Ascophyllm nodosum Extracts Improve Shelf Life and Nutritional Quality of
Spinach (*Spinacia oleracea* L.)

by

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "*Ascophyllm nodosum* Extracts Improve Shelf Life and Nutritional Quality of Spinach (*Spinacia oleracea* L.)" by Di Fan in partial fulfillment of the requirements for the degree of Master of Science.

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To

my parents, grandparents, and my boyfriend

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Abstract

In order to develop an environmentally friendly seaweed extract treatment which will benefit both pre- and post-harvest qualities of vegetables, the effects of pre-harvest application of the brown algae *Ascophyllum nodosum* extracts on the nutritional quality and post-harvest storability of spinach (*Spinacia oleracea* L.) was investigated. Plants treated with *A. nodosum* extracts accumulated higher concentrations of iron, potassium, total soluble protein, and total phenolics as compared to untreated controls. ^{1}H NMR and LC-MS analysis revealed a roughly 50% enhanced accumulation of the 9 flavonoids identified, which is partially confirmed by the elevated chalcone isomerase activity. *A. nodosum* extract treatment caused an increase in transcription of the genes related to plant growth, osmolyte accumulation, and antioxidative activities. Post-harvest analysis revealed that *A. nodosum* extract treatment caused an enhanced storability of spinach leaves in terms of visual quality, weight loss, and senescence. Lipid peroxidation and ascorbate content were correlated with visual quality during storage. Animal experiments using the *Caenorhabditis elegans* nematode model revealed that spinach extracts prolonged the life span of *C. elegans*, and *A. nodosum* extract-enhanced polyphenols exerted improved beneficial effects in *C. elegans* against oxidative and heat stresses. Taken together, the results suggest that *A. nodosum* extracts enhance both pre- and post-harvest quality of spinach through stimulation of flavonoid pathways, thus leading to accumulation of flavonoids and promotion of anti-radical capacity in spinach leaves, which may protect the plant tissue against reactive oxygen species and subsequent decay. Furthermore, the increased flavonoid content in spinach exerted beneficial effects in *C. elegans* against oxidative and heat stresses via different mechanisms.

Acknowledgements

Chapter 1

Introduction

Fruit and vegetables are essential for human nutrition and health. In addition to the major nutrients such as carbohydrate, protein, fat, and minerals, fruit and vegetables are also abundant in a number of essential phytochemicals such as ascobate, carotenoids, phenolics, glucosinolates and phytosterols (Kris-Etherton et al. 2002). Some of these phytochemicals possess antioxidant as well as antimicrobial or hormonal activities (Lampe 1999). Numerous epidemiological studies have shown that a diet rich in fruit and vegetables has beneficial effects on human health that reduce the risk of certain diseases like cardiovascular disease, heart disease, Alzheimer's disease, and some forms of cancers (Kang et al. 2005).

Fruit and vegetable production is an important part of the agricultural economy. An annual farm-gate value of $800 million worth of fruit and vegetables are produced commercially in Canada (http://www.thecanadianencyclopedia.com). However, the total production of fruit and vegetables are greatly reduced due to various biotic (e.g. fungi and pests) and abiotic stresses (e.g. drought, salinity, and air pollution), improper handling and poor storage conditions. Therefore, there have been many efforts such as conventional breeding and genetic engineering (Dalal et al. 2006) to improve crop yield and quality and to reduce post-harvest losses.

1.1 Oxidative Stress

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical ($^\cdot OH$) and superoxide ($O_2^{\cdot -}$), are produced in the respiratory chain in the mitochondria and are byproducts of normal cellular metabolism (Finkel and Holbrook 2000). Perturbed metabolism leads to accumulation of toxic levels of ROS, and oxidative stress results when the rate of generation of ROS exceeds the rate of their disposal (Hodges et al. 2004). Both biotic (e.g. pathogens) and abiotic factors (e.g. extreme temperature, light, storage duration, processing methods, and conditions accelerating water loss or/and ripening) can trigger excess production of ROS under growth and post-harvest conditions (Hodges 2003). Excessive ROS can react with various cellular components such as lipids, carbohydrates, proteins and nucleic acids,

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leading to lipid peroxidation, protein denaturation and mutagenesis (Hodges 2003). Senescence, a slow progress of cell death often symbolized by deterioration of cellular membranes, is largely related to excess ROS (Lurie 2003). Aerobic organisms including plants have developed antioxidant defense systems to regulate the concentrations of ROS in the cell (Hodges and DeLong 2007). These are generally composed of three classes: membrane-associated antioxidants (e.g. carotenoids and tocopherols), hydrophilic reductants (e.g. ascorbate, glutathione and flavonoids), and enzymatic antioxidants (e.g. catalase, superoxide dismutase, and enzymes of ascbate-glutathione cycle) (Lurie 2003).

If free radicals are not scavenged by antioxidants in animals, they may lead to aging and onset of stress-related diseases such as cancers, cardiovascular diseases, insulin resistance, and cataracts (Houstis et al. 2006; Lampe 1999). Thus, antioxidative phytochemicals in fruit and vegetables, such as carotenoids and polyphenols, provide protective effects against ROS damage. It should be noted that some compounds with antioxidative activities such as polyunsaturated fatty acids (PUFAs) and polyphenols may become transient radicals and can be regenerated by other antioxidants (Niki et al. 1995).

1.2 Health Effects of Bioactive Compounds

An inverse relationship between the consumption of fresh fruit and vegetables and numerous diseases has been demonstrated in many intervention studies. For example, studies have shown the relationship between a high risk of cardiovascular disease or certain kinds of cancer with a low intake of β-carotene, or flavonoids (Kritchevsk 1999; Neuhuser 2004; Arts and Hollman 2005). Epidemiological studies also revealed that whole fruit and vegetables were more efficient than its purified chemical component in reducing the risk of diseases (e.g. Holick et al. 2002). The difference between fresh whole fruit and vegetables with pure chemical component supplementation may be due to the interaction between bioactive components in the whole fruit and vegetables (Burri 1997). Moreover, many phytochemicals have recondite effects on human health which is dependent on their doses and this may in part explain the conflicting findings from epidemiological studies. More detailed and unbiased intervention trails are needed to

measure the beneficial effects and/or the optimum dosage of certain phytochemicals on humans.

1.3 Effects of Bioactive Compounds in Fresh Produce

Besides the positive effects on human health, bioactive compounds themselves may affect shelf life of fresh produce. Bergquist et al. (2006) found that the baby spinach, when harvested a few days earlier, had higher ascorbic acid, exhibited improved visual quality and better nutritional value during storage. Higher concentration of antioxidants at harvest ensured the ability of apples to reduce oxidative stress during subsequent storage (Hodges et al. 2004). It may thus be postulated that high concentrations of antioxidants can better protect fresh product against oxidative stress and the onset of senescence. Thus increasing the content of bioactive compounds in fruit and vegetables at harvest may not only have beneficial effects on human health, but may also improve the appearance, prolong shelf life, and reduce post-harvest losses of fresh produce.

1.4 Factors that affect Bioactive Compounds

The chemical composition of fruit and vegetables is affected by a number of pre- and post-harvest factors. The effects of some of the factors on certain compound(s) are clear, but others remain obscure.

1.4.1 Genetic Variation

Some of the bioactive compounds in fruit and vegetables are ubiquitous while others are unique to specific families, species, or even cultivars. Ascorbic acid and phenolic compounds are found in many fruit and vegetables but the concentrations vary among them (Kevers et al. 2007; Lin and Tang 2007). Flavonoid content has been shown to differ among spinach genotypes (Cho et al. 2008), and the flavonoid glycosides identified in spinach are rare and some are not present in other vegetables (Bergquist et al. 2005). Thus genetic factors have significant influences on the composition of bioactive compounds in fresh products.

1.4.2 Pre-harvest Factors

A number of pre-harvest factors such as temperature and light intensity during growth, water supply, and soil characteristics, affect the concentration of bioactive compounds in fruit and vegetables (Ferguson et al. 1999). For example, environmental conditions, such as air temperature (Lefsrud et al. 2005) and light condition (Bergquist et al. 2007a), affect the chemical composition in spinach. Shade netting decreased ascorbic acid concentration but increased the carotenoid content in spinach (Bergquist et al. 2007b). Shading the whole trees decreased the ascorbic acid in the peel and flesh of apple (Li et al. 2009). Nutrient availability and soil type may also affect bioactive compounds (Weston and Barth 1997).

Growth stage and maturity also affect the concentration of bioactive compounds. Mid-mature spinach leaves have higher total phenolics and flavonoids than immature and more mature leaves (Pandjaitan et al. 2005), whereas the highest ascorbic acid content was found in immature leaves (Bergquist et al. 2006). Carotenoid and/or flavonoids accumulate during fruit ripening to provide color to the ripe fruit (Kalt 2005). Reyes et al. (2007) reported that mechanical injuries at harvest may increase antioxidant content in fresh-cut produce. Moreover, the harvest time during the day may result in significant changes in the concentrations of bioactive compounds, possibly related with light intensity and water content (Veit et al. 1996).

1.4.3 Post-harvest Factors

The post-harvest quality of fruit and vegetables is dependent, in part, on the rate of depletion of food reserves and water, and can be influenced by factors like method of harvesting, CO_2 and O_2 partial pressure, ethylene, water vapour pressure, microbial decay, bruising, storage temperature and duration of storage (Kader 2002). Postharvest losses result from physiological changes accelerated by abusive conditions, such as improper storage conditions, low atmospheric humidity and high temperatures. Ascorbic acid decreases rapidly during storage in many plant produce while flavonoids and carotenoids are generally stable (Kalt 2005; Kevers et al. 2007). A lower storage temperature (2 °C) resulted in a smaller reduction in both visual quality and ascorbic acid content of baby spinach, probably due to the decrease in metabolic rates (Bergquist et al.

2006). Light condition is also of great importance, though affects in different ways (Bergquist et al. 2007b; Toledo et al. 2003).

In addition to physiological deterioration, diseases/decay caused by microorganisms as well as insect pests (i.e. biotic stress) also lead to considerable postharvest loss of fresh produce (Kader 2002). Chemical fungicides are often applied to reduce postharvest fungal decay; however, indiscriminate use of fungicides has led to the development of resistance in pathogen populations, as well as increasing consumer concern over pesticides residues in foods. Thus alternative methods such as thermal processing (Kader 2002), carbon dioxide treatments (Retamales et al. 2003), and irradiation treatments (Kader 2002) have been developed and/or increasingly used. Moreover, in order to compensate for limitations of these non-fungicidal treatments, natural products such as chitosan (Meng et al. 2008) and glucosinolates (Tripathi and Dubey 2004) have been exploited for effective and safe control of postharvest diseases, thus prolonging shelf life of fresh produce.

Unfavourable storage conditions can exacerbate oxidative stress. For example, less-than-optimal storage temperatures can induce oxidative stress, leading to such dysfunctions as loss of membrane integrity and accelerated senescence. When fresh produce was packaged or treated to reduce water loss, the development of chilling injury was repressed (Hodges et al. 2004). Controlled atmosphere storage has also been shown to influence antioxidant levels; for example, low O_2 levels impeded senescence by slowing down the rate of oxidative respiration in the mitochondria (Hodges and DeLong 2007). Besides temperature treatments, altering storage atmosphere, and chemical treatments such as cytokinin and ethylene application, soaks of exogenous antioxidants and coatings of edible materials which exert physical protection are also employed to reduce oxidative-associated injury (Toivonen 2003). As stated above, the concentration of bioactive compounds at harvest in fresh fruit and vegetables may as well play an important role in storability.

1.5 Nutrition

1.5.1 Ascorbic acid

L-ascorbic acid (vitamin C) is a ubiquitous sugar acid that exists in all cell compartments of plants (Debolt et al. 2007) with an intracellular concentration which is often in the mM range. When L-ascorbate performs its reducing (i.e. antioxidant) action, it is converted to oxidized ascorbate (L-dehydroascorbate; DHA). It can then be reduced back to ascorbate through a variety of enzymic and non-enzymic pathways (Hodges 2003).

Ascorbate has been characterized as a highly effective antioxidant, an enzyme cofactor for the biosynthesis of many biochemicals, and an electron donor/acceptor (Davey et al. 2000). Ascorbate also acts as a precursor in the formation of organic acids in plants (Debolt et al. 2007), and are involved in the regulation of cell division and elongation (Smirnoff 1996). Ascorbate is synthesized in plants through three different pathways (Valpuesta and Botella 2004).

Ascorbate is a common metabolite in plants, but it is an essential nutrient in humans as we lack the ability to biosynthesize it (González et al. 2005). Vitamin C deficiency causes physiological disorders such as scurvy, and in extreme cases can lead to morbidity. Vitamin C is required for the synthesis of collagen and neurotransmitter, and also has other biological activities in human body such as protecting plasma lipids through its radical scavenging activity (González et al. 2005) as well as enhancing the immune system. Since ascorbate is labile, it is often regarded as an indicator of the quality of fresh produce during postharvest storage (Podsędek 2007).

1.5.2 Phenolics

Phenolic compounds are widespread secondary metabolites in plants. Sub-classes include flavonoids, tannins, hydroxycinnamate esters and lignin. Phenolics possess a number of beneficial activities like antioxidant activity, capillary protective effects, and can inhibit malignant tumors (Podsędek 2007). They are efficient in quenching ROS due to its electron donor activity (Podsędek 2007). Polyphenols are considered as one of the major nutritional components for the claimed pharmacological benefits ascribed to fruit and vegetables.

Flavonoids are the most diverse group of polyphenols and are consist of a basic C_6-C_3-C_6 flavone skeleton. Six classes of flavonoids are widespread in most higher plant, and include the chalcones, flavanones, flavandiols, flavones, anthocyanins, catechins, and condensed tannins (Winkel-Shirley 2002). Flavonoids play roles in plants, such as pigmentation, protection against UV-radiation, reproduction, and regulation of plant growth (Winkel-Shirley 2002).

Flavonoids have significantly higher ROS scavenging activity, as compared to vitamin C and carotenoids (Podsędek 2007). Its antioxidative activity is dependent on the chemical structure, such as the number of hydroxyl groups substituted on the B ring (Rice-Evans et al. 1996). Intake of flavonoids have been associated with reduced incidences of cancer, heart disease, and various neurological disorders (Hodges and Kalt 2003). The beneficial effects exerted by fruit and vegetables on human health are, in part, associated with flavonoids. Spinach is a flavonoid-rich vegetable abundant in unique flavonoids glycosides that are not present in most fruits and vegetables (Edenharder et al. 2001).

1.5.3 Carotenoids and Tocopherols

Carotenoids are yellow-orange lipophilic pigments concentrated in chloroplast membranes and chromoplasts. Carotenoids function as accessory pigments in photosynthesis and protect photosynthetic reaction centers from light-induced oxidative damage (Paiva and Russell 1999). Carotenoids arise from 5-carbon isoprene units which are enzymatically polymerized to form 40-carbon structures with up to 15 conjugated double bonds. They are grouped into two classes, xanthophylls (oxygenated carotenoids, such as lutein, violaxanthin, zeaxanthin, and neoxanthin) and carotenes (pure hydrocarbons, such as α- and β-carotene and lycopene). Carotenes are the most common carotenoid in fruit and vegetables (Paiva and Russell 1999). About 10% carotenoids found in fruit and vegetables are precursors of vitamin A.

Carotenoids inhibit oxidation via ROS quenching activities. In animals, carotenoids are obtained *via* foods since they cannot be synthesized endogenously. Carotenoids have been shown to enhance immune response, eye health, and protect against DNA damage. Epidemiologic studies have demonstrated that higher intake of β-

carotene in non-smokers reduced the risk of some forms of cancer, particularly lung cancer (Cardozo et al. 2007). Besides β-carotene, lutein is also abundant in spinach. Lutein reduces the onset of cataract and macular degeneration in humans, possibly due to its role in the protection of oxidative stress and high-energy light (Ribaya-Mercado and Blumberg 2004).

Tocopherols are vitamin E analogues and occur in nature as 4 structurally related forms, α-, β-, γ-, and δ-tocopherol. They are membrane-soluble antioxidants and play an important role in integrity of cell membrane (Christen et al. 1997). γ-Tocopherol is the predominant form of vitamin E consumed. Besides antioxidant activity, tocopherols also show antiproliferative effects, anticlotting and immunoprotective functions (Dowd and Zheng 1995; Meydani 1995; Tasinato et al. 1995).

1.5.4 Non-Antioxidant Nutrients

Magnesium, one of the element deficient in human diets, is important in the regulation of blood pressure, improvement of serum lipid profile, prevention of stroke, and skeletal growth and development (Champagne 2008). Potassium is primarily used as a fertilizer as nitrate and chloride in agriculture, and exerts various vital roles in plant such as a cofactor for enzymes and the regulation of osmotic balance. As an essential mineral macronutrient in human diet, potassium is essential for neuron function, muscle contraction, maintaining fluid balance, and reducing the incidence of hypertension as well as heart disease (http://en.wikipedia.org/wiki/Potassium). Iron and zinc are important micronutrients in human diet. Iron is involved in psychomotor development, maintenance of physical activity, and resistance to infection, while zinc is needed for catalysis, stabilization of cell membranes and regulation of gene expression (Black 2003).

1.6 *Ascophyllum nodosum* (L.) Le Jol

Seaweeds are multicellular marine algae that consist of three major groups: the brown, green, and red algae. In addition to labile antioxidants such as ascorbate and glutathione, seaweeds also contain more stable molecules such as carotenoids, polyphenols, mycosporine-like amino acids, halogenated compounds, and polyketides (Cardozo et al. 2007). Seaweed possesses antimicrobial activity against bacteria, yeast

and moulds (Cardozo et al. 2007). It has been well-documented that the treatment of seaweed extracts of terrestrial crops can provide enhanced germination and seedling establishment, increased root growth (Metting et al. 1990), increased nutrient uptake, improved resistance to disease (Featonby-Smith et al. 1983), and improved resistance to abiotic stresses (Zhang and Ervin 2004).

Ascophyllum nodosum is a large, brown alga, extending from the zone of northern Atlantic Ocean (e.g. Nova Scotia) to Norway. It is often used as a fertilizer, animal feed supplement and also as a human nutritional supplement. *A. nodosum* is rich in minerals, cytokinins, betaines, polyamines, organic acids, oligosaccharides, amino acids, and proteins (Khan et al. 2009). Foliar and soil applications of *A. nodosum* extracts have been demonstrated to increase endogenous antioxidant activity and subsequent stress tolerance of several turfgrasses (Zhang and Ervin 2004). Application of ANE has been shown to increase the yield of cauliflower, lettuce, and maize (Abetz and Young 1983; Jeannin et al. 1991). Few investigations have been carried out into the potential use of ANE to increase nutritional quality and to minimize post-harvest losses in fruit and vegetables. Blunden et al. (1979) reported an increase of root sugar in sugar beet following application of ANE. Abdel-Hafeez (2005) showed that weight, firmness, total soluble solids and total acidity of pear fruit are notably increased by pre-harvest spraying with commercial ANE, and fruit decolouration was also delayed. Pre-harvest application of ANE significantly reduced nectarine weight loss and decay incidence during post-harvest handling (Norrie and Hiltz 1999).

1.7 *Spinacia oleracea* L.

Spinach (*Spinacia oleracea* L.) was first grown in Persia, southwestern Asia, about 2000 years ago, and was introduced to China as an herb in 647 B.C. and to Europe in the 12th century (Barzegar et al. 2007). Spinach is an important green, leafy vegetable that belongs to the Goosefoot family (Chenopodiaceae), and is cultivated in the temperate regions (Barzegar et al. 2007) (Fig. 1.1A). It is usually marketed fresh in polypropylene bags and is high in ascorbate, β-carotene, lutein, flavonoids, magnesium, folate, iron, potassium and unsaturated fatty acids (Gil et al. 1999; Barzegar et al. 2007). Spinach is highly perishable and should be stored at low temperatures, such as 4 °C, to slow down

its deterioration. In the present studies, we used the cultivar Unipack 12, a commercial processing hybrid which has been demonstrated to tast great (with a lower metallic oxalic acid), has round and smooth leaves, and demonstrates good bolt resistance.

