EXTRACTS OF THE BROWN SEAWEED, *ASCOPHYLLUM NODOSUM*, EFFECT
ARABIDOPSIS THALIANA – MYZUS PERSICAE INTERACTION

by

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ABSTRACT

An alkaline extract of the brown seaweed, *Ascophyllum nodosum* (ANE) increases plant growth and imparts resistance against biotic stresses. However, little is known of the effects of ANE on insects. *Myzus persicae,* green peach aphid (GPA), and Arabidopsis model were used to determine whether application of ANE confers protection from GPA infestation. GPA colonization increased in ANE treated plants, associated with improved biomass. However, ANE treated plants exhibited less cell death and also showed a greater ability to recover from GPA injury. Lower expression of *SAG13*, *SAG21* and *CHL1* and a higher expression of *ARR5* was observed in ANE treated plants. Taken together, gene expression along with lower cell death suggests ANE may delay senescence in Arabidopsis. Delayed senescence in Arabidopsis following ANE treatment may be a result of increased cytokinin activity. Increased GPA numbers could be, at least in part, due to delayed senescence in Arabidopsis following ANE treatment.

LIST OF ABBREVIATION AND SYMBOLS USED

α	alpha
ACT2	Actin 2
ANOVA	analysis of variance
ARR5	Arabidopsis response regulator 5
ANE	*Ascophyllum nodosum* extract
bp	base pair
^{o}C	degree Celsius
CLH1	Chlorophyllase 1
cDNA	complimentary deoxyribonucleic acid
DNA	deoxyribonucleic acid
df	degrees of freedom
g	centrifugation force
F	F-test statistic
GPA	green peach aphid
g/ L	grams per liter
h	hour
ι	iota
JA	jasmonic acid
kb	kilobase
λ	lambda
L	litre
LAR	local acquired resistance
μg	microgram
μL	microlitre
μM	micromolar
μm	micrometer
M	molar
Mg	milligram
min	minute(s)
ml	milliliter
ng	nanogram
LANS	Long Ashton standard nutrient solution
P	P value
PAD4	Phytoalexin deficient 4
%	percent
PR	pathogenesis-related proteins
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
SAG13	Senescence associated gene 13
SAG21	Senescence associated gene 21

SWC   seaweed extract concentrates
Taq   *Thermus aquaticus*

ACKNOWLEDGEMENTS

CHAPTER 1: INTRODUCTION

1.1 GENERAL INTRODUCTION

Ascophyllum nodosum (L.) Le Jol. is a dominant perennial brown seaweed found along the Atlantic coastlines that is used for production of commercial extracts (Ugarte and Sharp, 2001). *A. nodosum* extract (ANE) enhances plant growth, and increases tolerance/ resistance against various biotic and abiotic stresses (Craigie, 2010). It contains various bioactive polysaccharides such as laminarin, fucoidan, and alginates (Khan et al., 2009). Laminarins found in *A. nodosum* extracts (ANE) are considered to be potent elicitors of plant defense (Patier et al., 1993). Elicitors are chemicals that activate plant defense signaling pathways (Mejía-Teniente et al., 2010). *Laminaria digitata* (Hudson) J.V. Lamouroux, which also contains laminarin, activates salicylic acid (SA) and jasmonic acid (JA) defense pathways in tobacco (*Nicotiana tabacum*) (Klarzynski et al., 2000).

There are only a few published reports on the use of ANE as a plant protection agent against insects. Application of ANE for plants induced resistance against black bean aphid, *Aphid fabae* Scop. (Stephenson, 1966) and two-spotted red spider mite, *Tetranychus urticae* Koch. (Hankins and Hockey, 1990) were reported. However, the mechanisms underlying these effects are not well understood. One of the reasons is the lack of a good model system to study the interactions. Plant-insect model was used which consists of model plant *Arabidopsis thaliana* (L.) Heynh and green peach aphid (GPA), *Myzus persicae* (Sulzer), were used to investigate whether ANE enhances resistance or tolerance to insects. There are several reasons for using GPA as an insect model: it is an

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economically important insect pest with worldwide distribution (Blackman and Eastop, 2000) and it has rapid reproduction (Guerrieri and Digilio, 2008). Reasons for using Arabidopsis as a plant model are: small genome, (Meinke et al., 1998), availability of large genetic resources, a short life cycle and availability of numerous mutant strains (Somerville and Koornneef, 2002).

1.2 PROJECT HYPOTHESIS

A. nodosum extract (ANE) contains beneficial organic compounds that increase growth and yield, and improve resistance against biotic and abiotic stresses. It is hypothesized that organic compounds present in ANE will enhance vegetative growth and yield of *A. thaliana* and enhance resistance/ tolerance against green peach aphid (GPA), *M. persicae*. The objectives listed below were set to test these hypotheses.

1.3 OBJECTIVES

Major objectives of this study are:

➢ To examine the effect of *A. nodosum* extract on vegetative growth and yield of *A. thaliana*,

➢ To examine the effect of *A. nodosum* extract on resistance / tolerance in *A. thaliana* against *M. persicae*,

➢ To examine molecular changes in *A. thaliana* as affected by *A. nodosum* extract treatment under *M. persicae* infestation.

CHAPTER 2: LITERATURE REVIEW

2.1 *ASCOPHYLLUM NODOSUM* (L.) LE JOL.

Seaweeds are macroalgae found in shallow coastal waters, and are one of the prominent components of the marine environment. *Ascophyllum nodosum* is a dominant perennial brown seaweed found on intertidal shores of North America (Figure 2.1) and Europe, and it is commonly known as rockweed. It is one of the most predominant seaweed in Atlantic Canada. *A. nodosum* is widely used in the manufacturing of commercial seaweed products for use in agriculture (Ugarte and Sharp, 2001). Many marine algae contain industrially important polysaccharides including agar, carrageenans and alginic acids. Present research was conducted with alkaline *A. nodosum* extract (ANE) which contains different organic and inorganic components (Table 2.1.).

Figure 2.1 The brown seaweed, *Ascophyllum nodosum* in situ on a beach in the Annapolis Basin at Cornwallis, Nova Scotia.

Table 2.1 Composition of alkaline extract of ANE, a commercial product of Acadian Seaplants Limited (Dartmouth, Canada) derived from *Ascophyllum nodosum* (pH ~ 10.0).

Composition	Percentages from total dry powder of ANE
Organic matter content	**45-55 %**
Alginic acid	12-16 %
Fucose Polymers	13-17 %
Mannitol	4-6 %
Amino Acids	4-6 %
Other inorganic compounds	**10-12 %**
Ash	**45-55 %**
Nitrogen	0.8-1.5 %
Phosphorus	0.5-1.0 %
Potassium	14-18 %
Calcium	0.3-0.6 %
Iron	75-250 ppm
Magnesium	0.2-0.5 %
Manganese	8-12 ppm
Sodium	3.0-5.0 %
Sulfur	1.0-2.0 %
Zinc	10-25 ppm

There are many beneficial effects of ANE (Khan et al., 2009). Previous studies on different seaweed extracts showed that plants that received ANE had increased shoot growth and development (Pise and Sabale, 2010; Crouch, 1990), increased yield (Dobromilska et al., 2008; Temple and Bomke, 1989), increased carbohydrates, proteins, free amino acids and polyphenol, (Pise and Sabale, 2010), increased nutrient uptake

(Beckett and Staden, 1990), and improved resistance to biotic (Crouch and Staden, 1993) and abiotic stresses such as salinity and water stresses (Nabati et al. 1994., Nabati, 1991). Further, ANE has shown to elicit cytokinin like activity (Khan et al., 2011). In turf grass application of seaweed extracts delayed senescence and improved turf quality (Schmidt, 1990). ANE increased chlorophyll levels in tomatoes, beans, wheat, barley and maize. This increase in chlorophyll might be due to betines present in the seaweed extracts. Further, betaines can act as osmoprotectants by increasing osmotic pressure in cytoplasm and also stabilizing cell membrane (McNeil et al., 1999).

Elicitors are group of chemicals capable of inducing plant defense. Some elicitors present in seaweed extracts are complex polysaccharides such as alginates, fucans, laminarins and carrageenans (Vera et al., 2011). For example, a component of red seaweeds, λ-carrageenan was found to be a potential elicitor of defense response in tobacco against the black shank pathogen, *Phytophthora parasitica* (Mercier et al., 2001). Further investigation revealed that λ-carrageenan induced ethylene, jasmonic and salicylic acid defense signaling pathways in tabacco. In a recent study, Sangha et al. (2011) examined the effect of different carrageenan on Arabidopsis resistance against cabbage looper, *Trichoplusia ni*. The ɩ- carrageenan showed higher expression of plant defensin 1.2 (*PDF1.2*), pathogenesis-related gene 1 (*PR1*) and trypsin inhibitor protein 1 (*TI1*). ANE contains various bioactive polysaccharides such as laminarin, fucoidan, and alginates (Khan et al., 2009). Laminarins which are present in ANE, showed an elicitor activity of D-glycanase activities in *Rubus fruticosus* cell cultures (Patier et al., 1993). In a recent study, ANE treatment induced resistance of Arabidopsis to *Pseudomonas syringae* pv. tomato DC3000 (Subramanian et al., 2011). It was found that ANE activated

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plant defensin 1.2 (*PDF1.2*) which was activated mostly in jasmonic acid signaling pathway.

There are very few scientific reports on the effects of ANE on insects. Commercial products of ANE applied as leaf spray to strawberry plants, reduced the population of two-spotted red spider mites, *Tetranychus urticae* (Hankins and Hockey, 1990). ANE treatment induced resistance in broad beans against black bean aphid (*Aphid fabae*) (Stephenson, 1966). When ANE was applied to tomato plants, *Spodoptera exigua* larvae gained more body mass. Further, *Spodoptera exigua* larvae preferred root applied ANE treated plants than foliar applied plants (Reitz and Trumble, 1996).

2.2 *Myzus persicae* (Sulzer)

Green peach aphid (GPA), *Myzus persicae* belongs to the superfamily Aphidoidea, order Hemiptera. Aphids can feed on most plant parts including shoots, roots as well as bark (Guerrieri and Digilio, 2008). GPA is found worldwide and in most vegetable producing areas of Canada, especially in British Columbia and Southern Ontario, where they are an important pests to potatoes (Howard et al., 1994). GPA is a major pest known to attack over 400 plant species and 40 plant families (Blackman and Eastop, 2000). The Cruciferae are one of the most susceptible hosts of GPA. Direct damage to the plant by feeding can be significant, but perhaps the major threat of GPA infestation is transmission of plant viruses (Raccah and Fereres, 2009).

GPA is a useful insect model to examine plant insect interactions (De Vos et al., 2007; Pegadaraju et al., 2007; Pegadaraju et al., 2005). There are several reasons to use GPA as an insect model. It is an economically important insect pest which is distributed worldwide (Blackman and Eastop, 2000). In addition, it is easily cultured and reproduces

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quickly through asexual reproduction which makes the test population more homogeneous (Guerrieri and Digilio, 2008). A number of studies have also examined gene regulation in GPA, and the function of many GPA genes is known (De Vos et al., 2010; Ramsey et al., 2007).

2.2.1 LIFE HISTORY

Apterous (wingless) adults vary from 1.7 to 2.0 mm in length and are yellow to green in color (Figure 2.2 A). Alate (winged) adults tend to be darker in color (Figure 2.2 B). GPA nymphs are initially about 1 mm in length; they initially are green and then turn yellow (Figure 2.2 C). Nymphs can be produced sexually, but are often a result of parthenogenetic reproduction (without mating). Because color of GPA can vary, *M. persicae* is best distinguished from other aphids by the presence of convergent tubercles on the head and unevenly swollen moderately long cornicles on the abdomen (Blackman and Eastop, 2000). Winged forms usually appear when environmental conditions are unfavourable, and these forms mate and lay eggs, often during the late summer. However, winged forms may appear due to overcrowding on the host plant, and this enables dispersal to a larger area in search of host plants (Guerrieri and Digilio, 2008). Winged forms known as alate aphids have a black central dorsal patch on the abdomen (Figure 2.2 B) and the length varies to 1.2- 2.3 mm (Blackman and Eastop, 2000). Alate forms use visual sensors (Döring and Chittka, 2007), and also chemicals present in the plants (Pickett et al., 1992) to find the host plants.

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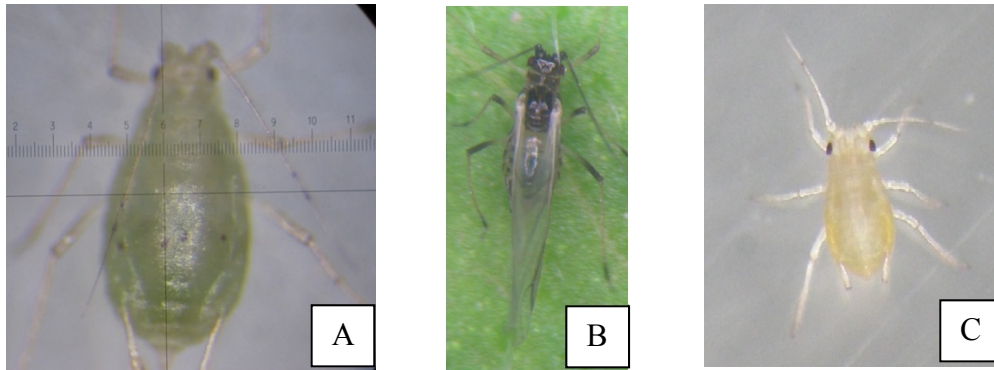
Figure 2.2 *Myzus persicae* apterous adult (A), alate adult (B), and nymph (C)

2.2.2 DAMAGE TO HOST PLANTS

Myzus persicae feeds on plant assimilates and is also capable being a vector for viruses (Kennedy et al., 1962). GPA is a phloem feeder that prefers young plant tissues. Feeding injury causes water stress, wilting and chlorosis (Deol et al., 2001), which can reduce growth and development of host plants (Petitt and Smilowitz, 1982). Aphids are also an important vectors of plant viruses (Pirone and Harris, 1977). Plant viruses spread by GPA in crucifers include: cucumber mosaic virus, turnip mosaic virus, and cauliflower mosaic virus (Cabrera y Poch et al., 1998). Examples of viruses which are spread by aphids in other crop families include: potato leaf roll virus (Ponsen, 1970), beet western yellow virus (Duffus and Gold, 1965), and lettuce mosaic virus (Moreno et al., 2007).

2.3 *ARABIDOPSIS THALIANA*

Arabidopsis thaliana, a small cruciferous weed, is a useful plant model for genetic and molecular studies. *A. thaliana* has a small genome (120 Mb) organized into five chromosomes (Meinke et al., 1998) and an estimated 27,416 protein-encoding genes (www.arabidopsis.org). The whole genome of Arabidopsis was sequenced in the year 2000 (The Arabidopsis Initiative, 2000). *A. thaliana* has many genetic and genomic

resources: it can easily be managed in small spaces, it has a relatively fast life cycle (around 8 weeks), it is easy to reproduce through seeds, and there is a large collection of mutants and transgenic plants that can be studied (Somerville and Koornneef, 2002). Arabidopsis growth can be separated into several stages such as seed germination, leaf development, rosette growth (Figure 2.2 A, B, C), inflorescence emergence (Figure 2.2 D), flower production (Figure 2.2 E), senescence completion (Figure 2.2 F), silique (Figure 2.2 G) and seed (Figure 2.2 H) production (Boyes et al., 2001). Many studies have used the Arabidopsis-green peach aphid (GPA) model to investigate plant-insect interactions at the molecular level (Louis et al., 2010; De Vos et al., 2007; Hunt et al., 2006; De Vos et al., 2005). Several accessions of *A. thaliana* were used to examine resistance to cabbage aphid in the field as well as laboratory conditions (Singh et al., 1994).
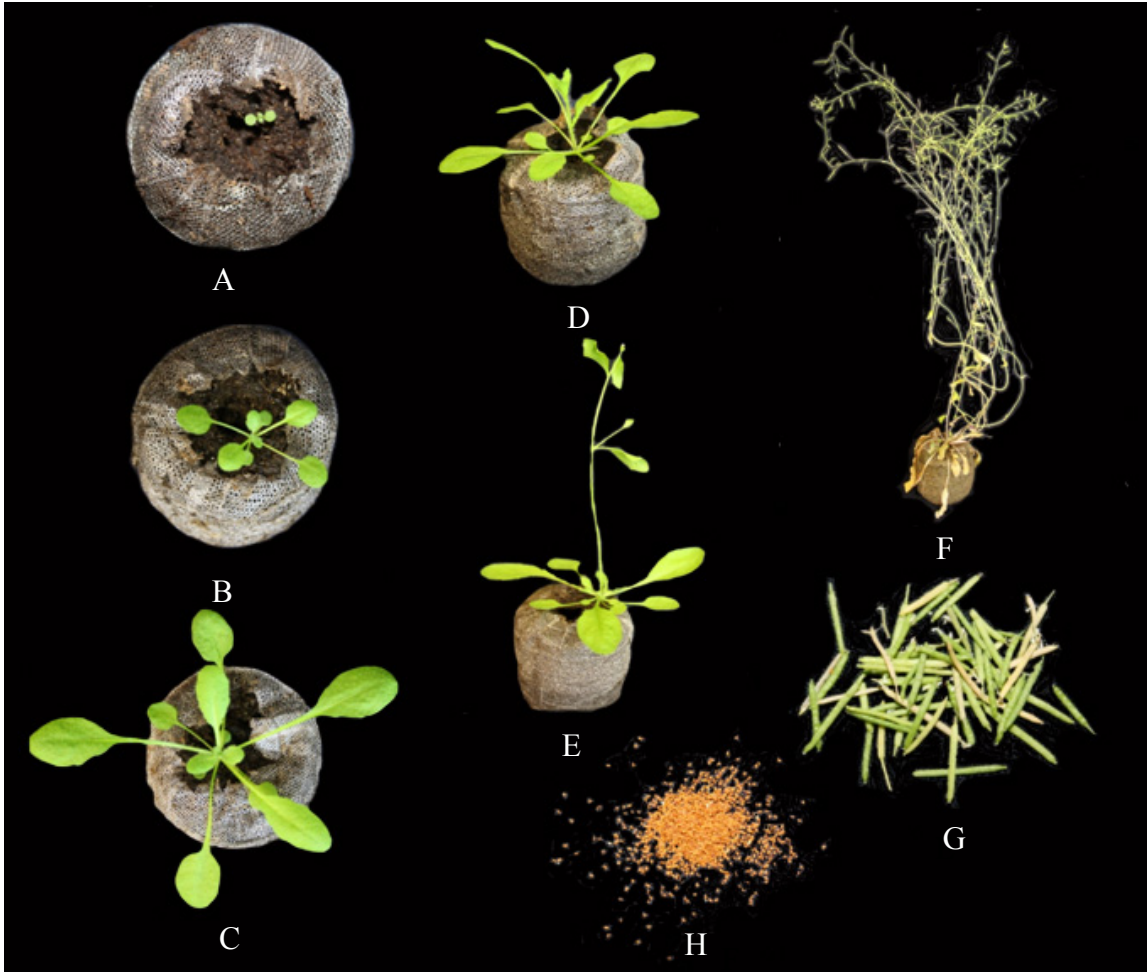
Figure 2.2 Different growth stages in *Arabidopsis thaliana*: (A) Arabidopsis at 4 day, (B) 13 day, (C) 21 days after germination, (D) first emergence of the peduncle, (E) first flower opens, (F) senescence completed, (G) siliques, (H) seeds.

