety Carson was chosen for this study because of its susceptibility to blight and reportedly profuse, strong top growth (Lada, 1999). Carson is a Danvers type variety, grown for the frozen food industry. It is a F1 hybrid, with Chantenay as one of its parents, and has longer, more slender roots compared to other popular varieties.

3.4.2 Treatment Imposition

3.4.2.1 2005 Field Experiment

The treatments were applied at either 4, 8 or 12 weeks after emergence using a standard block design (Table 3.1). The plants received, brushing (B), UV-C or trimming (Tr) treatments. Brushing involved a physical disturbance using a wooden hockey stick gently stroking 40 times (20 up the row, 20 down the row) just below canopy level. UV-C radiation was given using a UV-C mobile light (LiCor LI-188B Quantum Radiometer, Li Cor Inc. Lincoln, Nebraska, USA) for 30 s 1cm above canopy level. Both brushing and UV-C treatments were administrated to separate blocks at 4, 8 or 12 weeks post-emergence. Trimming treatments were applied at 12 weeks after emergence when the canopy growth covered the rows. There were five trimming treatments in total. Three of the treatments reduced the canopy tops by 25, 50 or 75%, respectively and the

remaining two treatments involved trimming the sides of the canopy. Specifically, the side canopy trimming treatments trimmed the carrot leaves to the original hill width where the leaf waste was allowed to either remain in the rows (TSLT) or removed (TSRT).

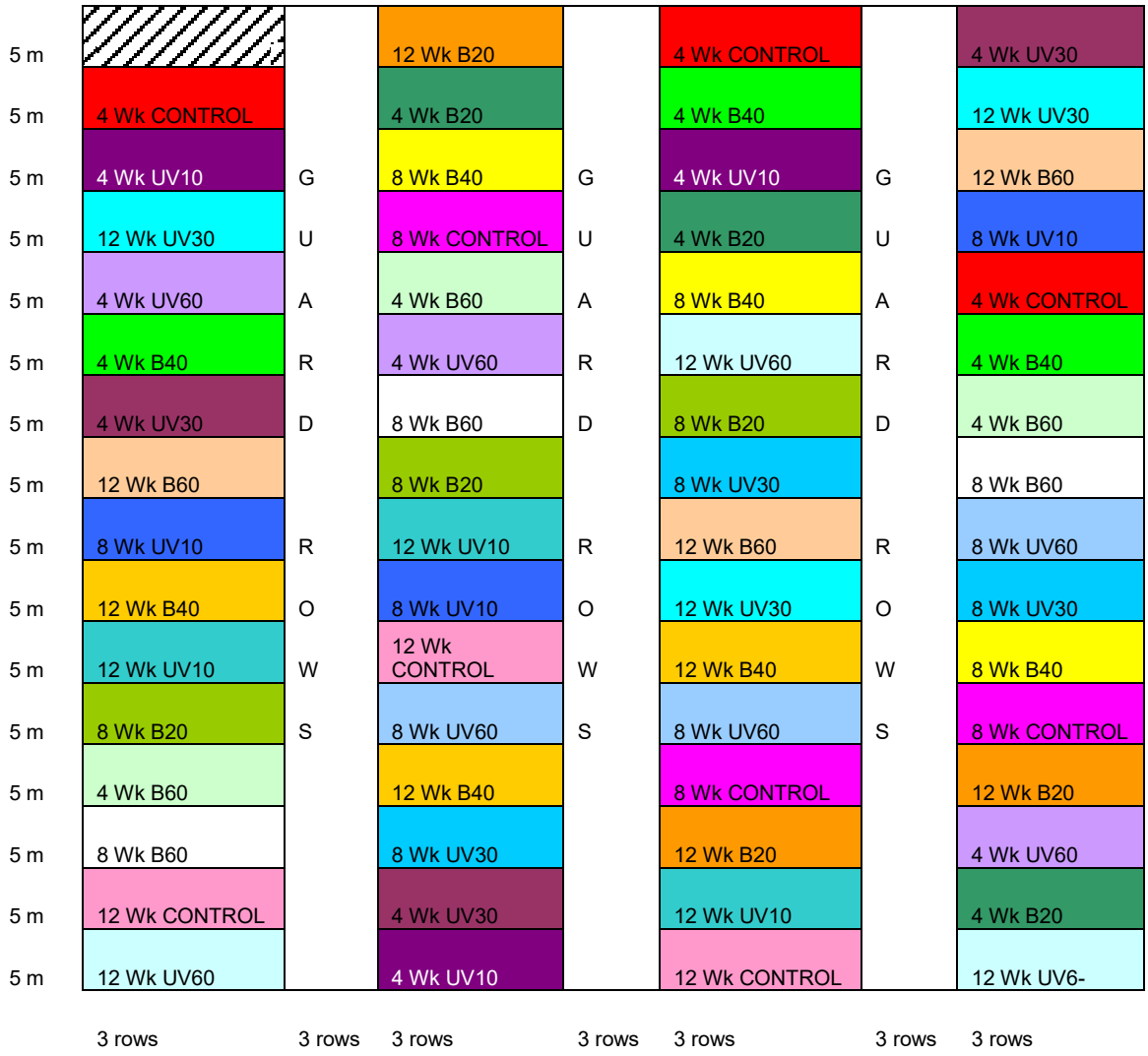
Table 3.1 2005 Field plan (field 201), located in Brookside, Truro, NS. Brushing (B), UV-C radiation (UV-C), TSLT (trimming side canopy at full cover and leaving trash), TSRT (trimming side canopy at full cover and removing trash), T (percent trimmed), Wk (week number after emergence).

5m	12Wk UV-C	G	TSRT	G	Control	G	TSRT
5m	4Wk B	U	TRLT	U	12Wk B	U	TSLT
5m	TSLT	A	4Wk UV-C	A	TSLT	A	12Wk B
5m	T75	R	12Wk UV-C	R	8Wk UV-C	R	4Wk B
5m	8Wk B	D	T75	D	TSRT	D	8Wk UV-C
5m	Control	R	12Wk B	R	12Wk UV-C	R	4Wk UV-C
5m	T25	O	Control	O	8Wk B	O	Control
5m	4Wk UV-C	W	8Wk B	W	T75	W	T25
5m	8Wk UV-C		T25		T50		12Wk UV-C
5m	T50		4Wk B		4Wk UV-C		8Wk B
5m	TSRT		8Wk UV-C		4Wk B		T50
5m	12Wk B		T50		T25		T75
	3 rows	3 rows	3 rows	3 rows	3 rows	3 rows	3 rows

3.4.2.2 2006 Field Experiment

At four weeks post-emergence the first round of treatments began; 20, 40 or 60 strokes of brushing (half up the row, half down the row) with a hockey stick at just below canopy level and 10, 30 or 60 s of UV-C radiation 1 cm above canopy level (LiCor LI-188B Quantum Radiometer, Li Cor Inc. Lincoln, Nebraska, USA). This was repeated at 8 weeks post-emergence and again at 12 weeks. No plots received more than one treatment. Table 3.2 depicts the 2006 field plan.

Table 3.2 2006 Field Plan (field 200), located in Brookside, NS. Brushing (B), UV-C radiation (UV-C), Wk (weeks after emergence)



3.4.3 Measurements

Blight occurrence was monitored throughout the growing season, but due to low disease indices, only post-harvest data was analyzed. The diseases, pests and physiological disorders measured at harvest were; blights (*A. dauci* and *C. carotae*), soft rot (*Erwinia carotovora* Winslow), white mold (*Sclerotinia sclerotiorum*), wireworm (*Agriotes sputator* L.), aster yellows and greenshoulders. Blight caused by *A. dauci* and *C. carotae* were each given a value based on a visual scale of 0 to 9, with zero being free of all visual symptoms and 9 as

complete leaf death due to disease. Figure 3.1 depicts this rating scale. Five random plants per plot were selected for blight monitoring. Blight from *C. carotae* was characterized as circular lesions found on the petioles and near the centre of the leaves (Figure 1.1), while blight from *A. dauci* was characterized as irregular lesions on the tips of the carrot leaves. Soft rot, white mold, wireworm, aster yellows and greenshoulders were all measured as number of roots per m infected with the disease. All disease measurements were performed within a week of harvest. Yield and harvest quality were also measured for all carrots in a 1 m row length. Measurements included the number of carrots and their mass, leaf fresh weight, number of leaves, and root length and girth. Carrot quality was then based on Bragg Lumber girth categories and recorded as: less than 0.75", 0.75-1.5", 1.5-2", 2-3" and greater than 3". Blemished, deformed or unmarketable carrots were called "culls" (Bragg Lumber, 1999).

a: Rate 1 (app. 1-11% BC)

b: Rate 2 (app. 12-22% BC)

c: Rate 3 (app. 23-33% BC)

d: Rate 4 (app. 34-44% BC)

e: Rate 5 (app. 45-55% BC)

f: Rate 6 (app. 56-66% BC)

g: Rate 7 (app. 67-77% BC)

h: Rate 8 (app. 78-88% BC)

i: Rate 9 (app. 89-100% BC)

Figure 3.1 Rating scale for blight from 1-9 (a-i). Images taken from 2007 field trials. Blight coverage (BC)

3.4.4 Statistical Design

3.4.4.1 2005 Field Experiment

This experiment was set up as a randomized complete block design (RCBD) with four replications. Two different statistical models were performed on

these data. All the treatments were analyzed using a one-way ANOVA model after verifying data normality, invoking procedures/statements of SAS version 8 (SAS, 2000). The means were separated using Tukey"s LSD test ($p<0.05$). The trimming treatments were only applied at the 12 week stage, but the UV-C and brushing treatments were applied at three different stages (4, 8 and 12 weeks); therefore, they were further subjected to a separate statistical analysis to elucidate the effects of "stage". This was accomplished by using a 3x3 factorial model. The two factors were treatments (UV-C radiation and brushing) and timing of treatments (4, 8 and 12 weeks). Since no negative or positive treatment effect on soft rot, wireworm, aster yellows or greenshoulders were detected; these variables were not further investigated.

3.4.4.2 2006 Field Experiment

In contrast to the 2005 experiment, the 2006 experiment was set up as a complete completely randomized design (CRD) with three replications. Two different statistical analyses were used on the 2006 data. All treatments were analyzed using a one-way ANOVA model after verifying data normality, invoking procedures/statements of SAS version 8 (SAS 2000). Means were separated using Tukey"s LSD test ($p<0.05$) and then further analyzed using a 3x3x3 factorial model, to determine the effects of "duration". The three factors were treatment (UV-C radiation, brushing and control) at three stages (4, 8 and 12 weeks) and three treatment durations (10, 30 or 60 s UV-C radiation and 20, 40 or 60 brushing strokes).

3.5 Results

3.5.1 2005 Field Experiment

3.5.1.1 Effect of UV-C, Brushing and Trimming Pretreatments on Blight

As described in the methods of this chapter, blight caused by *C. carotae* was distinguished from that caused by *A. dauci* by shape and location of the lesions on the carrot leaves. In the 2005 growing season, the blight incidence was abnormally low, yet both brushing and UV-C pre-treatments controlled blight

occurrence significantly (p=0.05) compared to the control. The only treatments that did not show significant reduction in blight symptoms at the 10% level was brushing at four weeks (B4) and reducing the canopy by 75% (T75, Fig.4.2). Plants given the earliest brushing treatment did not have any disease symptoms, but the timing of the other brushing treatments had an equal effect on blight control.

As described in section 3.4.4.1 (Statistical design), the UV-C and brushing treatments were subjected to a Tukey"s LSD test and then further analyzed using a 3x3 factorial model. The results of this analysis are shown in Table 3.3. Figure 3.2 and Table 3.3 clearly demonstrates the possible positive effects that UV-C and brushing treatments can have on common carrot diseases. Blight in field was a measurement of both *Alternaria* and *Cercospora* within the block, with treatment (brushing or UV-C) and stage (or timing of the treatment) both showing a clear significant (p<0.001) effect on these diseases. Figure 3.2 shows that UV-C and brushing had a significant positive effect in reducing both *Alternaria* and *Cercospora* blight diseases at the 5% level.

Table 3.3 The p values for the interaction effect of brushing and UV-C radiation treatment on certain pests and diseases in 2005. Bold indicates significance.

Effect	*Alternaria*	*Cercospora*	Blight in field	Aster Yellow	White Mold	Soft Rot	Wireworm
Stage	1.0000	**<.0001***	**<.0001***	0.8807	**<.0001***	0.8049	0.7602
Treatment	**<.0001***	**<.0001***	**<.0001***	0.8324	**0.0787****	0.7745	0.9817
Stage*treatment	**<.0001***	**<.0001***	**<.0001***	0.4052	**0.0467***	0.7745	0.2625

*5% significance level
**10% significance level

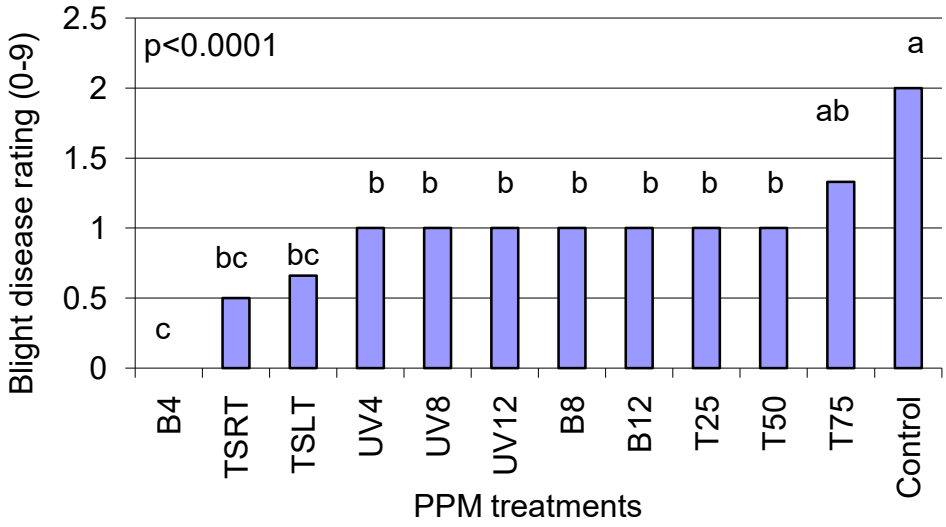


Figure 3.2 The effect of various PPM treatments on blight in research field 201 (Brookside, NS) at harvest (2005). Disease rating (0-9, 0 = no incident of blight, 9 = plant collapse due to blight) of *Alternaria* and *Cercospora* blight infection at harvest, following brushing (B), trimming (T% removed), UV-C radiation (UV), trimmed sides removing trash (TSRT) or trimmed sides leaving the trash (TSLT). Means were separated using Tukey"s test (p=0.05).