1.8 *Caenorhabditis elegans*

Caenorhabditis elegans is a lucent free-living soil nematode about 1 mm in length. It has become an important model organism for basic biological research due to easy culture conditions, short generation time (3 days) and short lifespan (2-3 weeks) (Kaletta and Hengartner 2006) (Fig. 1.1B). *C. elegans* is being used to study the theory of aging (Golden et al. 2002), nutrient sensing pathyways (Walker et al. 2005), the mechanisms of pharmacology (Kaletta and Hengartner 2006), and fat metabolism (Mullaney and Ashrafi 2009). *C. elegans* and humans share similar biological processes in aging (Herndon et al. 2002) and oxidative stress has been recognised as a major limiting factor in lifespan (Finkel and Holbrook 2000). Thus *C. elegans* is highly-valued for identifying compounds, genes, and mechanisms that may extend the longevity of humans.
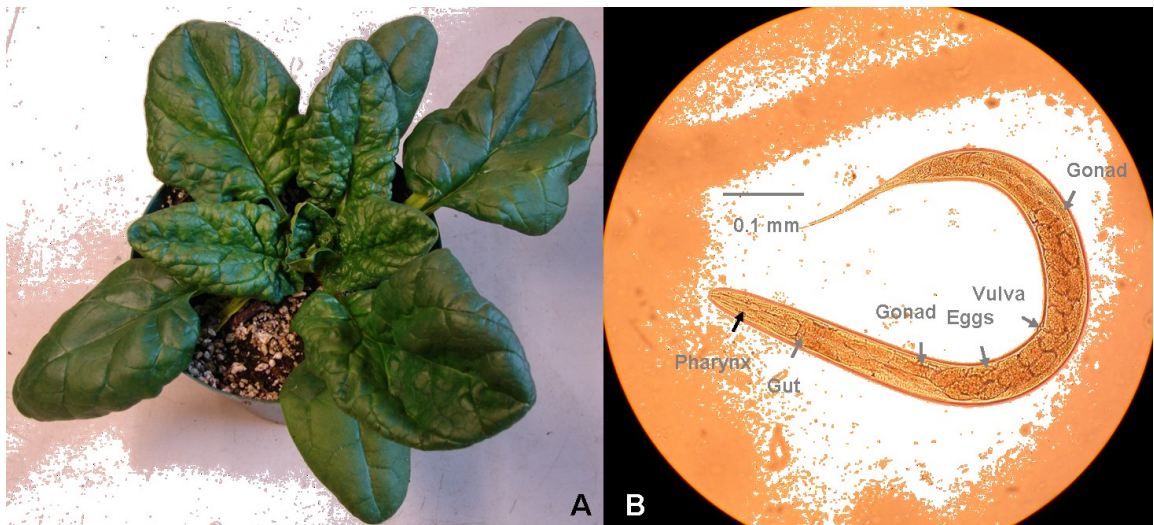
Figure 1.1. (A) 49-day-old spinach in growth chamber and (B) Light microscopic image of 5-day-old *C. elegans*.

1.9 Project Hypothesis

♦ *Ascophyllum nodosum* extracts improve the nutritional value and post-harvest quality of spinach (*Spinacia oleracea* L.)

1.10 Objectives

♦ To investigate the effects of *A. nodosum* extracts on nutritional quality of harvested spinach

♦ To determine the effects of *A. nodosum* extracts on storage quality of post-harvest spinach

♦ To study the effects of *A. nodosum* extracts on enzyme activities and gene expression in spinach

♦ To study the nutritional properties of *A. nodosum* extract treated spinach using *C. elegans* model

Chapter 2

Quality attributes of *Spinacia oleracea* L. at harvest as affected by *Ascophyllum nodosum* extract

2.1 Abstract

There is considerable interest to enhance the nutritional quality of fresh produce especially vegetables. There are very few studies on the effects of commercial extracts of the brown macro algae, *Ascophyllum nodosum* (ANE) on nutritional quality of vegetables. The effects of root treatment of spinach with ANE on nutritional qualities of spinach at harvest were investigated. At the concentration of 1.0 g/L, ANE-treatment significantly increased the content of K and Fe, total phenolic and flavonoids content, and total antioxidant activities in spinach leaves. For example, a 1.25-fold increase in the phenolic content and total antioxidant activity (as measured by DPPH (2,2-diphenyl-1–picrylhydrazyl) radical scavenging capacity) was observed. The total ascorbate and oxalate concentration was not affected. Moreover, the ^{1}H NMR metabolite profile of ANE-treated plants showed no significant differences by comparison with untreated control. The increased antioxidant activity appears to be largely associated with flavonoids, a result supported by ^{1}H NMR and LC-MS analyses. HPLC-MS analysis and 2D NMR spectra revealed that there were 9 major flavonoids in Unipack 12 cultivar and that the flavonoid content of plants treated with 1.0 g/L ANE increased by roughly 50%. Taken together, the results presented in this chapter suggest that ANE application can improve the nutritional content of spinach and may induce an adaptive response in spinach through stimulation of flavonoid synthesis.

2.2 Introduction

Reactive oxygen species (ROS) such as H_2O_2 and hypochlorous acid (HOCl), act as signaling molecules in cellular processes when at low concentration. Oxidative stress results when excessive amount of ROS are produced (Hodges et al. 2004). Excessive

ROS react with macro-molecules such as polyunsaturated fatty acids, proteins, and DNA, leading to oxidative degradation and subsequent senescence, diseases, and accelerated aging (Gutteridge and Halliwell 2000; Hodges and Lester, 2006). Plants have antioxidant defense systems including membrane-associated antioxidants, hydrophilic reductants, and oxidative enzymes to protect against free radical damage via scavenging radicals and to prevent the initiation of radical formation (Hodges and DeLong, 2007).

Considerable epidemiological studies exist for the role of a wide range of antioxidative constituents, such as vitamin C, vitamin E, β-carotene, and phenolic acids, present in fruits and vegetables in the maintenance of health. Also, antioxidants prevent a number of diseases associated with free radical attacks, such as cancer, cardiovascular and Alzheimer's disease (Ames et al. 1993; Hung et al. 2004; Wu 2006). Recently, there is increasing interest in the pharmacological effects of phenolic compounds present in foods such as black tea (Peng et al. 2009), grapes (Huntley 2007), strawberries and blueberries (Joseph et al., 1999), and spinach (Hait-Darshan et al. 2009). Besides the high antioxidative abilities, some researches suggest that part of the biological functions of polyphenols depend on their roles in modulating protein and lipid kinase signaling pathways (Williams et al. 2004).

Quality (appearance and nutritional value) of fresh produce is highly valued (Lampe, 1999). Conventional breeding and genetic engineering have been used to improve yield, quality, and resistance to environmental stresses in fruit and vegetables. For example, inter-specific crosses of strawberry were conducted to increase nutritional quality of the fruit (Capocasa et al. 2008). Transgenic tomato carrying anthocyanin-regulating snapdragon transcription factors, Del and RosI, enhanced the anthocyanin content in tomato fruits (Butelli et al. 2008). However, there are limitations to conventional breeding and the transgenic approach to increase the nutritional value of fruit and vegetables. Consumer's acceptance of transgenetic crops is becoming an issue, owing to concerns about the effect of transgenic products on human health. Therefore, improving the nutritional quality of fruit and vegetables via environmentally friendly methods would be an ideal approach.

Spinach (*Spinacia oleracea* L.) is a leafy green vegetable which contains high concentrations of bioactive phytochemicals and nutrients such as ascorbate, carotenoids,

tocopherols, phenolics, folate, and minerals (Gil et al. 1999). Ascorbic acid is a highly effective antioxidant involved in ROS scavenging and in the reduction of α-tocopherol radicals (Debolt et al. 2007). Polyphenols also possess antioxidant activities, and can regenerate ascorbic acid (Podsędek, 2007). Spinach contains large amounts of *p*-coumaric acid derivatives that exhibit strong antioxidant activity (Bergman et al. 2001). As one of the flavonoid-rich vegetables, spinach is also abundant in glucuronic acid derivatives and patuletin and spinacetin derivatives of flavonoids (e.g. patuletin and spinacetin) (Fig. 2.1) that are not common to most other vegetables (Pandjaitan et al. 2005). Spinach has traditionally been used as medicine in China. Spinach powder has been added in flour dough to enrich the nutrient levels in the product (Lee et al. 2002). Several researches have shown that spinach exert anti-aging, anti-inflammatory and anti-cancer properties in various biological systems (Bakshi et al. 2004; Joseph et al. 1998; Nyska et al. 2003). A recent study showed that green leafy vegetables, including spinach, lowered the hazard of diabetes among women (Bazzano et al. 2008).

Compound 1 "Patuletin" R1 = β-D-glucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = H; R3 = H
Compound 2 "Spinacetin" R1 = β-D-glucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = CH₃; R3 = H
Compound 3 "Patuletin" R1 = β-D-2" feruloylglucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = H; R3 = H
Compound 4 "Spinacetin" R1 = β-D-2" *p*-coumaroylglucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = CH₃; R3 = H
Compound 5 "Spinacetin" R1 = β-D-2"feruloylglucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = CH₃; R3 = H
Compound 6 "Spinacetin" R1 = β-D-glucosyl (1→6) β-D-glucoside; R2 = CH₃; R3 = H
Compound 7 "Jaceidin" R1= CH₃; R2 = CH₃; R3 = glucuronic acid

Compound 8 R1 = H; R2 = glucuronic acid
Compound 9 R1 = CH₃; R2 = glucuronic acid

Figure 2.1. Chemical structures of the nine major flavonoids in spinach (Pandjaitan et al. 2005)

Ascophyllum nodosum (L.) Le Jol., a perennial brown marine alga, is a widely-researched seaweed species traditionally used as a fertilizer, a soil conditioning agent, animal feed supplement and also as a human nutritional supplement. The application of brown seaweed extracts in agriculture and horticulture has been suggested to improve a

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number of physiological characteristics in plants, such as increased root growth, increased crop yield and performance, improved abilities to resist environmental stresses such as salinity and frost, and improved resistance to fungal diseases as well as insect infestation (Khan et al. 2009). A number of studies have shown that some of the physiological effects of *A. nodosum* extracts could be due to the elicitation of endogenous plant growth regulators such as β-D-(1,3) glucanases (Patier et al. 1993) and the up-regulation of antioxidant enzymes by the components present in commercial ANE such as betaines, hormone-like activities, oligosaccharides and laminarin (Patier et al. 1993; Whapham et al. 1993; Zhang and Schmidt 2000; Zhang and Ervin 2004). Although there are studies on increased productivity and nutritional quality via preharvest spraying with ANE (e.g. Abdel-Hafeez 2005; Fornes et al. 2002), there are only a few reports that deal with the effects of pre-harvest treatments of ANE on the physiological responses and nutritional quality parameters of harvested spinach (Cassan et al. 1992). Therefore, the present study is to investigate the impact of ANE on biomass production and nutritional qualities of spinach.

2.3 Materials and Methods

Seeds of spinach (*Spinacia oleracea* L., var. Unipack 12) were purchased from Stokes Seeds Co. (Thorold, ON, Canada). Soluble powder of *A. nodosum* alkaline extract (Acadian®) was obtained from Acadian Seaplants Limited (Dartmouth, NS, Canada). The organic and inorganic composition and elemental analysis of the extract is presented in Table 2.1 (Acadian Seaplants Limited, technical information). All the other chemicals and reagents were of analytical grade and were purchased from Sigma Aldrich (Oakville, ON, Canada), unless otherwise stated.

Table 2.1. The total organic and inorganic composition and elemental analysis of *Ascophyllum nodosum* extract (ANE).

Composition	ANE (dry powder)
Organic Matter	**45-55** %

Table 2.1. Cont.	
Alginic acid	12-16 %
Fucose Polymers	13-17 %
Mannitol	4-6 %
Amino Acids	4-6 %
Other Organic compounds	**10-12 %**
<u>Ash</u>	**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

2.3.1 Plant Culture and Treatment

Spinach seeds were planted at 1.5 cm deep in plastic pots (5" diameter; 3 seeds per pot) containing sweet soil (1 limestone:100 peatmoss:100 perlite:30 sand, w/v/v/v). Pots were placed in a growth chamber maintained at 18 °C and 95% relative humidity (RH) with a photo period of 10 h light/14 h dark under the light from fluorescent tubes and incandescent bulbs of approximately 350–400 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Fertilization with water-soluble $20N$-$20P_2O_5$-$20K_2O$ at the concentration of 200 ppm was initiated 3 weeks after planting and then applied every 4 days at the rate of 200 mL per pot. Plants were grown for 7 weeks.

Solutions of ANE were prepared by dissolving 0.1 g, 1.0 g, or 5.0 g soluble powder in 1.0 L distilled water with continuous stirring for 10 min. Spinach plants were irrigated with various ANE solutions (i.e. 0, 0.1, 1.0, or 5.0 g/L) at the rate of 50 mL per plant on day 14 and day 7 prior to harvest.

2.3.2 Preparation of Spinach Leaves

Leaves from the 5–8th positions counted from the bottom were cut at the petiole using a pair of sharp scissors and placed in plastic bags in a refrigerated cooler (4 °C) and

processed within 1 h of harvest. Leaf samples were de-ribbed and chopped into small pieces ($\approx$0.5–1 cm^2). Pieces were either thoroughly mixed and sub-samples immediately analyzed or flash-frozen in liquid nitrogen (N$_2$), freeze-dried and ground into a fine powder using a NQM-0.4 series planetary ball mill, and then stored in sealed plastic bags at –80 °C until analysis.

2.3.3 Plant Growth Assay

The growth of the control and ANE-treated spinach plants was measured in terms of appearance and leaf dry matter. Dry matter content (DMC) was calculated using the formula: DMC (%) = (DW/FW)$\times$ 100%, where FW is the fresh weight and DW is the dry weight. DW (g/100 g FW) was obtained after being heated in an oven set at 85 °C for at least 48 h.

2.3.4 Determination of Oxalic Acid

The total soluble oxalic acid (OA) concentration of spinach leaf tissues was determined following Xu and Zhang (2000) with some modifications. A fresh leaf sample (2.0 g) was extracted in 80 mL MilliQ water at 80 °C while shaken for 15 min. The extract was made up to 100 mL with MilliQ water in a volumetric flask. The suspension was centrifuged at 3,000$\times$g for 20 min and the supernatant was filtered through a Whatman No. 1 paper. The reaction mixture consists of 50 μL sample (or standard OA solution), 27.5 μL 1 mM bromophenol blue (BPB), 49.5 μL (1 M) sulfuric acid (H$_2$SO$_4$), 44 μL 100 mM potassium dichromate (K$_2$Cr$_2$O$_7$), and 1.2 mL MilliQ water. The mixture was vortexed and incubated in a water bath at 60 °C for 10 min, the reaction was quenched with 110 μL 2 M sodium hydroxide (NaOH). The absorbance was read at 600 nm against a blank (MilliQ water) in a spectrophotometer. OA concentration was expressed in mg/100 g FW by comparison with an OA standard curve (0.1–5 μg/mL).

2.3.5 Mineral Element Analysis

After harvesting, spinach leaves were washed twice with distilled water. Leaves were then de-veined, chopped into pieces, bagged in paper bags, and dried in an oven at 85 °C for 48 h.

The concentrations of minerals sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), Phosphorous (P), and boron (B) were determined by the AOAC method 968.08 (radial ICP) and for Nitrogen by the AOAC method 990.03 (LECO FP-528).

2.3.6 Determination of Total Protein

The extraction of total soluble protein was done using a methodology described by Gadzovska et al. (2007) with some modifications. Freeze-dried spinach leaf sample was extracted in 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 8.5), containing 5 mM 2-mercaptoethanol and 2% (w/v) polyvinlpyrrolidone (PVP), and centrifuged at 10,000×g for 20 min at 4 °C. The supernatant was collected for the determination of total protein concentration using the Coomassie Plus – The Better Bradford^{TM} Assay Kit (Pierce, Rockford, IL, USA). Bovine serum albumin (125–1500 μg/mL) was used as a standard.

2.3.7 DPPH Radical Scavenging Assay

The total antioxidant capacity of spinach leaves was determined using a DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate) assay following a method described by Brand-Williams et al. (1995) with minor modifications. The antioxidants react with DPPH$^\bullet$ and convert it to 1,1-diphenyl-2-picryl hydrazine with decoloration (from deep violet to light yellow). Fresh spinach leaf tissue (1.5 g) was homogenized in 15 mL pure methanol (MeOH) using a mortar and pestle. After centrifugation at 10,000×g for 10 min, the supernatant was recovered. The pellet was re-extracted with 10 mL MeOH. Supernatants were combined and the total volume was made up to 25 mL. The pellet was re-dissolved in 5 mL dichloromethane (DCM), homogenized and centrifuged at 10,000×g for 10 min. Pellet was re-extracted with 5 mL DCM, and supernatant was centrifuged, combined, and made up to 10 mL. Each extract was added to 2850 μL fresh DPPH$^\bullet$ solution (0.11 mM), and incubated for 6 h at 22 °C. Absorbance was then read at 515 nm against MeOH or DCM as a blank. The scavenging activity was calculated using the equation: Inhibition % = $[(A_b-A_s)/A_b]$ 100, where A_b is the absorption of blank sample and A_s is the absorption in the presence of test sample. The results were expressed in μM Trolox equivalents (TE, μM Trolox)/100 g FW through comparison against a Trolox standard curve (25–800 μM).

2.3.8 Ferrous Ion Chelating Ability

The Fe^{2+} chelating activity of spinach samples was determined according to Heimler et al. (2007). Each sample was diluted to 1.4 mL with 250 mM acetate buffer (pH 4.75) and mixed with 25 μL of 2 mM $FeCl_2$ and 1 mL of 70% MeOH. After 20-min incubation at room temperature, 100 μL of 5 mM ferrozine was added to initiate the reaction. The mixture was shaken vigorously, and the absorbance was read after 10 min at 562 nm against a blank (70% MeOH). The ability of disrupting the formation of the ferrozine-Fe^{2+} complex was calculated as follows: chelating activity % = $[(A_b-A_s)/A_b]$ 100, where A_b is the absorbance of the blank sample and A_s is the absorbance of the test sample. Ethylenediaminetetraacetic acid (EDTA) at 0.5 mM was used as a positive control.

2.3.9 Determination of Total Ascorbate

Total ascorbate was analyzed following the method of Hodges and Lester (2006) using L-ascorbic acid as a standard. MilliQ water was used throughout the assay. Fresh chopped leaves (5.0 g) were immediately ground in a mortar and pestle with inert sand and 15 mL ice-cold, freshly prepared 5% (w/v) *m*-phosphoric acid. The homogenate was centrifuged at 10,000×g for 15 min at 4 °C. Total ascorbate was determined by initially incubating 100 μL supernatant, 500 μL 150 mM KH_2PO_4 buffer (pH 7.4) containing 5 mM EDTA, and 100 μL 10 mM dithiothreitol (DTT) at room temperature for 50 min. Then 100 μL of 0.5% (w/v) N-ethylmaleimide (NEM) was added to remove excess DTT. In order to develop color in reaction mixtures, reagent solutions were added in the following order 400 μL 10% (w/v) trichloroacetic acid (TCA), 400 μL 44% *o*-phosphoric acid, 400 μL 4% (w/v) α-α^1-dipyridyl, and 200 μL 30 g/L ferric chloride ($FeCl_3$). The reaction mixtures were incubated at 40 °C for 60 min in a shaking water bath (Julabo Labortechnik, Seelbach, Germany), and absorbance was read at 525 nm. The results were expressed as μmol/g FW.