2.4 RESISTANCE MECHANISMS

Plants have different resistance mechanisms to defend themselves against aphids. These include antibiosis, antixenosis and tolerance (Painter, 1951). Antibiosis affects the life cycle or biology of the insect by reducing reproductive capacity of aphids (Panda and Khush, 1995), whereas antixenotic factors affect feeding preference of aphids (Kogan and Ortmann, 1978). These defense mechanisms can occur during various stages of aphid feeding. For example, volatiles and non-volatiles produced in glandular trichomes can

affect aphid behavior when they are probing a plant tissue and searching for sieve

elements (Neal et al., 1990). In Brassica species, it was found that the release of

thiocyanates is toxic to aphids (Rask et al., 2000). Sealing of xylem vessels and callose

deposition can affect aphid probing (Will and Bel, 2006). An antixenotic effect was

tested using polygodial, a plant derived sesquiterpenoid. Aphids quickly detected the

compound and it was found that aphid antenna tip was involved in the repellent activity

(Dawson et al., 1986). In another study, Arabidopsis was used to test repellent activity of

cis-jasmone, and the green peach aphid (GPA) was repelled, whereas the mustard aphid,

Lipaphis erysimi, was attracted (Bruce et al., 2008).

Plant tolerance is a mechanism by which plants grow or recover faster following

an insect infestation. It can be defined as the rapid accumulation of plant biomass in spite

of an insect herbivory (John and Schwenke, 1994). Plant tolerance is an important factor

in the management of insect pests (John and Schwenke, 1994). Several incidences of

plant tolerance to arthropods such as an increased level of photosynthesis (Deol et al.,

2001), increased growth (Brandt and Lamb, 1994) and increased yield (Jarvis et al.,

1991) have been reported. John and Schewenke (1994) reviewed the advantages and

disadvantages of plant tolerance. Plant tolerance does not affect insect biology, and there

has no detrimental effect on natural enemies because tolerance could maintain a

population of predators and parasitoids. Further, tolerance can increase the economic

injury levels (EIL), and it can reduce expensive chemical treatments. There are several

ways of quantifying tolerance. Visual ranking was used for assessing insect damage

(Heng-Moss et al., 2002). Dixon et al. (1990) proposed a tolerance index (TI) by dividing

the proportional weight loss of the plant by total number of insects at the end of the

experiment. Plant recovery from insect herbivory is another expression of having plant tolerance. Insect herbivory can reduce photosynthetic capacity of leaves, and it can affect the production of new leaves, storage organs and reproductive parts of plant (Meyer, 1998). There are several ways of recovering carbon gain capacity after insect herbivory. Delayed leaf senescence was described as one way of making the remaining leaves resistant after defoliation (Meyer, 1998).

2.5 PLANT RESPONSE TO INSECT HERBIVORY

Plants can incur damage from either chewing insects such as caterpillars or sucking insects such as aphids. Plant defense responses to insects can be classified into two categories: constitutive and inducible defense. Constitutive defenses are always present in the plant whereas inducible defenses are only produced after herbivore attack. There are various constitutive defenses in plants such as leaf cuticle (Zettler et al., 1969), thick cell walls, glandular trichomes (Musetti and Neal., 1997), epicuticular waxes (Alfaro-Tapia et al., 2007), and insecticidal allelochemicals (e.g. glucosinolates) (Kim et al., 2008). Metabolites present in glandular hairs produce chemicals (e.g. (E) - farnesene) (Gibson and Pickett, 1983) similar to alarm pheromones in aphids. This may affect the biology of insects (Walling, 2000) and play a role as a chemical constitutive defense response (Guerrieri and Digilio, 2008).

Inducible responses are elicited after infestation. Directly induced defense responses include digestive enzyme inhibiters such as protease inhibitors (Chen et al., 2005; Ussuf et al., 2001; Ryan, 2000) or reduced nutritional value (Hanhimäki, 1989). Moreover, plants synthesize insecticidal molecules such as phenolics, alkaloids, terpenoids (Karban and Baldwin, 1997) and glucosinolates. Glucosinolates are secondary

metabolites that are deterrents to generalist herbivores, whereas they act as an attractant to specialized herbivores (Rask et al., 2000). Glucosinolates themselves do not have insecticidal activity, but when they react with myrosinases, they produce the insecticidal compounds: nitriles, isothiocyanates, epithionitriles and thiocyanates (Rask et al., 2000). The stealthy feeding behavior of aphids minimizes the reaction of glucosinolate with myrosinase. However, indole glucosinolate breakdown products contribute to reduced reproduction in GPA on *A. thaliana* plants. Furthermore glucosinolate breakdown products have a deterrent effect on GPA (Kim et al., 2008). Indirect induced defenses include attracting natural enemies (Walling, 2008) such as predators and parasitoids (Van Poecke, 2007). Volicitin or N-(17-hydroxylinolenoyl)-L-glutamine, a chemical found in oral secretions of beet armyworms, applied to plants, it induces the production of volatiles that subsequently attracts natural enemies of beet armyworm (Alborn et al., 2000).

2.6 PLANT MOLECULAR RESPONSES TO APHID FEEDING

Plant defense responses to insects activate salicylic acid, jasmonic acid, and ethylene dependent defense pathways (Thompson and Goggin, 2006). The jasmonic acid signaling pathway is activated by wounding caused by chewing insects (Onkokesung et al., 2010), whereas salicylic acid signaling pathway is activated by sucking insects (Du et al., 2009; Zarate et al., 2007). However, there are "cross-talks" between defense signaling pathways (Koornneef and Pieterse, 2008; Pieterse and Dicke, 2007).

Salicylic acid (SA) is an important signaling molecule in induced plant resistance to pathogens. It plays a major role in LAR (local acquired resistance) (Wildermuth et al., 2001) as well as SAR (systemic acquired resistance) to pathogens (Ryals et al., 1996).

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SAR is a type of resistance which follows an earlier infection by a pathogen that can lead to systemic immunity to various pathogens in plants. It involves a burst of reactive oxygen species, cell death, endogenous hormonal activation and expression of defense genes (Choi and Hwang, 2011). LAR is a type of resistance acquired in the infected cells and nearby cells (Métraux, 2002). Accumulation of SA in SAR and LAR is important for the expression of pathogenesis-related proteins (PR) which suppress pathogen invasion. H_2O_2 also plays a role as a signaling molecule in PR protein expression (Chamnongpol et al., 1998). Structural and biochemical changes in the cell lead to activation of defense related genes (Kuc, 2006). Phenylalanine ammonia lyase (*PAL*) is one of the genes that activate after changes in cells (Klessig et al., 2000). *PAL* is involved in the biosynthesis of phytoalexins which are antimicrobial compounds (Klessig et al., 2000). A hypersensitive response (HR) includes rapid production of reactive oxygen species and eventually causes cell death (Mur et al., 2008).

PAD4 (phytoalexin deficient4) gene, which is involved in camelexin synthesis in SA signaling, is associated with plant resistance to pathogens (Glazebrook et al., 1997). It has a similar sequence to that of lipases (Feys et al., 2001) and encodes a nucleo-cytoplasmic protein. Moreover, the *PAD4* gene is expressed in GPA infested plants (Pegadaraju et al., 2007), and it can modulate GPA induced leaf senescence. Arabidopsis leaf senescence is characterized by chlorophyll loss, cell death and higher expression of senescence associated genes (*SAG*s). With GPA infestation, *PAD4* mutant plants exhibited delayed senescence compared to wild type Col-0 plants. Activation of leaf senescence is a defense response against GPA, whereas delayed senescence lowers plant's resistance to GPA (Pegadaraju et al., 2005). In addition, the Arabidopsis *PAD4*

mutant showed increased numbers of GPA compared to wild type plants (Pegadaraju et al., 2005). An electronic monitoring technique (EPG) showed that GPA prefers to feed longer on *PAD4* mutant plants compared to wild type plants. In choice experiments, GPA showed increased preference to *PAD4* mutants over wild type plants (Pegadaraju et al., 2007). Constitutive expresser of PR genes5 (*cpr5*) and suppressor of SA insensitivity2 (*ssi2*) that are considered as Arabidopsis hypersenescence mutant plants exhibits higher chlorophyll, higher cell death and higher expression of SAG genes showed low GPA reproduction as compared to wild-type (Col) plants (Pegadaraju et al., 2005).

2.7 CYTOKININS

Plant hormones are naturally occurring organic substances which affect plant physiological processes such as growth, differentiation and development at low concentrations (Davies, 2010). Plant hormones include auxins, abscisic acid, cytokinins, ethylene, gibberellins, brassinosteroids, jasmonic acid and salicylic acid (Santner et al., 2009). Cytokinins are one of the abundant plant growth regulators present in seaweeds (Senn, 1987). Naturally occurring cytokinins are adenine derivatives which have either isoprene-derived or an aromatic side chain at the *N6* terminus (Sakakibara, 2006). Some examples of synthetic and natural cytokinins are: diphenylurea, zeatin, kinetin (6-furfurylaminopurine). *Trans*-zeatin (Figure 2.4 C) is the most abundant naturally occurring cytokinin in Arabidopsis. Kinetin (Figure 2.4 A) and benzyladenines (Figure 2.4 B) are other examples of synthetic cytokinins.
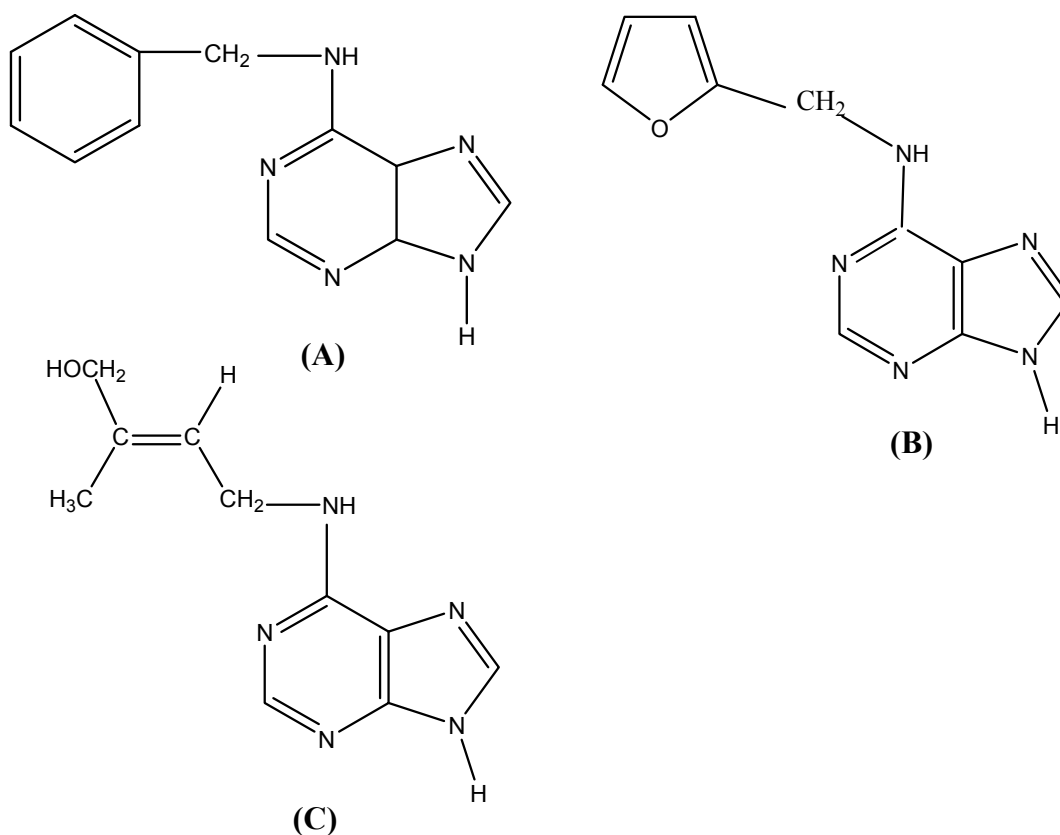
Figure 2.3 Chemical structures of selected cytokinins: (A) kinetin (6-furfurylaminopurine), (B) 6-benzylamino purine (BA), and (C) trans-zeatin.

Cytokinins play a major role in several physiological processes in plants, such as growth and development of cells, shoot initiation (Frett and McCardell, 1990), retarding senescence (Mok and Mok, 1994), increasing chlorophyll content, inhibiting carbohydrate translocation, mobilizing metabolites (Towne and Clenton, 1983) and increasing crop productivity (Ashikari et al., 2005). Root inhibition was observed *in vitro* with a high cytokinin to auxin ratio (Peres et al., 1999). *IPT*, a gene encoding isopentenyl transferase enzyme that catalyzes cytokinin biosynthesis was studied using senescence specific promoter P_{SAG12}-*IPT* in transgenic tobacco plants. It was found that endogenous

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cytokinins can regulate the leaf senescence (Gan and Amasino, 1996). These transgenic tobacco plants showed increased number of flowers, biomass and yield compared to wild type plants (Gan and Amasino, 1995). An increased concentration of cytokinin increases net CO_2 fixation in plants, due to increase assimilate transport and nutrient uptake (Satoh et al., 1977).

2.8 LEAF SENESCENCE

Leaf senescence is a complex process which involves changes in physiology, biochemistry and gene expression in the leaf cells (Lim et al., 2003). Leaf senescence in Arabidopsis depends on endogenous factors such as aging and hormones, but also on environmental factors such as stress and nutrient supply. Moreover, leaf senescence can be explained as a process which occurs in last stage of their development which leads to cell death. When leaf senescence begins, nitrogen, carbon and minerals will flow from mature to immature parts of the plants. Several events are involved in leaf senescence such as the degradation of chloroplasts, leaf proteins and chlorophylls (Buchanan-Wollaston, 1997). There are several genes involved in the senescence process which are referred to as senescence associated genes (*SAG*s) (Lim et al., 2003). *SAG13* and *SAG21* gene expression are induced by ozone treatment, and it was suggested that they are not associated with age dependent senescence, whereas *SAG12* was expressed during aging in *A. thaliana* leaves (Pegadaraju et al., 2005).

There are several plant hormones which regulate senescence. Ethylene, methyl jasmonate, brassinosteroids and salicylic acid were identified as plant hormones that accelerate senescence (Lim et al., 2003). Cytokinins were identified as plant hormones which can delay the leaf senescence (Gan and Amasino, 1995). Isolated cytokinin,

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kinetin (6-furfurylaminopurine) showed delayed senescence in excised wheat leaves (Mik et al., 2011). However, transgenic tobacco plants with delayed senescence produced more flowers (Gan and Amasino, 1995), increased chlorophyll, and there were fewer dead leaves compared to control plants (Ori et al., 1999). Furthermore, delaying senescence in plants resulted in better recovery after herbivory (Meyer, 1998).

2.9 LITERATURE REVIEW SUMMARY

This literature review focuses on effects of *A. nodosum extract* (ANE) on *M. persicae* (GPA) - *A. thaliana* interaction. The section outlines information on ANE, GPA and Arabidopsis, in order to build a platform to better understand the interaction. ANE has many beneficial effects that include enhanced growth and resistance/ tolerance against biotic and abiotic stresses. Plant resistance and tolerance can be achieved through elicitation of plant defense responses. However, there is little information on ANE effects on insects. To understand how plants cope with aphid attack, I discussed three major resistance mechanisms that plants use to defend themselves against herbivorous insects. Theses mechanisms include antibiosis, antixenosis and tolerance. The mechanisms were then discussed in terms of influence on the plant in response to insect herbivory.