3.5.1.2 Effect of UV-C, Brushing and Trimming Pretreatments on Other Carrot Diseases

There was a significant reduction in white mold (*Sclerotinia* sp.) due to certain pre-treatments compared to the control, $p<0.05$ (Figure 3.3). While control plants had a white mold infection rating of 2, the pre-treatment with UV-C applied at 4 weeks after emergence reduced the white mold incidence to zero (Figure 3.3). Brushing at 8 or 12 weeks after emergence and trimming of 75% were equally effective in controlling white mold. Trimming at 50% had the highest amount of white mold, which was similar to the control. Early UV-C exposure at 4 weeks was more effective than exposure at 12 weeks at controlling white mold. UV-C radiation treatments at 4 and 8 weeks, brushing at 8 and 12 weeks, reducing canopy by 75% and side trimming with leaving trash all had significantly less mold compared to the control group (Figure 3.3). These results also suggest that timing of a particular treatment may be an important factor in the control of

white mold. For instance, the earlier treatments of UV-C radiation (4 and 8 weeks) had equally reduced levels of white mold, whereas radiation at 12 weeks showed no significant reduction in mold. There was no significant difference between radiation treatments at 8 and 12 weeks, suggesting that for controlling white mold infection, the PPM treatments need to be addressed earlier in the season. In contrast, brushing the canopy later in the growth phase (8 and 12 weeks) showed a greater reduction (3-fold) in white mold compared to brushing at 4 weeks. However, there was no significant difference in white mold control due to timing of pre-treatment, as brushing (8 or 12 weeks), trimming 75% canopy and UV-C 4 weeks post-emergence were all equally effective in reducing white mold (Table 3.3). In contrast to blight and white mold, none of the PPM pre-treatments were effective in controlling wireworm, aster yellow or soft rot (Table 3.3).

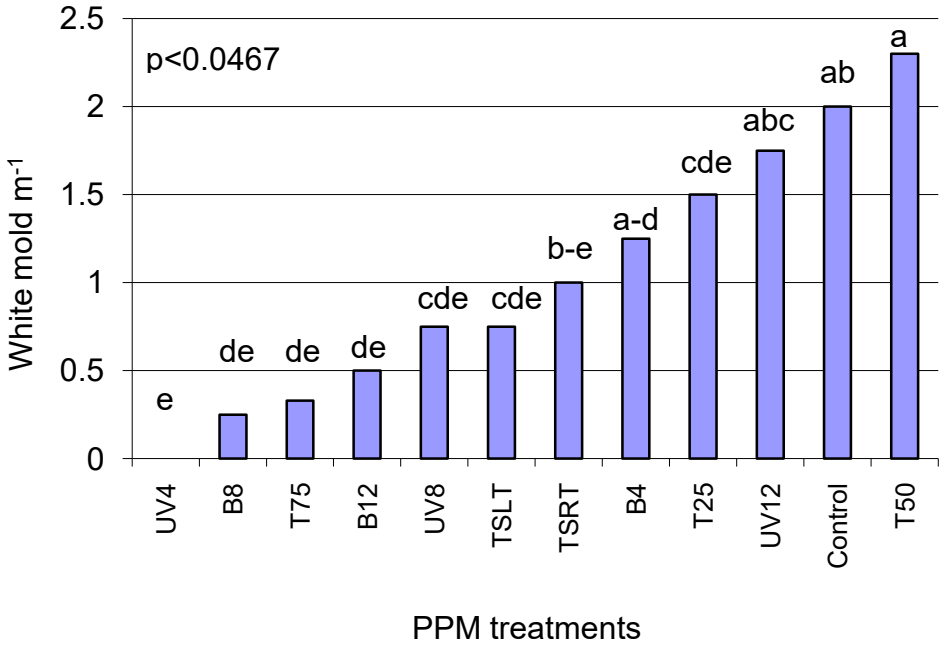


Figure 3.3 The effect of various PPM treatments on *Sclerotinia* white mold in research field 2001 (Brookside, NS) at harvest (2005). Treatments are brushing (B), trimming (T% removed), UV-C radiation (UV), trimmed sides removing trash (TSRT) or trimmed sides leaving the trash (TSLT). White mold was measured as number of infected roots per m. Means were separated using Tukey"s test (p=0.05).

3.5.1.3 Effect of UV-C, Brushing and Trimming Pretreatments on Carrot Quality and Yield

None of the treatments had any negative effect on carrot grades or yield (Tukey"s test, p=0.05). In general, there was no statistically significant difference among the treatment on either yield or different grades (Figure 3.4).

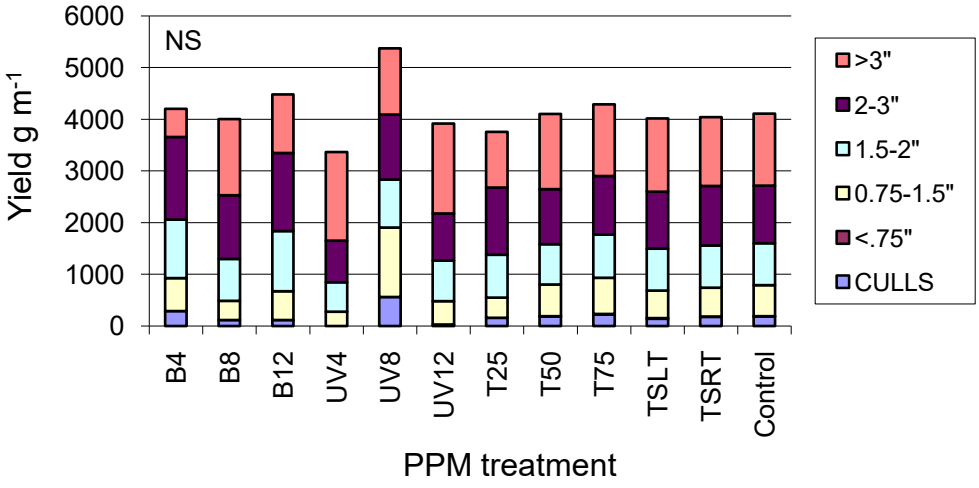


Figure 3.4 The effect of brushing and UV-C pre-treatment on yield (2005) and harvest quality represented as yield (g) per unit plot (m). Means were separated using Tukey"s test (p=0.05).

3.5.2 2006 Field Experiment

3.5.2.1 The Treatment Effect on Carrot Diseases

The 2006 field season was similar to the 2005 season, with a cool spring followed by a hot dry summer. The highest blight rating was 2.3 on the 0 – 9 scale. Approximately 20% of the leaves sampled had developed blight disease symptoms. Due to the low incidence of blight during treatment, other post-harvest diseases were also measured and analyzed (Table 3.4).

Table 3.4 The p value for the interactions of stage, treatment and duration on certain pests and diseases in 2006. Bold indicates significance.

Effect	*Cercospora*	*Alternaria*	Blight in field	Aster Yellows	Soft Rot	Wireworm	White Mold
Stage	0.4381	**0.0472***	**0.0365***	**0.0382***	**0.0058***	**0.0010***	0.2159
Treatment	0.2185	0.6224	0.3100	**0.0811****	0.1606	**0.0081***	0.7532
Duration	**0.0452***	**0.0827****	0.6357	0.4025	0.4134	1.0000	0.6733
Treatment *Stage	0.2708	**0.0804****	0.3340	**0.0680****	**0.0477***	0.1791	0.8853
Stage *Duration	0.0037*	0.4156	0.7023	0.7340	0.9478	**0.0144***	0.8073
Treatment *Duration	**0.0155***	0.2614	0.9358	0.5163	0.1875	0.6193	0.9903
Stage*Treatment *Duration	**0.0277***	**0.0934****	**0.0680****	0.4351	0.5368	**0.0071***	0.9997

*5% significance level
**10% significance level

3.5.2.2 *Cercospora* Leaf Blight

Cercospora (early blight – appearing as circular lesions on the petioles and centre of the leaves) had the most varied response to the treatments. Figure 3.5 shows the varying effect of UV-C and brushing treatments on the development of early carrot leaf blight. None of the treatments were significantly different from their respective controls. UV-C radiation for 30 s at 8 weeks had the highest incidence of disease of 1.33, however this value is not significantly different from the control. Similarly, UV-C radiation for 60 s at week 8 and brushing (both 20 and 40 strokes) at 12 weeks had the lowest incidence of early blight (0.33), but were also not significant, statistically.

Brushing at medium intensity (40 strokes) had a significantly lower degree of *Cercospora* blight than the control. In fact, low brushing duration (20 strokes)

and high UV-C radiation (60 seconds) both had lower blight than the mid-duration control (Figure 3.6).

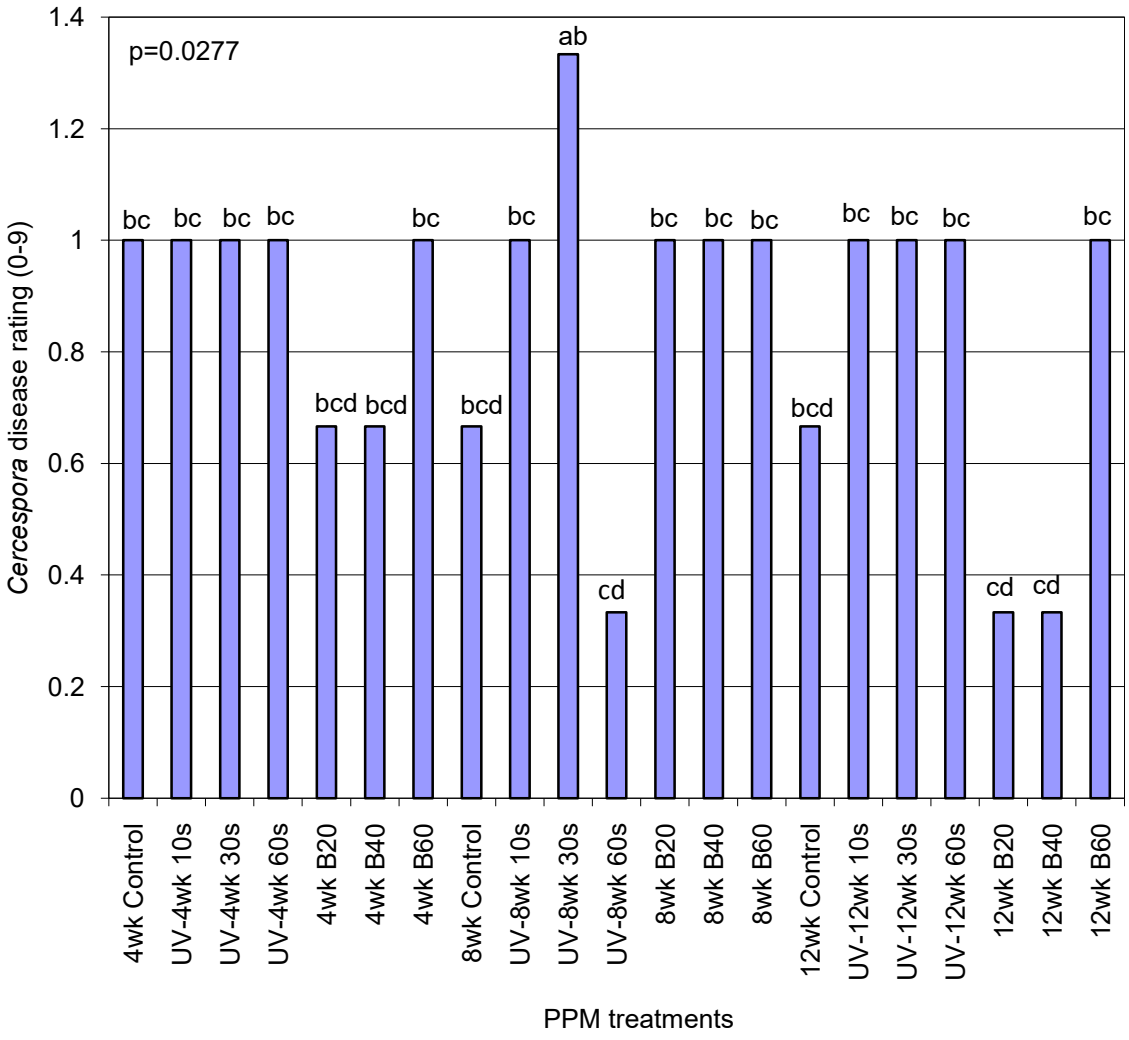


Figure 3.5 The effect of brushing and UV-C pre-treatments on *Cercospora* blight in research field 200 (Brookside, NS) at harvest (2006). Disease rating (0-9, 0 = no incidence of blight, 9 = plant collapse due to blight) of severity of *Cercospora* blight infection, following brushing (Bstroke#) and UV-C radiation (UV-duration) at the stage listed. Means were separated using Tukey"s test (p=0.05).