2.3.10 Determination of Total Phenolics

The total phenolic content was analyzed using the Folin-Ciocalteu method as described previously by Singh et al. (2002) with slight modifications. The fresh spinach

leaf tissues were extracted twice with 70% (v/v) aqueous MeOH at 40 °C for 2 h, and the extracts were centrifuged and combined. The extract was pipetted into 2 mL glass tubes and to each 1.58 mL water and 100 µL 2 N Folin-Ciocalteu reagent was added. After 8 min-incubation, 300 µL 20% (w/v) sodium carbonate (Na_2CO_3) was added to stop the reaction. The vortexed mixture was left at room temperature in dark for 2 h and the absorbance was read at 760 nm against a blank (70% MeOH). A calibration curve (20–500 mg/L) was prepared using the procedure as described above. Total phenolics were expressed as gallic acid equivalents (GAE, mg gallic acid/100 g FW).

2.3.11 Determination of Total Flavonoids

Total flavonoid content in spinach leaves was quantified following a colorimetric method described by Liu and Zhu (2007). The spinach extracts (in 70% MeOH) was mixed with 45 µL of 5% (w/w) sodium nitrite ($NaNO_2$) and 750 µL of 30% (v/v) ethanol (EtOH). After incubation at room temperature for 6 min, 45 µL of 10% (w/w) aluminium trichloride ($AlCl_3$) was added and mixed, and 6 min later 300 µL of 1 M NaOH was added. The mixture was then brought up to 1.5 mL with 30% EtOH, mixed well and incubated at room temperature for 15 min. Absorbance was measured against a blank (70% MeOH) at 510 nm. The total flavonoid content was expressed as mg of catechin equivalents/100 g FW by comparison with a catechin standard curve (5–100 mg/L).

2.3.12 NMR Spectrocscopy Analysis of Spinach Extracts

Spinach leaf extracts prepared from freeze-dried tissues as described in section 2.3.10 were dissolved to a concentration at 100 mg DW/mL in DMSO-d_6, and 750 µL of each sample was transferred to 5 mm NMR tube for both 1D and 2D analysis.

NMR spectra were acquired on a Bruker Avance *III* 600 MHz NMR spectrometer (Bruker Corporation, East Milton, ON, Canada) operating at 600.283 MHz ^{1}H observation frequency and a temperature of 25 ± 0.1°C. Spectra were obtained with a 5 mm gradient inverse probe (optimized for ^{1}H), auto tuned and matched and shimmed on each sample, at a flip angle of 30° pulse followed by a 2.66 s acquisition time and 2 s relaxation delay. Each spectrum consisted of 32 scans of 64 k data points with a spectral width of 12335.52 Hz. ^{1}H NMR chemical shifts in the spectra were referenced to δ 2.50

ppm solvent signal. The signals were acquired, processed and analyzed using TopSpin® NMR data acquisition and processing Software (Bruker Biospin Ltd, East Milton, ON, Canada) integrated with the spectrometer.

2.3.13 LC/MS Analysis of Spinach Extracts

Samples used for NMR profiling were subjected to LC-MS analysis. 100 μL of the DMSO-d_6 solution was diluted with MeOH (10 times), and then filtered through a 0.45 μm filter before loading to HPLC. The samples were analyzed on both Agilent 1100 and 1200 LC systems with Agilent Zorbax Eclipse XDB-C18 (PN 993967-902) 4.6 × 150mm, 5 micron column, 30 ºC, eluted with solvents A (water with 2.7% formic acid) and B (MeOH with 2.7% formic acid) with a linear gradient of 5–100% solvent B over 26 min and then held at 100% B until 45 min, at a low rate of 0.25 mL/min. Both DAD (monitored at 210, 254, and 320 nm with continuous recording of UV spectrum from 190–600 nm) and MSD (API-ES source with positive ion scan range of 100–1000 with a step size of 0.25 amu; the drying gas was set at 10 L/min and 350 ºC; the nebulizer pressure was 25 PSIG and the capillary was set at 4000V) were used for Agilent 1100 LC system, and the injection volume was 5 μL.

For LC-MS/MS analysis, AB/Sciex 4000 QTRAP was used in coupling with the Agilent 1200 LC system under the same chromatography conditions. The Turbo V IonSpray source was used in positive ion mode. Q1 scans were performed over the mass range 100-1200 amu with a 3 second scan period. The curtain gas was set at 10; the source temperature was 450 ºC, ion source gases 1 and 2 were both 40 and the source voltage was 5500V. The declustering potential was set at 100V. MS/MS experiments were performed using Information Dependent Acquisition (IDA) mode with the same source parameters as the scan runs. An enhanced MS scan was followed with enhanced resolution to determine charge state. Using collision assisted dissociation (CAD), enhanced product ion spectra were generated for the three most intense peaks in each scan using rolling collision energies.

2.3.14 Statistical Analysis

Experimental data were analyzed by one-way analysis of variance (ANOVA) and differences between control and ANE-treated plants were considered statistically significant at $P \leq 0.05$ using Tukeys Honestly Significant Differences (HSD) test of the COSTAT® statistical software. There were 5 spinach plants in each treatment, and each treatment group was replicated 3 times. This experiment was conducted in quadruplicate, unless otherwise stated. The plants irrigated with 0 g/L ANE which meant that they received an equal amount of distilled water in the extract treatment were considered as control plants.

2.4 Results

2.4.1 Plant Growth Assay

There were no discernable differences in appearance among the control and ANE-treated plants. There were no significant differences in the leaf dry weight among the control and treated plants (Fig. 2.2).

2.4.2 Determination of Oxalic Acid

Though a trend of increasing OA among ANE-treated spinach plants was observed, plants treated with ANE at the concentration of 0.1 or 1.0 g/L showed no significant differences in the accumulation of total soluble OA as compared to untreated controls, while those treated with 5.0 g/L ANE exhibited a significant increase in OA content over the control plants (Fig. 2.3). The oxalic acid content of control plants was 440 mg/100 g FW, whereas it was 500 mg/100 g FW in plants treated with 5.0 g/L ANE.
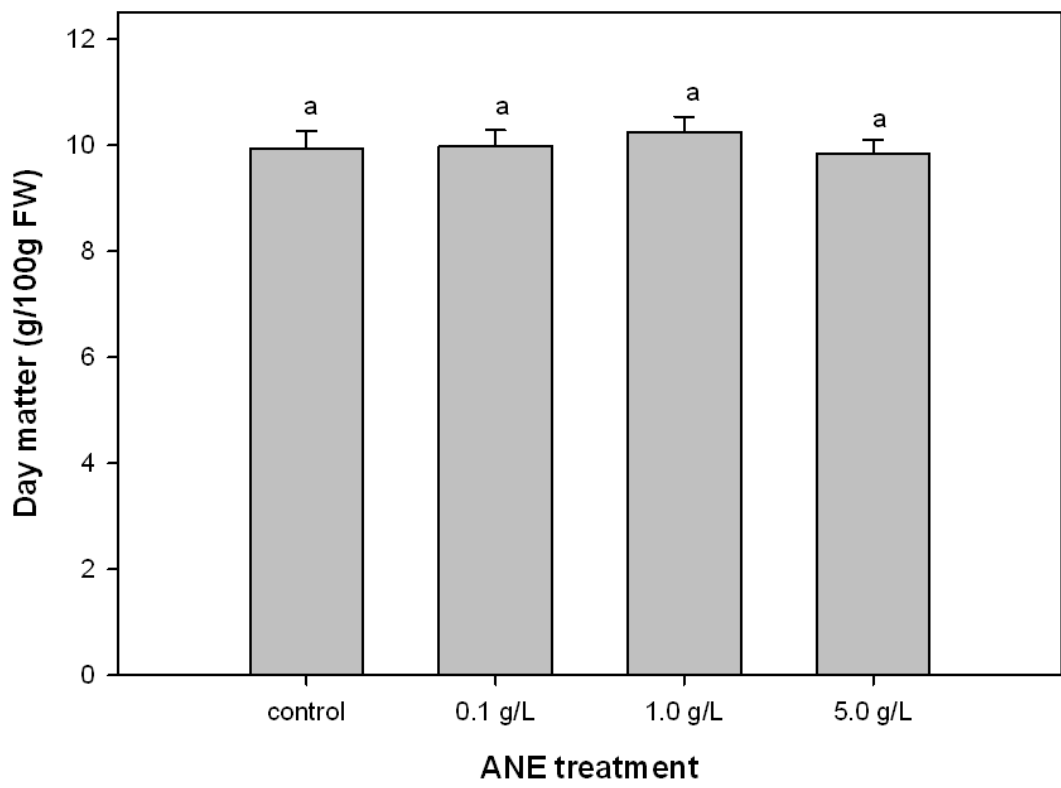
Figure 2.2. Leaf dry matter content of spinach (*Spinacia oleracea* L.) treated with different concentrations of ANE. Bars indicate mean ± standard error.
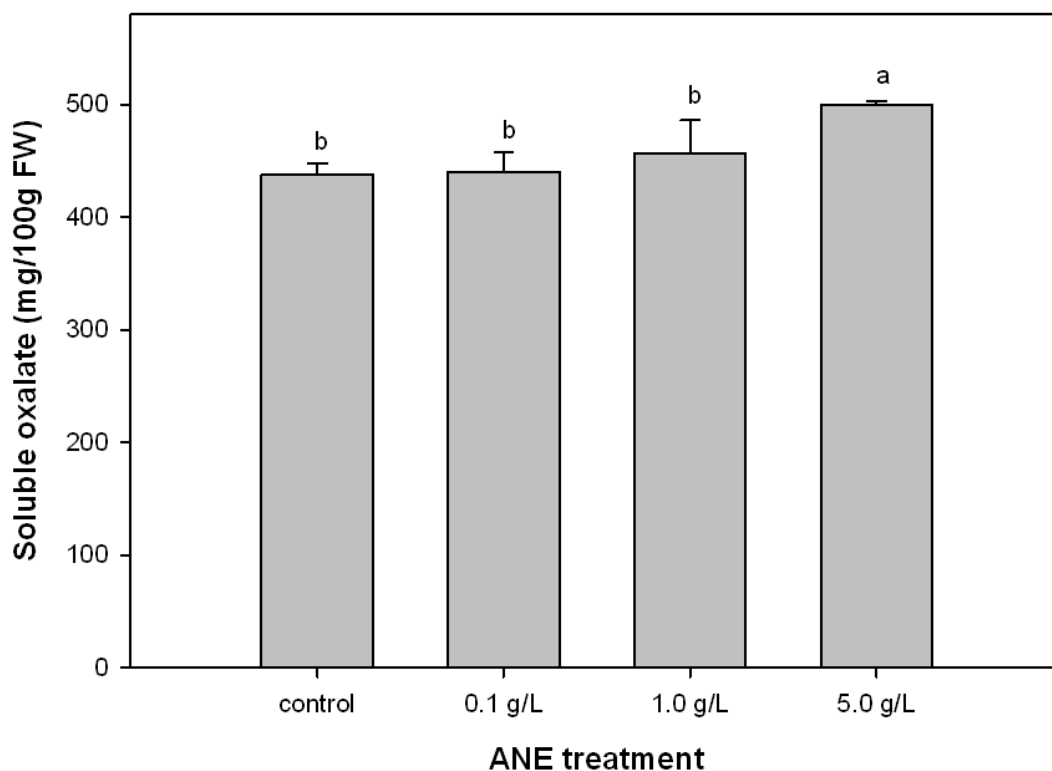
Figure 2.3. Oxalic acid content of spinach (*Spinacia oleracea* L.) treated with different concentrations of ANE. Bars indicate mean ± standard error.

2.4.3 Mineral Element Analysis

The macro and micro-element concentrations in ANE-treated and untreated spinach leaves are shown in Table 2.2. Compared to untreated control plants, spinach treated with 1.0 g/L ANE had significantly higher Fe content. The uptake of K was elevated with increased concentration of ANE. The 5.0 g/L ANE treatment significantly lowered the amount of P while notably increased the Zn levels as compared with untreated controls. The other macro- and micro-nutrients were not significantly influenced by ANE treatments.

Table 2.2. Effects of ANE on the concentrations of some mineral elements (dry weight basis) in spinach leaves[a]

Treatment	P (mg/g)	K (mg/g)	Na (mg/g)	Ca (mg/g)	Mg (mg/g)	Fe (µg/g)	Mn (µg/g)	Cu (µg/g)	Zn (µg/g)	B (µg/g)
Control	16.8a	62.4c	6.00a	4.76a	10.0a	104.4bc	364.3a	17.2a	139.6b	62.4a
0.1 g/L	17.0a	63.5bc	6.00a	4.93a	10.4a	106.8ab	399.4a	17.7a	146.2ab	63.5a
1.0 g/L	16.5ab	66.2ab	5.29a	4.64a	10.1a	107.7a	381.0a	17.9a	146.8ab	63.9a
5.0 g/L	15.9b	67.8a	5.24a	4.45a	10.1a	103.8c	369.8a	18.4a	153.5a	62.1a

[a] Values in the same column with the same letters are not significantly different ($P < 0.05$).

2.4.4 Determination of Total Protein

There were no differences in the nitrogen concentrations (65 mg/g DW) among control and treated plants. The soluble protein content was significantly higher in plants treated with 1.0 g/L ANE as compared to water controls and 0.1 and 5.0 g/L treated plants (Fig. 2.4).
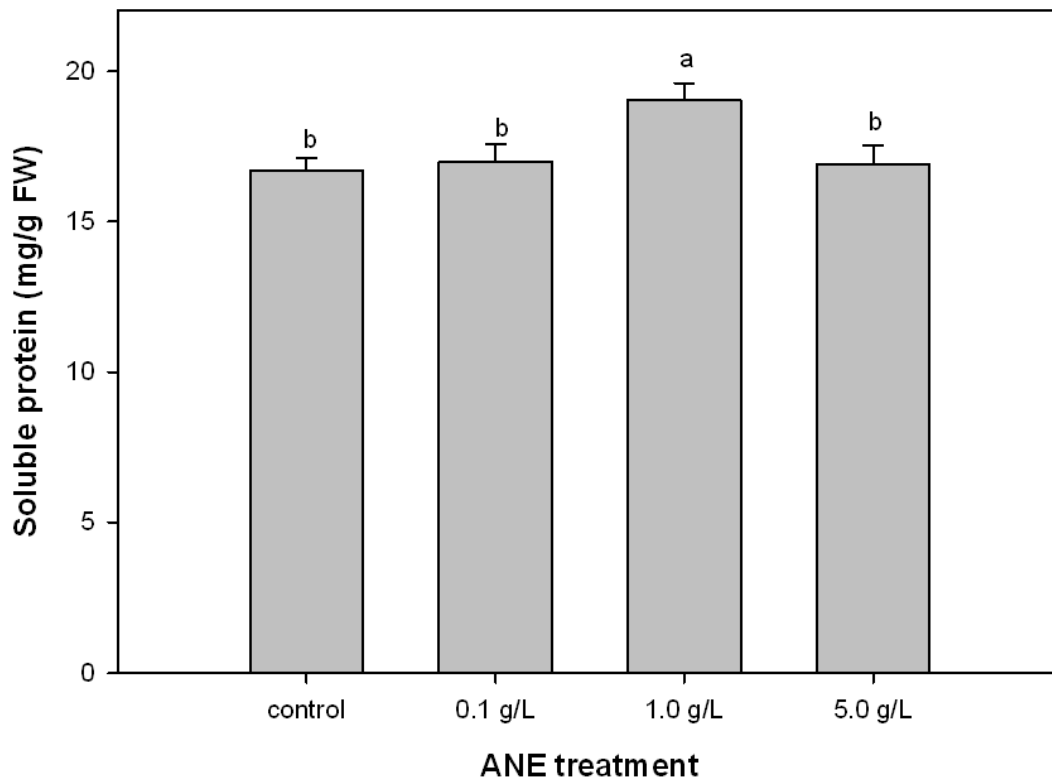
Figure 2.4. Total soluble protein of spinach (*Spinacia oleracea* L.) treated with different concentrations of ANE. Bars indicate mean ± standard error.

2.4.5 Antioxidant Content in Spinach

A 1.25-fold increase in the scavenging effect against DPPH radicals was observed in spinach leaves from plants treated with 1.0 g/L ANE (Fig. 2.5A). The Fe^{2+} chelating ability of extract of leaves from 1.0 and 5.0 g/L ANE treatments were significantly higher than in 0.1 g/L treated leaves or untreated controls. At 100 mg FW/mL, 1.0 and 5.0 g/L treated leaves chelated $32 \pm 1.6\%$ and $29 \pm 1.7\%$ of ferrous ions whereas control and leaves from 0.1 g/L treatment chelated $18 \pm 0.8\%$ and $21 \pm 1.9\%$, respectively (data not shown). There were no significant differences in total ascorbate concentrations among treated and control leaves (Fig. 2.5B). The leaves from 1.0 g/L ANE treatment had higher total phenolics (1.25-fold increase) as compared to untreated controls (Fig. 2.6A). Though total amounts of flavonoids in spinach increased in all ANE treatments, the 1.0 and 5.0 g/L treatments showed 1.5- and 1.2-fold higher than that of the controls (Fig. 2.6B), which were significant different.
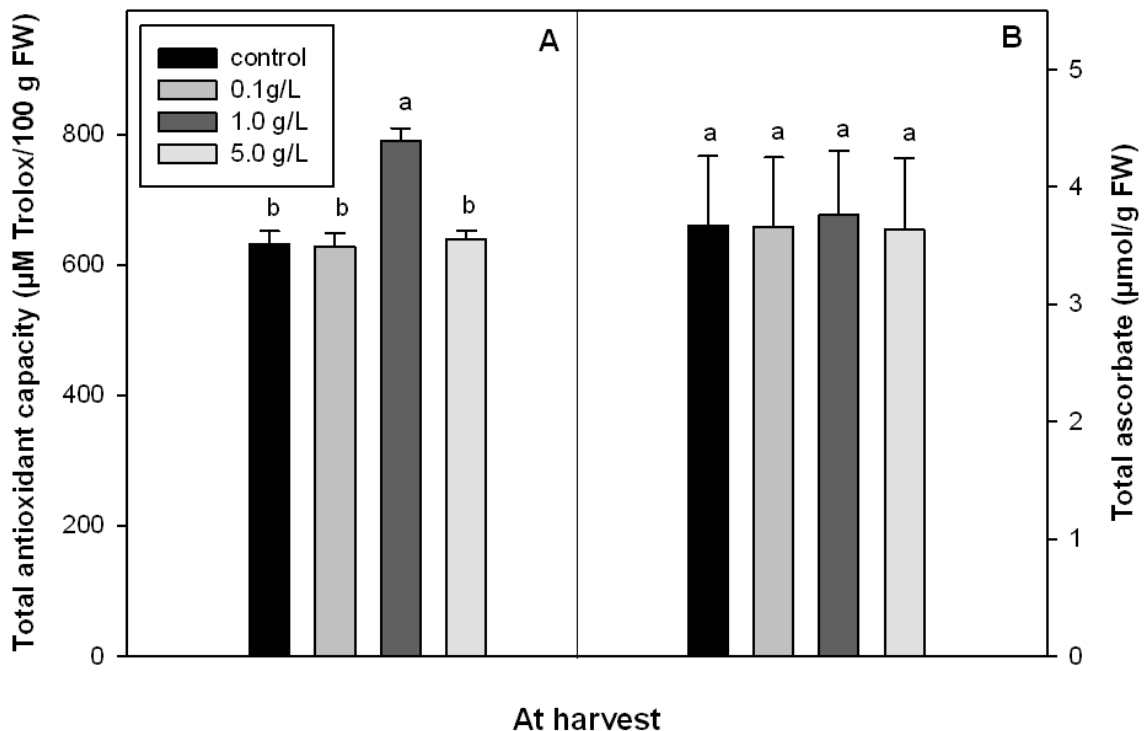
Figure 2.5. The effect of different concentrations of ANE on (A) total antioxidant capacity and (B) total ascorbate of spinach (*Spinacia oleracea* L.). Bars indicate mean ± standard error.