There are many reports on the promotion of plant growth following ANE treatment. Enhanced growth following ANE treatment may be due to activity of plant growth regulators. Among plant growth regulators, cytokinins play a major role in plant growth and development. Cytokinin activity increased following ANE treatment. Therefore cytokinins and their effect on plant growth and development may play an important role in this plant-insect interaction. The overall aim in this study was to understand more clearly the interaction of ANE on the GPA-Arabidopsis model.

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CHAPTER 3: EFFECT OF COMMERCIAL EXTRACT OF *ASCOPHYLLUM NODOSUM* ON GROWTH AND SEED YIELD OF *ARABIDOPSIS THALIANA*

3.1 ABSTRACT

Seaweeds and their extracts have been used as an organic soil amendment for centuries. *Ascophyllum nodosum* is brown macroalga found on northern Atlantic coasts and most commonly used in agriculture. As a model plant, *Arabidopsis thaliana* was used in this study in order to achieve rapid and systematic phenotypic analyses. *A. nodosum* extract (ANE) at 1.0 g/L was applied to Arabidopsis as leaf spray and soil drench. Control containing the same inorganic nutrient components in ANE was used. Leaf area was measured at 32nd day after planting. ANE applied as soil drench did not have an effect on dry weight, length of inflorescence and seed yield. However, there was a significant increase in dry weight, length of inflorescence and seed yield when ANE was applied as foliar treatment.

3.2 INTRODUCTION

Organic fertilizers can deposit organic matter and improve soil conditions for beneficial organisms relative to chemical fertilizers (Celik et al., 2004). In addition, they are non-toxic and cause less harm to the environment. To meet the increasing demand of organic fertilizers, several options have been explored. The application of seaweed extracts has been recognized as one of the better options. The use of seaweed extracts as organic fertilizers have many beneficial effects including enhanced yield (Dobromilska et al., 2008; Crouch, 1990) and their quality (Zodape, 2009), a delay of fruit senescence and an induced tolerance to biotic (Featonby-Smith and Van Staden, 1983) and abiotic stress conditions (Yan, 1993).

Ascophyllum nodosum (L.) Le Jol. is a brown macroalga found on intertidal shores of North America and Europe (Ugarte and Sharp, 2001). Numerous beneficial effects have been reported with *Ascophyllum* seaweed extracts (Khan et al., 2009). Extracts of marine algae contain secondary metabolites such as agar, carrageenan and alginic acid. Among organic components present in *A. nodosum* extracts (ANE), plant growth regulators have been studied to a large extent. Cytokinin is one of the most abundant plant growth regulators found in seaweeds (Senn, 1987) and plays a major role in several physiological processes in plants such as growth and development of cells, shoot initiation (Frett and McCardell, 1990), delayed senescence (Mok and Mok, 1994), increased photosynthesis, inhibition of carbohydrate translocation, mobilization of metabolites (Towne and Clenton, 1983) and increased crop production (Ashikari et al., 2005). Extracts from brown seaweed treated plants showed cytokinin-like activities (Khan et al., 2011). When seaweeds were applied to turfgrasses, it improved the turf

quality while delaying the senescence process in the treated grasses (Schmidt, 1990). Increased cytokinin levels in transgenic tobacco plants resulted in plants with increased biomass, flowers and seed yield compared to wild type plants (Gan and Amasino, 1995). Other than cytokinin, ethylene is major plant hormone involved in fruit ripening (Chaves and Mello-Farias, 2006). 1-aminocyclopropane-lcarboxylic acid (ACC) which is a precursor of the ethylene biosynthesis pathway was found in brown seaweed extracts (Nelson and Van Staden, 1985). Organic compounds present in seaweed extracts (SWE) might behave as a chelating agent for nutrients (Lynn, 1972). An extract of the red alga, *Kappaphycus alvarezii* increased nutrient uptake (N, P, K and S) in soybean plants (Rathore et al., 2009).

Arabidopsis was used in the present investigation in order to achieve rapid quantification of ANE effects. Since Arabidopsis is a model plant used in genetic studies, phenotypic analyses from this study will help to build a platform to interpret gene expression studies. Specifically, this study aimed to investigate the effects of leaf spray and soil drench applications on vegetative growth and yield of Arabidopsis.

3.3 MATERIALS AND METHODS

3.3.1 PLANT MATERIAL AND GROWTH CONDITIONS

Arabidopsis seeds (Lehle Seeds Company, Texas, USA) were seeded in peat pellets (Jiffy Co., Shippegan, New Brunswick, Canada) and grown under florescent bulbs at 16:8 h (day : night) cycle (100 μmol photons $m^{-2} s^{-1}$) in a growth room at 23 ± 2^0C. After germination, plants were thinned as one plant per peat pellet. Thirteen day-old plants were used in the initial treatments. Plants were watered regularly and fertilized using a standard fertilizer solution (20-20-20).

3.3.2 PREPARATION AND APPLICATION OF ANE AND INORGANIC CONTROL

An aqueous solution of ANE extract (Acadian Seaplants Limited, Dartmouth, NS, Canada) was prepared by dissolving the desired amount of ANE dry extract powder in distilled water by constant stirring by hand for 15 minutes. Modified Long Ashton solution (LANS) was used as a control, containing a similar inorganic composition as 1 g/L ANE dissolved in water (Appendix A). Two experiments were conducted using two application methods of ANE; leaf spray and soil drench. Freshly prepared ANE or control was applied as either a soil drench (10.0 ml applied to each peat pellet) or leaf spray (~ 500 μL) per plant. Plants were treated every three days starting at 13 days post germination. Beyond 22 days, plants were treated weekly. Ten plants were harvested on each of 25, 32 and 39 days after post-germination.

3.3.3 MEASUREMENTS OF GROWTH PARAMETERS

During harvest, Arabidopsis were cut at the bottom of the stem at the soil line. Plants were blotted very gently with paper towel to remove free moisture, and fresh weight per plant was measured. Length of inflorescence was measured from the base to

the terminal flower of the main bolt of Arabidopsis. Plants were later dried in an oven at 60^0C for three days, after which time dry weight was measured. At 35 day, before taking the dry weight of each plant, all leaves were separated and scanned with a scanner (Epson Expression 1000 XL). The digitalized image of each leaf was analyzed using image processing software (WinFOLIA 2008a, Regent Instruments Inc., Quebec, Canada) to measure total leaf area of plants at day 32 post-germination. Siliques from each plant were harvested on day 39. Siliques were oven dried at 60^0C for three days, and total dry weight of siliques on each plant was determined.

3.3.4 STATISTICAL ANALYSIS

As mentioned in section 3.3.2, two different experiments were conducted. Each experiment consisted of 30 plants arranged in a two factor factorial design with 10 replicates for each treatment at each time point. The two factors analyzed were treatments and harvest time. Treatments were considered as a fixed factor and time as a random factor. There are two levels for treatment (control and 1 g/L ANE) and three levels for harvest time (day 25, 32 and 39). The effects of these factors on fresh weight, dry weight and length of peduncle were analyzed using Minitab 15.0 (Minitab Inc. Pennsylvania, USA) and PROC MIXED in SAS version 9.2, Cary, NC, USA). Tukey's test was performed when treatment*time interactions were significantly different ($\alpha = 0.05$).

Leaf area of Arabidopsis was measured in day 32. Two experiments were conducted as mention in section 3.3.2. Each experiment was consisted of 20 plants in a completely randomized design with 10 replicates for each treatment. Data were analyzed using ANOVA ($\alpha = 0.05$) in Minitab 15.

The experiment on seed yield of Arabidopsis was conducted as completely randomized block design. Two runs of experiments were conducted (July-August 2011 and January-February 2012) each constituting a block. Data were analyzed using ANOVA ($\alpha = 0.05$) in Minitab 15.

3.4 RESULTS

3.4.1 EFFECT OF ANE ON FRESH WEIGHT OF ARABIDOPSIS

There was no difference between control and ANE treatment on fresh weight of Arabidopsis for leaf spray (Table 3.1) and soil drench application (Table 3.2) of ANE. There was a significant effect of harvest time on fresh weight for both leaf spray (Table 3.1) and soil drench application methods (Table 3.2) of ANE. Plants were heavier at late harvest dates. There was no interaction between treatments*harvesting time on fresh weight of Arabidopsis for leaf spray (Table 3.1) and soil drench application (Table 3.2) of ANE.

Table 3.1 ANOVA results for an analysis of the effects of leaf sprays of *Ascophyllum nodosum* extract (ANE) and harvest time on fresh weight of Arabidopsis.

Effect	df	F value	Pr> F
Treatment	1	1.72	0.19
Harvest time	2	55.13	<0.01*
Harvest time x treatment	2	0.02	0.98

* Significant at $\alpha = 0.05$

Table 3.2 ANOVA results for an analysis of the effects of soil drench of *Ascophyllum nodosum* extract (ANE) and harvest time on fresh weight of Arabidopsis.

Effect	df	F value	Pr> F
Treatment	1	0.08	0.78
Harvest time	2	134.17	<0.01*
Harvest time x treatment	2	0.15	0.86

* Significant at $\alpha = 0.05$

3.4.2 EFFECT OF ANE ON DRY WEIGHT OF ARABIDOPSIS

Leaf treatment of ANE significantly increased dry weight of Arabidopsis (Figure 3.1, Table 3.3), and there was a significant interaction between ANE leaf treatment and harvest time. Moreover, leaf spray application of ANE had significant effects on length of inflorescence of Arabidopsis on day 39 post-germination ($P<0.05$).

Soil application of ANE did not have a significant effect on the dry weight of Arabidopsis, and there was no significant interaction between ANE soil drench treatment and harvesting time (Table 3.4). However, dry weight in both ANE treated and control plants significantly increased over time (Table 3.4).

Table 3.3 ANOVA results for an analysis of the effects of leaf sprays of *Ascophyllum nodosum* extract (ANE) and harvest time on dry weight of Arabidopsis.

Effect	df	F value	Pr> F
Treatment	1	7.59	0.01*
Harvest time	2	139.71	<0.01*
Harvest time x treatment	2	6.91	<0.01*

* Significant at $\alpha = 0.05$

Table 3.4 ANOVA results for an analysis of the effects of soil drench application of *Ascophyllum nodosum* extract (ANE) and harvest time on dry weight of Arabidopsis.

Effect	df	F value	Pr> F
Treatment	1	0.95	0.33
Harvest time	2	152.49	<0.01*
Harvest time x treatment	2	0.96	0.39

* Significant at $\alpha = 0.05$
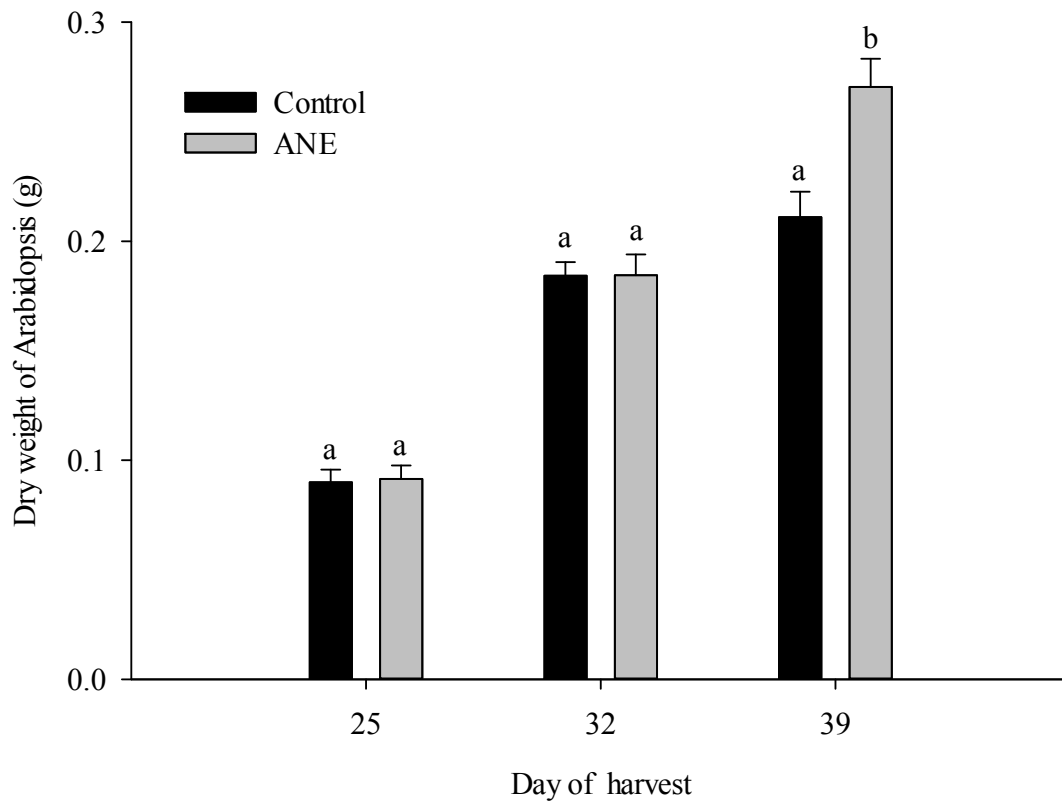
Figure 3.1 Effect of leaf sprays of *Ascophyllum nodosum* (ANE) on dry weight (g) (± SE) of Arabidopsis. Different letters above bars indicate treatments are significantly different on a given day (Tukey's test, $\alpha = 0.05$).

3.4.3 EFFECT OF ANE ON LENGTH OF INFLORESCENCE OF ARABIDOPSIS

Leaf treatment of ANE significantly increased the length of inflorescence of Arabidopsis (Figure 3.2, Table 3.5), and there was a significant interaction of ANE leaf treatment with harvest time, as the treatment effect was only seen in plants harvested on day 39 (*P<0.05*).

Soil drench of ANE did not have significant effect on the length of inflorescence of Arabidopsis and there was no significant interaction of ANE soil drench treatment with harvest time (Table 3.6). However, dry weight in both ANE treated and control plants were significantly increased over time (Table 3.6).

Table 3.5 ANOVA results for an analysis of the effects of leaf sprays of *Ascophyllum nodosum* extract (ANE) and harvest time on length of inflorescence of Arabidopsis.

Effect	df	F value	Pr> F
Treatment	1	8.61	0.01*
Harvest time	2	138.37	<0.01*
Harvest time x treatment	2	3.50	0.04*

* Significant at α = 0.05

Table 3.6 ANOVA results for an analysis of the effects of soil drench of *Ascophyllum nodosum* extract (ANE) and harvest time on length of inflorescence of Arabidopsis.

Effect	df	F value	Pr> F
Treatment	1	0.05	0.83
Harvest time	2	234.97	<0.01*
Harvest time x treatment	2	0.10	0.91

* Significant at α = 0.05
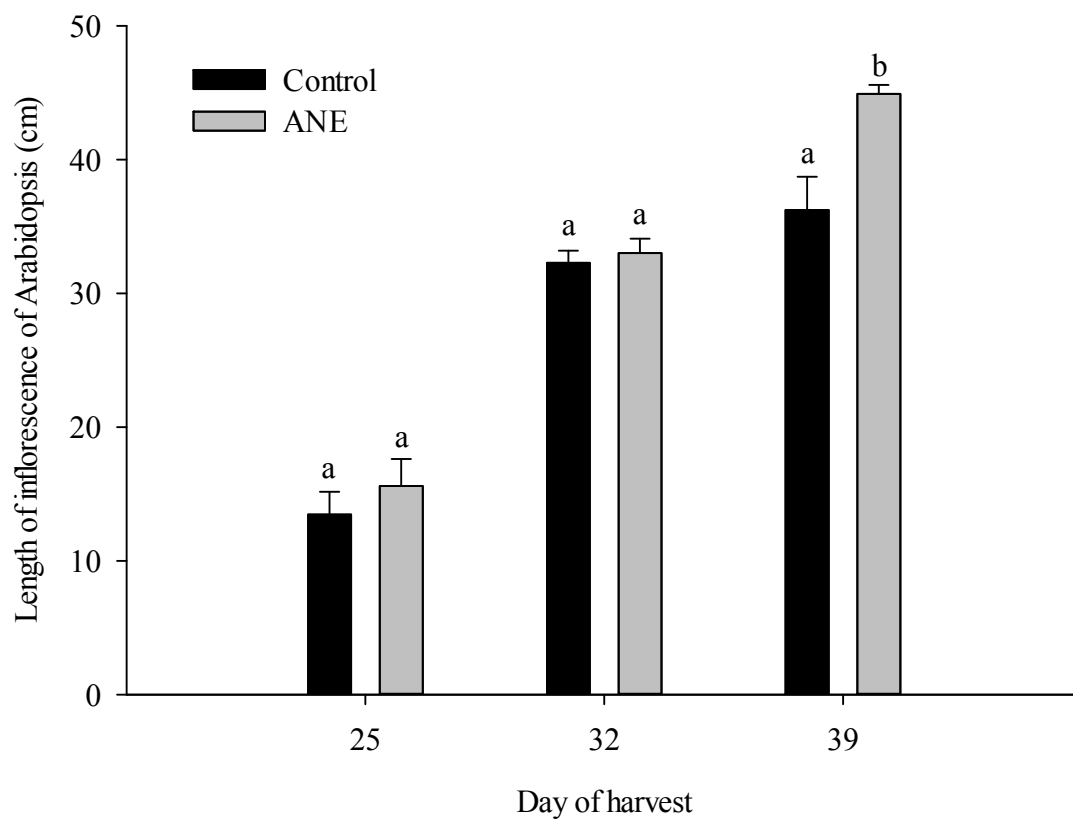
Figure 3.2 Effect of foliar application of *Ascophyllum nodosum* (ANE) on length of inflorescence (cm) (± SE) of Arabidopsis. Different letters above bars indicate treatments are significantly different on a given day (Tukey's test, α = 0.05).