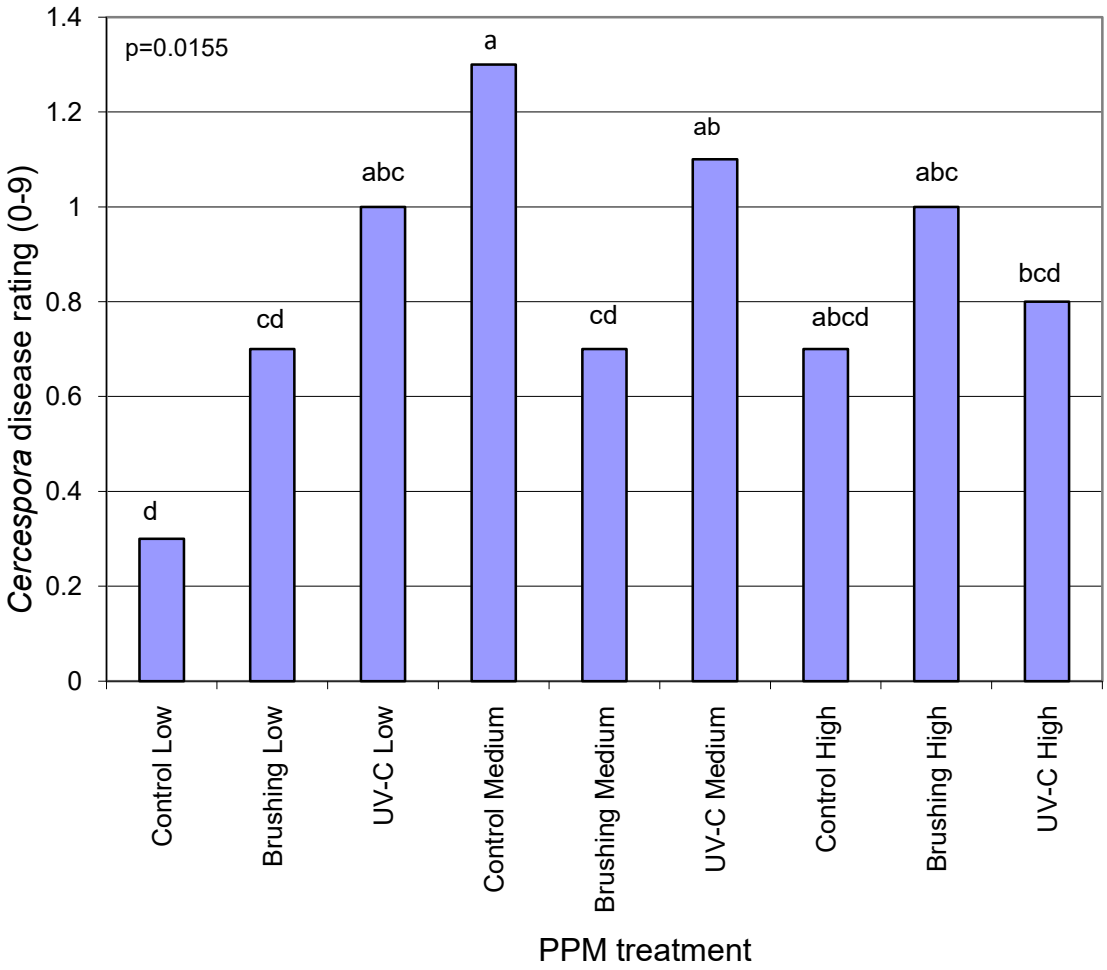


Figure 3.6 The effect of brushing and UV-C pretreatments on *Cercospora* disease in research field 200 (Brookside, NS) at harvest (2006). Disease rating (0-9, 0 = no incident of blight, 9 = plant collapse due to blight) of severity of *Cercospora* blight infection, following brushing and UV-C radiation at the duration listed, averaged over all time points. Means were separated using Tukey‟s test (p=0.05).

3.5.2.3 *Alternaria* Leaf Blight

Blight caused by *Alternaria* was identified as irregular lesions on the edges of the carrot leaves. As shown in Table 3.4 there was a three-way interaction effect on the occurrence of *Alternaria* leaf blight. Figure 3.7 depicts the most informative points of these results, the two-way treatment x stage effect.

Overall, Figure 3.7 shows that the incidence of *Alternaria* leaf blight increases as the treatments were performed later in the growing season. The exception was with brushing at 12 weeks, which had a lower disease incidence

compared to its control, and UV-C radiation at 4 weeks, which also had a lower disease rating than the 12 week control, although neither was statistically significant.

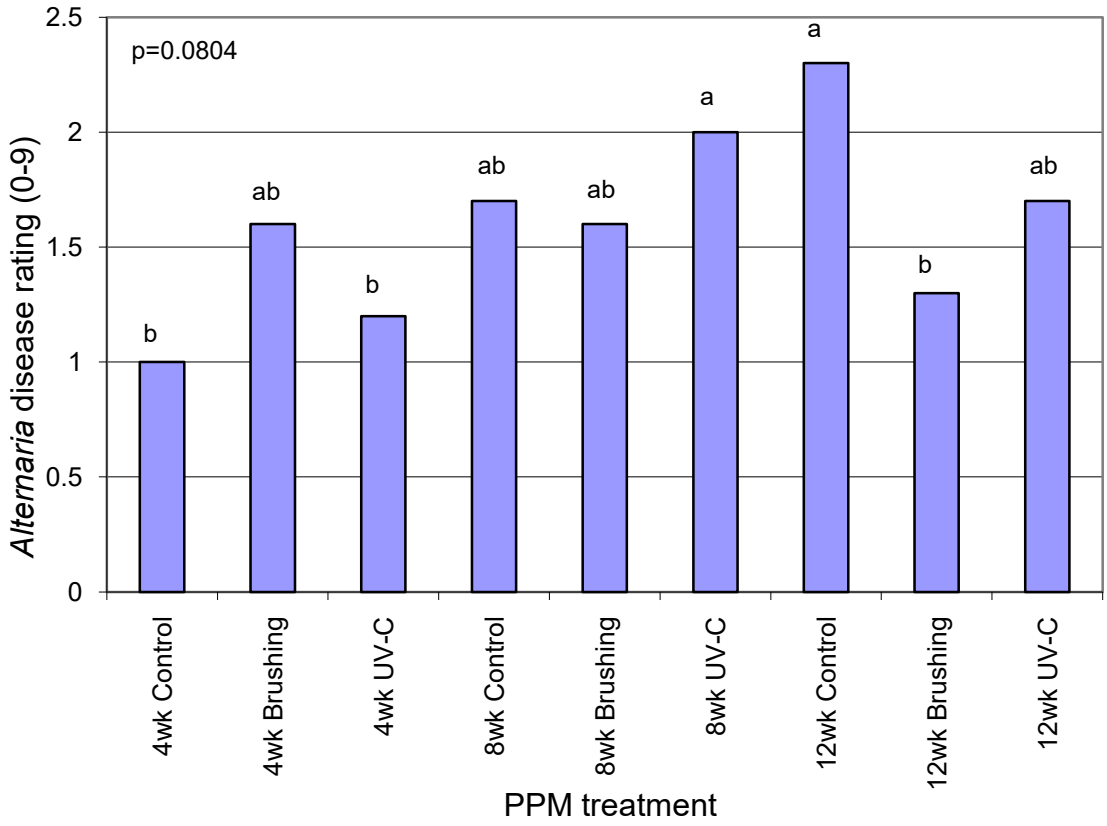


Figure 3.7 The effect of brushing and UV-C pretreatments on *Alternaria* disease in research field 200 (Brookside, NS) at harvest (2006). Disease rating (0-9, 0 = no incident of blight, 9 = plant collapse due to blight) of severity of *Alternaria* blight infection, following brushing (B duration) and UV-C radiation (UV-duration) at the stage listed. Means were separated using Tukey"s test (p=0.05).

3.5.2.4 Other Carrot Diseases

3.5.2.4.1 Aster Yellow

Table 3.4 demonstrates that there is no significant duration effect on aster yellow development, but that there is a significant treatment x stage effect. PPM treatment has no positive control over aster yellows regardless of timing of treatment imposition. Both UV-C and brushing treatment had an apparent adverse effect on the control of aster yellows when applied mid and late season (Figure 3.8).

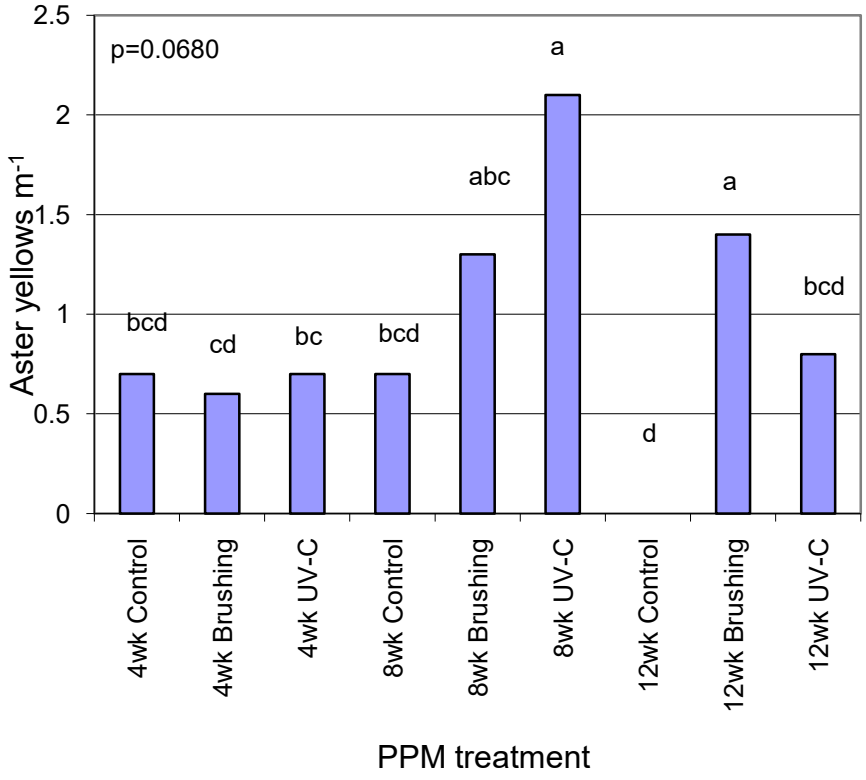


Figure 3.8 The effect of brushing and UV-C pre-treatment on aster yellows in research field 200 (Brookside, NS) at harvest (2006). PPM treatments imposed were brushing and UV-C radiation applied on the week (wk) listed. Aster yellows were measured as the average number of roots infected per m harvested. Means were separated using Tukey"s test (p=0.05).

3.5.2.4.2 Soft Rot

In general, there was very little soft rot infection of carrot roots in 2006, with less than 1.5 carrot roots infected per metre harvested (Figure 3.9). Table 3.4 showed that there was a treatment x stage effect, but not significant duration effect. At 4 and 12 weeks there was no difference between the controls and the treatments. However, there was a significant difference between brushing and UV-C radiation treatments and their respective control in week 8.

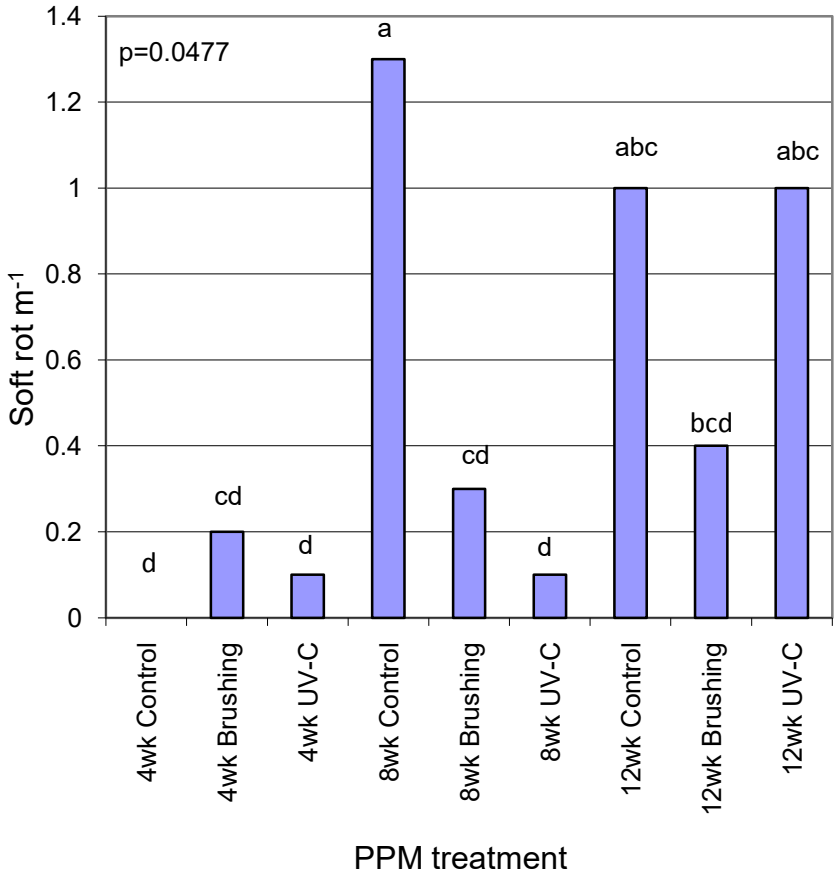


Figure 3.9 The effect of brushing and UV-C pre-treatments on soft rot in research field 200 (Brookside, NS) at harvest (2006). PPM treatments imposed were brushing and UV-C radiation applied at the stage listed. Soft rot was measured as the average number of roots infected per m harvested. Means were separated using Tukey"s test (p=0.05).

3.5.2.4.3 Wireworm Damage

Table 3.4 showed that wireworm damage was only affected by treatment, and not by timing or intensity. Therefore, only the effect of treatment was examined in Figure 3.10. Statistically, both brushing and UV-C radiation reduced the incidence of infection. There is no difference between treatments as both caused a 4.5 fold reduction in wireworm damage.