2.4.6 Linear Regression Analysis

In order to determine the extent to which phenolic compounds contribute to total antioxidant capacity in spinach, linear regression was performed with data from both control and treated plants. A positive, linear relationship ($R^2 = 0.933$) between total phenolic content and DPPH measurements was observed (Fig. 2.7). The higher phenolic content was correlated with a stronger antioxidant capacity.
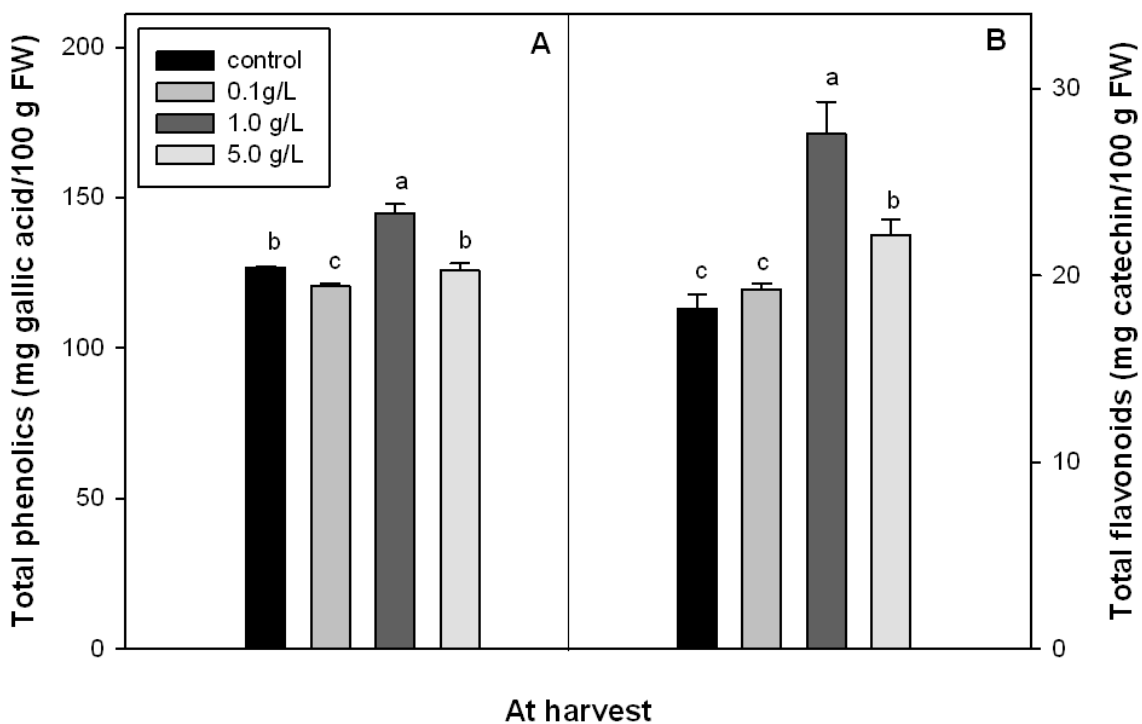
Figure 2.6. The effect of different concentrations of ANE on (A) total phenolics and (B) total flavonoids of spinach (*Spinacia oleracea* L.). Bars indicate mean ± standard error.

2.4.7 NMR Spectrocscopy Analysis of Spinach Extracts

The proton NMR spectra of 6 representative samples of spinach extracts were compared. In general, no major differences were observed (Appendix A-A, 1-3: control;

4-6 1.0 g/L ANE-treated, data of 0.1 and 5.0 g/L ANE-treatment not shown). As phenolics have been documented as one of the major bioactive components in spinach, the aromatic proton region of the spectra were expanded (Appendix A-B). It appeared that the samples have very similar phenolic component profiles. Clearly, the signals within the aromatic proton region indicated the presence of flavonoids as the dominant phenolics. With the assistance of ^{1}H-^{1}H COSY spectrum (Appendix A-C) and the NMR data reported previously (Ferreres et al. 1997; Edenharder et al. 2001), the peaks at δ7.58 ppm (d, J=2.2 Hz), 7.53 ppm (dd, J=8.7; 2.2 Hz), and 7.24 (d, J=8.7 Hz) can be assigned to the protons of H-2', H-6', and H-5' in B-ring. H-8 proton in A-ring for 6:7-methylenedioxy flavononoids appeared at δ 7.01 or 6.96 ppm as singlet; while in other 5,6,7-oxygenated substituted flavonol derivatives the H-8 singlet is at δ 6.62-6.54 ppm. The two protons in methylenedioxy moiety are shown at δ 6.18 ppm.
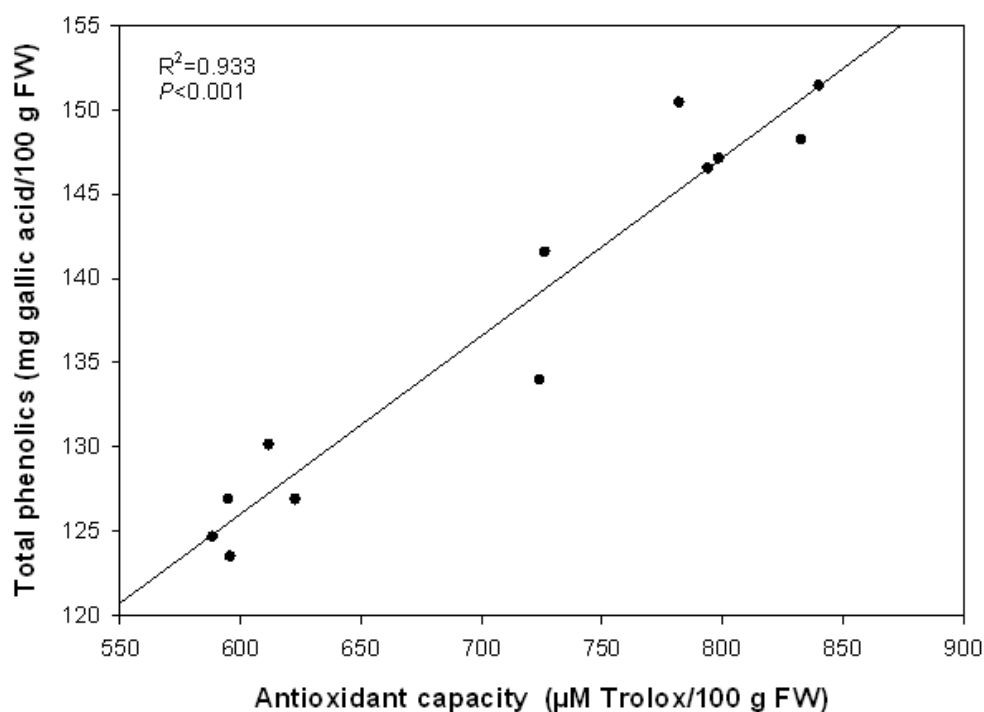
Fig. 2.7. Correlation between total phenolic content and antioxidant capacity of spinach (*Spinacia oleracea* L.).

2.4.8 LC/MS analysis of spinach extracts

In order to investigate the quantitative changes of major flavonoids induced by ANE treatment, 12 representative spinach samples were analyzed by HPLC-MS/MS. As shown in Appendix B-A (1-3: control; 4-6 1.0 g/L ANE-treated, data of 0.1 and 5.0 g/L ANE-treatment not shown), the HPLC profiles of these samples were similar. Sample 5 was used as a representative sample for further LC-MS/MS analysis. Based on our MS/MS data and the MS data in literature (Bergquist et al. 2005; Cho et al. 2008), the major flavonoids in the spinach samples were tentatively identified as: Peak 1, patuletin-3-glucosyl-(1→6)[apiosyl(1→2)]-glucoside; peak 2, spinacetin-3- glucosyl-(1→6)[apiosyl(1→2)]-glucoside and patuletin-3-(2''-feruloylglucosyl)-(1→6)[apiosyl(1→2)]-glucoside; peak 3, spinacetin-3- (2''-feruloylglucosyl)-(1→6)[apiosyl(1→2)]-glucoside glucoside and spinacetin-3- (2''-coumaroylglucosyl)-(1→6)[apiosyl(1→2)]-glucoside; peak 4, spinatoside; peak 5, jaceidin-4'-glucuronide; peak 6, 5,3',4'-trihydroxy-3-methoxy-6:7-methylendioxyflavone-4'-glucuronide; and peak 7, 5,4'-dihydroxy-3,3'-dimethoxy-6:7-methylendioxyflavone-4'-glucuronide (Appendix B-B and Appendix C). The changes of these flavonoids from the control and ANE-treated samples were based on the peak areas of HPLC. The results indicated an overall increase from 36–51% in 1.0 g/L ANE treatment as compared to untreated control (Appendix C). As for 0.1 and 5.0 g/L ANE treatments, the changes of the 9 major flavonoids, as compared to control samples, were minor. In 0.1 g/L treated plants, Spinacetin-3- glucosyl-(1→6)[apiosyl(1→2)]-glucoside and Patuletin-3-(2''-feruloylglucosyl)-(1→6)[apiosyl(1→2)]-glucoside (peak 2), Spinatoside (peak 4) and Jaceidin-4'- glucuronide (peak 5) were decreased significantly by 12%, 22%, and 15%, respectively, while 5.0 g/L ANE treatment showed no significant differences when compared to controls in regard to the 9 major flavonoids (Appendix C).

2.5 Discussion

The results show that *A. nodosum* extracts induce biochemical changes resulting in increased nutritional content in spinach. The ANE treatments were applied in the root which had led to changes in the biochemical components in the leaf suggests a systematic nature of the effect.

The commercial extract of the brown seaweed *A. nodosum* is rich in minerals, proteins, phenols, polyphenols, and vitamins (Cardozo et al. 2007), and also has a number of hormone-like elicitors (unpublished data). The mechanisms by which the commercial *Ascophyllum* extracts affect the growth of plants appear to be associated with the effects of oligosaccharides, hormone-like elicitors, betaines, and minerals present in the extract. These components, such as hormone-like elicitors, promote cell division and protein synthesis, often associated with improvement in stress tolerance (Allen et al. 2001; Fike et al. 2001). Field trials have shown that ANE application improved the yield of fruit and vegetables (Norrie and Hiltz 1999). In the present work, no significant effect of ANE was found in regard to appearance, DW, or vitamin C of spinach between treated and untreated controls. The difference between previous reports (e.g. Zodape et al. 2008) and our findings here may be due to the fact that ANE application was initiated at an early growth stage of plants while in the current study ANE was applied at the late growth stage prior to harvest. It has been shown that timing of application of ANE influence the plant yield (Chouliaras et al. 1997).

In this study, the protein content in 1.0 g/L ANE-treated spinach was found to be significantly higher than those in other treatments. Zodape et al. (2008) also reported that using a liquid seaweed fertilizer from fresh *Kappaphycus alvarezii* increased protein content in okra (*Abelmoschus esculentus* L.). This increase might be a result of an increase in protein biosynthesis stimulated by ANE, but the underlying mechanism(s) are not known. Spinach is a good source of important minerals. ANE treatment has been shown to increase leaf tissue N, P, K and Fe content in turfgrass (Butler and Hunter 2006), and K, Fe, and Cu levels in olive leaves (Chouliaras et al. 2009). In the present study, ANE application at 1.0 g/L was found to significantly increase K and Fe concentrations in spinach leaves whereas treatment at high concentration (5.0 g/L) decreased Fe content. Turan and Köse (2004) found that seaweed extract could significantly increase Cu uptake

of grapevine. Our results showed that the mean value of Cu concentration was higher in ANE-treated plants than control but was not statistically different. Earlier experiment in our laboratory showed that ANE significantly decreased Na in the leaves of *Arabidopsis thaliana* (unpublished data); I also observed a trend of decreasing Na concentration in ANE-treated spinach. The K/Na ratio in spinach treated with 5.0 g/L ANE (13.6) was significantly higher when compared with control plants (10.8). This is of interest from the point of view of nutrition and health, since high Na intake with a low K/Na ratio has been demonstrated to increase the incidence of hypertension. It appears that ANE might stimulate the Na^+/K^+ antiporters in spinach to accelerate the Na^+ secretion (Zhu 2002). In okra, lower to moderate concentration of ANE application was more effective in increasing nutrition quality as compared to lower or higher concentrations; this was in line with the current results. The differences in the changes of growth, weight, vitamin C and minerals from ANE-treatment in various plants are probably dependent on time, method, concentration, or cultivar of plant used in the study.

Oxalic acid is an organic acid that occurs naturally in many plants. It binds to Ca^{2+}, Fe^{2+}, and Mg^{2+} and interferes with the bioavailability of these minerals. Spinach contains relatively high levels of oxalate as compared to other vegetables (Savage et al. 2000). The oxalate levels observed in this cultivar is low as compared to most other cultivars of spinach, which is usually in the range of 400–900 mg/100 g FW (Savage et al. 2000). It is interesting that ANE treatments at low concentrations did not influence oxalate content in spinach leaves. However, 5.0 g/L ANE significantly increased the oxalate content, possibly due to the inhibited oxalic acid oxidase activity, and reduced degradation or the stimulation of the synthesis of oxalate.

Some studies have shown that exogenous application of ANE increased endogenous antioxidant activity in plants, such as increased amounts of non-enzymatic antioxidant compounds (α-tocopherol, ascorbate and β-carotene) and enhanced activities of antioxidant enzymes including ascorbate peroxidase, glutathione reductase, and superoxide dismutase (Allen et al. 2001; Zhang and Schmidt 2000; Zhang and Etvin 2004). High antioxidant amounts/activities following ANE treatment are important for improving the nutritional value of fruit and vegetables as well as prolonging shelf life, thus enhancing the overall quality and marketable value of fresh produce. In this study,

an increase in the phenolic content and total antioxidant capacity of spinach treated with 1.0 g/L ANE was found. Specifically, 9 major flavonoids found in spinach leaf tissues were increased following ANE treatment. The detected flavonoids corresponded to those reported by Howard and Pandjaitan (2008). Flavonoids commonly found in many other vegetables, such as quercetin, kaempferol, luteolin, and myricetin were not detected in this study; these results were similar to studies reported by Bergquist et al. (2005).

The significant increase in concentrations of phenolics and total antioxidant capacity specifically obtained from a 1.0 g/L ANE treatment may partly be attributed to the increase of endogenous plant hormones stimulated by ANE. For example, elicitors causing cytokinins-like reponses which are present in ANE have been shown to increase endogenous antioxidant activity and subsequent stress tolerance of creeping bent-grass (Zhang and Ervin 2004). In this study, there was no effect with respect to total ascorbate concentration in all ANE treatments. This suggests that ANE treatment through root drench may induce specific systemic physiological responses, including eliciting the phenylpropanoid and flavonoid pathways, thus leading to promotion of anti-radical capacity in spinach leaves. It should be noted that the content of certain minerals and total phenolics, total antioxidant abilities in spinach treated with 5.0 g/L exhibited same levels or lower values as compared to control or lower concentration (e.g. 1.0 g/L) of ANE. High concentration of ANE may retard the development of plants, probably owing to the high salt index in the extracts which may affect plant growth (Abetz 1980).

Recent research has identified certain chemicals in ANE that impart heat tolerance or increased chlorophyll content. For example, elicitors that result in cytokinin-like activities have been shown to increase endogenous antioxidant activities (such as α-tocopherol and superoxide dismutase) and subsequent stress tolerance in several turf-grasses (Zhang et al. 2000, 2004, 2008). Betaines present in ANE significantly enhanced chlorophyll contents in the cotyledons of cucumber (Whapham et al. 1993). However, there are no reports on the specific chemical compounds within ANE that elicit the phenylpropanoid and flavonoid pathways in plants. This chapter shows that ANE applied during the late growth stage of spinach was effective in enhancing nutritional qualities in spinach. The use of natural, environmentally friendly, safe extracts to increase nutritional values, especially antioxidant level of vegetables would be ideal, thus it would be

beneficial to elucidate the mechanism(s) by which ANE may exert such effects on spinach.

Chapter 3

In vitro studies of effects of commercial extract of *Ascophyllum nodosum* on *Spinacia oleracea* L.

3.1 Abstract

Extracts of the brown marine alga *Ascophyllum nodosum* (ANE) have been widely used as a biostimulant to enhance plant growth, yield, and quality in various crops. There are only a few reports on the effects of ANE on the yield and nutritional status of spinach. In the previous chapter it was demonstrated that ANE treatment applied at the late growth stage of selected plants significantly increased the nutritional quality and total antioxidant capacities of spinach leaves. In this study, the biochemical and molecular changes in spinach affected by ANE treatment *in vitro* was investigated. Increases in biomass, chlorophyll and antioxidant activities in spinach leaves were obtained with 0.1 g/L ANE treatment. The shoot fresh weight, dry matter content as well as total soluble protein showed 1.6, 1.2, and 1.5-fold increase, respectively. Total chlorophyll increased by 30% and the levels of total antioxidant capacity, phenolics and flavonoids all increased by at least 33%. Moreover, a 1.4-fold increase in chalcone isomerase activity was observed whereas the activity of phenylalanine ammonia lyase was not influenced by ANE treatment. Moreover, the differences between control plants and ANE-treated plants in the transcript levels of antioxidant enzymes and those involved in the synthesis of protein, sucrose and glycine betaine were studied. mRNA levels of cytosolic glutamine synthetase (GS1), related to protein synthesis, betaine aldehyde dehydrogenase (BADH) and choline monooxygenase (CMO), involved in betaine biosynthesis, and glutathione reductase (GR), an important antioxidant enzyme, increased in plants treated with 0.1 g/L ANE, while the transcripts of enzymes involved in the ascorbate-glutathione cycle (except for GR) were either up-regulated or unaffected. Taken together, the data from this study demonstrate a noticeable activation of the plant growth, pigmentation and antioxidant metabolism in spinach when the 0.1 g/L ANE treatment was started at the early growth stage of plants.

3.2 Introduction

The public has realized that there are linkages between diet and disease risk. The bioactive constituents of foods consumed have been demonstrated to be highly responsible for positive changes in human health. Numerous attempts via genetic engineering have been widely conducted to manipulate the synthesis of health-promoting chemicals so as to enhance the nutritional quality of crops such as increased Fe concentration in rice grains (Goto et al. 1999) and induced anthocyanins in tomato fruits (Butelli et al. 2008). With increasing concerns regarding environmental and health risks that genetic engineering may pose, an environmentally friendly and safe alternative would be highly desirable in order to enhance nutrient content in plant food products.

Many seaweeds have been directedly consumed and used as raw materials in food and pharmaceutical products for a number of years (Rupérez 2002). Seaweeds contain large quantities of macronutrients and micronutrients as well as other bioactive compounds (Whapham et al. 1993). Extracts derived from the brown alga *A. nodosum* (ANE) have been widely used in agriculture and horticulture as complementary fertilizers due to their beneficial effects on crop production, nutrient uptake, stress resistance, and quality of products after harvest (Abdel-Hafeez 2005; Fornes et al. 2002; Khan et al. 2009; Norrie and Hiltz 1999).