3.4.4 EFFECT OF ANE ON LEAF AREA OF ARABIDOPSIS

There was no significant ANE effect on leaf area of Arabidopsis on day 32 post-germination by either leaf spray ($F = 0.13$; $df = 1, 18$; $P = 0.13$; Figure 3.3), or soil drench ($F = 0.12$; $df = 1, 18$; $P = 0.73$; Figure 3.3).
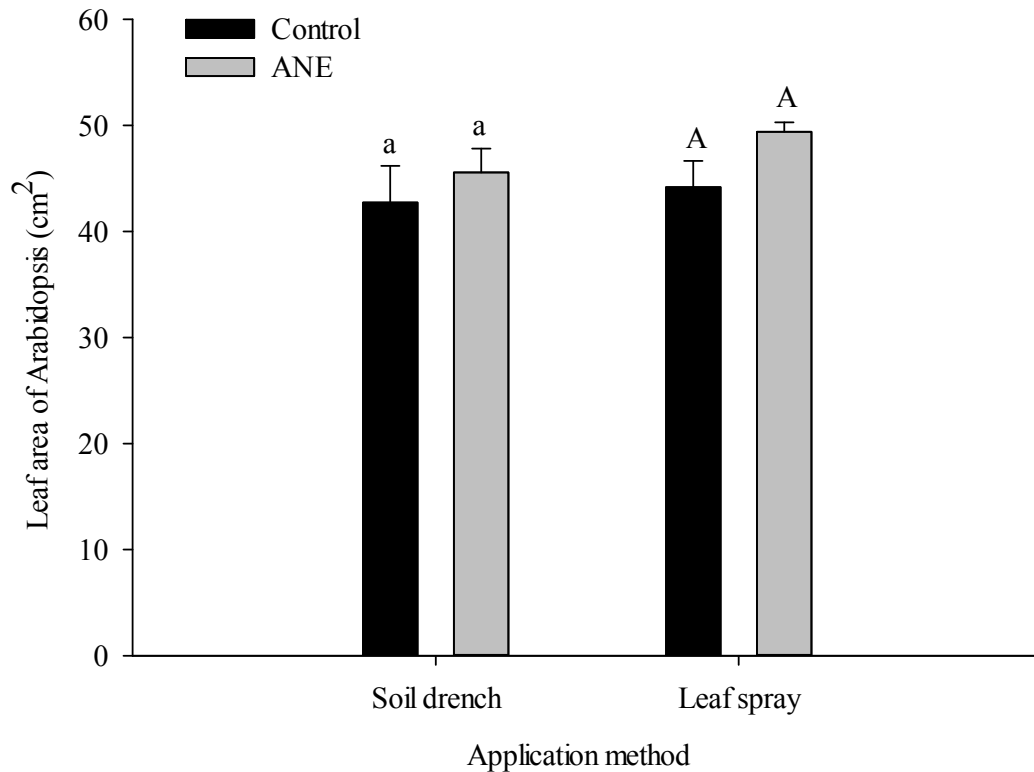
Figure 3.3 Effect of two application methods of *Ascophyllum nodosum* (ANE) (soil drench and leaf spray) on leaf area (cm^2) (± SE) of Arabidopsis. Same case of same letters above bars indicate treatments are not significantly different (ANOVA, $\alpha = 0.05$)

3.4.5 ANE EFFECT ON DRY WEIGHT OF SILIQUES OF ARABIDOPSIS

There was no significant effect of soil drench of ANE on dry weight of siliques of Arabidopsis on day 39 ($F = 0.83$; $df = 1, 36$; $P = 0.37$; Figure 3.4). Leaf application of ANE enhanced the dry weight of siliques ($F = 8.49$; $df = 1, 36$; $P < 0.01$; Figure 3.4).
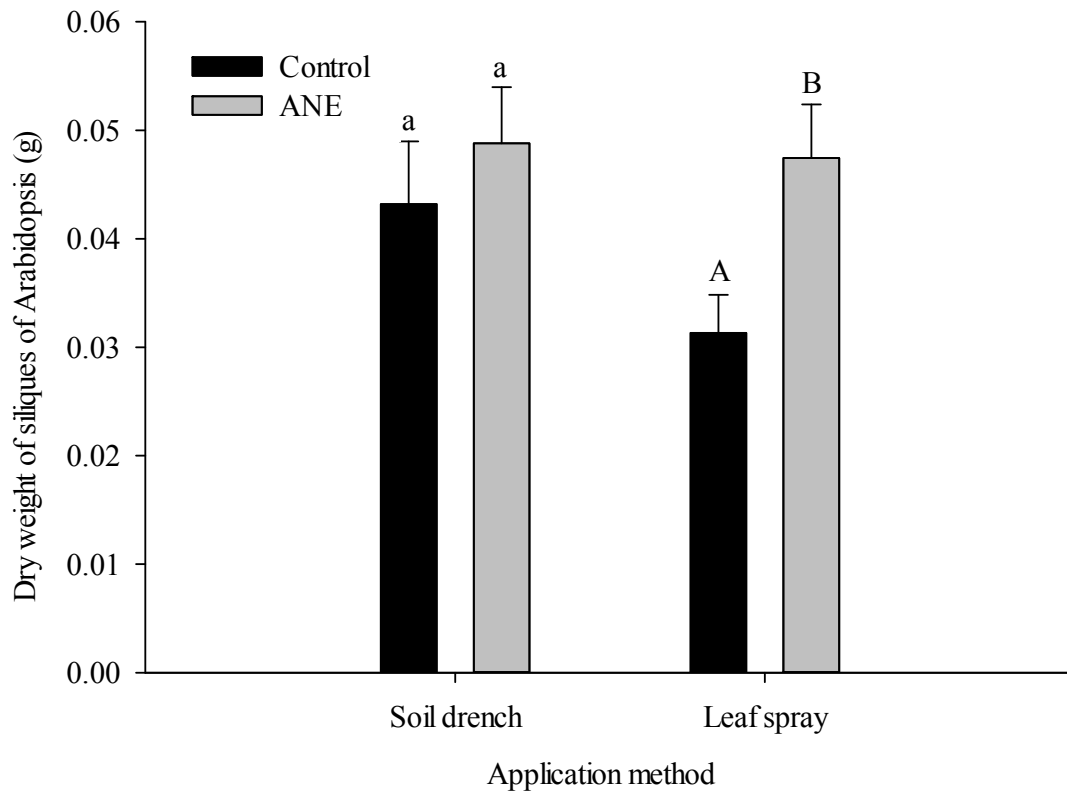
Figure 3.4 Effect of two application methods of *Ascophyllum nodosum* (ANE) (soil drench and leaf spray) on dry weight of siliques (g) (± SE) of Arabidopsis. Same case of different letters above bars indicate treatments are significantly different (ANOVA, α = 0.05)

3.5 DISCUSSION

Results from this study showed that the leaf spray treatment of *A. nodosum* extract (ANE) increased whole plant dry weight, length of peduncle and dry weight of siliques of Arabidopsis at last harvest time (day 39). This may be due to increased activity of ANE treated plants in flowering stage. Previous reports have shown improved plant growth and yield increase following treatment with seaweed extracts. Soil drench application of extracts of the brown alga, *Ecklonia maxima* (Osbeck) Papenfuss enhanced the growth of tomato plants and reduced the infestation of root-knot nematode, *Meloidogyne incognita* (Crouch and Staden, 1993). Yield of 'Thompson seedless' grape (*Vitis vinifera* L.) increased 60 % over three years with application of ANE, giving an increase in: number of berries per bunch, berry size, length of rachis and number of primary bunches (Norrie and Keathly, 2006). Early ripening of the tomato was observed after application of seaweed extract concentrates (SWC). Total fruit fresh weight increased by 17% with the SWC. By the end of the experiment, plants treated with foliar application of 0.4% SWC had 70 % more flowers than control plants (Crouch and Staden, 1992). Overall, foliar spray had a greater effect on fruit yield compared to soil drenching with SWC.

In this present study, the controls contained the same mineral composition as that present in 1 g/L ANE. Thus any results indicating a beneficial increase in growth and yield will be due to organic components present in ANE. Enhanced fruit growth could be caused by organic plant growth promoting activity following the application of ANE. Seaweed extract (0.4 %) prepared from *Ecklonia maxima* increased the yield of lettuce plants by 14 %, and increased the amounts of Ca, K and Mg in leaves of lettuce. This was due, at least in part, to increased nutrient uptake due to application of *E. maxima* extract

(Crouch et al., 1990). Increased nutrient uptake could be a result of chelating activity of organic compounds present in seaweeds (Lynn, 1972). Increased flower and fruit weight might be a result of increased plant growth or the activity of hormones present in extracts (Crouch and Staden, 1992). Cytokinins, auxins, gibberellins and ethylene are important for increased flowering, and increased yield may be a result of increased cytokinin activity following SWC treatment (Crouch and Staden, 1992). Higher cytokinin levels were found at the fruiting stage of tomatoes and beans (Featonby-Smith, 1984). Seaweed extract treated plants showed more cytokinins in roots, which would be translocated to the developing fruits (Vonk, 1979; Davey and Van Staden, 1978) and seeds (Davey and Van Staden, 1979). On the other hand, higher seed yield may be the result of early fruit ripening, which has economic importance as earlier ripened fruit get better market prices (Crouch and Staden, 1992).

The observed different physiological processes affected by the application of seaweed extracts may explain the presence of other beneficial plant growth regulators in the extracts (Nelson and Van Staden, 1985). Ethylene is a phytohormone responsible for plant growth and developmental processes including fruit ripening. It can be active at very low concentrations (Chaves and Mello-Farias, 2006). Precursor of ethylene biosynthesis (1-aminocyclopropane-lcarboxylic acid) was found in commercial extracts of brown seaweed *Ecklonia maxima* (Nelson and Van Staden, 1985). There is an antagonistic effect between production of cytokinin and ethylene. Thus, while plants are under stress, there is a reduction in cytokinins, and an increase in the levels in ethylene (Hare and Van Staden, 1997). However, the effect of seaweed extracts on plant growth

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has a greater influence than when cytokinin applied alone and this greater effect may due

to different plant growth regulators present in the seaweed extracts (Allen et al., 2001).

CHAPTER 4: EFFECT OF *ASCOPHYLLUM NODOSUM* EXTRACT TREATMENT ON *ARABIDOPSIS THALIANA – MYZUS PERSICAE* INTERACTION

4.1 ABSTRACT

An extract of the brown macroalga, *Ascophyllum nodosum* (ANE) contains biological elicitors that impart plant resistance against various biotic and abiotic stresses. A green peach aphid (GPA) (*Myzus persicae*)-Arabidopsis model was used to examine how application of ANE affects the interaction of Arabidopsis with GPA. Antibiosis, antixenosis and tolerance mediated resistance were examined following treatment of plants with ANE. Application of ANE did not induce antibiosis or antixenosis in treated plants. GPA numbers were higher in ANE treated plants, which was also associated with higher plant biomass. However, ANE treated plants were significantly more tolerant of GPA feeding. Treated plants did not exhibit higher chlorophyll content as compared to control. ANE treated plants recovered quickly from GPA damage resulting significantly higher seed yield. As compared to control, the expression of the cytokinin response gene (*ARR5*) was higher (~ two fold) in ANE treated plants and without the presence of GPA (~ half fold). The expression of one of the chlorophyll degradation gene (*CLH1*) was lowered (half fold) in ANE treated plants with GPA whereas slightly higher expression was observed without GPA (~ quarter fold). Senescence associated gene 13 (*SAG13*) transcript level was lower (~ quarter fold) at 24 and 48 h with GPA and (~ half fold) at 48 and 72 h without GPA. Senescence associated gene 21 (*SAG21*) transcript level was suppressed (~ quarter fold) at all the time points without GPA but there was no change occurred in the presence of GPA. Expression of phytoalexin deficient gene (*PAD4*) did not change in any of the treatments. ANE-treated plants showed less trypan staining indicating reduced cell death. Taken together, these results suggest that ANE treatment activates delayed senescence in Arabidopsis, which may lower resistance to GPA but result in enhanced recovery of the plant after GPA feeding, which may be partly, mediated by increased cytokinins in the ANE treated plants.

4.2 INTRODUCTION

The world population estimated to exceed 8.9 billion by 2050 and it is essential to increase food supply to feed an increasing population (Cohen, 2003). At the same time there is increased competition for land, water and energy (Godfray et al., 2010). Crop losses due to insects are estimated to be between 26-40 % of total production in sugar beet, soybean, wheat, cotton, maize, potatoes and rice (Oerke and Dehne, 2004). Minimizing the losses due to insect damage has potential to achieve an increase food production without increasing the arable land area (Oerke and Dehne, 2004). The use of insecticides to control insects is costly and undesirable due to the development of pesticide resistance, damage to non-target organisms, and various unknown effects on animals. Induction of plant innate defense pathways through elicitors is one potential solution (Hammond-Kosack and Parker, 2003).

Elicitors are chemicals from biological or synthetic sources that activate or enhance the endogenous defense pathways within plants, resulting in increased tolerances towards a vast array of stressors (Mejía-Teniente et al., 2010). *Ascophyllum nodosum* (L.) Le Jol. is a brown macroalga found along the shores of the North Atlantic Ocean (Ugarte and Sharp, 2001). It is the most commercially applied macroalga in agriculture (Craigie, 2010). Macroalgae are a rich source of biological elicitors (Cluzet et al., 2004). Brown macroalga contains bioactive compounds including: alginates, laminarans and sulfated fucans (Rioux, 2007; Chevolot et al., 2001; Marais and Joseleau, 2001). These are considered to be effective elicitors of plant defenses (Craigie, 2010). However, there are only few reports on the effect of *A. nodosum* extracts (ANE) on plant resistance to insects. In this research, plant-insect model was utilized which consists of the model plant

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Arabidopsis thaliana and the green peach aphid (GPA), *Myzus persicae* to investigate whether ANE could enhance resistance or tolerance to insects.

Plant resistance against insect pests can be grouped into three categories: antibiosis, antixenosis and tolerance (Painter, 1951). Antibiosis affects the development or physiology of insects (Panda and Khush, 1995) whereas antixenotic factors can deter settling and feeding of pests on resistant plants (Kogan and Ortmann, 1978). Plant tolerance is a mechanism which allows the plant to grow or recover quickly under the pressure of insect pest populations (Painter, 1951). These defense mechanisms can occur during various stages of aphid feeding (Neal et al., 1990). Antibiosis and antixenosis mechanisms can be tested by no-choice and choice feeding experiments (Smith, 2005).

A. nodosum extract (ANE) treated plants showed cytokinin-like activities (Khan et al., 2011). Cytokinins play a major role in several physiological processes in plants such as growth and development of cells, shoot initiation (Frett and McCardell, 1990) and retarding senescence (Mok and Mok, 1994). Leaf senescence is a complex process which involves various changes in cellular physiology, biochemistry and gene expression. It can be explained as a process which occurs in the final stages of the plant life cycle leading to cell death. When leaf senescence begins, nitrogen, carbon and minerals in mature leaves will flow from mature parts to immature parts of plants (Buchanan-Wollaston, 1997). Leaf senescence in Arabidopsis is characterized by chlorophyll loss due to up regulation of chlorophyll degradation gene *CLH1* (Tsuchiya et al., 1999), up regulation of senescence associated genes (*SAG*s), and a higher rate of cell death (Pegadaraju et al., 2005; Lim et al., 2003; Satoko, 2003). Leaf senescence is one key mechanism to defend against GPA (Pegadaraju et al., 2005). Leaf senescence in

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Arabidopsis can reduce GPA growth whereas delaying senescence can lower the resistance to GPA (Pegadaraju et al., 2005).

The phytoalexin deficient gene (*PAD4*), which is associated with camelexin synthesis and salicylic acid (SA) signaling, is associated with plant resistance to pathogens (Glazebrook et al., 1997), but it is also expressed in GPA infested plants (Pegadaraju et al., 2007). *PAD4* mutant plant which is the deficient in camelexin synthesis and salicylic acid (SA) signaling was used in an insect choice experiment (Pegadaraju et al., 2007). GPA showed an increased preference for *PAD4* mutants over wild type plants. A number of plant hormones regulate the senescence process. Cytokinin is a plant hormone which can delay the leaf senescence (Gan and Amasino, 1995). Cytokinin primary response gene, *ARR5* is a gene which responds to exogenous application of cytokinins (Kieber, 2002). *CLH1* encodes for the first enzyme involved in chlorophyll degradation pathway and it can play a significant role in plant defense signaling pathways by detoxifying free chlorophylls after tissue damages. Moreover this can avoid accumulation of reactive oxygen species in plant cells (Kariola et al., 2005).

ANE contains biological elicitors which can activate plant defense signaling pathways that can lead to elicitation of resistance or tolerance in Arabidopsis against GPA. To test this hypothesis, experiments were conducted examining the development of antibiosis, antixenosis and tolerance mechanisms following Arabidopsis treatment with ANE.