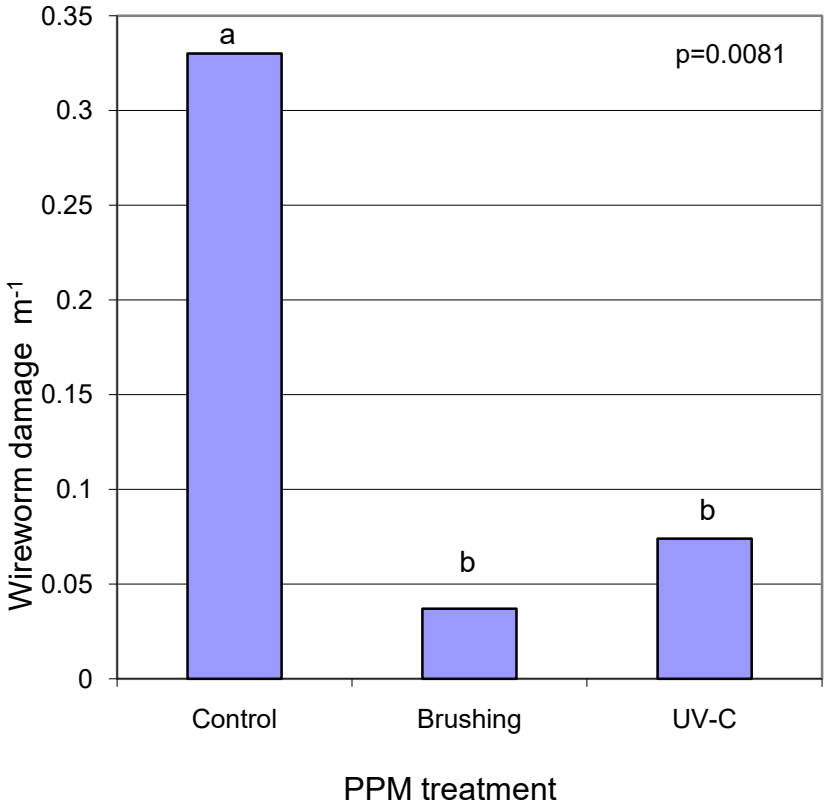


Figure 3.10 The effect of brushing and UV-C pre-treatments on wireworm damage in research field 200 (Brookside, NS) at harvest (2006). Wireworm was measured as the average number of roots infected per m harvested. Means were separated using Tukey"s test (p=0.05).

3.5.2.4.4 Carrot Quality and Yield

The overall yield and quality of the harvested carrot roots are depicted in Figure 3.11. There was no treatment, stage or duration effect on carrot quality or yield.

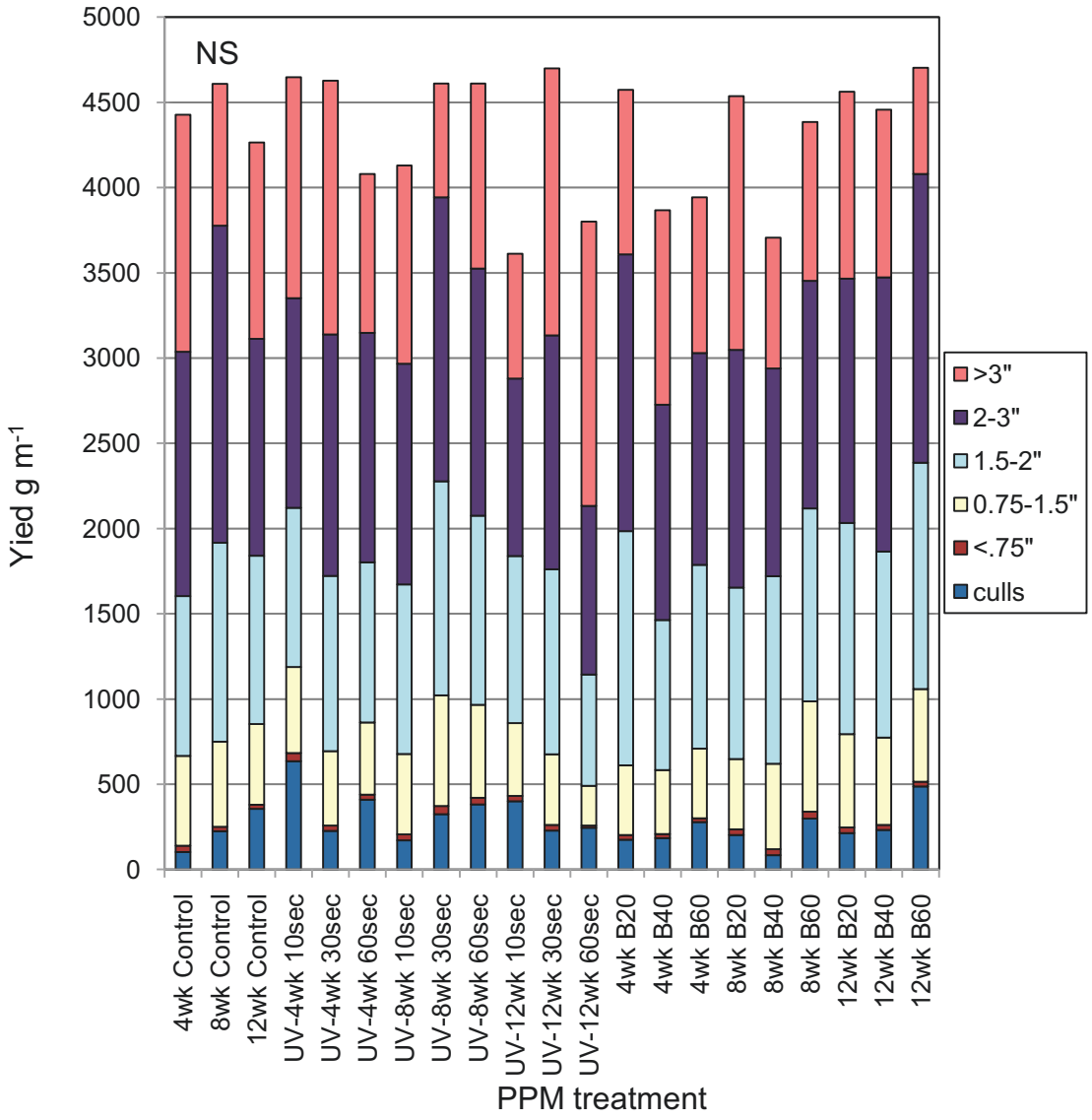


Figure 3.11 The effect of various PPM treatments on yield (2006) and harvest quality represented as yield (g) per unit plot (m). PPM treatments were brushing and UV-C radiation at the stage listed. Means were separated using Tukey‟s test (p=0.05).

3.6 Discussion

3.6.1 2005 Field Experiment

3.6.1.1 Blight

Carrots can be grown continuously throughout the year in more temperate regions, and without proper rotation practices and debris removal the disease can remain in the field for subsequent production cycles. Pryor *et al.* (2002) examined the persistence of *A. dauci* in two very different agricultural environments, California and Florida, and demonstrated the range of conditions within which blight can survive and thrive. Spores and/or conidia can over-winter on volunteer plants, leaf debris or weeds and can withstand both flooding and drought conditions. The relationship between rate of decomposition and the survival of blight causal agents is linear; as the host material degrades, there are fewer leaves for the spores to survive on. Pryor *et al.* (2002) stressed the importance of leaf debris removal and tilling to increase host matter degradation. In Canada, only one carrot harvest can be done per year, allowing the host material to decompose for several months (October to May) before a new crop of carrots are planted.

In the case of field 201 at the NSAC used in this study, the previous crop was a cereal grain, with a carrot field only a few meters away. It is possible that the carrot debris from 2004 did not contain sufficient amounts of infected host plants to produce a high spore load in 2005. However, it should be noted that the 2005 growing season for carrots in Nova Scotia was atypical, with low blight levels reported on many farms. Since blight was not a decisive measurement for the effect that PPM treatments may have against diseases, other physiological measurements made as possible indicator of effects.

The 2005 season had a late seeding (June 2) due to wet and cold weather, which was followed by a dry summer. Table 3.5 depicts the environmental conditions of field 201 in 2005 compared to historical Environment Canada (http://climate.weatheroffice.ec.gc.ca/climate_normals/index_e.html) weather data for Truro, NS. It is possible that due to late seeding, carrot seedlings were not affected by early blight, which typically infects carrots in mid

July. *Cercospora* is considered the more damaging blight in Atlantic Canada (Davis and Raid, 2002). It is also possible that without the early stress of *Cercospora*, the carrots were better able to defend themselves from further *Alternaria* infection later in the growing season. Although most of the treatments had less blight at harvest than the control ($p<0.05$), a disease incidence of two is generally not high enough to warrant fungicidal spraying. Research from previous years suggests that brushing at 8 weeks and UV-C radiation at 4 weeks significantly reduced blight infections ($p<0.05$) (Lada, 1999), with 2004 having higher overall levels of blight (disease rating >3.5).

Table 3.5 The temperature averages and rainfall totals for 2005 in field 201 compared to the averages for Truro, NS from years 1982 to 2000 (Environment Canada, http://climate.weatheroffice.ec.gc.ca/climate_normals/index_e. html).

	High Temperature (°C)		Low Temperature (°C)		Total Precipitation (mm)	
Month	2005	Historical	2005	Historical	2005	Historical
May*	13.8	15.6	5.9	3.8	39.2	91.6
June	20.4	20.7	8.8	8.7	61.6	85.1
July	23.8	24.1	12.7	12.7	101	89.8
August	25.1	23.5	12.5	12.1	47.2	85.4
September	20.9	19.2	9.3	7.7	132.2	101.3
October	14.1	12.9	5.6	2.5	251.1	104.6

*Records for field 201 in 2005 began on May 20

In 2005, brushing and UV-C treatments resulted in less blight than the control, although only brushing at 8 weeks and UV-C radiation at 4 weeks had significantly less blight. There were no patterns from 2004 (Figure 1.7) to suggest that timing of UV-C radiation or brushing has an effect over the control of blight, as they are not significantly different from each other (Lada, 1999). The same results were found in 2005, except that brushing at 4 weeks exhibited a greater reduction in blight over other brushing treatments and the control. Due to the lack of patterns with timing of treatments and control of disease development,

these results indicate that timing of treatment may not be as important for the strength of induced resistance in blight.

The average disease cycle of *A. dauci* is 8 to 12 days under field conditions (Strandberg, 1988; Rotem, 1994; Ben-Noon *et al.*, 2001). Ben-Noon *et al.* (2001) determined that foliar applications of fungicide provided the best disease control when applied two to three disease cycles (16 to 36 days) prior to actual blight symptoms appearing. This does not seem to be consistent for all carrot producers. In Eastern Canada, spraying for disease control tends to occur after symptoms reach a critical level of 25% of scouted leaves showing any, or even minor blight (Bragg Lumber, 1999). The difference in best-defence practices may be due to location and environment. Due to the many factors that are involved with blight infection and development, such as temperature, wind, rainfall, humidity and even rotation, the effect of PPM may vary among locations and even seasons.

3.6.1.2 Effect of PPM on Certain Carrot Pests and Diseases

To carrot producers, perhaps the most influential consequences of certain pests and disease is root quality. Marketability of the root is of critical importance, and the various rots, molds and scabs that affect the roots can greatly decrease the value of a field of carrots. For instance, *Sclerotinia* white mold is a common and injurious disease of carrots that usually occurs late in the season and can be very destructive in storage. Molloy *et al.* (2004) demonstrated that treatment with chitosan, a putative stimulus and signal for pathogen defence, might induce host resistance of carrots to white mold. An *in vivo* study did not show reduced *S. sclerotiorum* growth when carrots or other fruits and vegetables were treated with chitosan, but when applied post-harvest, created a protective environment around the carrots (Molloy *et al.*, 2004). Molloy *et al.* (2004) suggested that chitosan might be endohydrolased by the plant, stimulating the release of oligosaccharides (known to be a signal for plant disease defence). Many plant signals are known to travel in a bidirectional manner between leaves and roots; therefore, it is possible that disturbing the

leaves of carrots produces a mobile signal that would also prime the root for defence.

In the present study, PPM treatments significantly reduced *Sclerotinia* white mold compared to the controls ($p<0.05$, Figure 3.3). The UV-C radiation treatment at 4 weeks significantly reduced white mold, yet the 12 week treatment did not. This apparent decrease in effectiveness with later treatments suggests that the defence compounds induced by UV-C radiation take several months to reach the critical level necessary to effectively control white mold. The brushing treatment effect does not follow this same pattern, lending support to the idea that the brushing and UV-C radiation treatments may induce different defence mechanisms and may work through different pathways. If the PPM treatments induce different defence pathways, then they may not protect the carrots in the same way. Additionally, these findings indicate that proper timing of the appropriate PPM technology may be just as important as the defences they induce.

3.6.2 2006 Field Experiment

This experiment was designed to test both the effects of timing and of varying durations of two PPM treatments (brushing and UV-C) on carrot diseases. To achieve this, three duration treatments (low, medium and high) were applied to field grown carrots over the course of the growing season (4, 8 and 12 weeks) in Truro, NS. Trimming treatment was omitted in the 2006 study due to conflicting and less than encouraging results in 2004 (Figure 1.7) and 2005 (Figure 3.2) trials. In general, the disease indices for each disease were atypical of the NS carrot growing season, with very little chemical control needed to suppress blight and insects. The data analysis was further complicated by the fact that controls were randomly assigned to meet the statistical requirements for RBCD and 3x3 factorial tests. In addition, there was a high degree of variability between the controls for all diseases examined. Figure 3.6 demonstrates the variability between controls. For instance, control medium treatments showed the highest incidence of blight (1.3) while control low had the lowest (0.3). As a

result, a number of treatments that appeared to affect disease incidence were not found to be statistically significant.