During the last 30 years, numerous experiments have been conducted to study the impact of ANE on the nutritional levels in food crops. ANE application has been shown to improve yield and/or size in many fruit and vegetables such as apple, grape, pepper, carrot, and potato (Norrie and Hiltz 1999), lettuce and cauliflower (Abetz and Young 1983) and olives (Chouliaras et al. 2009). Bluden et al. (1979) reported that seaweed extract application produced significantlt increase in root sugar content in sugar beet. A study from Abdel-Hafeez (2005) showed that weight, firmness, total soluble solids and total acidity of pear (*Pyrus leconte*, Rehd) were notably increased by pre-harvest spraying with commercial ANE, and fruit decolouration was also delayed. More recently, Zodape et al. (2008) reported that applications of a liquid fertilizer produced from fresh *Kappaphycus alvarezii* not only increased the yield but also elevated the content of protein, dietary fiber and minerals in okra (*Abelmoschus esculentus* L.). Moreover, an *in-vitro* study by Rayorath et al. (2008) showed that ANE significantly promote root and

shoot growth of *Arabidopsis thaliana*. There are some studies that show that ANE could induce levels of antioxidative molecules such as α-tocopherol, β-carotene, vitamin C, superoxide dismutase, glutathione reductase and ascorbate peroxidase in plants, which could benefit their growth and resistance to stresses (Allen et al. 2001; Zhang et al. 2003; Zhang and Schmidt 2000; Zhang and Ervin 2008). Unfortunately, there are only a few reports on the effects of pre-harvest treatment of ANE on the physiological responses and nutritional quality parameters of harvested spinach. Cassan et al. (1992) reported that foliar sprays increased the fresh weight of spinach (*Spinacia oleracea* L. cv. Monstrueux De Viroflay and cv. Polka) leaves by 12–15%. As such, the use of ANE application to improve production and nutritional levels in fresh crop products may be ideal.

Recently, it has been shown that ANE, when applied 5 and 6 weeks after sowing through root irrigation, elicited specific systemic physiological responses leading to improved nutritional values in spinach leaves after 7 weeks of culture (Fan et al. 2011). However, the effect of ANE when applied from the early growth stage of spinach is not known. Therefore, the purpose of this study is to investigate the effect of early-applied ANE on the growth, antioxidant response and transcription of pertinent genes in spinach.

3.3 Materials and Methods

Seeds of spinach (*Spinacia oleracea* L., var. Unipack 12) were purchased from Stokes Seeds Co. (Thorold, ON, Canada). Soluble powder of *A. nodosum* alkaline extract (Acadian®) was obtained from Acadian Seaplants Limited (Dartmouth, NS, Canada). All the other chemicals and reagents were of analytical grade and were purchased from Sigma Aldrich (Oakville, ON, Canada), unless otherwise stated.

3.3.1 Plant Culture and Treatment

Spinach seeds were surface sterilized in 70% ethanol (EtOH) for 1 min and then shaken for 30 min at 100 rpm in 50 mL 2.6% (w/v) sodium hypochlorite containing 100 µL Tween-20. The seeds were then rinsed in 5 changes of sterile distilled water. Seeds were planted evenly on half-strength Murashige and Skoog basal medium (pH 5.8) containing 1% (w/v) sucrose and 0.8% (w/v) agar in Petri dishes (9 cm diameter), at a rate of 6 seeds per plate.The Petri dishes were incubated at 20 °C ± 2 °C in the dark for 3

days to facilitate germination and then stacked vertically under a cool fluorescent light (100 μmol·m^{-2}·s^{-1}) for 6 days, until the cotyledons were fully developed. The seedlings were then carefully transferred to Magenta jars containing solid medium (with 1% sucrose and 0.8% agar in half-strength MS basal medium) supplemented with different concentrations of ANE (i.e. 0, 0.1, or 0.5 g/L). The required concentrations of extract was added to molten agar medium (55 °C) prior to pouring into jars. Plants were harvested at the four-leaf stage after 21 days of growth on treatment medium.

3.3.2 Determination of Plant Growth

The growth of spinach plants was assessed in terms of fresh weight (FW), dry weight (DW) and dry matter content (DMC). Whole shoots were harvested and weighed, and per seedling fresh weight was obtained. Dry matter content was assessed by drying shoot samples at 85 °C in an oven for 48 h.

3.3.3 Determination of Total Protein

Total soluble protein was analyzed following the protocol described in section 2.3.6. Fresh leaf samples (the 3rd and 4th leaves) were extracted in 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 8.5), containing 5.0 mM 2-mercaptoethanol and 2% (w/v) PVP, and centrifuged at 10,000×g for 20 min at 4 °C. The supernatant was collected as the crude enzyme. Protein concentration was measured using the Coomassie Plus – The Better Bradford^{TM} Assay Kit (Pierce, Rockford, IL, USA). Bovine serum albumin (125–1500 μg/mL) was used as a standard reference.

3.3.4 Pigment Measurements

All extraction procedures were carried out under dim light on ice. After harvesting, leaf tissue (0.2 g) was immediately homogenized using a mortar and a pestle in cold methanol (MeOH) in the presence of a small amount of inert sand. After centrifuging at 10,000×g at 4°C for 10 min, the pellets were re-extracted in MeOH until all color was removed. The volume of combined extracts was increased up to 10 mL in a volumetric flask. Absorbance was measured at 652, 665, and 750 nm using a spectrophotometer. The total chlorophyll content was calculated according to Ritchie (2008).

3.3.5 DPPH Radical Scavenging Assay

The total antioxidant capacities of spinach leaves were performed following the protocol described in section 2.3.7. Fresh leaf tissue (0.2 g) was homogenized in 4 mL MeOH using a mortar and pestle. After centrifugation at 10,000×g for 10 min, the supernatant was recovered. Pellet was re-extracted with 3 mL MeOH. Supernatants were combined and the total volume was made up to 7 mL. The pellet was re-dissolved in 0.5 mL dichloromethane (DCM), re-homogenized and centrifuged again at 10,000×g for 10 min. Pellet was re-extracted with 0.5 mL DCM, and supernatant was centrifuged, combined, and made up to 1 mL. Each extract was added to 2850 µL fresh DPPH$^{\bullet}$ solution (0.11 mM), and held incubated for 6 h at 22 °C. Absorbance was then read at 515 nm against MeOH or DCM as a blank. The scavenging activity was calculated according to the equation: Inhibition % = [(A$_b$−A$_s$)/A$_b$] 100, where A$_b$ is the absorption of blank sample and A$_s$ is the absorption in the presence of test sample. The results were expressed in µM Trolox equivalents (TE, µM Trolox)/100 g FW through comparison against a standard curve (25–800 µM Trolox).

3.3.6 Determination of Total Phenolics

The total phenolic content was analyzed following the protocol described in section 2.3.10. Fresh leaf tissue of spinach plants (0.5 g) was extracted twice with 70% (v/v) MeOH at 40 °C for 2 h, and the extract was centrifuged, combined, and made up to 5 mL. Spinach extract was pipetted into 2 mL tubes and to each 1.58 mL water and 100 µL 2 N Folin-Ciocalteu reagent was added. After 8 min-incubation, 300 µL 20% (w/v) sodium carbonate (Na$_2$CO$_3$) solution was added to stop the reaction. The vortexed mixture was left at room temperature in the dark for 2 h and the absorbance was read at 760 nm against a blank (70% MeOH). A standard calibration curve (20–500 mg/L) was prepared under the same procedure as above. Total phenolics were expressed as gallic acid equivalents (GAE, mg gallic acid/100 g FW).

3.3.7 Determination of Total Flavonoids

Total flavonoid content in spinach leaves was quantified following the protocol described in section 2.3.11. The extracts (in 70% MeOH) of fresh leaf tissues of spinach

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seedlings or standard solutions, were mixed with 45 µL of 5% (w/w) sodium nitrite (NaNO$_2$) and 750 µL of 30% EtOH. After incubation at room temperature for 6 min, 45 µL of 10% (w/w) aluminium trichloride (AlCl$_3$) was added and mixed, and 6 min later 300 µL of 1 M sodium hydroxide (NaOH) was added. The mixture was then brought up to 1.5 mL with 30% EtOH, mixed well and incubated for 15 min. Absorbance was measured at 510 nm against a blank (70% MeOH). The total flavonoid content was expressed in mg of catechin equivalents/100 g FW by comparison with a catechin standard curve (5–100 mg/L).

3.3.8 Estimation of Phenylalanine Ammonia Lyase and Chalcone Isomerase Activities

Enzyme extraction was conducted following the protocol described in section 3.3.3. The phenylalanine ammonia lyase (PAL, EC 4.3.1.5) assay was based on the method of Gadzovska et al. (2007), with modifications. The 1 mL reaction mixture, containing 10 μmol of L-phenlalanine, 50 μmol of borate buffer at pH 8.8, and crude enzyme extract, was incubated at 40 °C for 60 min. The absorbance of the solution was determined at 290 nm. The concentration of cinnamic acid was calculated using a cinnamic acid standard curve (5–50 mg/L). PAL activity was defined as μmol of cinnamic acid/h/mg protein.

Chalcone isomerase (CHI, EC 5.5.1.6) activity was estimated following a method of Terai et al. (1996), with minor modifications. Briefly, to 2.5 mL of 0.1 M potassium phosphate buffer (pH 8.5), was added 20 µL of 1 mg/mL isoliquiritigenin. Crude enzyme extract was added to initiate the reaction. After incubation at 30 °C for 60 min, the absorbance at 400 nm was measured. Enzymatic activity was expressed as nmol isoliquiritigenin/min/mg protein.

3.3.9 *Ascophyllum nodosum* Induced Gene Expression Analysis

The effect of ANE on the expression of genes known to be involved in nutritional quality and antioxidant levels in spinach was studied. The 3rd and 4th leaves of 30-day old spinach plants grown with or without ANE were harvested by sharp knives and immediately frozen in liquid N$_2$ and stored at −80 °C until use. Frozen leaf material was

ground with a mortar and pestle in liquid N_2 and approximately 100 mg was used for total RNA extraction, using the RNeasy Plant Mini Kit (QIAGEN, Mississauga, ON) according to the manufacturer's instruction. Total RNA was quantified with a Nanodrop ND-1000 spectrophotometer at 260 nm. To compare differential gene expression, a 'semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)' was used. The first-strand cDNA synthesis was carried out using 2 µg of each total RNA sample and the Retroscript® Reverse Transcription Kit (Ambion Inc., Austin, TX). The cDNA product was used as a template in a standard PCR reaction containing *Taq* polymerase and gene specific primers (Table 3.1.) to amplify the transcripts of the genes of interest. The differential levels of gene expression were compared on 1.5 % (w/v) agarose gels using a UV light transilluminator. 18s rRNA was used as an internal standard.

Table 3.1. Primers and key PCR conditions used in this study

Gene	Gene Locus	Primer Sequence (5'–3')	T_m (°C)	Cyl (No.)
SPS	S54379	TCGCGCTGCATCAACTCGCA	64	30
		TCGCGTTCCACATGGCGTTT		
BADH	FJ595952	ATGCCGGCTTGGCCCTGTTA	62	30
		ATGCTCAGGACGGGAACCTCCA		
CMO	EF362838	TGGCATGGACGGATCACTTGCG	64	30
		AGCTGATAAGAACGAACGGCCCC		
GS1	EU057984	TGCTGCAGACGAGGTGTGGGTA	64	30
		TGTGAGCACCAGCACCATTCCA		
GS2	EF143582	TGGGGTGTTGCCAATCGTGGT	64	30
		TGTCTCCGCAAGCAAACCCGT		
GR	SPIGLRE	TGGCGGTGTTGTAGCTCGTACC	60	30
		ACCTTGACAGCTCCTGTTCGGTC		
DHAR	EU247959	ATTCCACCGCCGCAATCGCA	64	30
		GGACAATCGCCAAGCTTGTTGGG		
MDHAR	AB063289	TGGCAACAGGCTGCACAGCTA	64	30
		ACCGCCCACAATCACCACCT		
tAPX	SPICPAP	TGCTGGACCTCCTTCACCTGCT	64	30
		TGCCCCAACCACTGCGTTCA		
sAPX	SPICPSAP	TGCTGGACCTCCTTCACCTGCT	64	30
		TGCCCCAACCACTGCGTTCA		

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3.3.10 Statistical Analysis

The experimental data were analyzed by ANOVA and differences between control and ANE-treated plants were considered statistically significant at $P \leq 0.05$ using Tukeys HSD test of the COSTAT® statistical software. Three Magenta jars (2 seedlings per jar) were used in each treatment group. The experiment was conducted as a complete randomized design with 3 replicates. The whole experiment was run in triplicate. The seedlings grown with 0 g/L ANE were considered as control plants.

3.4 Results

3.4.1 Determination of Plant Growth

Addition of ANE to the growth medium was found to significantly enhance spinach shoot growth. At 21 days after treatment, 0.1 g/L of ANE significantly increased the FW and DMC by 58% and 23%, respectively, while 0.5 g/L ANE showed a 12% increase in DMC, with no significant effect on FW (Table 3.2.).

Table 3.2. Shoot fresh weight, dry weight and dry matter content of spinach (*Spinacia oleracea* L.) grown with different concentrations of ANE[a]

Biomass	control	0.1 g/L	0.5 g/L
Fresh weight (g per plant)	0.47b	0.74a	0.57b
Dry weight (g per plant)	0.016b	0.031a	0.022b
Dry matter content (% w/w)	3.46c	4.26a	3.89b

[a] The means within each row followed by different letters are statistically different at $P \leq 0.05$.

3.4.2 Determination of Total Protein

Spinach plants grown with 0.1 g/L ANE showed a significant increase in protein concentration as compared to control (Fig. 3.1). The ability of ANE to improve leaf total soluble protein levels was at least partially correlated to the increased expression of cytosolic glutamine synthetase (GS1) (Fig 3.6). Plastid glutamine synthetase (GS2) mRNA was not significantly influenced by ANE treatments (Fig 3.6).
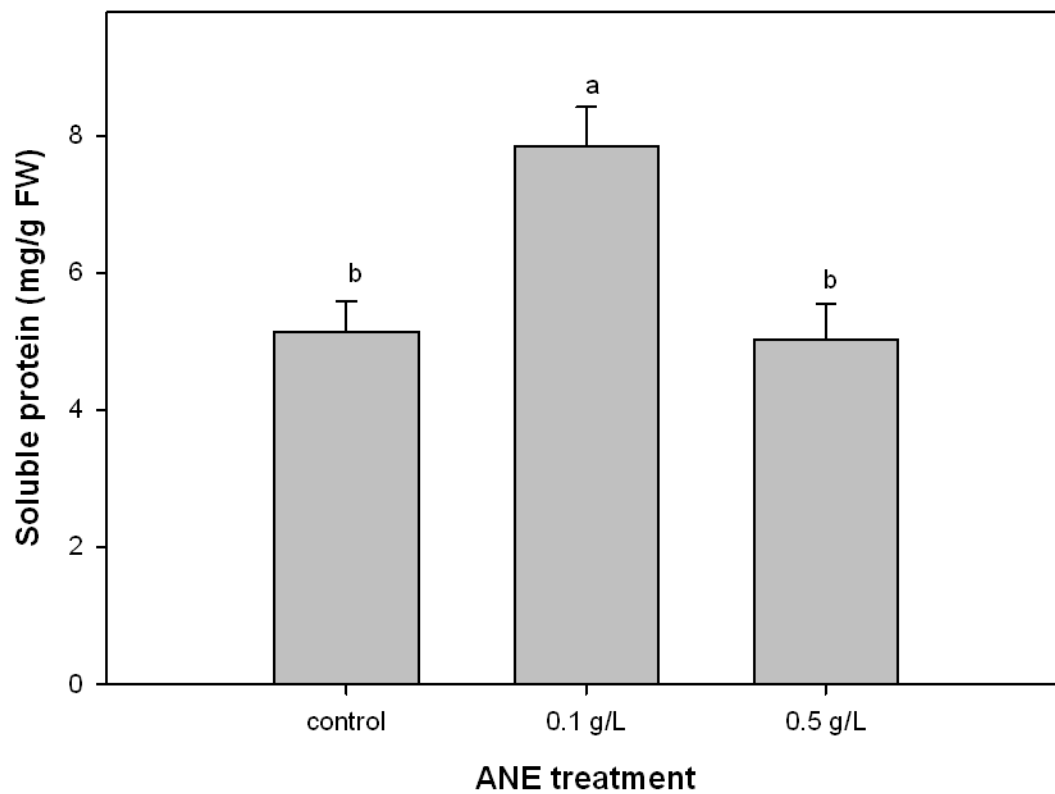
Figure 3.1. The effect of different concentrations of ANE on total soluble protein of spinach (*Spinacia oleracea* L.) seedlings. Bars indicate mean ± standard error.

3.4.3 Pigment Measurements

There was an increase in the chlorophyll content in the leaves of plant grown in ANE. The chlorophyll content in the control leaves was 350 µg/g FW. While those grown in 0.1 g/L ANE exhibited significantly higher total chlorophyll content (1.3-fold increase) as compared to control ones (Fig. 3.2). Leaves from 0.5 g/L ANE-treated plants exhibited lower concentrations of chlorophyll, even when compared to the untreated controls (Fig. 3.2).
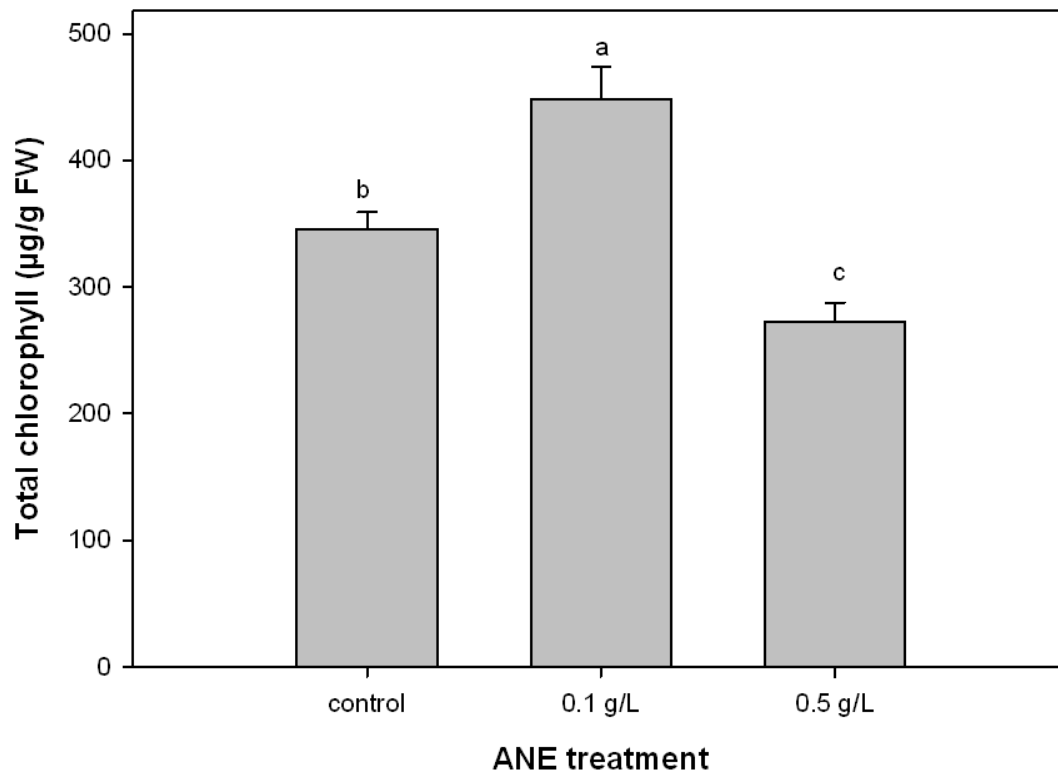
Figure 3.2. The effect of different concentrations of ANE on total chlorophyll of spinach (*Spinacia oleracea* L.) seedlings. Bars indicate mean ± standard error.