4.3 MATERIALS AND METHODS

4.3.1 BIOLOGICAL MATERIALS AND CHEMICALS

Seeds of *A. thaliana* ecotype Columbia (Col-0) (Lehle seed company Texas, USA) were used for all the experiments. Arabidopsis mutant, *cpr5* (constitutive expresser of pathogenesis-related gene 5) plants have elevated levels of salicylic acid (SA) that leads to chlorotic lesions and spontaneous cell death (Bowling *et al*., 1997) was used in a cell death visualization experiment. Seeds of Arabidopsis mutant *cpr5* (CS 3770) were obtained from Arabidopsis biological resource center, Ohio State University, Columbus, USA. Arabidopsis seeds were planted in peat pellets (Jiffy Co., Shippegan, New Brunswick, Canada) and grown under florescent bulbs at 16:8 h (day: night) cycle (100 μmol photons m^{-2} s^{-1}) and 23 ± 2^0C in a growth room. After germination, plants were thinned so that one plant per peat pellet remained. Thirteen-day-old plants were used at the beginning of all experiments.

A GPA colony was started from a single adult, reared on the leaves of potato plants (Kennebec potatoes, Fred A. Duplessis, New Brunswick, Canada). GPA cultures were maintained in a greenhouse under 16:8 h day: night cycle (150 μmol photons m^{-2} s^{-1}) and 23 ± 2^oC. Apterous GPA adults were used at the start of all experiments.

Chemicals used in the experiments were of analytical grade purchased from Sigma Aldrich (ON, Canada) unless otherwise stated.

4.3.2 PREPARATION AND APPLICATION OF ANE AND CONTROL

An aqueous solution of ANE (Acadian Seaplants Limited, Dartmouth, NS, Canada) was prepared by dissolving the desired amount of extract powder in distilled water by constant stirring by hand for 15 min. Modified Long Ashton standard nutrient

solution (LANS) was used as control. Mineral composition in LANS was adjusted as same inorganic composition as in 1.0 g/L ANE (Appendix A.), but does not contain the organic components in ANE. Freshly prepared ANE or control solution was applied as either a soil drench application (10.0 ml) or leaf spray (~ 500 µL) per plant. Leaf spray applications of treatments were sprayed to Arabidopsis rosette until dripping. Arabidopsis were treated with either ANE or control three times, every three days starting at 13th day after germination. Beyond day 22, plants were treated weekly.

4.3.3 GPA INFESTATION

Twenty-one-day old treated Arabidopsis were infested with 15 adult GPA by placing with a paint brush on the center of rosettes of Arabidopsis. Each infested plant was kept in a transparent plastic container covered with a nylon mesh to retain GPA and prevent immigration of other insects on to test plants. All experiments (without no-choice experiments using clip cages) studied GPA reproduction on wild type Col-0 Arabidopsis through the method described by Pegadaraju et al. (2005).

4.3.4 ANE EFFECT ON ANTIBIOSIS MECHANISMS IN GPA

4.3.4.1 NO-CHOICE EXPERIMENTS USING CLIP CAGES

Two no-choice experiments were conducted using clip cages to study GPA reproduction with two different application methods: leaf spray and soil drench. Either leaf spray or soil drench application of water and ANE with three concentration levels (1.0, 2.0 and 3.0 g/L) were used as different treatments. A second whorl leaf of each Arabidopsis was clipped using a clip cage sized 36.5 x 25.4 x 9.5 mm (BioQuip Products, CA, USA). Each clip cage contained one second instar of GPA. The total number of GPA

within clip cage was counted 9 days after infestation. Each treatment consisted of nine replicates.

4.3.4.2 NO-CHOICE EXPERIMENTS USING WHOLE ARABIDOPSIS

Two no-choice experiments were conducted to study GPA population growth with two types of ANE application methods: leaf spray and soil drench. Either leaf spray or soil drench application of water and ANE with three concentration levels (1.0, 2.0 and 3.0 g/L) were used as different treatments. Each Arabidopsis received 15 adult apterous GPA at the center of the rosettes. Each treatment consisted five replicates. The experiments were conducted using a method described by Pegadaraju et al. (2005) with minor modifications. In this experiment, GPA infestation was done at day 21 post-germination of Arabidopsis and total number of GPA was counted 5 days after infestation.

4.3.4.3 EFFECT OF ORGANIC COMPOUNDS PRESENT IN ANE ON GPA REPRODUCTION

To distinguish the effect of organic components of ANE on GPA number, inorganic control was used. Soil drench of ANE (1.0 g/L) and inorganic control were used as treatments. Same procedure was followed as mention in section 4.3.4.2 and total GPA number was counted five days after infestation.

4.3.4.4 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSES

The experiments were conducted using a completely randomized design (CRD). Before performing ANOVA, three assumptions were tested. The assumption of normality was checked using the normal probability plot of residuals (Anderson-Darling normality test); homogeneity of variance was checked by plotting the residuals vs. fitted values; independence was assumed through randomization of GPA infested Arabidopsis.

ANOVA was performed to test the effect of ANE on number of GPA. All analyses were performed at $\alpha = 0.05$. Tukey's test was performed to compare treatments when mean effects were significantly different. All analyses were done by using Minitab 15.0 (Minitab Inc. Pennsylvania, USA) and SAS version 9.2 software (Cary, NC, USA).

4.3.5 ANE Effect on GPA settling

4.3.5.1 Multiple-choice Feeding-preference Experiments

Multiple-choice feeding-preference experiments were conducted to test antixenotic mediated resistance in Arabidopsis following leaf spray and soil drench treatments of ANE. Two choice experiments were conducted to study GPA settling with two types of ANE application methods: leaf spray and soil drench. Twenty apterous adult GPA were introduced approximately same distance to each leaf in the middle of plastic disk (Figure 4.1). Water, LANS (inorganic control) and ANE at three concentrations (1.0, 2.0 and 3.0 g/L) were selected as choices for GPA. Each arena was placed in an insect cage (BioQuip Products, CA, USA). The total number of GPA was counted in treated Arabidopsis plants after 48 h GPA infestation. Percentage settling was calculated for all five treatments. Each setup was replicated five times. The method used in this experiment (Castle et al., 1998) was carried out with a minor modification. Treated Arabidopsis leaves were not covered with a plexiglas cylinder.
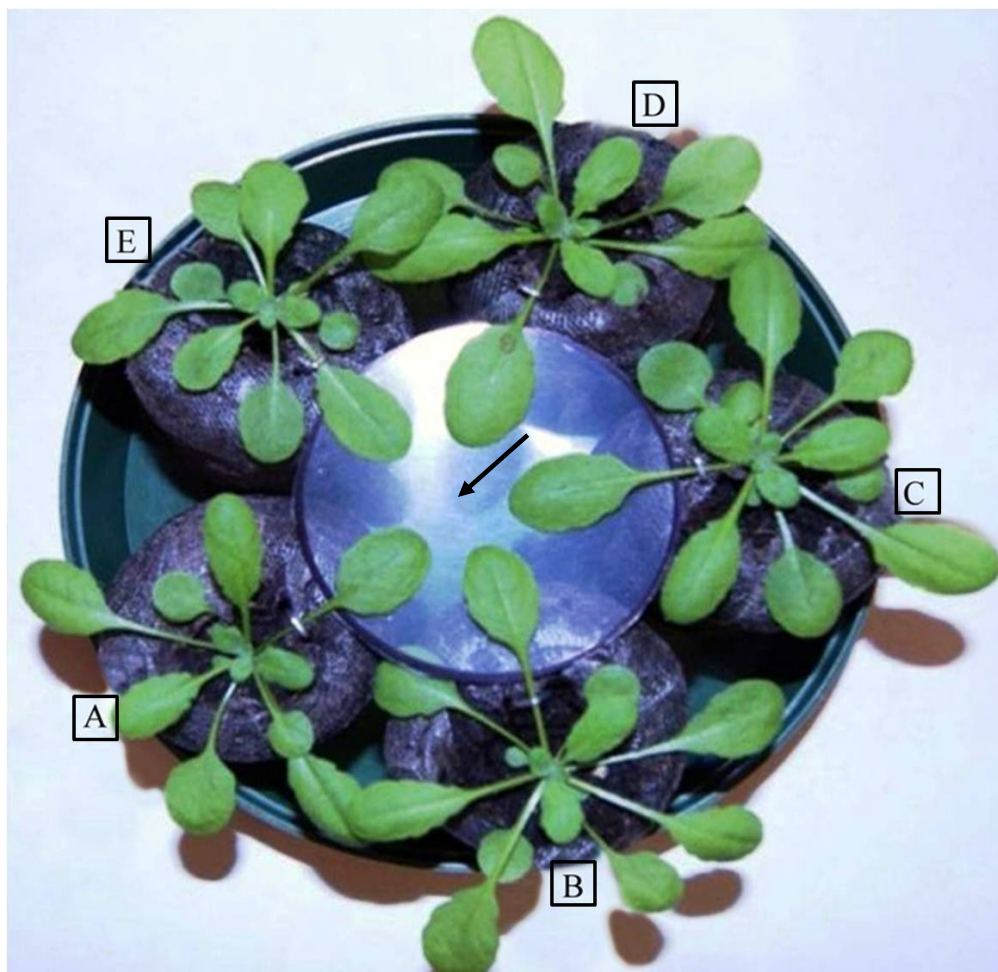
Figure 4.1 Multiple-choice feeding-preference set-up to observe *Myzus persicae* (GPA) settling on different treatments, (A) water, (B) inorganic control and ANE at three concentrations (C) 1.0, (D) 2.0 and (E) 3.0 g/L. Treated Arabidopsis leaves were randomly placed on a plastic disk. Arrow indicates the spot where the GPA was released.

4.3.5.2 PAIRED CHOICE EXPERIMENTS USING CLIP CAGES

One concentration of ANE (1.0 g/L) and the inorganic control were selected as treatments for paired choice experiments. Two paired choice experiments were conducted to study GPA settling with two types of ANE application methods: leaf spray and soil drench. Paired choice experiments were carried out using clip cages (36.5 x 25.4 x 9.5 mm; BioQuip Products, CA, USA) (Figure 4.2). Treatment either consisted of 1.0 g/L ANE or control. Second whorl leaves of Arabidopsis were clipped after placing 10

apterous adult GPA on each leaf. Each setup was replicated ten times. Total GPA

numbers within clip cages were counted after 48 h and the percentage settling was

calculated.
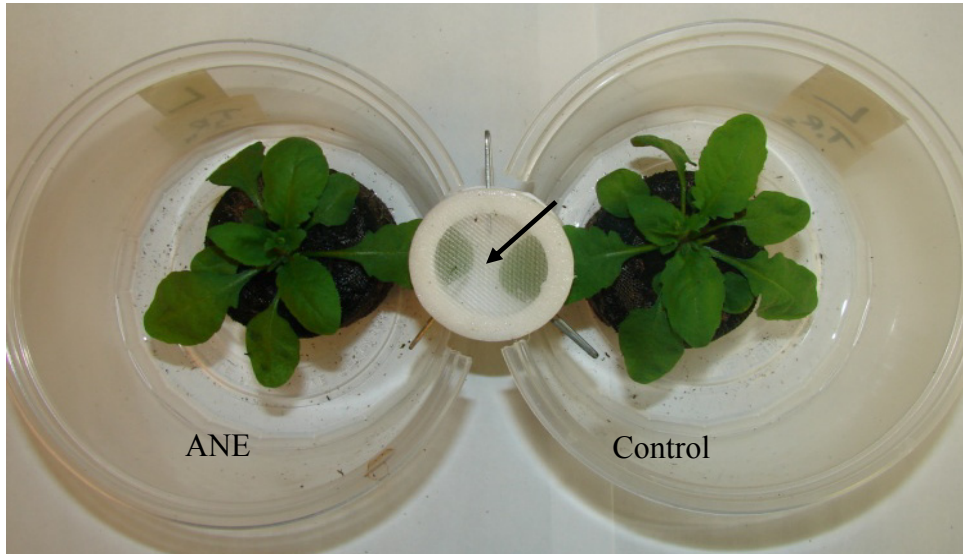
Figure 4.2 Paired choice test to observe *Myzus persicae* (GPA) settling of control and ANE treated Arabidopsis leaves after clipping. Arrow indicates the spot where the GPA was released in the clip cage.

4.3.5.3 PAIRED CHOICE EXPERIMENTS USING WHOLE ARABIDOPSIS PLANT

Paired choice experiment was conducted using whole Arabidopsis plant instead of

clip cages. Paired choice experiments were carried out using methods described by

Pegadaraju et al., (2007) with modifications. Rectangular plastic containers (13.5 X 9.0

cm) were used for potting Arabidopsis with peat pellets. The nearest leaf of ANE (1.0

g/L) and control plants in each replicate pair were spaced approximately 1 cm apart in

these plastic containers. Twenty-five GPA were released on sides of the rectangular

container which consists of one ANE treated and one control Arabidopsis. Each pair was

replicated ten times. The number of GPA counted after 48 h and 72 h. The percentage

settling of control vs. ANE was calculated.
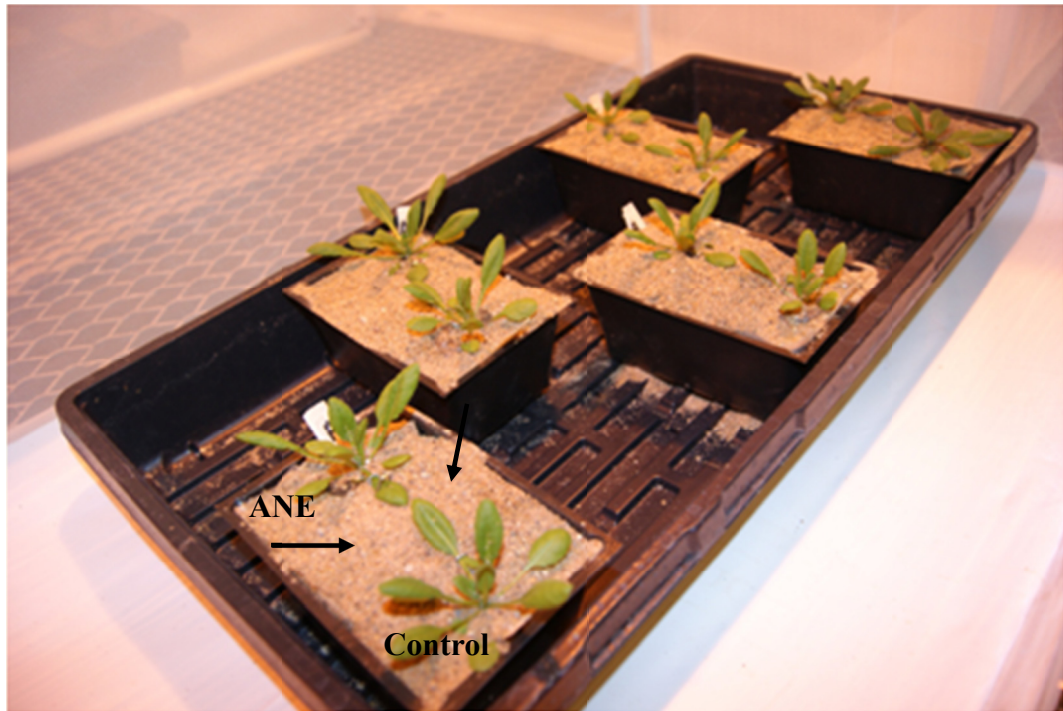
Figure 4.3 Paired choice test to observe *Myzus persicae* (GPA) settling on control and ANE treated whole Arabidopsis plants. Arrow indicates the place where the GPA was released.

4.3.5.4 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSES

All data were checked to meet the three assumptions of the ANOVA tests. All choice experiments were conducted under a completely randomized design. ANOVA was performed to test the effects of ANE on percentage settling of GPA in Multiple-choice feeding-preference experiments. A paired t-test was performed to test the effects of ANE on percentage settling of GPA in paired choice experiments using clip cages. In paired choice experiment using whole Arabidopsis plants, repeated measures analyses were performed to test the ANE on percentage settling of GPA over time (PROC MIXED procedure in SAS 9.2 software.

4.3.6 ANE EFFECT ON PLANT TOLERANCE AGAINST GPA

4.3.6.1 MEASUREMENT OF FRESH WEIGHT AND ESTIMATION OF CHLOROPHYLL PIGMENTS

Soil drench application of either 1.0 g/L ANE or control treatments were conducted as in section 4.3.2. Fifteen adult GPA were released at the center of the rosettes of Arabidopsis. Fresh weight of all above ground parts of five Arabidopsis plants were measured prior to chlorophyll analyses. After taking the fresh weight, each Arabidopsis plant leaf samples infested with GPA were collected and analyzed for chlorophyll a, b and total chlorophyll during three different harvesting time points (8, 11, 14 days). Analysis of chlorophyll was carried out by the method described by Pegadaraju et al (2005). All extractions carried out at 4 $^{\circ}$C under low light conditions. One hundred milligram of leaf sample (from second whorl) of *A. thaliana* were crushed until leaf tissues were turn into colorless using 1.0 ml of extraction buffer consisted of 85 % acetone and 15 % 1.0 M Tris-HCl (pH 8.0). The extract was centrifuged at 12, 000 x g for 5 min, at 4 ^{0}C. Supernatant was collected and diluted five times to measure the absorbance against the blank (consists of extraction buffer). Absorbance was measured at 664 nm, 647 nm using a microplate reader (BioTek Power XS2, VT, USA). Calculation of chlorophyll a, chlorophyll b and total chlorophyll was done by using the following formula described by Lichtenthaler (1987).