3.6.2.1 *Cercospora* Leaf Blight

In general, there was no significant positive or negative treatment effect on early blight, except for medium duration of brushing. This was probably due to the unusually low incidence of blight in 2006, as every block was between 0.3 and 1.4 on a scale of 9. Had the average early blight of the controls been around 5, we may have seen a greater spread in the data, which may have resulted in more statistically significant results. With that in mind, one cannot rule out the possibility that PPM treatments would not have had an effect on blight, regardless of blight intensity.

3.6.2.2 *Alternaria* Leaf Blight

Similar to what was observed with *Cercospora*, there was little statistical difference between treatments and control for *Alternaria,* except for brushing at week 12. Even though incidences of late blight were not as low as early blight, it still was not severe enough to warrant any chemical control for surrounding non-experimental fields. Again, this may have complicated the statistical analysis as all were on the extreme low end of the blight rating scale. As with *Cercospora*, we cannot dismiss the possibility that these treatments, regardless of intensity or timing, had no effect on the severity of *Alternaria*.

Although UV-C treatments applied in this study had no statistically significant effect, previous data from 2005 (Figure 3.2) did show a significant benefit in reducing blight with UV-C treatments. We cannot rule out that some PPM treatments may have a positive effect on reducing blight in carrot fields, leaving the possibility that further studies could support this theory.

3.6.2.3 Aster Yellow

The occurrence of aster yellows in any field is due to the feeding habit of leaf hoppers, which are the vector of this disease. Wally *et al.* (2008)

hypothesized that once a carrot plant has been infected with the aster yellow phytoplasmas, treatments become ineffective. For a treatment to succeed in reducing aster yellows, it must reduce the chance that a leaf hopper will feed on the carrots. To that end, the present study supports this theory by demonstrating that treatments applied later in the season did not significantly reduce aster yellows (Figure 3.8). It is therefore possible that early treatments were negatively influencing the feeding patterns of leaf hoppers thus reducing the severity of aster yellows observed at the end of the growing season. It is also possible that the vectors did not carry the aster yellow virus, thus leaf hopper feeding would not result in aster yellow symptoms.

3.6.2.4 Soft Rot

Timing of the treatments is of prime importance for the resistance to soft rot. Figure 3.9, depicts the effect of treatment timing has on soft rot. The mid-season treatments (in direct contrast to aster yellows, Figure 3.8) did have an effect on reducing root damage. That was complicated by the dissimilarity within the three controls, which varied between no recorded soft rot to almost 1.4 affected-roots per metre affected. However, even taking this into consideration, the 8 week treatments (both brushing and UV-C radiation) did have a positive effect on the reduction of soft rot. The defence pathways that the PPM treatments might be initiating are likely time dependent. For instance, treating the crop too early may lead to early triggering. In contrast, starting them too late may lead to missing the window for successful defence. The data from this experiment suggest that soft rot can be more successfully reduced when treatments are given early to mid season. In general, this reduction of soft rot due to PPM treatment is in agreement with a recent study that showed that wounding increased the immunity of *Arabidopsis thaliana* Heynh. to soft rot (Chassot *et al.*, 2008).

3.6.2.5 Wireworm Damage

The results of treatment on wireworm show that sometimes neither timing nor duration of the PPM treatment effect the level of damage. It may be that any

directed disturbance to the carrots will confer control against wireworm. The control in Figure 3.10 shows that wireworms were affecting carrots in the research plot, therefore it can be concluded that PPM may have a positive effect on deterring wireworms from damaging carrot roots.

3.7 Conclusion

This chapter has provided evidence that certain PPM technologies may have reduced the incidences of carrot blight and other pests and diseases, and that timing of treatment is more important than the duration. However, these conclusions were limited by the low incidence of blight and other diseases during the 2005 and 2006 growing seasons. In addition, there were a number of conflicting results between 2005 and 2006 field trials. For example, white mold was the only disease that had a treatment effect in 2005, yet was the disease that did not have a treatment effect in 2006 (Tables 4.2 and 3.5). Similarly, brushing at 4 weeks resulted in significantly less blight in 2005 (Figure 3.2), yet the same treatment conditions led to no statistical difference in *Alternaria* in 2006 (Figures 4.5 and 4.7). However, the 2006 *Alternaria* data (along with soft rot and aster yellows) shows a decreasing trend when treatments were applied at early stages. This trend was in contrast with *Cercospora* which showed a decreasing trend with treatments applied at the later stages. Future field trials or controlled laboratory trials (as outlined in Chapter 2) may help to solidify the results and provide a more solid basis for the conclusions provided in this chapter. In addition, it should be noted that in the 2006 experiment, the experimental design resembled a CRD rather than a CRBD. This means that the 2006 field study was not ideally suited for the factorial analysis conducted as described in this chapter. For instance, controls were not properly assigned to separate blocks, which means that the analyses did not adequately address the effect of field. However, even with the CRD design and field layout, alternative statistical analyses could have been, used such as contrasts analysis. This would have allowed pooling of the control groups, and specific inter-treatment comparison which may have led to statistical analyses with more meaningful biological interpretation. However, by pooling all the control would have allowed an assumption that the disease and

pest occurrences are similar in all stages of crop growth, which was not so; and this was the reason why there was a control for each stage rather than for each block. The current statistical method employed on the 2006 data is statistically valid and is consistent with not only the initial experimental plan but also with the 2005 data analyses. Furthermore, the general biological trends and conclusions are limited regardless of the statistical method due to the low incidence of carrot pests and other diseases during the 2006 field trial. Nevertheless, the evidence provided from the experiments described in this chapter suggests that PPM treatments may contribute to the reduction of certain pests and diseases of great ecological and economical significance to carrot producers of Nova Scotia.

Chapter IV: Quantification of salicylic acid from carrot (*Daucus carota* var. Sativus cv. Carson.) seedlings subjected to photo, physical and mechanical (PPM) treatments

4.1 Introduction

Salicylic acid (SA) is a simple phenol (Figure 1.4) involved in several metabolic functions within plants, including thermogenesis, flowering and defence mechanisms (Raskin, 1992). Recently, it has been shown that SA is a key component in the activation of systemic acquired resistance (SAR) in plants (Segarra *et al.*, 2006; Bari and Jones, 2009), with methyl salicylic acid (MeSA) its likely mobile signal (Park *et al.*, 2007). The increase of concentration of SA is also linked to the activation of pathogenesis related (PR) protein expression, as well as key components in pathogen defence (Segarra *et al.*, 2006; Bari and Jones, 2009). Exogenous applications of SA have been shown to induce resistance in plants to various biotic (Loake and Grant, 2007; Bari and Jones, 2009) and abiotic (Horvath *et al.*, 2007) stresses. It has also been applied to carrots to determine its effectiveness in reducing boron and salt stress, although it did not reduce the stress caused by these agents (Eraslan *et al.*, 2007). However, that study did demonstrate that SA increased reactive oxygen species (ROS) generation, which is an asset in pathogen defence (Bari and Jones, 2009).

It is hypothesized that photo, physical and mechanical (PPM) pre-treatments on carrot seedlings will induce SAR and thereby protect carrots from future pathogen attack. To further this understanding of the potential benefits of PPM treatments on carrots, the accurate detection and quantification of SA is required. To that end, there have been a number of analytical methods used to detect SA in a variety of plant species. High performance liquid chromatography (HPLC) has been used by Verberne *et al.* (2002) and Chaman *et al.* (2003) to quantify SA in tobacco (*Solanum lycopersicum* L.) and barley (*Hordeum vulgare* L.*)*, respectively. Other analytical methods have also been employed to measure SA, such as gas chromatography – mass spectrophotometry (GC-MS) (Metraux *et al.*, 1990; Muljono *et al.*, 1998; Muller *et al.*, 2002; Waller *et al.*, 2006) and liquid chromatography – mass spectrophotometry (LC-MS) (Segarra *et al.,* 2006). Recently, an alternative non-analytical method has been developed using bacterial biosensors to semi-quantitatively detect SA in plant tissue (DeFraia *et*

al., 2008). However, none of the previous authors have reported a method for the accurate quantification of SA from carrot tissue.

In the present study, an experiment was designed to develop a reliable method for detection of SA from carrot seedlings using HPLC in combination with a two-phase extraction method. This was done as part of a strategy for future biochemical characterization of the stress response in carrots to diseases such as blight.

4.2 Hypothesis

Salicylic acid concentrations in carrot seedlings treated with moderate levels of UV-C radiation or brushing will increase.

4.3 Objectives

The goal of this experiment is to establish an analytical method to detect salicylic acid and then to determine if PPM treatments can increase the concentration of SA in carrot seedlings.

4.4 Materials and Methods

4.4.1 Chemicals

3,4-dihydroxybenzylamine (DHBA), salicylic acid (SA) and all other chemicals used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.4.2 Plant Material

Daucus carota var. Sativus cv. Carson seeds were sown into 5 cm pots in Promix BX (Premier Horticulture Inc., Red Hill, PA, USA) and thinned to ten plants per pot one week after emergence. Pots were kept in a growth chamber at 24ºC/12ºC day/night temperature, with 94±2% relative humidity and a 16/8 h light cycle. Pots were rotated counter-clockwise every three days to reduce localized chamber fluctuations.

4.4.2.1 Treatment of Seedlings

Three weeks after emergence, the carrot seedlings were divided into two treatments and two control groups; UV-C for 30 s, UV-C control, brushing 40 strokes and brushing control. Each group contained 42 pots (approximately 420 seedlings). The UV-C for 30 s group was treated with 30 s of UV-C mobile light radiation (LiCor LI-188B Quantum Radiometer, Li Cor Inc. Lincoln, Nebraska, USA) at just above (<3 cm) canopy level. The brushing 40 strokes group was subjected to 40 strokes (20 strokes from the left and 20 strokes from the right directions) using a 30 cm wooden ruler.

4.4.2.2 Harvesting of Treated Seedlings

Each of the four treatments and control groups were divided into three replicates at seven different harvesting times; immediately post treatment (0 h), 3, 6, 12, 24, 48 and 72 h post treatment. All the above ground seedling material was removed and placed in small, labeled plastic tissue containers for each pot. One pot represents one repetition of a treatment or control group at a specific harvesting time. The containers were immediately dropped into a cylinder of liquid nitrogen. Once the material was completely frozen, it was placed at -20°C until further analysis.

4.4.3 Extraction Method

The following methodology was modified from Verberne *et al*. (2001). Frozen leaf material was ground into a fine powder and transferred to a 1.5 mL microtube, to which 1 mL of 90% methanol and 2.5 µL internal standard (DHBA, 10 µg uL^{-1}) was added. The solution was then vortexed 1 min, sonicated for 5 min and then centrifuged at 13,000 rpm for an additional 5 min. The supernatant was collected in a 2 mL microtube. The pellet was re-suspended in 0.5 mL 100% methanol, then sonicated and centrifuged for 5 min. At this point the supernatant were combined and then centrifuged another 5 min. The resulting supernatant solution was collected and then evaporated under a stream of nitrogen gas.

After all liquid had evaporated, 250 µL of 5% trichloroacetic acid (TCA) (in water) was added and vortexed. To this was added 800 µL ethyl acetate:cyclohexane (1:1). The solution was allowed to settle and the upper phase (containing free SA) was separated from the lower phase. Another aliquot of 800 µL ethyl acetate:cyclohexane was added to the lower phase, and another separation of phases was conducted. The two upper phases containing free SA were combined and evaporated to dryness. The final concentrate was re-suspended with 600 µL of 0.2M sodium acetate (pH 5.5) in methanol:water (1:1) and placed at 4°C for 16 h.

4.4.4 HPLC Analysis

A 10 µL aliquot of each sample was injected into a Beckman HPLC (Beckman System Gold, Beckman Coulter Canada Inc. Mississauga, ON) with a PA-1 CarboPac column (Dionex, Sunnyvale, CA, USA). A flow rate of 0.8 mL min^{-1} was used with a eluant solution consisting of 0.2M sodium acetate (pH 5.5) in methanol:water (9:1).

4.4.5 Statistical Analysis

DHBA and SA standard curves (loading concentration vs. peak area) were generated and analyzed using standard regression analysis with Minitab 13 software.

4.5 Results

4.5.1 HPLC Detection of Commercial SA

To verify that the HPLC setup could effectively detect SA, a commercial SA was passed through the HPLC using the system described in section 4.4.4. A peak corresponding to SA was detected at approximately 15 min (Figure 4.1). The response of the SA detection was linear with respect to concentration (data not shown). Similarly, the internal standard (DHBA) was detected at approximately 8 min (Figure 4.2) and it was also linear with respect to concentration (data not shown).

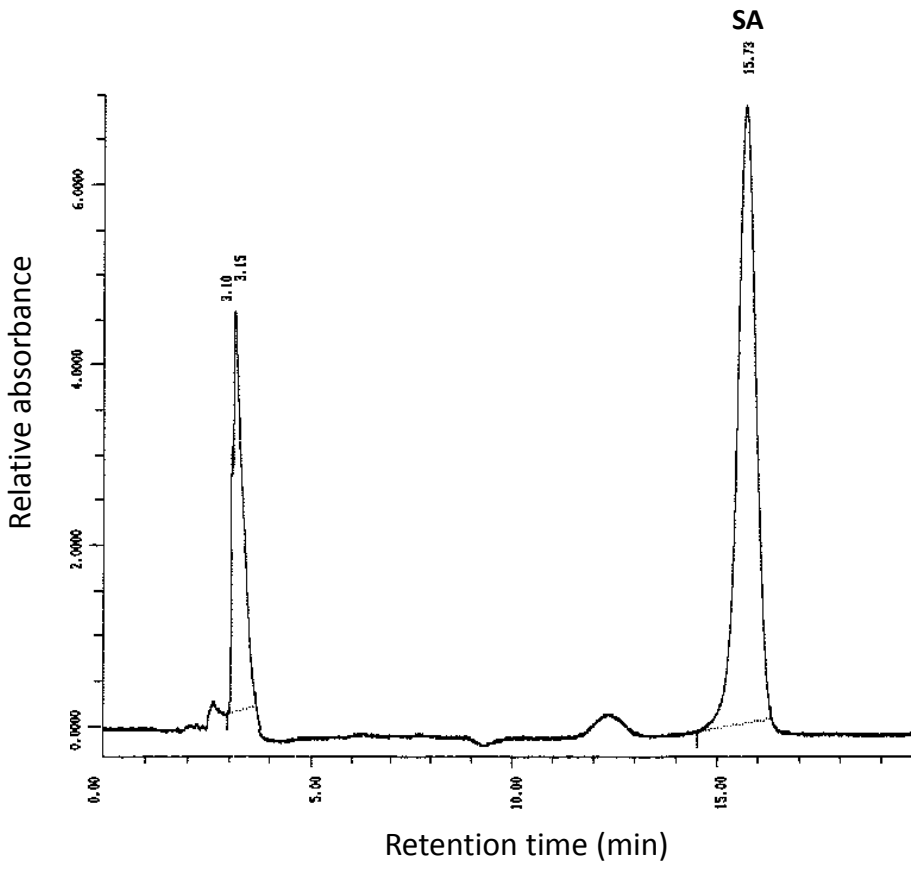


Figure 4.1 HPLC chromatographic trace of commercial salicylic acid (SA) eluting at approximately 15 min.