3.4.4 Antioxidant Content

A 1.5-fold increase in the scavenging effect against DPPH radicals was observed in leaves from spinach grown with 0.1 g/L ANE (Fig. 3.3). ANE treatment at 0.5 g/L did not significantly influence the total antioxidant capacity. The leaves from the 0.1 g/L ANE treatment also exhibited higher total phenolic content (1.3-fold increase) and higher total flavonoids (1.3-fold increase), when compared with the untreated control (Fig. 3.4). Leaves treated with 0.5 g/L ANE showed the same level of total phenolics and flavonoids as did the control.
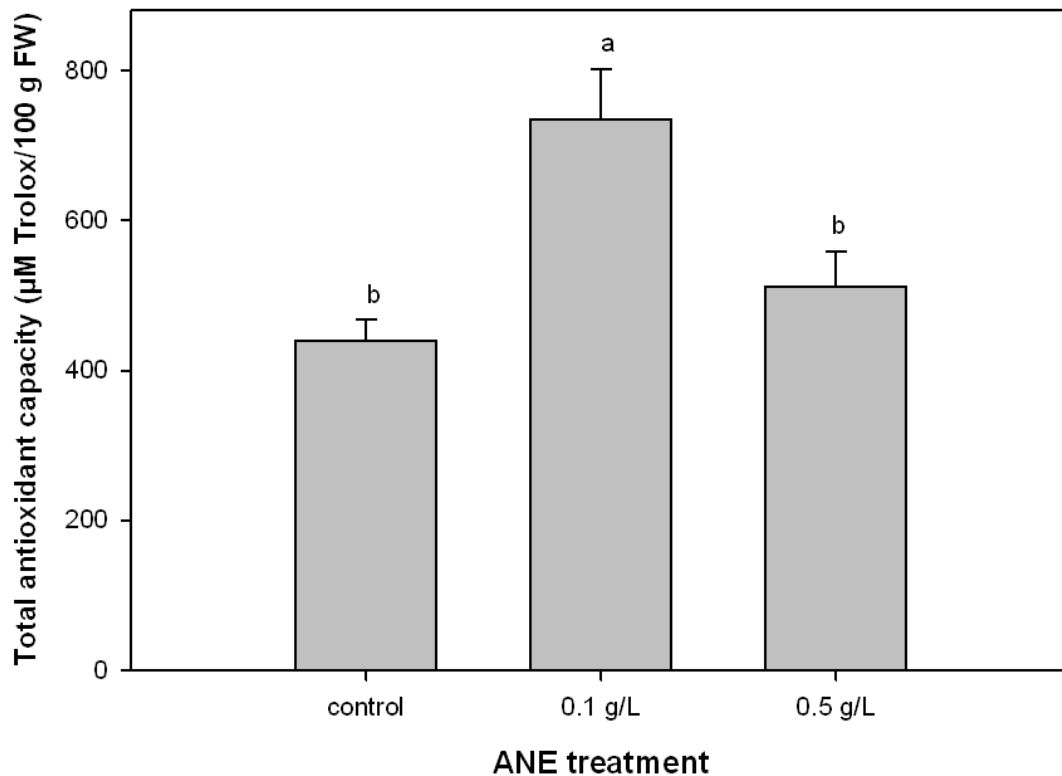
Figure 3.3. Total antioxidant activity of spinach (*Spinacia oleracea* L.) seedlings grown with different concentrations of ANE. Bars indicate mean ± standard error.
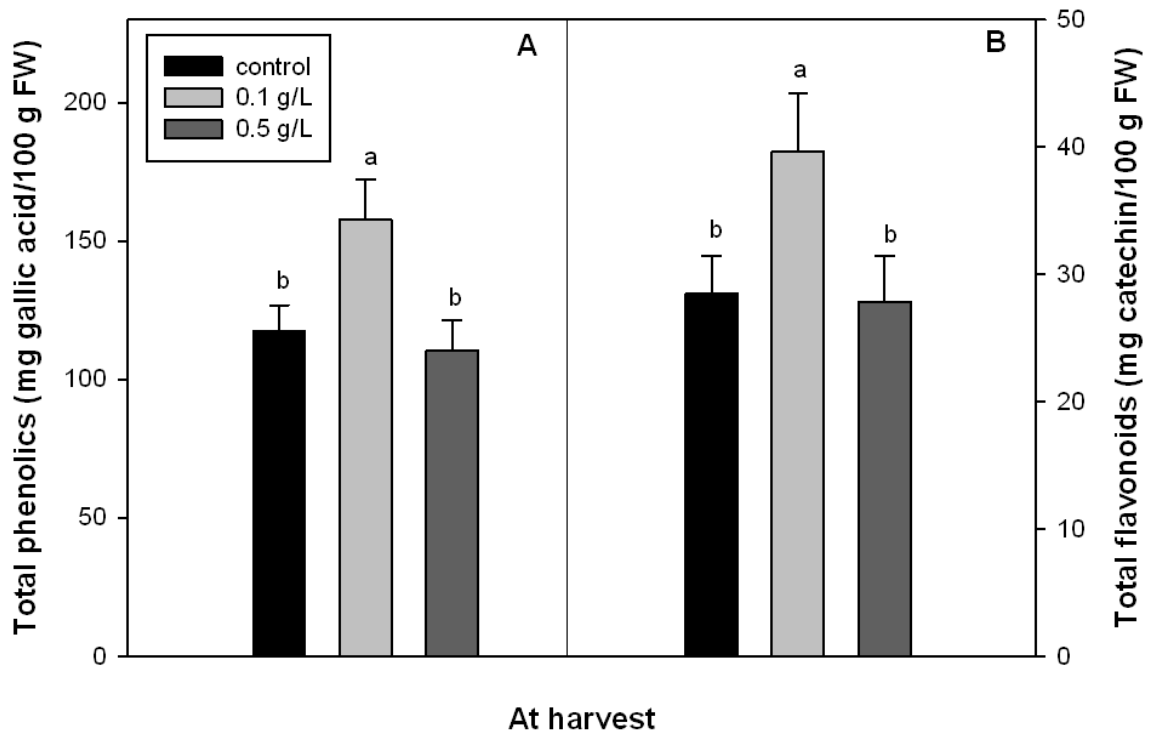
Figure 3.4. The effect of different concentrations of ANE on (A) total phenolics and (B) total flavonoids of spinach (*Spinacia oleracea* L.) seedlings. Bars indicate mean ± standard error.

3.4.5 Estimation of Phenylalanine Ammonia Lyase (PAL) and Chalcone Isomerase (CHI) Activities Isomerase Activities

Treatments with ANE did not effect the activity of PAL in the spinach leaves (Fig. 3.5A). However, plants treated with 0.1 g/L ANE showed significant enhanced activity of CHI as compared to the control ones (Fig. 3.5B) while higher concentration of ANE (i.e. 0.5 g/L) recorded an CHI activity comparable to the control.
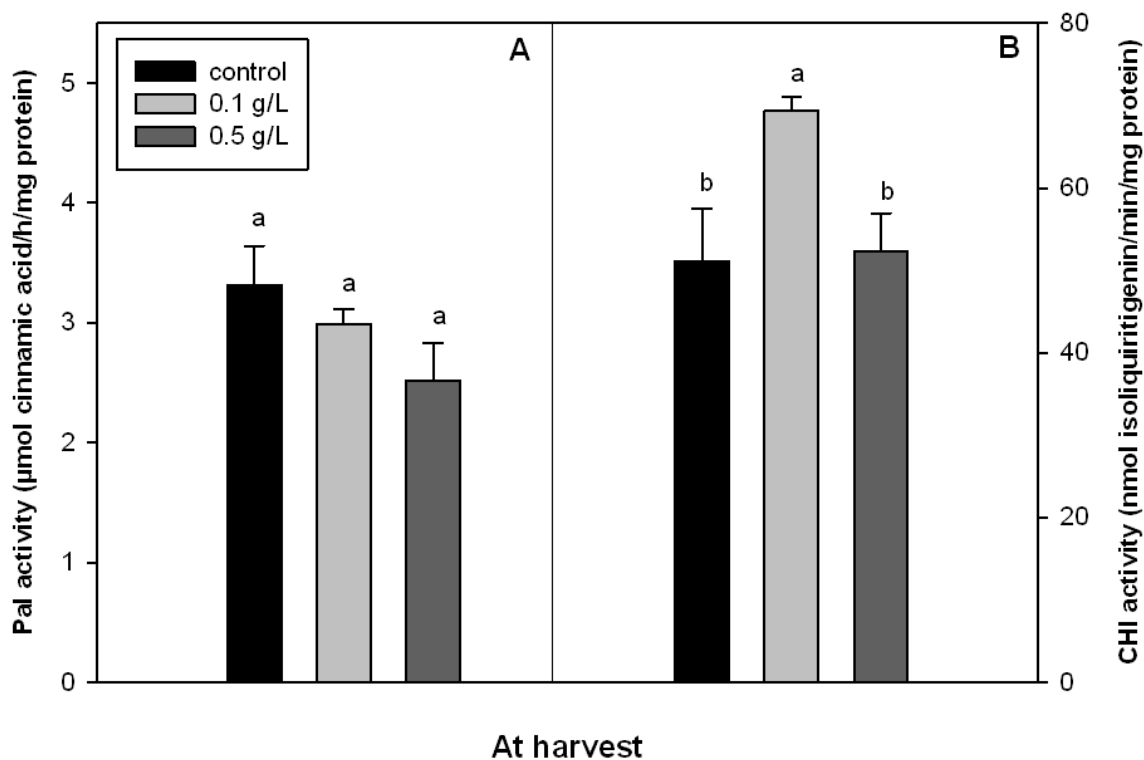
Figure 3.5. The effect of different concentrations of ANE on (A) PAL (B) CHI activities of spinach (*Spinacia oleracea* L.) seedlings. Bars indicate mean ± standard error.

3.4.6 *Ascophyllum nodosum* Extract Induced Gene Expression Analysis

The analysis of transcriptional levels of genes involved in biosynthesis and antioxidative activities is shown in Fig. 3.6. The transcript abundance of cytosolic glutamine synthetase (GS1), betaine aldehyde dehydrogenase (BADH), and choline monooxygenase (CMO) in spinach leaves increased in 0.1 g/L ANE treatment over that of the control and 0.5 g/L treatment. In contrast, the transcripts of sucrose phosphate synthase (SPS; which is involved in carbon assimilation), and plastid glutamine synthetase (GS2) were not affected by the ANE treatments. The influence of ANE on the mRNA levels of the enzymes involved in ascorbate-glutathione cycle (Fig. 3.7) was

somewhat complicated. The transcriptional level of glutathione reductase (GR) that maintains the reduced glutathione in the cytosol and chloroplast was up-regulated by 0.1 g/L the ANE treatment in this study. However, the transcript of dehydroascorbate reductase (DHAR; which reduces dehydroascorbate (DHA) to ascorbate,) was not affected by ANE treatments while that of thylakoid-bound ascorbate peroxidase (tAPX; which oxidizes ascorbate to monodehydroascorbate (MDA)), was up-regulated. The transcriptional level of monodehydroascorbate reductase (MDHAR; which reduces MDA to ascorbate), was also up-regulated. The mRNA level of stromal APX (sAPX) was not influenced by the ANE treatments.
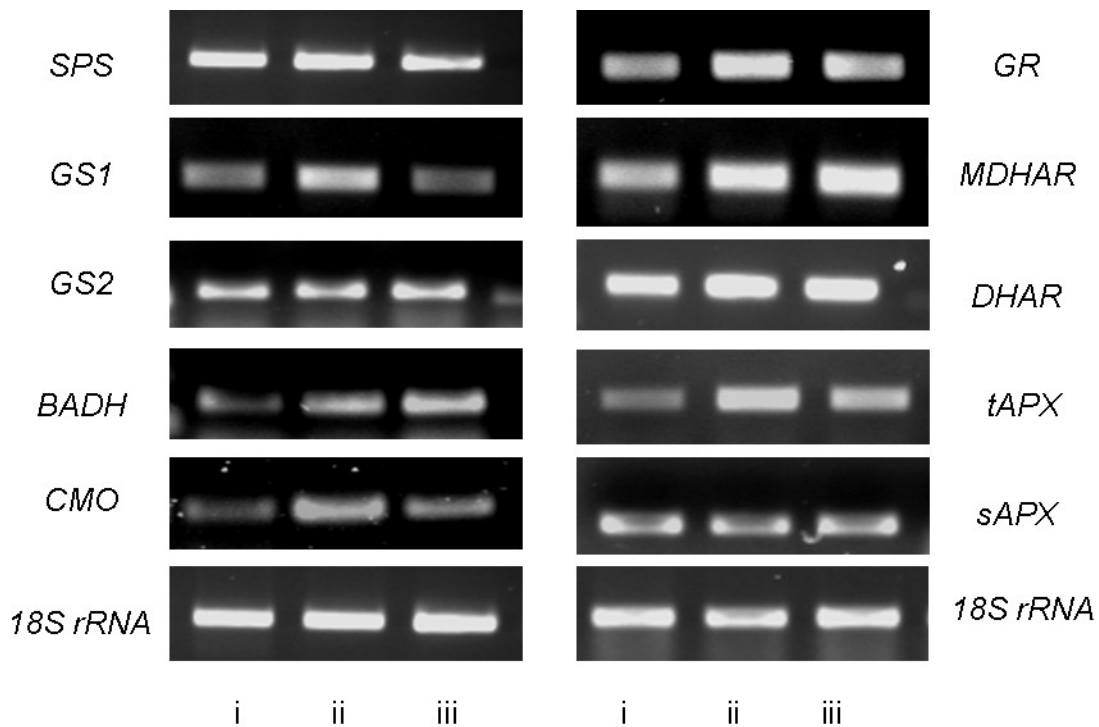
Figure 3.6. RT-PCR analysis of transcript abundance of pertinent genes in (i) control and (ii) 0.1 g/L ANE or (iii) 0.5 g/L treated spinach plants.
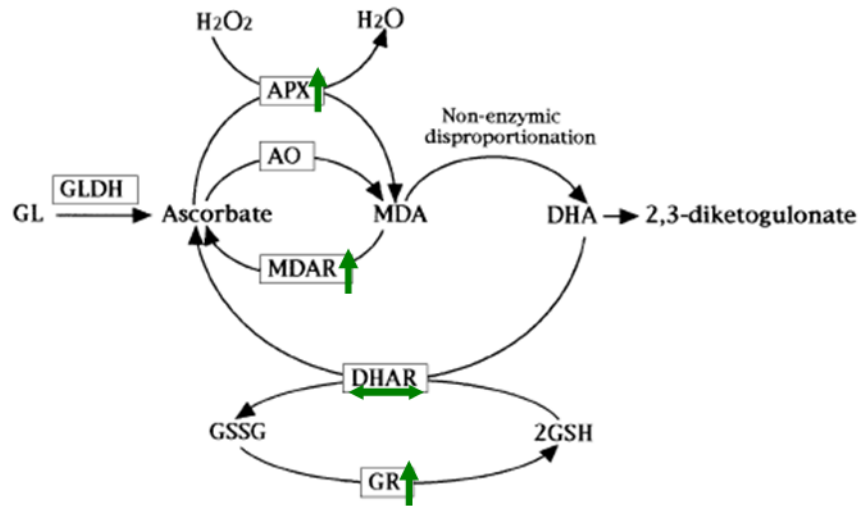
Figure 3.7. The ascorbate metabolism in plants (Nishikawa et al. 2003, with minor modifications). Not all reactions are depicted stoichiometrically. Ascorbate is synthesized from L-galactotno-1,4-lactone (GL) by GL dehydrogenase (GLDH) which is assumed to be in mitochondria. Ascorbate is oxidized to monodehydroascorbate (MDA) by ascorbate peroxidase (APX) located in the cytosol and chloroplasts and by ascorbate oxidase (AO) located in the cytosol or apoplast. MDA is converted to ascorbate by MDA reductase (MDAR) found in the cytosol and chloroplasts. MDA disproportionates non-enzymatically to ascorbate and dehydroascorbate (DHA), if not rapidly reduced by monodehydroascorbate reductase (MDHAR). DHA is hydrolysed to 2,3-diketogulonate unless reduced by dehydroascorbate reductase (DHAR) appearing in the cytosol and chloroplasts, using glutathione (GSH) as the reductant. Oxidized glutathione (GSSG) is reduced by glutathione reductase (GR) in the cytosol and chloroplasts.

3.5 Discussion

The results described in this chapter reveal that *in vitro* ANE treatment at 0.1 g/L induced specific physiological changes resulting in an increase in biomass, pigmentation and total antioxidative capacities in spinach. The ANE treatments were applied from the early growth stage of the plants and also as a root irrigation which induced enhanced yield and antioxidant capacities in the shoots of spinach.

Commercial extract of the brown seaweed *A. nodosum* is rich in major and minor plant nutrients, trace minerals, mycosporine-like amino acids, phycocolloids and polyphenols (Cardozo et al. 2007). The extrct may also have a number of hormone-like effect elicitors (unpublished data). The mechanisms by which ANE affects the growth of

plants appear to be associated with the effects of oligosaccharides, hormone-like elicitors, betaines and minerals that promote cell division, protein synthesis, and improve stress tolerance (Allen et al. 2001; Fike et al. 2001). A number of studies have shown that ANE improve yield of sugar beet (Blunden et al. 1979), lettuce (Abetz and Young 1983), apple, grape, and tomato (Norrie and Hiltz 1999). For instance, foliar spray of ANE increased fresh weight of maize seedlings by 15−25% (Jeannin et al. 1991). In the present work, we found that 0.1 g/L ANE treatment, initiated at the early growth stage of seedlings, significantly increased the yield and DW of spinach as compared to control plants. This contrasts to research from the previous chapter that no significant differences were obtained in DW between the control and ANE-treated plants, indicating that the timing of application of ANE may affect on biomass production in spinach.

Glutamine synthetase which catalyzes inorganic nitrogen (ammonium) to glutamine, an organic form, plays an essential role in nitrogen metabolism and is a key enzyme controlling nitrogen assimilation (Oliveira et al. 2002). It has been reported that over-expressed GS1 significantly increased biomass, leaf protein and photosynthesis in transgenic tobacco (*Nicotiana tabacum*; Fuentes et al. 2001; Oliveira et al. 2002). Over-expression of sucrose phosphate synthase (SPS) significantly longthened fibers and increased total dry biomass relative to the control tobacco (*Nicotiana tabacum* cv. Xanthi) plants (Park et al. 2008). In the present study, we found that total soluble protein content in 0.1 g/L ANE-treated spinach was significantly increased than those in either control plants or 0.5 g/L ANE-treated plants. This is in accordance with the results presented in the previous chapter as well as other publications (e.g. Zodape et al. 2008). The transcriptional abundance of GS1 was significantly higher in 0.1 g/L ANE-treated plants whereas transcripts of GS2 and SPS were not affected. Thus, it seems that ANE-induced increases in FW, DW, and protein concentrations are, at least in part, due to the elevated transcription of GS1. Moreover, ANE also may promote the synthesis of protein in spinach through increasing activity of nitrate reductase, an enzyme that catalyzes the reduction of nitrate to nitrite which is the first committed step of the nitrate assimilation pathway (Durand et al. 2003).

It has been reported that application of ANE increase the chlorophyll content of tomato leaves, cucumber cotyledons, and dwarf French Bean leaves (Whapham et al.