Chlorophyll a = 12.25 * A_{663} - 2.79 * A_{647}

Chlorophyll b = 21.50 * A_{647} - 5.10 * A_{663}

Total chlorophyll content = Chlorophyll a + Chlorophyll b

4.3.6.2 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSES

A plant tolerance study was conducted using a completely randomized design with five replicates for each treatment. All data were checked to meet the three assumptions of ANOVA and analyzed using repeated measures in PROC MIXED procedure in SAS 9.2 software ($\alpha = 0.5$) followed by Tukeys test.

4.3.7 VISUALIZATION OF CELL DAMAGE

Cell death visualization experiment was conducted to determine whether the senescence phenomenon was associated with the application of ANE. Arabidopsis constitutive expresser of *PR* gene5 (*CPR5*) mutant plant which can accumulate more salicylic acid (SA), and undergoes more cell death as well as ANE treated and control Arabidopsis Col-0 type plants were used. Soil drench applied to either 1.0 g/L ANE or inorganic control was used as treatments. A second whorl leaf of either the Arabidopsis ecotype Col-0 or the *CPR5* leaf was clipped using a clip cage with five apterous adult GPA. Leaf samples were taken seven days after release of GPA. All treatments were consisted of three replicates. Cell damage was visualized using a method described by Rate et al. (1999). The cellular clearing step was modified by using chloral hydrate solution. Lactophenol blue solution was prepared by mixing 10:10:10 mL; glycerol, lactic acid, phenol. Ten mg of trypan blue powder was dissolved in 10.0 mL of distilled water and then added to lactophenol solution to a final concentration of 0.025%. Stained leaf samples were cleared by placing the leaf samples overnight in chloral hydrate solution. Chloral hydrate solution was prepared by adding 25.0 g of chloral hydrate to 10.0 mL of distilled water with constant stirring. Cleared leaf samples were mounted in 70 %

glycerol, and dead cells were observed using a dissecting microscope. Cell death was observed in all treatments.

4.3.8 EXPERIMENT ON RECOVERY OF ARABIDOPSIS FROM GPA DAMAGE AFTER REMOVAL OF GPA

GPA feeding reduces photosynthetic capacity and prolonged infestation can reduce biomass of plants. A recovery experiment was conducted to determine whether ANE treated plants can recover better following GPA infestation than non ANE treated plants. Soil drenches applications of either 1.0 g/L ANE or control solution were followed as in section 4.3.2. Fifteen adult GPA were placed at the center of rosettes of each Arabidopsis. Seven days after infestation, with GPA, all insects were removed carefully with a fine paint brush. Treatment of ANE or control was continued every seven days until harvesting the siliques. Dry weight of each Arabidopsis rosettes and total dry weight of siliques were measured at 16th day of after removing GPA.

4.3.8.1 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSES

The experiment on recovery of Arabidopsis from GPA damage was conducted using a completely randomized design. All data were checked to meet the three assumptions of the ANOVA tests. ANOVA was performed to test effect on ANE on dry weight of each Arabidopsis plant and total dry weight of siliques from each plant. Results were analyzed using ANOVA at $\alpha = 0.05$ level by using Minitab 15.0.

4.3.9 QUANTITATIVE GENE ANALYSES OF ARABIDOPSIS AS AFFECTED BY ANE

4.3.9.1 ISOLATION OF RNA

Soil drench application of either 1.0 g/L ANE or control treatments were followed as in section 4.3.2. Fifteen GPA were released at the center of the rosettes of either ANE or inorganic control solution treated Arabidopsis plants. A similar setup was kept without GPA for treatment comparison. All the leaves from GPA infested and uninfested Arabidopsis plants were collected at 24, 48 and 72 h. Three plants were pooled as one biological replicate for analyses. Leaf samples were collected and snap frozen in liquid nitrogen and then lyophilized. Leaf samples were ground at 3000 rpm for 15 seconds using a Tomy MicroSmash^{TM} MS-100 micro homogenizing system (Tokyo, Japan). RNA was extracted using Trizol (Invitorgen, USA) following manufactures instructions. Isolated RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Isolated RNA quality was confirmed by visualizing RNA bands after resolving on 0.7% agarose gel.

4.3.9.2 PURIFICATION OF RNA AND CDNA SYNTHESIS

Isolated RNA was treated with RQ1 DNAse (Promega Inc., USA) following the instructions provided by the manufacturer. Purified RNA was reverse transcribed using a high capacity cDNA reverse transcript kit (Applied Biosystems, ON, Canada) following the instructions provided by the manufacturer.

4.3.9.3 REAL TIME PCR ANALYSES

Real time PCR was performed using StepOne™ Real-Time PCR System (Applied Biosystems, CA). Ten microliter of total reaction mixture contained 50 ng of cDNA, 20 ng of gene specific primers, 5 µL of 2X SYBR green reagent and 2.5 µL DEPC water.

Reaction products were confirmed with the melt curve and running a 1 % agarose gel.

Transcript levels of each gene were normalized to the expression of *ACT2* (actin 2) gene.

Fold change for GPA infested Arabidopsis was relative to GPA uninfested control plants.

PAD4 (phytoalexin deficient 4) (Wang et al., 2010), *CLH1* (chlorophyllase1), *SAG21*

(senescence associated gene 21), *SAG13* (senescence associated gene 13), *ARR5*

(Arabidopsis response regulator 5) (Takei et al., 2004a) and *ACT2* (Takei et al., 2004b)

were selected for quantitative PCR. Real Time PCR conditions for gene specific primers

were as follows; heat activation at 95$^{\mathrm{o}}$C for 10 min, denaturation at 95$^{\mathrm{o}}$C for 15 s,

annealing and final extension at 60$^{\mathrm{o}}$C for 1 min followed by 40 cycles. Relative transcript

levels were analyzed using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). The gene specific primers

used in this study are given in Table 4.1.

Table 4.1 Primer sequences used in the study

Gene	Locus	Gene specific primers
AtCLH1	At1g19670	F5' GAGTGTAAGGCGACGAAAGC-3' R5' AGAAACGCAACCACAATTCC-3'
AtSAG21	At4g02380	F5' CATTCCTCGGATATATCTCTCCTT-3' R5' TTCTTGAAAAGAGAGAAAACAGACTTT-3'
AtSAG13	At2g29350	F5' AGGAAAACTCAACATCCTCGTC-3' R5' GCTGACTCGAGATTTGTAGCC-3'
AtARR5	At3g48100	F5' TTAAAAGCTCAAAGATTCACACACA-3' R5' ATCAGCAAAAGAAGCCGTAATGT-3'
AtPAD4	At3g52430	F5' TCTTAGCCGAGCCACTCGAC-3' R5' TTCTCGCCTCATCCAACCAC-3'
AtACT2	At3g18780	F5' TTGTTTGTTTCATTTCCCTTTTTG-3' R5' GCAGACGTAAGTAAAAACCCAGAGA-3'

4.4 RESULTS

4.4.1 NO-CHOICE EXPERIMENTS USING CLIP CAGES

No-choice experiments were conducted to test antibiosis mediated resistance in Arabidopsis following leaf spray and soil drench treatment of ANE. There were no significant effect of ANE treatment on total GPA numbers, whether by leaf spray ($F = 0.45$; $df = 3, 30$; $P = 0.72$) and soil drench ($F = 0.80$; $df = 3, 26$; $P = 0.50$).

4.4.2 NO-CHOICE EXPERIMENTS USING WHOLE ARABIDOPSIS

Compared to water controls, GPA numbers were higher in leaf spray applications ($F = 5.58$; $df = 3, 16$; $P < 0.01$) and soil drench applications ($F = 5.15$; $df = 3, 16$; $P = 0.01$) of ANE. For both application methods, ANE concentrations (1.0 and 2.0 g/L) had significantly higher GPA numbers ($P<0.05$), but GPA numbers for the 3.0 g/L were not significantly different as compared to control (Figure 4.4).

Compared to the inorganic control, GPA numbers were higher in the soil drench application of ANE 1.0 g/L ($F = 8.73$; $df = 1, 8$; $P = 0.02$).
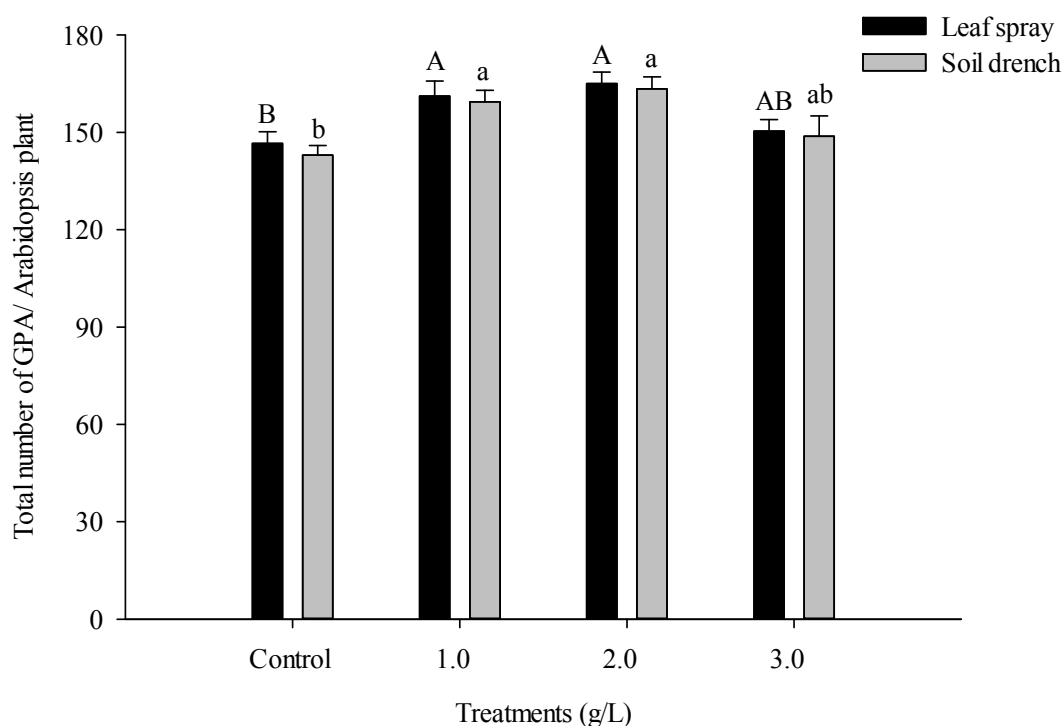
Figure 4.4 Comparison of total *Myzus persicae* (± SE) (GPA) five days after release of 15 adult GPA each on Arabidopsis plants following leaf spray and soil drench treatment of *Ascophyllum nodosum* extract (ANE). Bars with same case different letters indicate that each treatment is significantly different (Tukey's test, α = 0.05)

4.4.3 ANE EFFECT ON GPA SETTLING

In multiple-choice feeding-preference experiments, there were no significant effects of ANE on percentage settling of GPA for both application methods of ANE: leaf spray ($F = 0.79$; $df = 4, 20$; $P = 0.55$) and soil drench ($F = 1.40$; $df = 4, 20$; $P = 0.27$).

In paired choice experiments using clip cages, there were no significant differences between control plants and those treated with ANE in the number of GPA recovered, whether for the leaf spray ($P = 0.63$) or soil drench ($P = 0.53$) application methods.

In whole plant paired choice experiments, there were no significant differences between control plants and those treated with ANE in the number of settling GPA (leaf

spray, $F = 0.96$; $df = 1, 18$; $P = 0.34$; soil drench ($F = 0.82$; $df = 1, 18$; $P = 0.38$). There was no ANE effect on GPA settling over time (leaf spray, $F = 0.01$; $df = 1, 18$; $P = 0.91$; soil drench, $F = 0.08$; $df = 1, 18$; $P = 0.78$) and between treatment * time (leaf spray, $F = 0.44$; $df = 1, 18$; $P = 0.51$; soil drench $F = 1.51$; $df = 1, 18$; $P = 0.24$).

4.4.4 EFFECT OF ANE ON CHLOROPHYLL A, B AND TOTAL CHLOROPHYLL CONTENT AND FRESH WEIGHT OF ARABIDOPSIS INFESTED WITH GPA

ANE treatment did not affect the level of chlorophyll 'a' ($F = 0.88$; $df = 1, 8$; $P = 0.38$), chlorophyll 'b' ($F = 2.37$; $df = 1, 8$; $P = 0.16$) and total chlorophyll ($F = 1.24$; $df = 1, 8$; $P = 0.30$). Chlorophyll in both ANE treated and control plants were significantly reduced over time with GPA infestation, chlorophyll a ($F = 39.62$; $df = 2, 16$; $P < .01$); chlorophyll b ($F = 36.62$; $df = 2, 16$; $P = <.01$) and total chlorophyll ($F = 41.16$; $df = 2, 16$; $P = <.01$). There were no significant interaction effects between treatment*harvest time on chlorophyll 'a' ($F = 0.28$; $df = 2, 16$; $P = 0.76$); chlorophyll 'b' ($F = 0.04$; $df = 2, 16$; $P = 0.96$) and total chlorophylls ($F = 0.13$; $df = 2, 16$; $P = 0.88$).

Application of ANE reduced the total fresh weight loss in Arabidopsis following GPA infestation ($F = 7.27$; $df = 1, 8$; $P = 0.03$, Figure 4.5). Biomass of both ANE and control were significantly reduced over time with GPA infestation ($F = 22.10$; $df = 2, 16$; $P < 0.01$, Figure 4.5). However there were no significant interaction effects between treatment*harvesting time ($F = 0.11$; $df = 2, 16$; $P = 0.90$, Figure 4.5).

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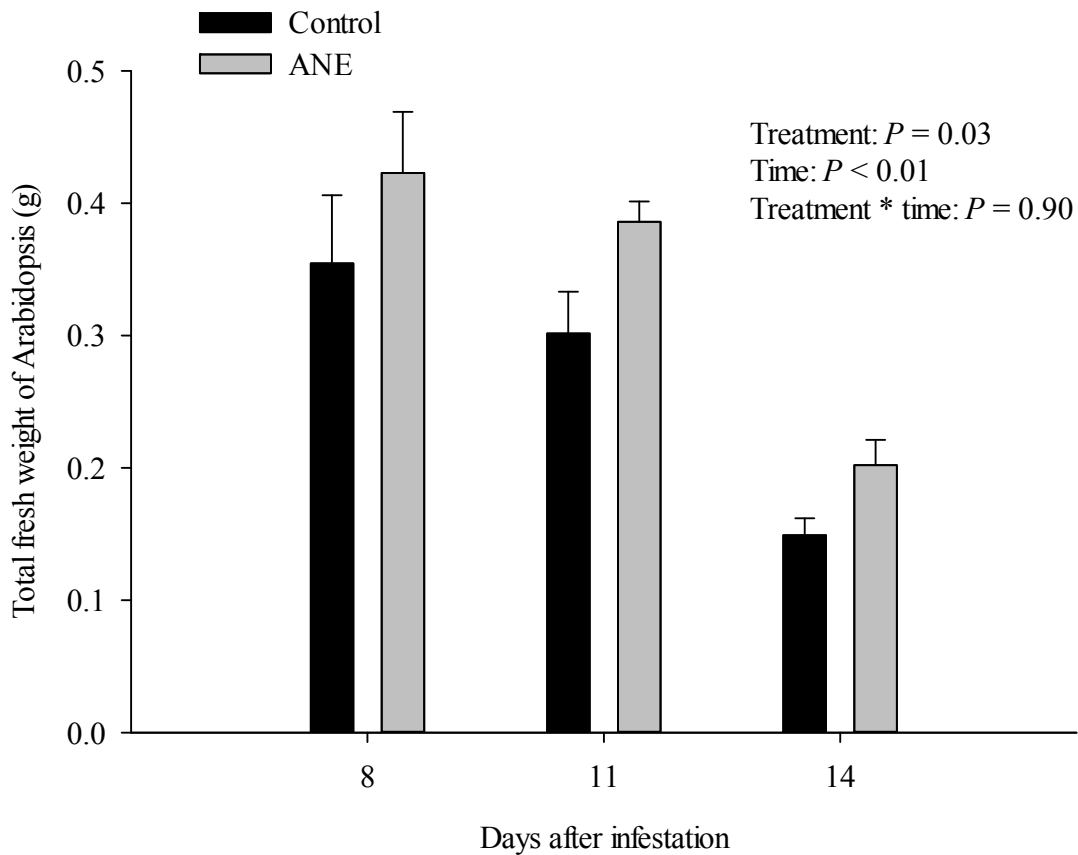
Figure 4.5 Comparison of total fresh weight (± SE) content in Arabidopsis with or without treatment with *Ascophyllum nodosum* extract (ANE) at 8, 11 and 14 days after *Myzus persicae* infestation. Overall, treatments are significant different (α = 0.05).