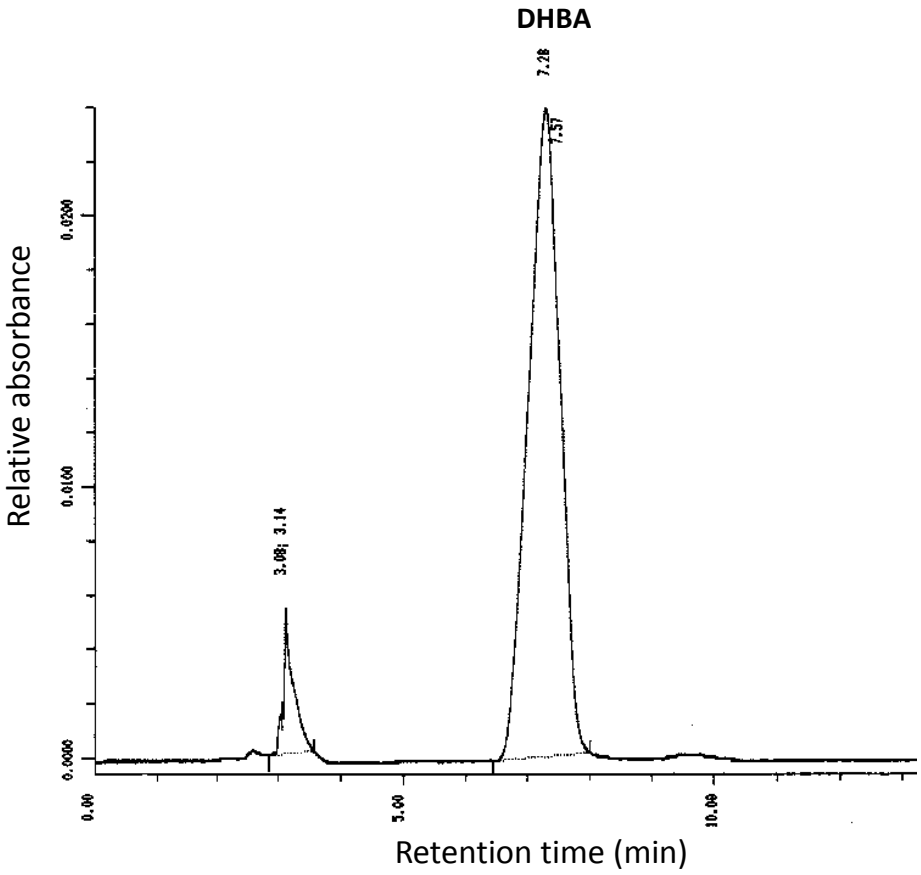


Figure 4.2 HPLC chromatographic trace of the internal standard 3,4-dihydroxybenzylamine (DHBA) eluting at approximately 8 min.

4.5.2 HPLC Detection of SA in Sample Tissues

To test the effectiveness of the extraction and analysis protocols employed in this study, approximately 0.5 g of carrot leaf tissue was taken from the field, irrespective of treatment, and analyzed. Using this test material, SA was easily detected using the HPLC setup as described in sections 3.4.3 and 3.4.4 (Figure 4.3). This showed that natural SA can be detected in carrots and analyzed in a similar fashion as commercial SA. However, when experimental samples (controls, brushing and UV-C treatments) were examined using approximately 0.25 g of material the SA signal was low and co-elution with unidentified compounds was apparent (Figure 4.4).

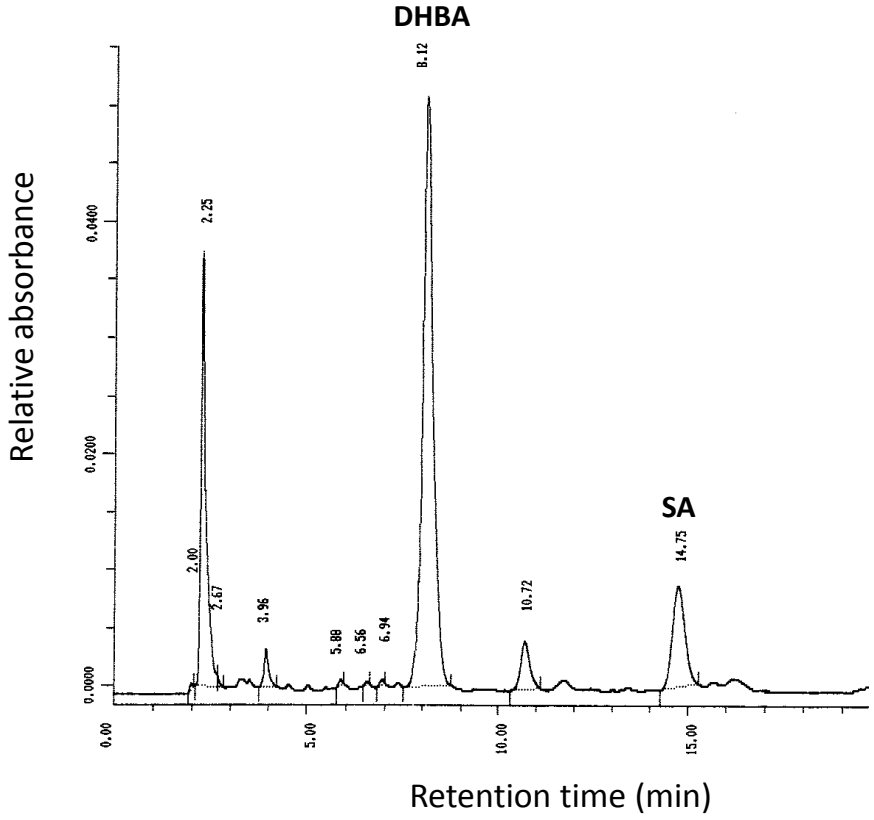


Figure 4.3 HPLC chromatographic trace of compounds extracted from sample carrot leaf material showing the internal standard 3,4-dihydroxybenzylamine (DHBA) and salicylic acid (SA).

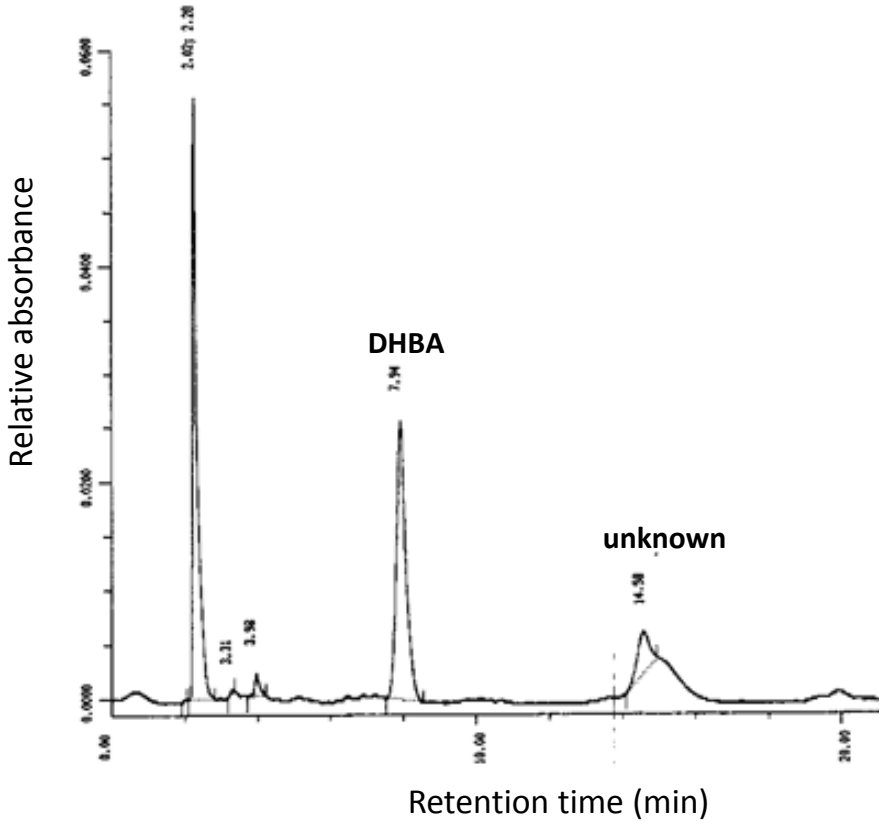


Figure 4.4 HPLC chromatographic trace of compounds extracted from a representative experimental sample showing the internal standard 3,4-dihydroxybenzylamine (DHBA) and a cluster of co-eluting compounds (unknown).

4.5.2.1 Effect of Treatment and Sampling Time on Detection of SA

Tissue from plants that had two different treatments applied to them was tested to determine whether co-elution of peaks was a treatment effect. The two treatments (brushing and UV-C), along with the control plants were examined over seven sample times (0, 3, 6, 12, 24, 48 and 72 h). Regardless of treatment and time, the detection of SA was severely limited due to low peaks and co-elution as shown in Figure 4.4.

4.6 Discussion

4.6.1 Extraction Methods and HPLC Detection

A standard phase separation technique common to HPLC and other chromatographic analyses was employed in this study. The combination of methanol and ethyl acetate was effective at extracting the polar compounds (including SA) from the non-polar compounds of carrot seedling leaf tissue. This was supported by the detection of SA using HPLC analysis. A peak corresponding to SA was found at 14-15 min, which is consistent with chromatograms obtained with standard SA. These results suggest that a suitable two-phase separation technique and HPLC detection method for SA in carrots was established.

4.6.2 Experimental SA Detection

Two treatments and seven sampling times were used in an attempt to test for SA variation in relevant field studies. The amount of tissue was halved to 0.25 g in an attempt to divide the collected tissue (0.5 g) for both SA and future jasmonic acid (JA) analysis. Unfortunately, this reduction led to nearly undetectable SA peaks using the HPLC setup described. Furthermore, the SA peaks co-eluted with unknown compounds, further complicating detection. Low peaks and co-elution was common to all treatments and collection times, suggesting that this difficulty was due to technical difficulties (i.e. experimental setup) rather than biologically meaningful phenomena, although the possibility that treatments could have lower SA cannot be ruled out. No definitive trends between treatment or sample times could be observed due to these difficulties.

4.6.3 Future Recommendations

Given that the initial testing was successful using 0.5 g of tissue and that difficulties were only later observed using 0.25 g of tissue, obvious recommendations would be to either collect more tissue at the time of harvest or to not divide original amounts for the purposes of other experiments (for example, future jasmonic acid experiments). This change would help to account for low

detection peaks for SA. Alternatively, more samples could be loaded into the HPLC; however, the issue of co-elution would persist. The difficulty in separating the SA peaks from other unknown compounds could be resolved in a number of ways. Flow-rate is an obvious condition to vary that could help separate the co-eluted peaks and resolved SA. To this end, a range of flow-rates was tested but with limited success (data not shown). Similarly, eluant concentration is often commonly varied to remedy peak co-elution. In this experiment, a range of eluant concentration was employed, but again with limited success in separating SA from a group of unknown compounds. It is possible that better detection of SA would be achievable with a different HPLC instrument, column, or eluant type, and this warrants future experimentation. In addition, better separation and quantification of SA may be achieved using alternative analytical techniques, such as GC-MS (Muljono *et al.*, 1998; Metraux *et al.*, 1990; Muller *et al.*, 2002; Waller *et al.*, 2006), LC-MS (Segarra *et al.,* 2006) and even bacterial bioreactors (DeFraia *et al.*, 2008).

4.7 Conclusion

The evidence collected in this experiment suggests that it is possible to quantify SA from three week-old carrot seedlings using a simple two-phase extraction technique and common HPLC detection method. Applying this system to field-grown carrots was shown to be theoretically possible provided the proper amount of starting material is used and further experimentation with HPLC peak separation is conducted. The findings of this work can be used in future SA-based experiments designed to investigate the effects of PPM treatment on blight and other carrot diseases.

Chapter V: Conclusion

5.1 General Discussion

This thesis investigated the effects of PPM technology on carrot (*Daucus carota* var. Sativus cv. Carson) seedlings as a means to induce resistance to pathogens such as *Alternaria* and *Cercospora*. Photo (UV-C), physical (brushing) and mechanical (trimming) treatments were applied over a variety of intensities, durations and time periods during two separate field trials in 2005 and 2006 (Chapter 3). The 2005 field trial examined treatment and timing, while the 2006 field trial focused on treatment, timing and duration. Broadly, the results of these experiments suggest that timing and treatment are more important for reducing disease than duration. The exception was the incidence of *Cercospora*, which was reduced by brushing treatments of medium duration in 2006. In 2005 all treatments of UV-C and brushing significantly reduced blight, regardless of timing. In contrast, white mold was reduced when UV-C was applied in early and mid season (four and eight weeks) only, suggesting that timing contributes to the effect of treatment. Similarly, brushing at mid and late season was more effective at reducing white mold than early application. Timing of a particular treatment was also shown to play an important role in the 2006 experiment as both *Alternaria* and Aster yellows were reduced when treatment was applied early or mid season, although this trend was not statistically significant. As a further example of the effect of timing in 2006, both treatments applied at eight weeks significantly reduced soft rot. In general, the data suggest that treatment applied earlier in the season may increase disease resistance. In contrast, both brushing and UV-C treatment significantly reduced wireworm damage in 2006 regardless of either duration or timing of treatment, suggesting that certain treatments may help induce defense against wireworm feeding.