1993; Blunden et al. 1997). Our results also showed that 0.1 g/L ANE treatment significantly enhanced the chlorophyll content of spinach plants. It was shown that cytokinin-like effect and betaines in ANE might be responsible for this effect (Whapham et al. 1993). Interestingly, we observed that the transcription of betaine aldehyde dehydrogenase (BADH) and choline monooxygenase (CMO) were higher in 0.1 g/L ANE-treated leaves as compared to the control. These enzymes catalyze a two-step metabolic pathway in glycine betaine synthesis in plants (Russell et al. 1998). The results presented here show that ANE may induce endogenous accumulation of betaine which may act additively with betaine present in ANE and furthermore, may "arm" plants with enhanced resistance to osmotic stress. ANE has been demonstrated to mediate increase in osmolyte (e.g. proline) accumulation in *Arabidopsis thaliana* during freezing stress (-2 °C; Rayorath 2009) but whether the effect on induced proline content exists under normal growth conditions is not known.

The studies (Allen et al. 2001; Zhang and Schmidt 2000; Zhang and Ervin 2004; Zhang and Ervin 2008) have shown that exogenous application of ANE containing cytokinins-like elicitors increased endogenous antioxidant activity in plants, such as increased amounts of non-enzymatic antioxidant compounds (α-tocopherol, ascorbate and β-carotene) and enhanced activities of antioxidant enzymes including ascorbate peroxidase, glutathione reductase, and superoxide dismutase under water stress In the present study, we found increased total phenolic and flavonoid content and total antioxidant capacity of spinach treated with 0.1 g/L ANE. These may partly be attributed to the increase of endogenous plant hormone activities stimulated by ANE. A higher transcript abundance of glutathione reductase (GR) was observed in the ANE treatment, indicating a high reduced GSH pool could be maintained, thus enhancing the antioxidative capacity in cells. We observed that both ANE treatments had no effect on the activity of phenylalanine ammonia lyase (PAL), but 0.1 g/L treatment significantly increased the activity of chalcone isomerase (CHI), being one of the key enzymes for the biosynthesis of flavanone precursors and phenylpropanoid plant defense compounds. This suggests that ANE supplemented into the solid growth medium may induce specific systemic physiological responses, including eliciting the phenylpropanoid and flavonoid pathways. If so, this would lead to promotion of anti-radical capacity in spinach leaves.

This is in accord with the results in the previous chapter. Although ANE treatment enhanced the transcription of genes involved in the ascorbate cycle, the effect did not lead to significant differences in the total ascorbate content in plants (data not shown). The higher concentration (i.e. 0.5 g/L) of ANE exerted no positive effects as compared to the low concentration (i.e. 0.1 g/L) demonstrated that a high "load" of ANE may negatively affect plant growth. This is possibly due to the high salt index; the same pattern was also observed in the previous chapter.

Taken together, the results presented in this chapter show that extracts of *A. nodosum*, when applied to the early growth stage of spinach, can induce specific systemic physiological responses, probably the phenylpropanoid and flavonoid pathways. In doing so, this resulted in significant activation of plant growth and antioxidative activities in spinach. These data further confirm the results obtained in the previous chapter. The study of physiological mechanisms responsible for the beneficial effects of ANE is on the horizon and mode of action of the compounds contained in ANE needs future study.

Chapter 4

Effects of *Ascophyllum nodosum* extract on post-harvest storage of spinach (*Spinacia oleracea* L.)

4.1 Abstract

Fresh leaves of spinach (*Spinacia oleracea* L.) contain high levels of beneficial phytochemicals. However, spinach is highly perishable especially during post-harvest storage and handling. In the previous chapters, it has been shown that application of commercial *Ascophyllum nodosum* extract (ANE) improves the nutritional quality in spinach, but the effect of pre-harvest ANE-treatment on post-harvest quality of spinach is largely unknown. In this study, the quality, total chlorophyll content, ascorbic acid, shelf life and status of oxidative stress were determined during storage in order to assess the influence of pre-harvest ANE treatment on senescence and decay of fresh-cut spinach leaves during a 35-day post-harvst storage period. ANE-treatment did not affect the dry weight, chlorophyll, ascorbate, and lipid peroxidation at the time of harvest. However, the loss in fresh weight and visual quality of spinach leaves was reduced by pre-harvest application of ANE after day 21 and day 14 during the 35-day storage, respectively. Those ANE-treated leaves a retained better color and turgor than the untreated control leaves. The total chlorophyll content in the control and treated leaves was identical over the storage period and decreased at a similar rate. Lipid peroxidation was significantly reduced in ANE-treated leaves. The ascorbate content decreased during storage in both ANE-treated and untreated spinach at a similar rate. A negative correlation was found between visual quality and lipid peroxidation. Taken together, the results showed that pre-harvest ANE application through root drench, especially at 1.0 g/L, enhances post-harvest storage quality of spinach leaves by reducing the senenscence rate.

4.2 Introduction

The incidence of various human chronic diseases, such as cancer and cardiovascular disease, is high and increasing. The public has realized that a nutritious diet can promote the maintanence of good health and reduce the incidence of certain

diseases. A number of epidemiological studies have documented that a high intake of fruit and vegetables is associated with a lower occurence of chronic disease (Hung et al. 2004). As a result, there is an understandable increased demand for high quality food products, such as fresh fruit and vegetables that are rich source of bioactive compounds particularly antioxidative phytochemicals.

Previous studies have revealed that the external quality (e.g. color and firmness), and often the nutrient content, of fruit and vegetables decrease during post-harvest storage (Buescher et al. 1999). Pre- and post-harvest physiological changes in fresh produce, physical damage, undesirable environmental factors, microbial pathogens and insect pests contribute to a decrease in quality and consequent reduction of shelf life (Kader 2002). Post-harvest quality loss in fruit and vegetables can often be related to a number of pre-harvest factors (e.g. temperature and light intensity during growth, water supply, and application of agro-chemicals). Furthermore, it is commonly considered that stresses during the pre-harvest period reduce post-harvest quality (Hodges et al. 2004). For example, Wurr et al. (2002) reported that in broccoli (*Brassica oleracea* var. *italica*) onset of water stress after head initiation led to reduced head weight and diameter as well as shortened shelf life. Different species and cultivars within a species vary in their ability to respond to stresses (Hodges and Lester 2006; Howard et al. 2002). The growth stage/maturity of fruits and vegetables at harvest can also influence response to abiotic stress during storage. For example, Bergquist et al. (2006) found that harvesting baby spinach a few days earlier than normal improved visual quality and nutritional value during their storage. Reyes et al. (2007) reported an increased antioxidant content in response to wounding of fresh-cut produce was dependent on the vegetable and the tissue selected. Since there may be significant changes in the concentrations of bioactive compounds throughout the day of harvest, possibly related to light intensity and water content (Veit et al. 1996), timing during the day of harvest might also be a factor that impacts on quality of fresh produce. Higher content of antioxidants at harvest ensured the ability of apples from later harvests to reduce the influence of oxidative stress during subsequent storage (Hodges et al. 2004).

Post-harvest losses may also result from abusive storage conditions which can manifest as loss of flavor, texture and pigments; softening of tissues, induction of decay,

dehydration/weight loss and a decline in nutrient levels (Kader 2002; Nishikawa et al. 2003). In this regard, unfavourable storage conditions can exacerbate oxidative stress. For example, less-than-optimal storage temperature can induce oxidative stress, leading to such dysfunctions as loss of membrane integrity and accelerated senescence. Senescence is a slow progress of cell death often symbolized by deterioration of cellular membranes and is largely related to excess reactive oxygen species (ROS; Hodges, 2003). Oxidative stress-associated post-harvest senescence (e.g. the repression of enzyme activities, loss of pigments, and decomposition of membranes) is often manifested as shortened shelf life and nutritional quality decline (Hodges et al. 2004).

It has been documented that post-harvest losses are one of the major problems in modern agriculture and it has been estimated that approximately 25–50% of fresh fruit and vegetables are lost annually between the field and consumer (Kader 2002). Considerable work is being conducted to maintain quality and reduce losses in visual quality and quantity and quality of bioactive compounds in order to retain overall storage quality of the commodity. There are several physical (e.g. low/high temperature and ultraviolet and gamma irradiation) and chemical (e.g. methlyl jasmonic acid and ethylene) treatments (Toivonen 2003) that reduce post-harvest loss. For instance, cold storage is often used for leafy vegetables because they are highly prone to degradation at room temperature (Kader 2002). Ethylene treatment was found to reduce chilling injury in honeydew melons (*Cucumis melo* L., Inodorus Group; Toivonen 2003). It should not be overlooked that some of these treatments can induce the synthesis of phytochemicals in fruit and vegetables. The extent of such benefits differs greatly between genotypes and is dependent on the phyto-chemical compound itself.

The brown alga *Ascophyllum nodosum* is a widely-researched seaweed species traditionally used as a fertilizer and soil conditioning agent. The application of *Ascophyllum nodosum* extract (ANE) has been suggested to improve a number of physiological characteristics in plants, such as increased root growth, elevated capsidiol concentration (Norrie and Hiltz 1999) and improved resistance to environmental stresses (Khan et al. 2009). A few investigations have been carried out into the potential use of ANE to minimize postharvest losses in fruit and vegetables. The deterioration of watermelon and nectarines was delayed and their shelf life was significantly prolonged in

ANE-treated fruits than that of control ones over an 18-day storage (Norrie and Hiltz 1999). Abdel-Hafeez (2005) reported that pre-harvest application of ANE significantly reduced pear fruit weight loss and decay incidence during postharvest handling.

Spinach is an annual, cool season, green, leafy vegetable which is high in bioactive compounds such as vitamins A and C, and flavonoids (Conte et al. 2008). Spinach has a relatively high respiration and water-loss rates, and is prone to tissue decay (Hodges and Forney 2003), microbial growth (Conte et al. 2008), and loss of nutrients (Bergquist et al. 2006; Pandrangi and Laborde 2004), all of which lead to a low storage potential. Cold storage at ~7.5 °C with a high relative humidity (RH) (>90%) can significantly improve the shelf life of spinach (Kader 2002). In Nova Scotia, Canada, spinach accounts for $20 million worth of vegetables production. Recently, it was observed that ANE treatments may induce specific, systemic physiological responses, including elicitation of the phenyl-propanoid and flavonoid pathways of spinach leaves, thus leading to promotion of nutritional quality and anti-radical capacity (Fan et al. 2011). There are very few reports that deal with the effects of ANE application on physiological responses and nutritional quality of harvested spinach (Cassan et a1. 1992). This study was undertaken to investigate the influence of pre-harvest ANE-treatment on the postharvest senescence, the retention of vitamin C and the maintenance of other quality attributes in spinach during storage.

4.3 Materials and Methods

Seeds of spinach (*Spinacia oleracea* L., var. Unipack 12) were purchased from Stokes Seeds Co, Thorold, Ontario, Canada. Soluble powder of *Ascophyllum nodosum* alkaline extract (Acadian[®]) was obtained from Acadian Seaplants Limited, Dartmouth, Nova Scotia, Canada. All the other chemicals and reagents were of analytical grade and were purchased from Sigma Aldrich (Oakville, Ontario, Canada) unless otherwise stated.

4.3.1 Plant Culture and Treatment

Spinach seeds were planted at 1.5 cm deep in plastic pots (5" diameter; 3 seeds per pot) containing sweet soil (1 limestone:100 peatmoss:100 perlite:30 sand, w/v/v/v). Pots were placed in a growth chamber maintained at 18 °C and 95% relative humidity

(RH) with a photo period of 10 h light/14 h dark under light from fluorescent tubes and incandescent bulbs of approximately 350–400 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Fertilization with water-soluble $20N-20P_2O_5-20K_2O$, at a concentration of 200 ppm was initiated 3 weeks after sowing and then applied every 4 days at a rate of 200 mL per pot. Plants were grown for 6 weeks.

Solutions of *A. nodosum* extract (ANE) were prepared by dissolving 0.1 g, 1.0 g, or 5.0 g soluble powder in 1.0 L distilled water with continuous stirring for 10 min. Spinach plants were root drenched with various ANE solutions (i.e. 0, 0.1, 1.0, or 5.0 g/L) at the rate of 50 mL per plant on days 21, 14 and 7 prior to harvest.

4.3.2 Sample Preparation and Storage

Leaves from the 5–7th positions counted from the bottom were manually cut using a pair of sharp scissors at a petiole length of three-fourths of the leaf blade length. These were placed in plastic bags in a refrigerated cooler (4 °C) within 1 h of harvest.

For the studies on changes during post-harvest storage, randomly selected spinach leaves for each treatment were placed into perforated plastic bags (50 g per bag), sealed and then stored in a dark, controlled storage room maintained without light at 10 °C with a relative humidity (RH) of $\geq$ 95%. A long cold storage period (i.e. 5 weeks) was investigated. Samples were removed from storage on day 0, 7, 14, 21, 28, and 35. The leaves were de-veined and chopped into small pieces ($\approx$0.5–1 cm^2). Pieces were either thoroughly mixed and sub-samples immediately extracted for total ascorbate, malondialdehyde (MDA) and total chlorophyll assays, or bagged, flash-frozen in liquid nitrogen and lyophilised to be stored in sealed plastic bags at –80 °C for later use.

4.3.3 Weight Loss

To determine the weight loss of each treatment during storage, the perforated plastic bags containing spinach leaves were weighed on day 0, and the leaves were weighed on the day of removal. Fresh weight loss was expressed as a percentage of the initial fresh weight.

To determine changes in dry weight (DW) during storage, on each sampling day, de-veined leaves were dried in an oven at 85 °C for 48 h, and expressed as g/100 g fresh weight (FW).

4.3.4 Visual Quality Analysis

Spinach leaf quality was assessed in fresh tissue at harvest and after 7, 14, 21, 28, and 35 days of storage. The visual quality (color and turgor) of spinach leaves was evaluated on a 15 to 2 scale (Appendix D).

4.3.5 Determination of Total Chlorophyll

Total chlorophyll was analyzed as described in section 3.3.4. All extraction procedures were carried out under dim light and on ice. Chlorophyll was analyzed on the day after harvest, and after 7, 14, 21, 28 and 35 days of storage in all treatments. Fresh leaf tissue (0.8 g) was immediately ground using a mortar and a pestle with cold methanol (MeOH) in the presence of a small amount of inert sand. After centrifuging at 10,000×g at 4°C for 10 min, the pellets were re-extracted in MeOH until all color was removed. The volume of combined extracts was made up to 50 mL in a volumetric flask. Absorbance was measured at 652, 665, and 750 nm using a spectrophotometer. The total chlorophyll content was calculated according to Ritchie (2008).

4.3.6 Lipid Peroxidation Analysis

The malondialdehyde (MDA) concentration was assayed using a modified method after Hodges et al. (1999). Fresh leaf samples (2 g) were homogenized in 8 mL 80% ethanol (EtOH), followed by centrifugation at 3,000×g at 4 °C for 10 min. The supernatent (100 μL) and 900 μL distilled water were added to a test tube with 1 mL of either (i) –TBA (thiobarbituric acid) solution which comprised 20% (w/v) trichloroacetic acid (TCA) and 0.01% (w/v) butylated hydroxytoluene (BHT), or (ii) +TBA solution containing the above plus 0.65% (w/v) TBA. The mixture as then mixed vigorously, heated at 95 °C in a dry bath for 25 min, cooled and centrifuged at 3,000×g for 10 min. Absorbances were measured at 440, 532, and 600 nm. MDA equivalents were calculated using the following formula: (1) [(Abs 532_{+TBA})-(Abs 600_{+TBA})-(Abs 532_{-TBA}-Abs600_{-}

$_{TBA}$)] = A, (2) [(Abs 440$_{+TBA}$-Abs 600$_{+TBA}$) 0.0571] = B, (3) MDA equivalents (nmol/mL) = $10^6 \times$[(A-B)/157 000].

4.3.7 Determination of total ascorbate

Total ascorbate was analyzed as described in section 2.3.9. Fresh chopped leaves (5 g) were immediately ground in a mortar and pestle with inert sand and 15 mL ice-cold, freshly prepared 5% (w/v) *m*-phosphoric acid. The homogenate was centrifuged at 10,000×g for 15 min at 4 °C. Total ascorbate was determined by initially incubating 100 µL supernatant, 500 µL 150 mM KH$_2$PO$_4$ buffer (pH 7.4) containing 5 mM EDTA, and 100 µL 10 mM dithiothreitol (DTT) at room temperature for 50 min. Following then 100 µL of 0.5% (w/v) N-ethylmaleimide (NEM) was added to remove excess DTT. In order to develop color in reaction mixtures, reagent solutions were added in the order of 400 µL 10% (w/v) TCA, 400 µL 44% *o*-phosphoric acid, 400 µL 4% (w/v) α-α^1-dipyridyl, and 200 µL 30 g/L ferric chloride (FeCl$_3$). The reaction mixtures were incubated at 40 °C for 60 min in a shaking water bath, and absorbance was taken at 525 nm. The results were expressed as µmol/g FW.

4.3.8 Statistical Analysis

The effects of treatment were analyzed by ANOVA and differences were considered statistically significant at $P \leq 0.05$ using Tukeys Honestly Significant Differences (HSD) test of the COSTAT$^®$ statistical software. Twenty spinach plants were used in each treatment group. This experiment was repeated in quadruplicate. The plants irrigated with 0 g/L ANE which meant that they received an equal amount of distilled water in the extract treatment were considered as control plants.

4.4 Results
4.4.1 Weight Loss

The weight loss of spinach leaf in storage is shown in Fig. 4.1. The loss of fresh weight of the samples increased with storage time. The weight loss of the leaves from the control plants was higher than that of the leaves from ANE-treated spinach. Treatment with 1.0 g/L ANE significantly reduced the weight loss as compared with control and

other treatments after day 21 during the 35-day storage. An average daily loss of 0.6% was observed in control plants while the loss from 1.0 g/L ANE treatment was 0.4%. The dry matter content of the leaves from different treatments did not change significantly during storage or between treatments and the control (Table 4.1.).
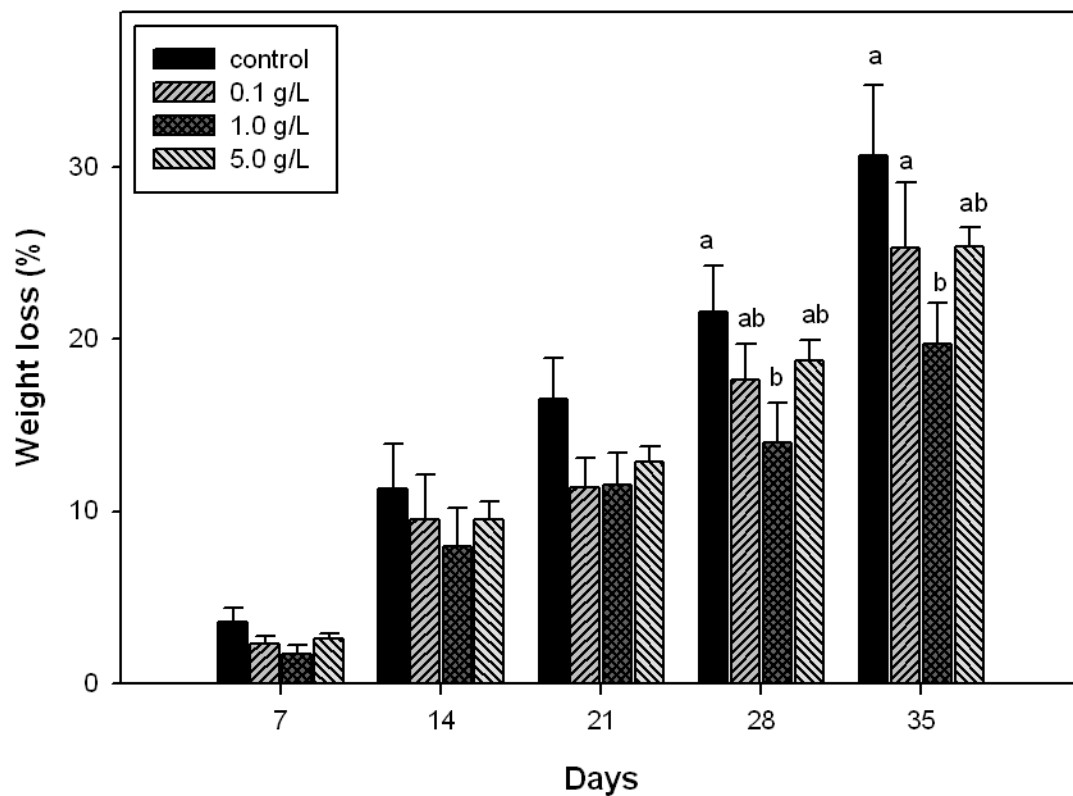
Figure 4.1. The effect of different concentrations of ANE on fresh weight loss of spinach leaves held over 35 d post-detachment at 10 °C in the dark with RH ≥ 95%. Bars indicate mean ± standard error.