4.4.5 DRY WEIGHT OF ROSETTES AND SILIQUES OF ARABIDOPSIS RECOVERY FOLLOWING GPA INFESTATION

In the experiment examining plant recovery after removal of GPA, there were no significant differences between dry weight of rosettes of ANE treated plants and control plants after 16 days of GPA recovery period ($F = 2.59$; $df = 1, 33$; $P = 0.12$). When siliques from Arabidopsis were weighed after a recovery following removal of a GPA infestation, ANE treated Arabidopsis had significantly higher seed yields than control plants ($F = 4.69$; $df = 1, 33$; $P = 0.04$, Figure 4.6).
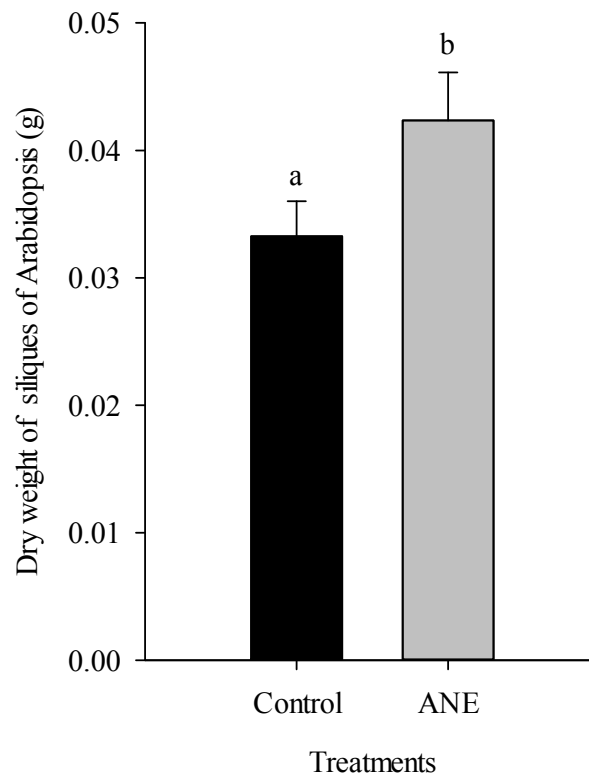
Figure 4.6 Comparison of total dry weight (± SE) of siliques of Arabidopsis with or without treatment with *Ascophyllum nodosum* extract (ANE) following 16 days Arabidopsis recovery. Different letters above them bars indicate that each treatment is significantly different (ANOVA, α = 0.05)

4.4.6 VISUALIZATION OF CELL DAMAGE

Greater trypan staining was observed more in *CPR5* mutant plants (Figure 4.7.) than the control Arabidopsis Col-0 plants (Figure 4.8). ANE treated leaves exhibited much lower trypan blue staining (Figure 4.9).
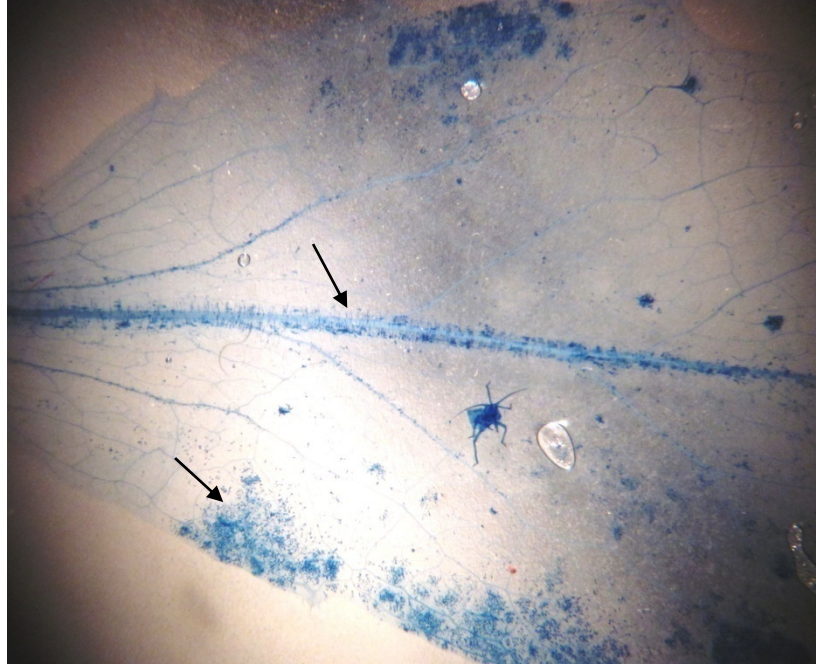
Figure 4.7 Trypan blue stained *Myzus persicae* infested Arabidopsis mutant *CPR5* leaf observed under dissection microscope. Arrows indicate the intensely stained clusters of dead cells.
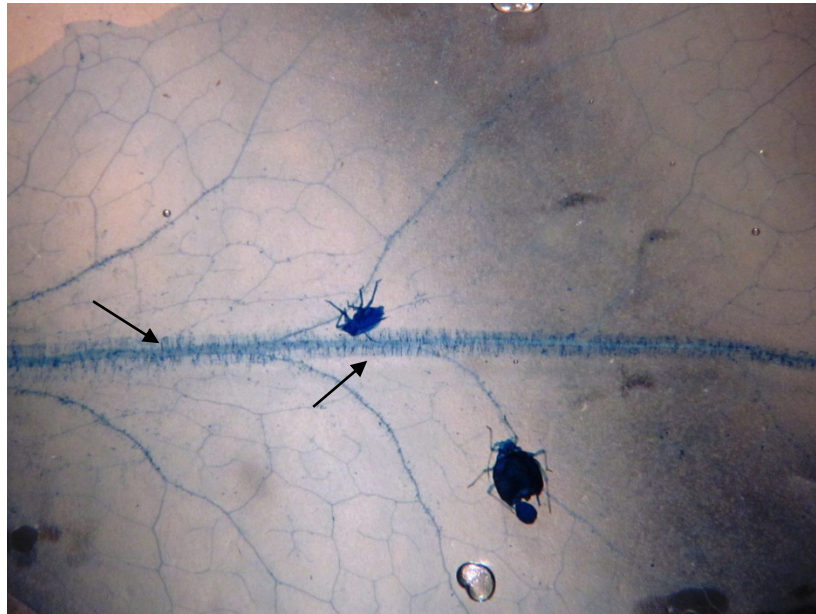
Figure 4.8 Trypan blue stained *Myzus persicae* infested Arabidopsis Col-0 (control) leaf observed under dissection microscope. Arrows indicate the intensely stained clusters of dead cells.
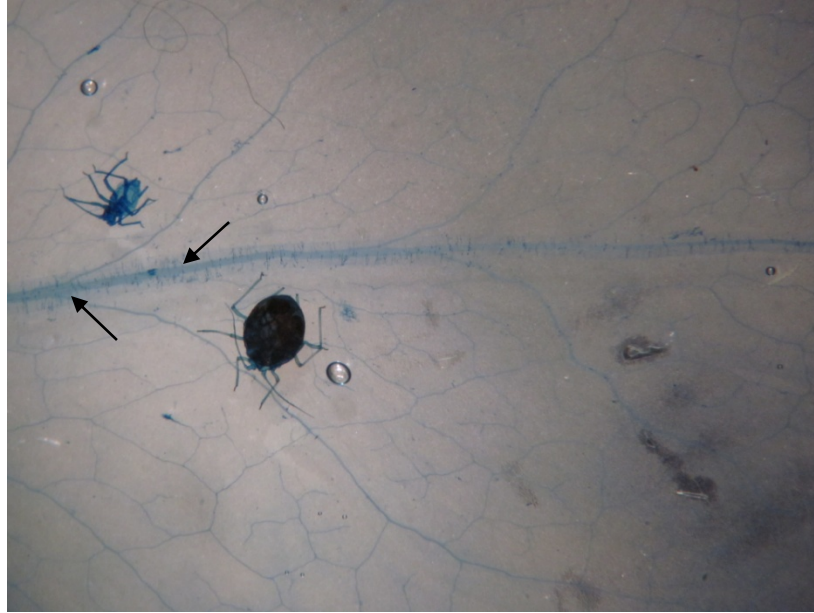
Figure 4.9 Trypan blue stained *Myzus persicae* infested *A. nodosum* extract (ANE) treated Arabidopsis Col-0 leaf observed under dissection microscope. Arrows indicate the intensely stained clusters of dead cells.

4.4.7 GENE EXPRESSION STUDY IN ARABIDOPSIS AS AFFECTED BY ANE

Observation of higher numbers of GPA and decreased cell death (section 4.4.6) in ANE treated plants suggests that ANE treatment may lead to delayed senescence in Arabidopsis. Further, gene expression studies were conducted to validate the phenotypic observations.

Expression of *ARR5,* a gene which is considered to be one of cytokinin primary responsive genes, was examined following the application of ANE (Figure 4.10). Approximately 50% increase in the expression of *ARR5* was observed after 24 and 72 h without GPA infestation as compared to uninfested control. Application of ANE also increased expression of *ARR5* in Arabidopsis during GPA infestation at 24 h (~ two fold). However, expression of *ARR5* decreased at 48 h and 72 h after GPA infestation.
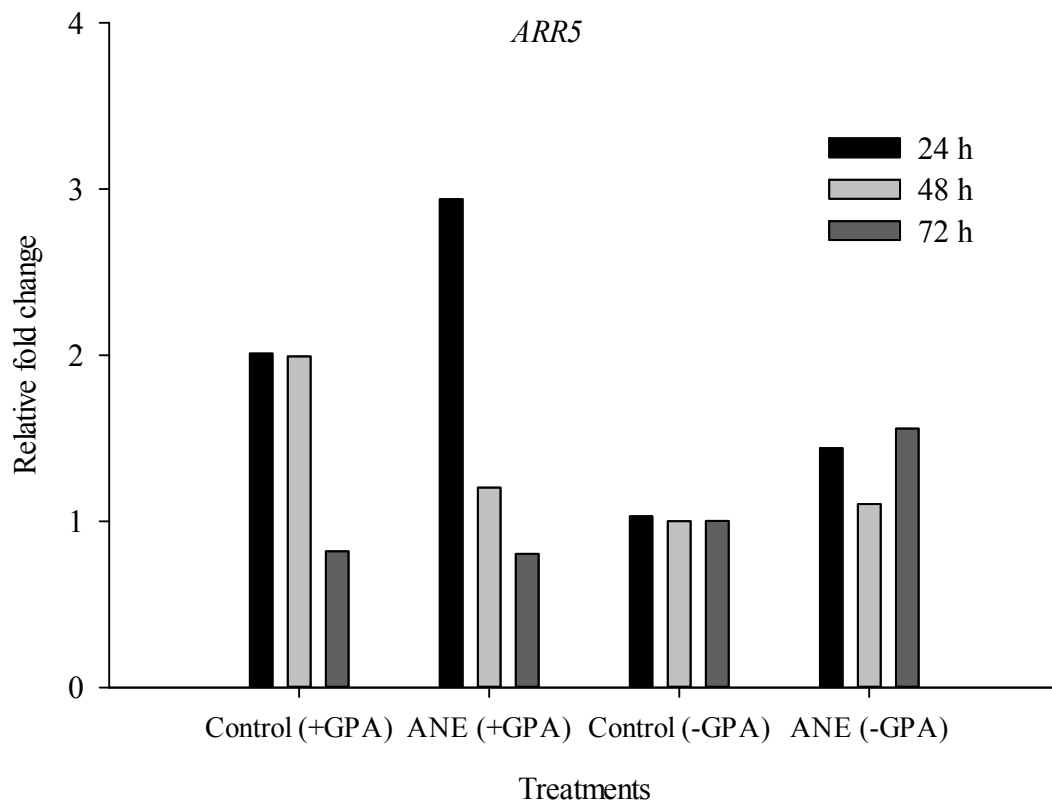
Figure 4.10 Quantitative expression of *ARR5* gene in Arabidopsis at 24, 48 and 72 h with and without infestation by *Myzus persicae*, or treatment with *Ascophyllum nodosum* extract (ANE)

CLH1 was suppressed (~ half fold) at 48 and 72 h following GPA infestation (Figure 4.11). ANE did not affect the level of expression of *CLH1* at 24 and 72 h without GPA. However, ANE treatment suppressed the expression of *CLH1* at 48 h without GPA infestation.
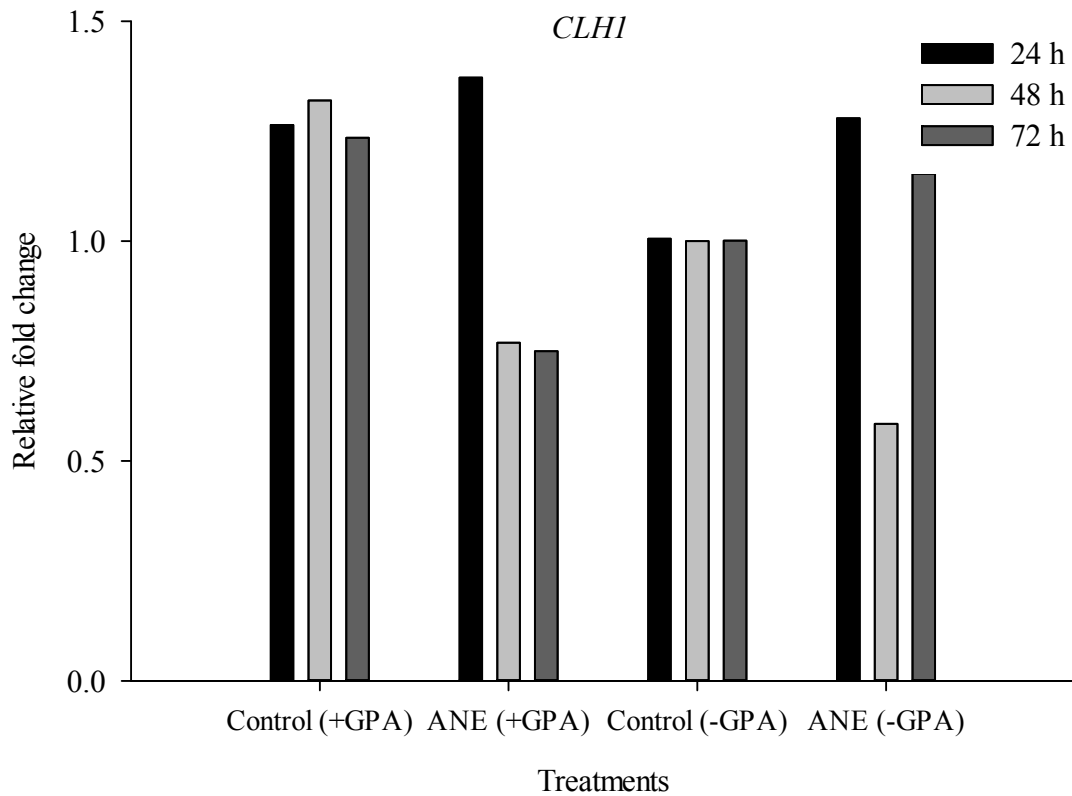
Figure 4.11 Quantitative expression of *CLH1* gene in Arabidopsis at 24, 48 and 72 h with and without infestation by *Myzus persicae*, or treatment with *Ascophyllum nodosum* extract (ANE)

Expression of *SAG21*, a gene which is involved in leaf senescence in Arabidopsis, was not affected by ANE treatment (with GPA infestation) at 24, 48 and 72 h (Figure 4.12). However, expression of *SAG21* was lowered (~ quarter fold) in ANE treated plants without GPA at 24, 48 and 72 h as compared to uninfested control plants.
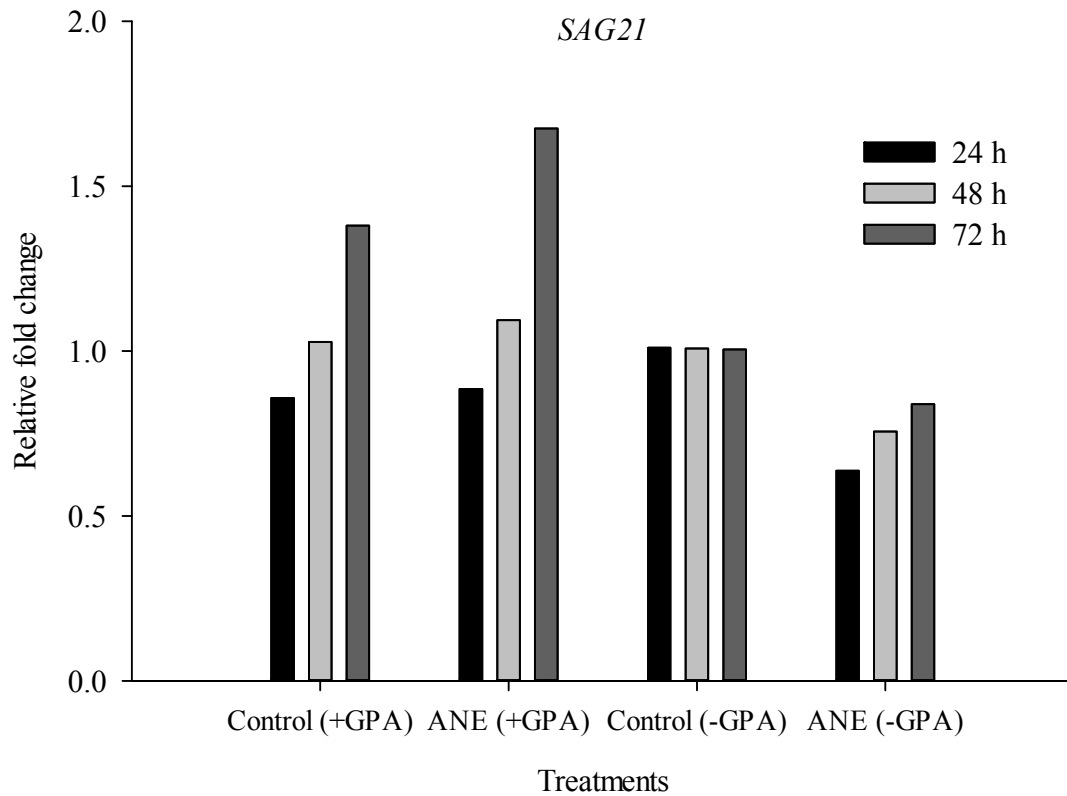
Figure 4.12 Quantitative expression of senescence associated gene 21 (*SAG21*) in Arabidopsis at 24, 48 and 72 h with and without infestation by *Myzus persicae*, or treatment with *Ascophyllum nodosum* extract (ANE)