Taken together, the data collected from both the 2005 and 2006 field experiments provide evidence that some PPM treatments are effective at inducing resistance in carrots to blights, white mold, soft rot and wireworm when compared to controls (summarized in Table 5.1). However, these trends were not always significant due to statistical limitations associated with the experimental set up (e.g. high variability in controls). This can be partially

attributed to the use of a CRD rather than a more appropriate CRBD in the 2006 experiment. This change in design meant that the effect of field was not taken into account, which led to inconsistent control ratings for all pests and diseases. Using alternative statistical mean comparisons (e.g. contrasts) may have to overcome these difficulties by allowing the controls to be pooled prior to comparison, which can allow for more meaningful biological interpretations. However, there are limitations in doing contrast comparisons well since the controls were set to the stages as it is important to understand the changes in pest and disease occurrence, which depend on the stage of plant growth and treatment application. Pooling controls would be inappropriate and could lead to an erroneous interpretation. In addition to statistical analyses, the typically low incidences of disease during the 2005 and 2006 growing seasons further complicated the analysis and interpretation of the experimental results. To address this problem, a set of experiments was conducted to establish blight on carrot seedlings in a controlled laboratory setting (Chapter 2). This was met with limited success; however these experiments provided clues for the successful establishment of blight in a laboratory setting. These include the maintenance of high humidity and obtaining highly virulent viable starting cultures of either *Alternaria* or *Cercospora*. The future examination of PPM on carrot seedlings from either the field or controlled laboratory may be enhanced by the detection of the widely-studied and well-established defence hormone, salicylic acid (SA). To that end, an additional set of experiments was conducted to establish a method of quantifying SA in the leaves of carrot seedlings (Chapter 4). Although a successful extraction and quantification method was established using test material, issues arose when experimental samples of PPM-treated samples were used. The difficulties were attributed to low amounts of starting material and probably the co-elution of SA with unknown compounds. Nevertheless, an effective extraction method for SA from carrot seedlings was established and through future experimental modification an effective HPLC-based quantification method may also be established. Specific recommendations for optimizing HPLC conditions are outlined in the discussion of Chapter 4.

Table 5.1 Summary of treatments along with their duration and/or timing that significantly reduced certain pests and disease incidence of field grown carrots in 2005 and 2006.

Pest/Disease (year)	Treatment	Timing (weeks)
Blight (2005)	Brushing	4, 8, 12
	UV-C	4, 8, 12
	TSLT	12
	TSRT	12
	Trimming 25%	12
	Trimming 50%	12
Cercospora (2006)	Brushing (medium)	
Alternaria (2006)	Brushing	12
White mold (2005)	Brushing	4, 8, 12
	UV-C	4, 8
	TSLT	12
	TSRT	12
	Trimming 25%	12
	Trimming 75%	12
Soft rot (2006)	Brushing	8
	UV-C	8
Wireworm damage (2006)	Brushing	
	UV-C	

5.2 Future Recommendations

To verify and build on the conclusions from PPM-treatments of field-grown carrots, more field experiments are necessary. This could be a single additional field study similar to those conducted in 2005 and 2006 outlined in this thesis, or multiple additional field studies. If multiple additional field studies are used, it would be ideal to use field sites at multiple locations to increase the probability of blight and other carrot diseases. Higher incidence of pathogens would undoubtedly enhance the statistical analysis and overcome the limitations outlined in this thesis. Alternatively, field trials could be replaced or supplemented with large-scale laboratory experiments where pathogens such as

Alternaria and *Cercospora* could be directly applied to carrot seedlings to establish high levels of blight. The information collected in Chapter 2 of this thesis will help to establish such laboratory experiments.

Any experimental PPM-treated carrot leaf tissue collected from field or laboratory-based experiments should be used for SA quantification, using a similar experimental setup as outlined in Chapter 4. Care should be taken to collect more tissue than was used for experimental samples in Chapter 3 as the analytical results were limited by SA peak size due to low amounts of starting material. Expanding the HPLC analysis to include other markers of the defence response, such as jasmonic acid (JA), will help to better understand the biochemical response associated with the various PPM treatments. Similarly, it may be possible to use a molecular approach in the future, where the gene products (i.e. transcripts) of various defence-related enzymes can be examined. The combination of quantifying defence compounds along with understanding of defence gene regulation should help to resolve the underpinning mechanism of the defence strategy of carrots in response to PPM treatments. All of these supplemental analyses will require additional tissue to be collected from the field or laboratory and care must be taken to ensure that enough tissue is collected to satisfy the experimental requirements of each analysis.

Finally, the information gathered from this experiment along with anticipated future experiments should not only contribute to the understanding of pest and disease resistance in crop plants, but also help agronomists prepare field-based strategies for efficiently treating carrot crops in preparation for pathogen attack. The latter effort will require the consideration of many factors including determining the PPM treatment that is most effective at reducing disease in carrots (e.g. brushing) along with considering the costs associated with realistic application of the PPM treatment to the crop. The development of a suitable strategy to control pests and diseases in an eco-friendly way, will undoubtedly involve the cooperation and careful planning of many scientists, agronomists and engineers. However, the proper application of such non-

chemical, environmentally friendly approaches to pathogen management could help sustain and even enhance the viability of the carrot industry of Nova Scotia.

References

Agrios, G. 2004. Plant Pathology, 5th ed. Elsevier Academic Press, MA, USA.

Alasalvar, C., Grigor, J., Zhang, D., Quantick, P. and Shahidi, F. 2001. Comparison of volatiles, phenolics, sugars, antioxidant vitamins, and sensory quality of different colored carrot varieties. J. Agric. Food Chem., 49:1410-6.

Arimura, G., Kost, C. and Boland, W. 2005. Herbivore-induced, indirect plant defences. Biochimica et Biophysica Acta, 1734:91-111.

Bach, M., Schnitzler, JP. and Seitz, H. 1993. Elicitor-induced changes in Ca^{2+} influx, K^{+} efflux, and 4-hydroxybenzoic acid synthesis in protoplasts of *Daucus carota* L. Plant Physiol., 103:407-12.

Bais, H., Prithiviraj, B., Jha, A., Ausubel, F and Vivanco, J. 2005. Mediation of pathogen resistance by exudation of antimicrobials from roots. Nature, 434:212-7.

Baldwin, I., Zhang, Z., Diab, N., Ohnmesis, T., McCloud, E., Lynds, G. and Schmelz, E. 1997. Quantification, correlations and manipulations of wound-induced changes in jasmonic acid and nicotine in *Nicotiana sylvestris*. Planta, 201:397-404.

Baldwin, I. 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proc. Natl. Acad. Sci. USA, 95:8113-8.

Bari, R. and Jones, J.D.G. 2009. Role of plant hormones in plant defence responses. Plant Mol. Biol., 69:473-88.

Ben-Noon, E., Shtienberg, E., Shlevin, E. and Vintal, H. 2001. Optimization of chemical suppression of *Alternaria dauci*, the casual agent of Alternaria leaf blight in carrots. Plant Dis., 85:1149-1156.

Birkenmeier, G. and Ryan, C. 1998. Wound signaling in tomato plants: Evidence that ABA is not a primary signal for defence gene activation. Plant Physiol., 117:687-93.

Bragg Lumber, 1999. Carrot grower guide. Oxford frozen foods, Ltd, NS.

Brodeur, C., Carisse, O. and Bourgeois, G. 1998. *Cercospora* leaf blight of carrot control strategies. Agriculture and Agri-Food Canada, Horticultural Research and Development Centre, Saint-Jean-sur-Richelieu, Que., Canada.

Carisee, O. and Kushalappa, A. 1989. Effect of media, pH and temperature on spore production and of inoculum concentration on number of lesions produced by *Cercospora carotae.* Phytoprotection, 70:119-24.

Carisse, O., Kushalappa, A. and Cloutier, D. 1993. Influence of temperature, leaf wetness, and high relative humidity duration on sporulation of *Cercospora carotae* on carrot leaves. Phytopathology, 83:338-43.

Chaman, M., Copaja, S. and Argandona, V. 2003. Relationship between salicylic acid content, phenylalanime ammonia-lyase (PAL) activity, and resistance of barley to aphid infection. J. Agric. Food Chem., 51:2227-31.

Chao, W., Gu, Y., Pautot, V., Bray, E. and Walling, L. 1999. Leucine aminopeptidase RNAs, proteins, and activities increase in response to water deficit, salinity, and the wound signals systemin, methyl jasmonate, and abscisic acid. 1999. Plant Physiol., 120:979-92.

Chassot, C., Buchala, A., Schoonbeek, H., Metraux, J.P. and Lamotte, O. 2008. Wounding of Arabidopsis leaves cause a powerful but transient protection against *Botrytis* infection. Plant J., 55:555-67.

Constabel, C. 1999. A survey of heribivore-inducible defence proteins and phytochemicals. Induced Plant Defences Against Pathogens and Herbivores: Biochemistry, Ecology, and Agriculture. APS Press, MN, USA.

Costa H., Gallego, SM. and Tomaro, ML. 2002. Effect of UV-B radiation on antioxidant defence system in sunflower cotyledons. Plant Sci., 162:939-945.

Creelman, R., Tierney, M. and Mullet, J. 1992. Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc. Natl. Acad. Sci. USA, 89:4938-41.

Danon, A. and Gallois, P. 1998. UV-C radiation apoptotic-like changes in *Arabidopsis thaliana*. FEBS Letter, 437:131-6.

Davis, R. and Raid, R. 2002. Compendium of Umbelliferous Crop Diseases. APS press, MN, USA.

Daub, M. and Ehrenshaft, M. 2000. The photoactivated *Cercospora* toxin cercosposporin: Contribution to plant disease and fundamental biology. Annu. Rev. Phytopathol., 38:461-90.

DeFraia, C.T., Schmelz, E.A. and Mou, Z. 2008. A rapid biosensor-based method for quantification of free and glucose-conjugated salicylic acid. Plant Methods, 4:28.

De Moraes, C.M., Lewis, W.J., Pare, P.W., Alborn H.T. and Tumlinson, J.H. 1998. Herbivore-infested plants selectively attract parasitoids. Nature, 393:570-3.

Doares, S., Syrovets, T., Weiler,E. and Ryan, C. 1995. Oligogalacturonides and chitosan activate plant defence genes through the octadecanoid pathways. Proc. Natl. Acad. Sci. USA, 92:4095-8.

Dugdale, L., Mortimer, A., Isaac, S. and Collin, H. 2000. Disease response of carrot and carrot somaclones to *Alternaria dauci.* Plant Path., 49:57-67.

Durrant, W.E. and Dong, X. 2004. Systemic Acquired resistance. Annu. Rev. Phytopathol., 42:185-209.

Environment Canada. (2007) *National Climate Data and Information Archive.* Retrieved from http://climate.weatheroffice.ec.gc.ca/climate_normals/index_e.html

Eraslan, F., Inal, A., Gunes, A., and Alpaslan, M. 2007. Impact of exogenous salicylic acid on the growth, antioxidant activity and physiology of carrot plants subjected to combined salinity and boron toxicity. Scientia Hort., 113:120-8.

Fancelli, M.I., Kimati, H. 1991. Occurrence of iprodione resistant strains of *Alternaria dauci.* Summa Phytopathologica, 17: 135-146.

Farmer, E. and Ryan, C. 1992. Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell, 4:129-34.

Fitzgerald, A. 2000. Basic techniques in plant pathology: An introduction to methods. Nova Scotia Agriculture College Press, Nova Scotia, Canada.

Glabgen, W.E., Rose, A., Madlung, J., Kock, W., Gleitz, J. and Seitz, H. 1998. Regulation of enzymes involved in anthocyanin biosynthsis in carrot cell cultures in response to treatment with ultraviolet light and fungal elicitors. Planta, 204:490-8.

Gleitz, J and Seitz, H.A. 1989. Induction of chalcone synthase in cell suspension cultures of carrot (*Dacus carota* L. ssp. *sativus*) by ultraviolet light: evidence for two different forms of chalcone synthase. Planta, 179:323-30.

Gundlach, H., Müller, M., Kutchan, T. and Zenk, M. 1992. Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc. Natl. Acad. Sci. USA, 89:2389-93.

Hammerschmidt, R. 1999. Phytoalexins: What have we learned after 60 years. Annu. Rev. Phytopathol., 37:285-306.

Hara-Nishimura, I and Matsushima, R. 2003. A wound-inducible organelle derived from endoplasmic reticulum: a plant strategy against environmental stresses? Curr. Opin. in Plant Biol., 6:583-8.

Hildmann, T., Ebneth, M., Pefia-Cortés, H., Sanchez-Serrano J.J., Willmitzer L. and Prat, S. 1992. General roles of abscisic and jasmonic acid in gene activation as a result of mechanical wounding. Plant Cell 4: 1157-70.

Hirner, A.A., Veit, S. and Seitz, H. U. 2001. Regulation of anthocyanin biosynthesis in UV-A-irradiated cell cultures of carrot and in organs of intact carrot plants. Plant Sci., 161:315-22.

Horvath, E., Szalai, G. and Janda, T. 2007. Induction of abiotic stress tolerance by salicylic acid signaling. J. Plant Growth Regul., 26:290-300.