Unlike results for *SAG21*, expression of *SAG13*, a gene which is involved in leaf senescence in Arabidopsis, was suppressed with ANE at 24, 48 h with GPA (Figure 4.13). However, *SAG13* expression was up regulated in ANE treatment at 72 h. Similar to *SAG21*, *SAG13* was suppressed at 48 and 72 h without GPA.
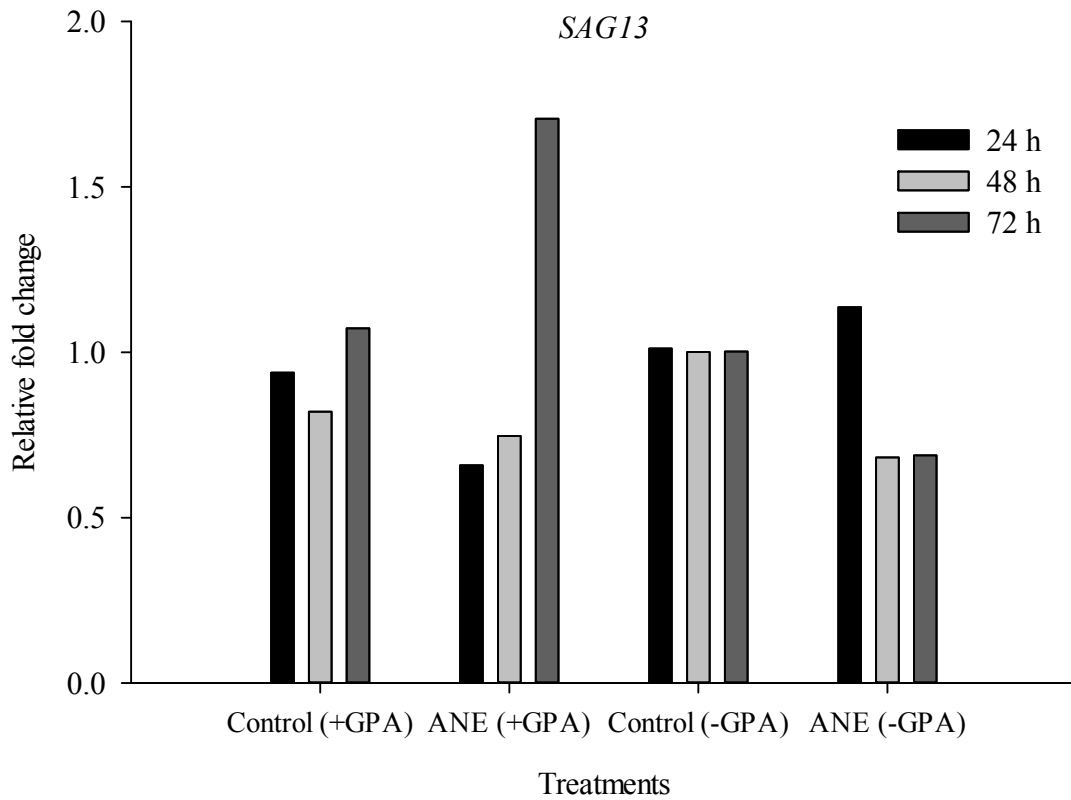
Figure 4.13 Quantitative expression of senescence associated gene 13 (*SAG13*) in Arabidopsis at 24, 48 and 72 h with and without infestation by *Myzus persicae,* or treatment with *Ascophyllum nodosum* extract (ANE)

Expression of *PAD4*, a gene which is involved in antixenosis mediated resistance in Arabidopsis against GPA (Pegadaraju et al. 2005) increased with increasing time of GPA infestation (Figure 4.14). However, application of ANE had no effect on gene expression. However, ANE increased the expression of *PAD4* (~ half fold) at 72 h after without GPA as compared to uninfested control plants.
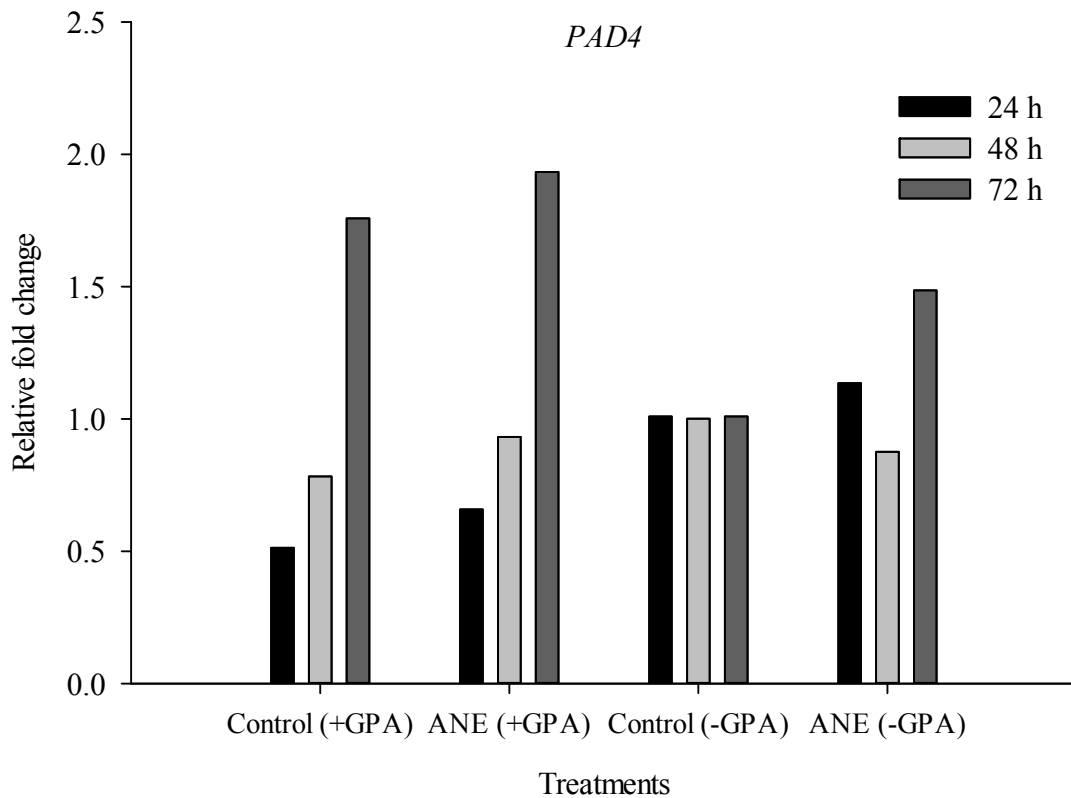
Figure 4.14 Quantitative expression of phytoalexin deficient 4 (*PAD4*) gene in Arabidopsis at 24, 48 and 72 h with and without infestation by *Myzus persicae*, or treatment with *Ascophyllum nodosum* extract (ANE)

4.5 DISCUSSION

      This study demonstrated that green peach aphid (GPA), *Myzus persicae* numbers increased following application of ANE. However, in the first set of experiments with clip cages, there was no significant difference in GPA numbers between treated and untreated plants. Clip cages for insect experiments are advantageous in that they can be fixed to the same leaf of the plant and counting is easier than counting on a whole plant. However, there was no treatment effect on GPA reproduction in no-choice experiments using clip cages.

      In no-choice experiments using whole plants, an increase in the GPA numbers with 1.0 and 2.0 g/L ANE concentrations occurred as compared to water control. However, the 3.0 g/L ANE concentration was not significantly different when compared to the control. This may be a result of high amounts of potassium (14-18%) and sodium (3.0-5.0%) in higher concentrations of ANE (Acadian Seaplants Limited, Canada). Since water was used as a control, a higher alkalinity may have affected the health of Arabidopsis and thereby GPA numbers did not increase with 3 g/L ANE treatment. Because inorganic or organic components present in the ANE extracts might have affected the response of aphids, I also used an inorganic control containing the same inorganic composition as in 1.0 g/L ANE. Since, higher numbers of GPA were observed in ANE treated plants, the effect observed was likely due to organic components present in ANE. The same type of inorganic control was used in a previous study to test the effect of organic components of seaweed extract on chlorophyll content of tomato, wheat, dwarf French bean, barley and maize, and no significant differences were found between inorganic control and water control (Blunden, 1997). There are few scientific reports on

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the effects of seaweed extracts on insects. Plants treated with ANE exhibited resistance to the two-spotted red spider mite, *Tetranychus urticae* (Hankins and Hockey, 1990). When ANE was applied to broad beans, black bean aphid, *Aphid fabae* the population was reduced (Stephenson, 1966). Cytokinin containing ANE applied as a foliar spray resulted in heavier *Spodoptera exigua* larvae (Reitz and Trumble, 1996). As the fecundity of *S. exigua* larvae is correlated with body mass (Rothschild, 1969), it suggests that ANE could increase *S. exigua* pest populations (Reitz and Trumble, 1996). Similar effects were observed in no-choice experiments where significantly more GPA was found on Arabidopsis plants following ANE treatment.

The second set of experiments focused on whether or not ANE increases antixenosis mediated resistance in Arabidopsis when infested with GPA. No effect on GPA settling was observed in choice experiments. Initially, all five treatments (water, inorganic control and ANE-1.0, 2.0 and 3.0 g/L) were used in a multiple-choice feeding-preference experiment. There were no significant effects on GPA settling (sections 4.4.3) following either foliar or soil drench application of ANE. Because high variability was observed in these multiple-choice feeding preference experiments, subsequently a paired choice experiment was conducted using 1 g/L concentration of ANE and an inorganic control. However, there were also no significant effects on GPA settling following ANE treatment in the paired experiments. Gene expression studies were conducted to determine the mechanisms behind the phenotypic observations. *PAD4* gene, which is associated with camelexin synthesis, showed antixenosis mediated resistance in Arabidopsis against GPA (Pegadaraju et al., 2007; Pegadaraju et al., 2005). No differences in expression of *PAD4* gene were observed in ANE treated plants as

compared to controls. This is in agreement with phenotypic observations in the choice experiments. There were no significant differences in GPA preference to ANE treated plants as compared to control plants. However, there is some evidence of ANE affecting antixenotic resistance with other insects. Cytokinine containing ANE increased feeding preference for tomato foliage by *Spodoptera exigua* larvae following root application, although foliar application did not alter the feeding preference (Reitz and Trumble, 1996). In another study, ANE deterred the herbivorous snail, *Littorina littorea,* when it was provided an artificial diet containing polyphenols (Geiselman and McConnell, 1981). ANE has polyphenol compounds in its extracts, which might provide chemical defenses against herbivores (Geiselman and McConnell, 1981).

Since neither antibiosis nor antixenosis mediated resistance was observed in Arabidopsis infested with GPA, the next sets of experiments focused on plant tolerance. The plant tolerance study consisted of time course analyses of fresh weight (g), chlorophyll a, b, and total chlorophyll (μg/ mg fresh weight). There were no significant differences in chlorophyll content between ANE and control plants at any harvest time. However, treated plants maintained good biomass under GPA infestation. Since the control treatment contained all the same inorganic mineral constituents as ANE, it is likely that the effects are largely due to the organic fraction of the extracts. This might be associated with beneficial growth due to plant growth regulators present in ANE. In the gene experiments, higher cytokinins were observed following ANE treated GPA infested plants at 24 h , and these are considered to be one of the plant growth regulators activates following ANE treatment (Khan et al., 2011). Cytokinins play a major role in reducing chlorophyll loss under stress (De Oliveira et al., 2008), and delaying the senescence

process (Gan and Amasino, 1995). A gene (*IPT*) encoding for isopentenyltransferase enzyme which is involved in cytokinin biosynthesis, was studied using a senescence specific promoter P_{SAG12}-*IPT* in tobacco plants and it was reported that endogenous cytokinins can regulate the leaf senescence (Gan and Amasino, 1996). Betaines are also present in seaweeds and increased the chlorophyll content of tomato, wheat, barley and maize (Blunden, 1997).

In summary, increased GPA numbers could be partially due to delayed senescence in Arabidopsis. ANE treated plants had a decrease in cell death, and suppression of *SAG* genes. ANE effect on chlorophyll was not statistically significant. However, suppression of chlorophyllase gene 1 (*CLH1*) was observed. Decreased cell death and suppression of *SAG* genes suggests that ANE may delay plant senescence, resulting in increased GPA numbers in treated plants. Senescence is one of the key characteristics which enhance resistance to GPA (Pegadaraju et al., 2005). The Arabidopsis mutant *PAD4* had delayed senescence and had decreased cell death due to GPA feeding (Pegadaraju et al., 2005). In contrast, the hypersenescent mutant, Arabidopsis constitutive expresser of *PR* gene5 (*CPR5*) had greatly reduced aphid numbers, while exhibiting a much higher degree of visible cell death (Pegadaraju et al., 2005). Delaying leaf senescence may be one way of recovery from insect herbivory (Meyer, 1998). This paralleled the GPA recovery experiment and it was found that significantly high seed yield was observed in ANE applied plants. Less cell death due to GPA infestation in ANE treated plants may result in faster recovery than control.

CHAPTER 5: GENERAL CONCLUSIONS

Minimizing losses caused by insect pest would increase crop production; an important goal to feed the increasing population without increasing the use of arable land area (Oerke and Dehne, 2004). Traditional use of pesticides is still important in pest management, but the usefulness of pesticides in agriculture is limited by insect resistance and potentially harmful effects on the environment. Use of biological elicitors to induce resistance in crop plants has potential to control insect pests. *Ascophyllum nodosum* extracts (ANE) contains several elicitors that activate plant defences (Vera et al., 2011). Further, ANE enhances the growth and development of crop plants and imparts resistance to biotic and abiotic stresses (Khan et al., 2009). The objectives of this research were to evaluate the effect of ANE on growth of plants and then examine the effect of ANE on resistance/ tolerance of Arabidopsis towards GPA, a plant-insect model. I also wanted to examine the molecular changes in Arabidopsis as affected by ANE treatment on GPA infested plants.

There was a significant effect of ANE on dry weight and length of the inflorescence and dry weight of siliques following leaf treatment. Fresh weight, dry weight and length of inflorescence did not change following soil drench application of ANE. However, ANE application through both soil and foliage at the same time might be a more effective method of application. Since an inorganic control was used, increase in seed yield may be an effect of organic compounds present in the extracts. This might possibly be due to different plant growth regulators which are acting at different stages of growth in the Arabidopsis plant. Therefore detail quantitative chemical analysis of plant

growth regulators following treatment with ANE would facilitate to clear understanding effects of ANE on plants. Further, the results of this study can lead to further investigation in the future, to evaluate the field efficacy of ANE application on economically important crop species.

In the second set of experiments, The ANE effect on GPA was evaluated through three different insect resistance mechanisms; antibiosis, antixenosis and tolerance. ANE did not induce either antibiosis or antixenosis mediated resistance in Arabidopsis against GPA. Moreover, ANE treated plants exhibited a greater number of colonizing GPA. However, ANE enhances tolerance to biomass loss following GPA infestation. Since inorganic control was used, enhanced growth effect might be an effect of organic compounds present in the ANE extracts. Therefore, further research will be required to determine the different organic compounds in ANE which lead to enhance the plant tolerance to insects. This will provide a better strategy to manage insect populations in agriculture fields without increasing the resistant in insect pest populations. Further this could help to develop new seaweed extract products for eco-friendly management of insect pests.

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APPENDIX A: MINERAL COMPOSITION OF LONG ASHTON SOLUTION

(LANS) AND INORGANIC CONTROL

Compound	LANS 0.1x	Modified LANS (inorganic control) 0.1x
	Final conc'n (ppm=mg/L)	Final conc'n (ppm=mg/L)
KNO_3	K = 15.6 N = 5.6	K = 15.6 N = 5.6
$Ca(NO_3)_2\ 4H_2O$	Ca = 16.6 N = 11.2	Ca = 3.3 N = 2.2
$MgSO_4\ 7H_2O$	Mg = 3.6 S = 4.8	Mg = 3.6 S = 4.8
$NaH_2PO_4\ H_2O$	P = 4.1 Na = 3.1	P = 4.1 Na = 3.1
Fe-citrate H_2O	Fe = 0.28	Fe = 0.28
$MnSO_4\ H_2O$	Mn = 0.05	Mn = 0.05
$CuSO_4\ 5H_2O$	Cu = 0.003	Cu = 0.003
$ZnSO_4\ 7H_2O$	Zn = 0.013	Zn = 0.013
H_3BO_3	B = 0.032	B = 0.032
$Na_2MoO_4\ 2H_2O$	Mo = 0.005	Mo = 0.005
NaCl	Cl = 0.36 Na = 0.23	Cl = 0.36 Na = 0.23
$CoSO_4\ 7H_2O$	Co = 0.0012	Co = 0.0012
KCl	-	K = 65 Cl = 60
KOH	-	K = 65

Source: Acadian Seaplants Limited, Dartmouth, Canada