Jain, K., Kataria, S. and Guruprasad, KN. 2003. Changes in antioxidant defences of cumcumber cotyledons in response to UV-B and to the free radical generating compounds AAPH. Plant Sci., 165:551-7.

Jansen, M., van den Noort, R., Tan, M., Prinsen, E., Lagrimini, M. and Thornely, R. 2001. Phenol-oxidizing peroidases contribute to the protection of plants from ultraviolet radiation stress. Plant Physiol., 126:1012-23.

Karban, R and Kuć, J. 1999. Induced resistance against pathogens and herbivores: An overview. Induced Plant Defences Against Pathogens and Herbivores: Biochemistry, Ecology, and Agriculture. APS Press, MN, USA.

Korthari, I. and Patel, M. 2004. Plant Immunization. Indian Journal of Experimental Biology, 42:244-52.

Kuchitu, K., Kikukawa, M. and Shibuya, N. 1993. N-Acetylchitooligosaccharide, biotic elicitor for phytoalexin production, induce transient membrane depolarization in suspension-cultured rice cells. Protoplasma 174: 79-81.

Kurosaki, F and Nishi, A. 1983. Isolation and antimicrobial activity of the phytoalexin 6-methoxymellein from cultured carrot cells. Phytochemistry, 22:669-72.

Lada, R. 1999. Processing Carrot Research Program: A study of the performance of various slicer and dicer varieties in Nova Scotia. Nova Scotia Agriculture College, NS.

Lada, R. 2004a. Technology development grant proposal. Nova Scotia Agriculture College, Nova Scotia, Canada.

Lada, R. 2004b. Technology Development Interim Report December, 2004. Nova Scotia Agriculture College, NS.

Li, J., Ou-Lee, T., Raba, R., Amundson, R and Last, R. 1993. Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. Plant Cell, 5:171-9.

Li, L., Li, C., Lee, G. and Howe, G. 2002. Distinct roles for jasmonate synthesis and action in the systemic wound response of tomato. PNAS, 99:6416-21.

Loake, G. and Grant, M. 2007. Salicylic acid in plant defence – the players and protagonists. Curr. Opi. Plant Biol., 10:466-72.

Ma, Z. and Michailides, T.J. 2004. Characterization of iprodione-resistant *Alternaria* isolates from pistachio in California. Pestic. Biochem. Phys., 80:75-84.

Ma, Z., Yoshimura, M., Holtz, B., and Michailides, T. 2005. Characterization and PCR-based detection of benzimidazole-resistant isolates of *Monilinia laxa* in California. Pest Mgt. Sci., 61:449-457.

Mackerness, SAH., Johm, CF., Jordan, B. and Thomas, B. 2001. Early signaling components in ultraviolet-B responses: distinct roles for different reactive oxygen species and nitric oxide. FEBS Lett. 489:237-42.

MacNab, A., Sherf, A. and Springer, J. 1983. Identifying diseases of vegetables. The Pennsylvania State University, PA, USA.

Madison, G. and Stevenson, W.R. 2008. Evaluation of QoI Fungicide Application Strategies for Managing Fungicide Resistance and Potato Early Blight Epidemics in Wisconsin. Plant Dis., 92:561-8.

Mercier, J. and Arul, J., 1993. Induction of systemic disease resistance in carrot roots by pre-inoculation with storage pathogens. Can. J. Plant Pathol., 15:281–3.

Mercier, J and Kuć, J. 1996. Induced systemic resistance to *Cercospora* leaf spot of carrot by inoculation with *Cercospora carotae*. J. Phytopathology, 144:75-7.

Mercier, J and Kuć, J. 1997. Elicitation of 6-methoxymellein in carrot leaves by *Cercospora carotae*. J. Sci. Food Agric., 73:60-2.

Metraux, J.P., Singer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W. and Inverardi, B. 1990. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science, 250:1004-6.

Molloy, C., Cheah, L. and Koolaard, JP. 2004. Induced resistance against *Sclerotinia sclerotiorum* in carrots treated with enzymatically hydrolysed chitosan. Postharvest Biol. Tec., 33:61-5.

Muljono, R.A.B., Looman, A.M.G., Verpoorte, R. and Scheffer, J.J.C. 1998. Assay of salicylic acid and related compounds in plant cell cultures by capillary GC. Phytochem. Anal., 9:35-8.

Muller, A., Duchting, P., and Weiler, E.W. 2002. A multiplex GC-MS/MS technique for the sensitive and quantitative single-run analysis of acidic phytohormones and related compounds, and its application to *Arabidopsis thaliana*. Planta, 216:44-56.

Narváez-Vásquez, J., Pearce, G., Orozco-Cárdenas, M., Franceschi, V. and Ryan, C. 1995. Autoradiographic and biochemical evidence for the systemic translocation of systemin in tomato plants. Planta 195: 593-600.

Nojiri, H., Sugimori, M., Yamane, H., Nishimura, Y., Yamada, A., Shibuya, N., Kodama, O., Murofushi, N and Omori, T. 1996. Involvement of jasmonic acid in elicitor-induced pytoalexin production in suspension-cultured rice cells. Plant Physiol., 110:387-92.

Park, S.W., Kaimoyo, E. and Kumar, D. 2007. Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. Science, 318:113-6.

Pautot, V., Holzer, F., Walling, L. 1991. Differential expression of tomato proteinase inhibitor I and II genes during bacterial pathogen invasion and wounding. Mol Plant-Microbe Interact., 4: 284-92.

Peña-Cortés, H., Albrecht, T., Prat, S., Weiler, E. and Willmitzer, L. 1993. Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta, 191:123-8.

Pettipas, F.C., Lada, R., Caldwell, C.D. and Miller, C. 2006. Leaf tissue testing and soil and plant tissue relationships for nitrogen management in carrots. Commun. Soil. Sci. Plan., 37:1597-609.

Prell, H. and Day, P. 2000. Plant-Fungal Pathogen Interaction: A Classical and Molecular View. Springer, NY, USA.

Pryor, B.M., Strandberg, J.O., Davis, R.M., Nunez, J.J. and Gilbertson, R.L. 2002. Survival and persistence of *Alternaria dauci* in carrot cropping systems. Plant Dis., 86:1115-1122.

Raskin, I. 1992. Role of salicylic acid in plants. Annu. Rev. Plant Physiol. Plant Mol. Bio., 43:439-63.

Rasmussen, J.B., Hammweschmidt, R. and Zook, M.N. 1991. Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae syringae.* Plant Physiol., 97:1342-7.

Reyes, L. and Cisneros-Zevallos. 2003. Wounding stress increases the phenolic content and antioxidant capacity of purple-flesh potatoes (*Solanum tuberosum* L.). J. Agric. Food Chem., 51:5296-300.

Rotem, J. 1994. The Genus Alternaria. Biology, Epidemiology, and Pathogenicity. APS Press, MN, USA.

Rubatzky, V.E., Quiro, C.F. and Simon P.W. 1999. Carrots and related vegetable Umbelliferae. Crop Production Science in Horticulture 10. CABI Publishing, Oxon, UK.

Ryan, C. 1990. Protease inhibitors in plants: Genes for improving defences against insects and pathogens. Annu. Rev. Phytopathol., 28:425-49.

SAS Institute Inc. 2000. *SAS Onlinedoc*, Version 8. Cary, SAS Institute Inc., NC, USA.

Saskia, C., Chang, H., Zhu, T. and Glazebrook. 2003. Characterization of the early response of Arabidopsis to *Alternaria brassicicola* infection using expression profiling. Plant Physiol. 132:606-17.

Segarra, G., Jauregui, O., Casanova, E. and Trillas, I. 2006. Simultaneous quantitative LC-ESI-MS/MS analyses of salicylic acid and jasmonic acid in crude extracts of *Cucumis sativus* under biotic stress. Phytochem., 67:395-401.

Segarra, G., Casanova, E., Bellido, D., Odenam, M.A., Oliveira, E. and Trillas, I. 2007. Proteome, salicylic acid, and jasmonic acid changes in cucumber plants inoculated with *Trichoderma asperellum* strain T34. Proteomics, 7:3943-52.

Seljasen, R., Bengtsson, G., Hoftun, H. and Vogt, G. 2001. Sensory and chemical changes in five varieties of carrot (*Daucus carota* L.) in response to mechanical stress at harvest and post-harvest. J. Sci. Food Agr., 81:436-47.

Solel, Z., Timmer, L.W. and Kimchi, M. 1996. Iprodione resistance of *Alternaria alternata* pv. *citri* from Minneola tangelo in Israel and Florida. Plant Dis., 80: 291–3.

Srivastva, L. 2001. Plant Growth and Development. Hormones and Environment. Academic Press, MO, USA.

Sticher, L., Mauch-Mani, B and Métraux, J.P. 1997. Systemic acquired resistance. Annu. Rev. Phytopathol., 35:235-70.

Strandberg, J. O. 1988. Establishment of Alternaria leaf blight on carrots in a controlled environments. Plant Dis., 72:522-6.

Takaichi, M and Oeda, K. 2000. Transgenic carrots with enhanced resistance against two major pathogens, *Erysiphe heraclei* and *Alternaria dauci*. Plant Sci., 153:135-44.

Takeda, J., Ito, Y., Maeda, K. and Ozeki, Y. 2002. Assignment of UVB-responsive *cis*-element and protoplastization-(dilution-) and elicitor-responsive ones in the promoter region of a carrot phenylalanine ammonia gene (gDcPAL1). Photochem. Photobio. 76:232-8.

Teklemariam, T and Blake, T. 2004. Phenylalanine ammonia-lyase-induced freezing tolerance in jack pine (*Pinus banksiana*) seedling treated with low, ambient levels of ultraviolet-B radiation. Physiol. Plant., 122:244-53.

Teklemariam, T and Blake, T. 2003. Effects of UVB preconditioning on heat tolerance of cucumber (*Cucumis Sativus* L.). Environ. Exp. Bot., 50:169-82.

Teklemariam, T and Blake, T. 2004. Phenylalanine ammonia-lyase-induced freezing tolerance in jack pine (*Pinus banksiana*) seedling treated with low, ambient levels of ultraviolet-B radiation. Physiol. Plant., 122:244-53.

Thomma, B., Eggermont, K., Penninckx, I., Mauch-Mani, B., Vogelsang, R., Cammue, B. and Broekaert, W. 1998. Separate jasmonate-dependent and salicylate-dependent defence-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. USA, 95:15107-11.

van Delden, A. and Carisse, O. 1993. Effect of plant age, leaf age and leaf position on infection of carrot leaves by *Cercospora carotae*. Phytoprotection, 74:75-87.

van Kan, J., Cozijnsen, T., Danhash, N. and de Wit, P. 1995. Induction of tomato stress protein RNAs by ethephon, 2,6-dichloroisonicotinic acid and salicylate. Plant Mol. Biol., 27:1205-13.

van Wees, S., Chang, H., Zhu, T and Glazebrook, J. 2003. Characterization of the early response of Arabidopsis to *Alternaria brassicicola* infection using expression profiling. Plant Physiol., 132:606-17.

Veit, S., Wörle, J., Nürnberger, T., Koch, W. and Seitz, H. 2001. A novel protein elicitor (PaNei) from *Pythium aphanidermatum* induced multiple defence responses in carrot, Arabidopsis, and tobacco. Plant Physiol., 127:832-41.

Verberne, M., Bouwer, N., Drlbianco, F., Linthorst, H., Bol, J. and Verpoorte, R. 2002. Method for the extraction of the volatile compound salicylic acid from tobacco leaf material. Phytochem. Anal., 13:45-50.

Vesonder, R.F., Peterson, R.E., Labeda, D. and Abbas H.K. 1992. Comparative toxicity of the fumonisins, AAL-toxin and yeast sphingolipids in *Lemna minor* L. (Duckweed) . Archives of Environmental Contamination and Toxicology 23:464-7.

Vintal, H., Ben-Noon, E., Shlevin, E., Yermiyahu, U., Shtienberg, D., and Dinoor, A. 1999. Influence of rate of soil fertilization on *Alternaria* leaf blight (*Alternaria dauci*) in carrots. Phytoparasitica, 27:193-200.

Waller, F., Muller, A., Chung, K., Yap, Y., Nakamura, K., Weiler, E. and Sano, H. 2006. Expression of a WIPK-activated transcription factor results in increase of endogenous salicylic acid and pathogen resistance in tobacco plants. Plant Cell Physiol., 47:1169-74.

Wally, O., Hadrami, A.E., Khadhair, A.H., Adam, L.R., Shinners-Carnelley, T., Elliott, B. and Daayf, F. 2008. DNA sequencing reveals false positives during the detection of aster yellows phytoplasmas in leafhoppers. Scienta Hort., 116:130-37.

Wang., N., Xu, F., Zhang, Z., Yang, C., Sun, X. and Li, J. 2008. Simultaneous determination of sipyrodamole and salicylic acid in human plasma by high performance liquid chromatorgraphy-mass spectrometry. Biomed. Chromatogr., 22:149-56.

Wasternack, C. and Parthier, B. 1997. Jasmonate-signalled plant gene expression. Trends Plant Sci., 2:302-7.

Wildermuth, M., Dewdney, J., Gang, W. and Ausubel, F.M. 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature, 414:562-5.

Xu, Y., Chang, P., Liu, D., Narasimhan, M., Raghothama, K., Hasegawa, P. and Bressan, R. 1994. Plant defence genes are synergistically induced by ethylene and methyl jasmonates. Plant Cell, 6:1077-85.

Yalpani, N., Enyedi, Leon, J. and Raskin, I. 1994. Ultraviolet light and ozone stimulate accumulation of salicylic acid, pathogensis-related proteins and virus resistance in tobacco. Planta, 193:372-6.

Yannarelli, GG., Gallego, SM. and Tomaro, ML. 2005. Effect of UV-B radiation on the activity and isoforms of enzymes with peroxidase activity in sunflower cotyledons. Env. Environ. Exp. Bot., 56:174-81.