Table 4.1. Dry matter (g/100g FW) of spinach leaves held over 35 d post-detachment at 10 °C in the dark with RH ≥ 95%[a]

	Days of storage					
Treatment	**0**	**7**	**14**	**21**	**28**	**35**
control	10.75a	10.55a	10.96a	11.11a	11.34a	11.52a
0.1 g/L	10.96a	10.96a	10.76a	10.74a	10.98a	11.12a
1.0 g/L	11.06a	10.59a	11.11a	11.03a	10.89a	11.62a
5.0 g/L	11.23a	10.74a	10.84a	11.04a	11.16a	11.39a

[a]Values with the same letters are not significantly different ($P < 0.05$).

4.4.2 Visual Quality

Stored spinach leaves were examined for changes of visual (color and turgor) quality over a 35-day period (Fig 4.2). The fresh leaves scored between 15 and 6 for total quality assessment, where 15 was the best. The visual quality gradually decreased during storage in all the treatments (Fig 4.3). The quality score was significantly higher in ANE-treated leaves than untreated controls on each sampling date during post-harvest storage. It was showed that both color and turgor decreased during storage, but ANE treatments, especially 1.0 g/L, significantly reduced the degradation and retarded the onset of quality loss (Fig 4.3).

Control leaves started to curl and yellow after day 7 and became notably wilted after day 14 with approximately 25% of the leaves showing signs of yellowing and the onset of yellowing and rolling accelerated with further storage (Fig 4.4). The loss of turgor also increased during storage in all leaves and was significantly less in 1.0 g/L ANE-treated leaves on day 35 (Fig 4.2B). Overall, the visual quality reduced significantly in all leaves at the end of 35 days versus the start. Moreover, 1.0 g/L treatment significantly better maintained the visual quality of spinach leaves after day 14 during the 35-day storage period.
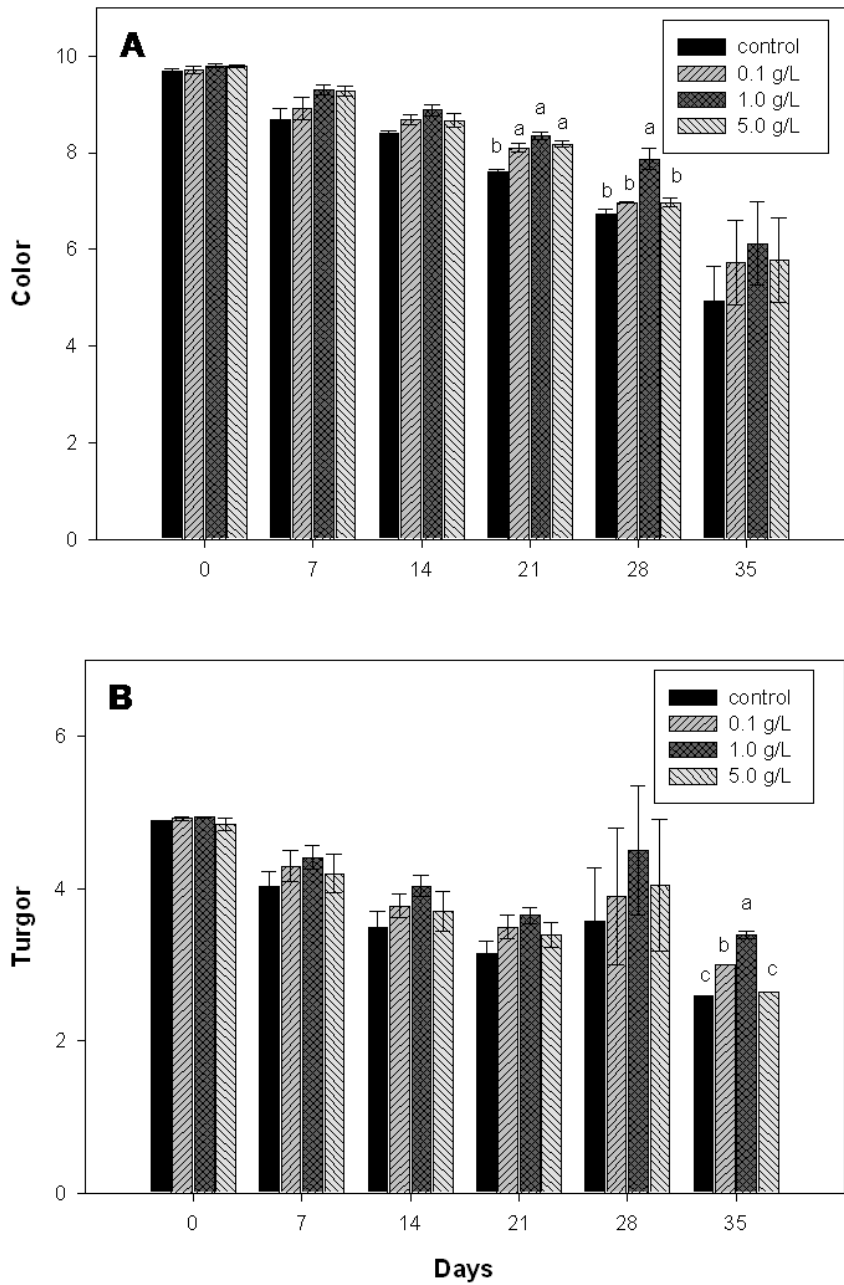
Figure 4.2. The effect of different concentrations of ANE on (A) color and (B) turgor of spinach leaves held over 35 d post-detachment at 10 °C in the dark with RH ≥ 95%. Bars indicate mean ± standard error.
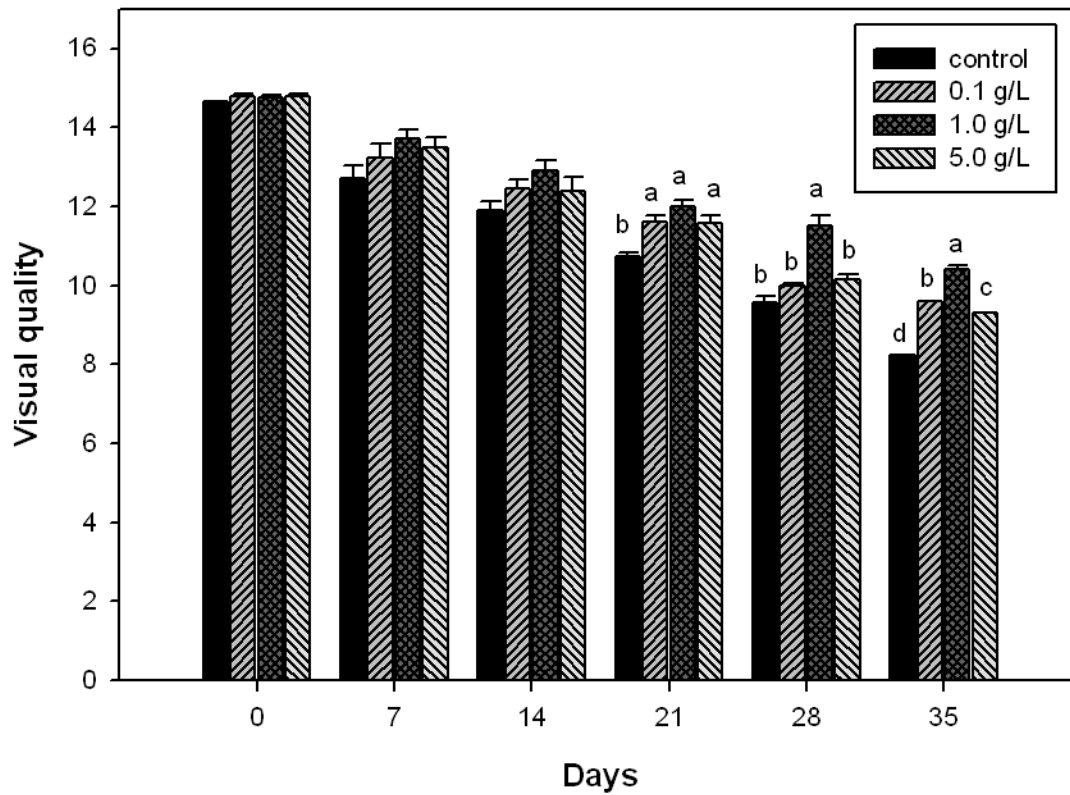
Figure 4.3. The effect of different concentrations of ANE on visual quality of spinach leaves held over 35 d post-detachment at 10 °C in the dark with RH ≥ 95%. Bars indicate mean **±** standard error.

Days of storage: 7 14 21 28 35

control

0.1 g/L

1.0 g/L

5.0 g/L
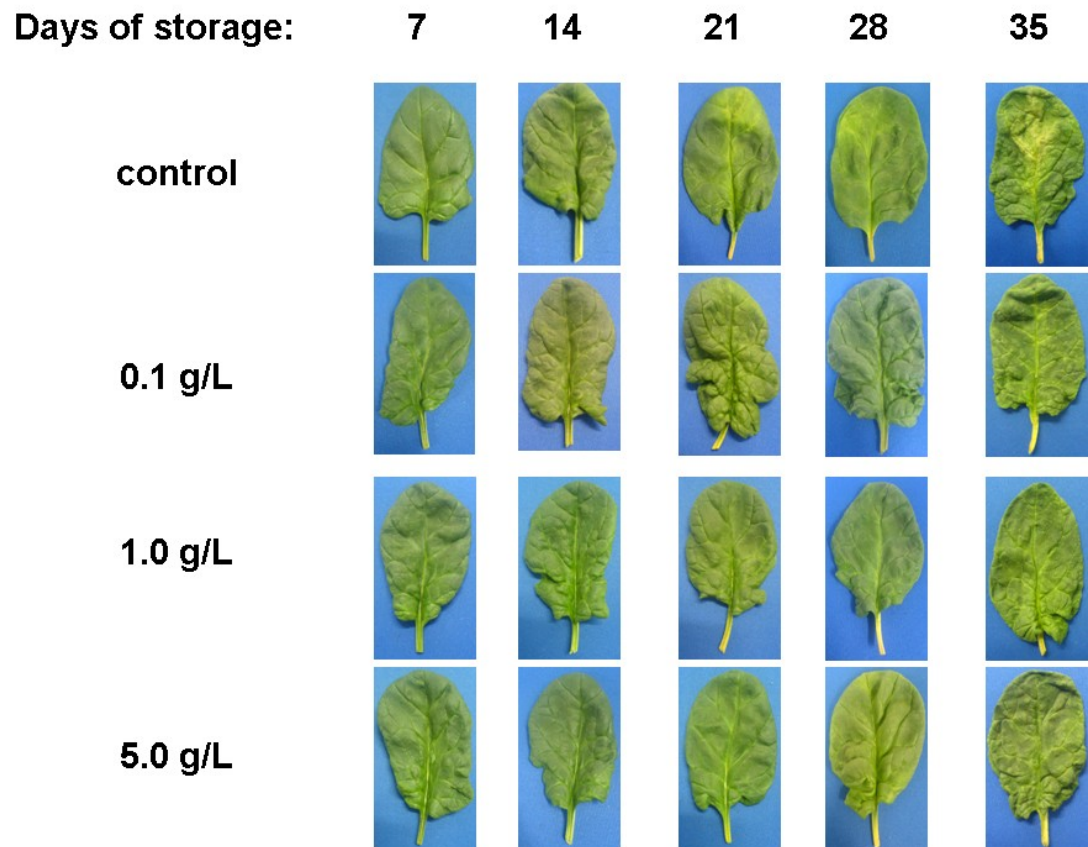
Figure 4.4. Leaves of ANE-treated and untreated spinach over 35 d post-detachment at 10 °C in the dark with RH ≥ 95%.

4.4.3 Determination of Total Chlorophyll

The total chlorophyll content of spinach leaves was 7 mg/g DW and there was no significant difference between ANE-treated and untreated leaves (Fig. 4.5). Little change in chlorophyll content was observed until after 14 days of storage (Fig. 4.5). No significant difference in the loss of chlorophyll was found between control and treated leaves at each of the sampling time during post-harvest storage (Fig. 4.5). The average loss of total chlorophyll was roughly 85 µg/g DW per day.
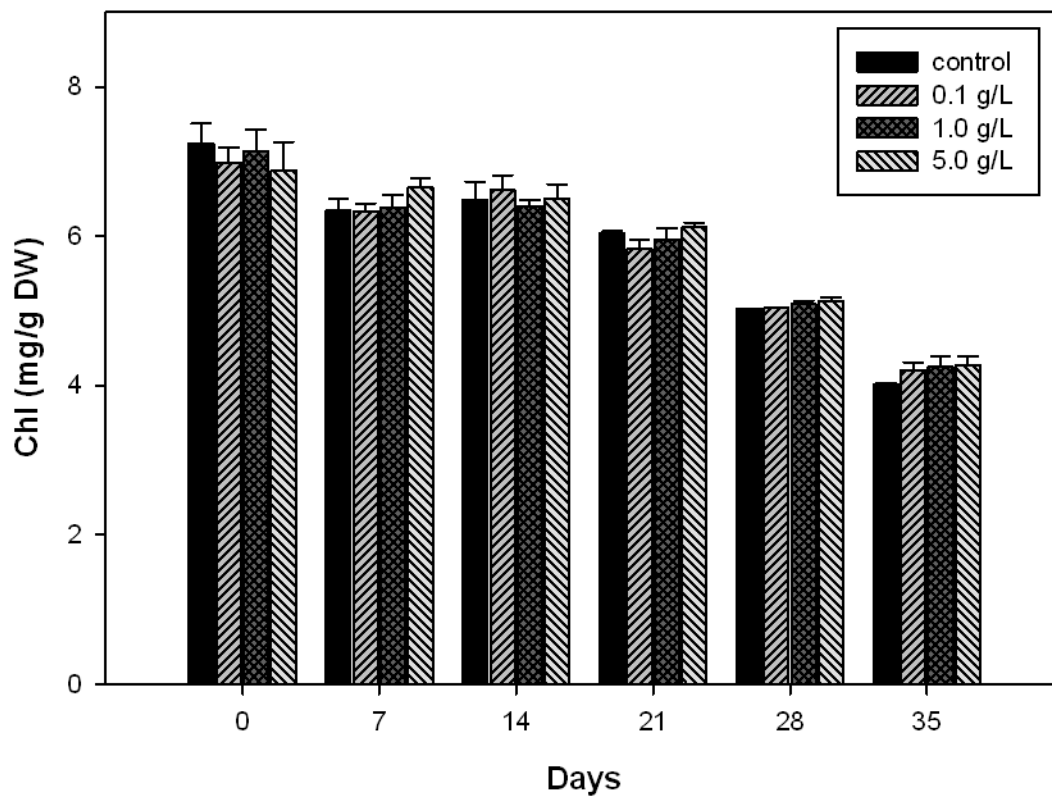
Figure 4.5. The effect of different concentrations of ANE on total chlorophyll content of spinach leaves held over 35 d post-detachment at 10 °C in the dark with RH ≥ 95%. Bars indicate mean ± standard error.

4.4.4 Lipid Peroxidation Analysis

Oxidative stress, as estimated by malondialdehyde (MDA) content, dramatically increased after day 7 in the detached spinach leaves from all treatments during the course of the experiment (Fig. 4.6). The initial levels of MDA were not significantly different between control and treated leaves, which were found to be around 4 nmol/g DW. As compared with control and 0.1 and 5.0 g/L treatment, ANE treatment at 1.0 g/L significantly reduced lipid peroxidation on day 21, 28, and 35. The concentration of MDA in the control leaves increased to over 8 nmol/g FW compared with 1.0 g/L treatment that recorded values below 6 nmol/g FW at day 21, and over 13 nmol/g DW in controls compared with below 10 nmol/g DW in ANE-treated leaves at day 35. Overall, control leaves showed a significant increase after day 14 and exhibited the highest level of lipid peroxidation throughout the storage period. MDA content was significantly higher in all leaves at the end of removal versus the start.
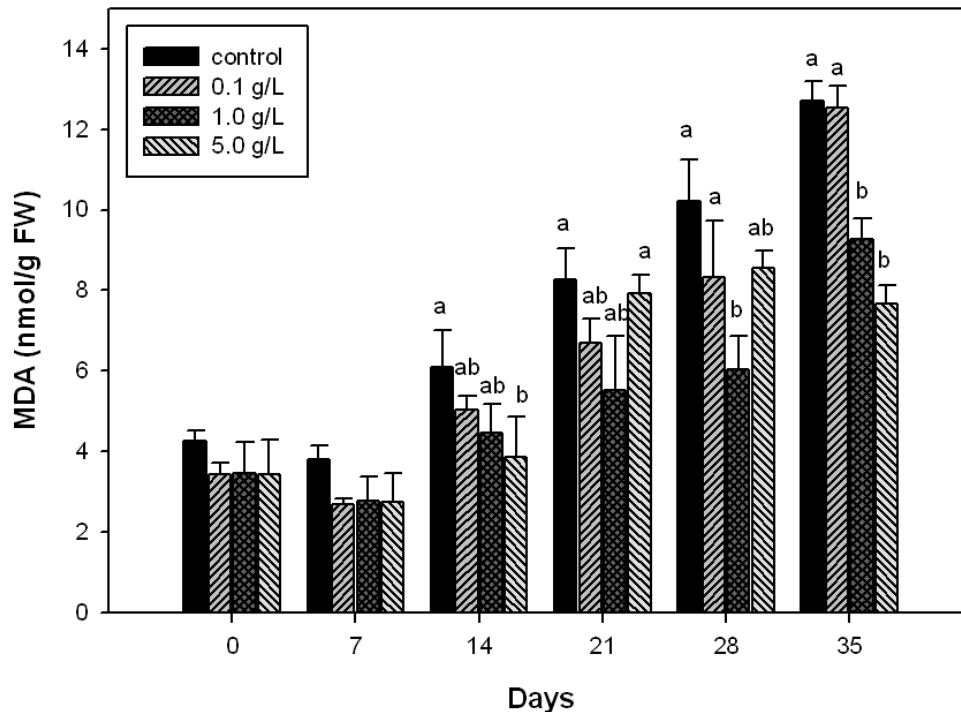
Figure 4.6. The effect of concentration of ANE on MDA content levels of spinach leaves held over 35 d post-detachment at 10 °C in the dark with RH ≥95%. Bars indicate mean ± standard error.

4.4.5 Ascorbate Loss

As shown in Fig. 4.7, there were no significant differences in total ascorbate content in leaves between control and treatments on day 0. There was a rapid decrease in ascorbate content in all leaves for the first 21 days. The total ascorbate content declined from 3.6 to 1.3 µmol/g FW during 35 days of storage. No differences in total ascorbate between control and treated leaves were noted throughout the post-harvest storage period.
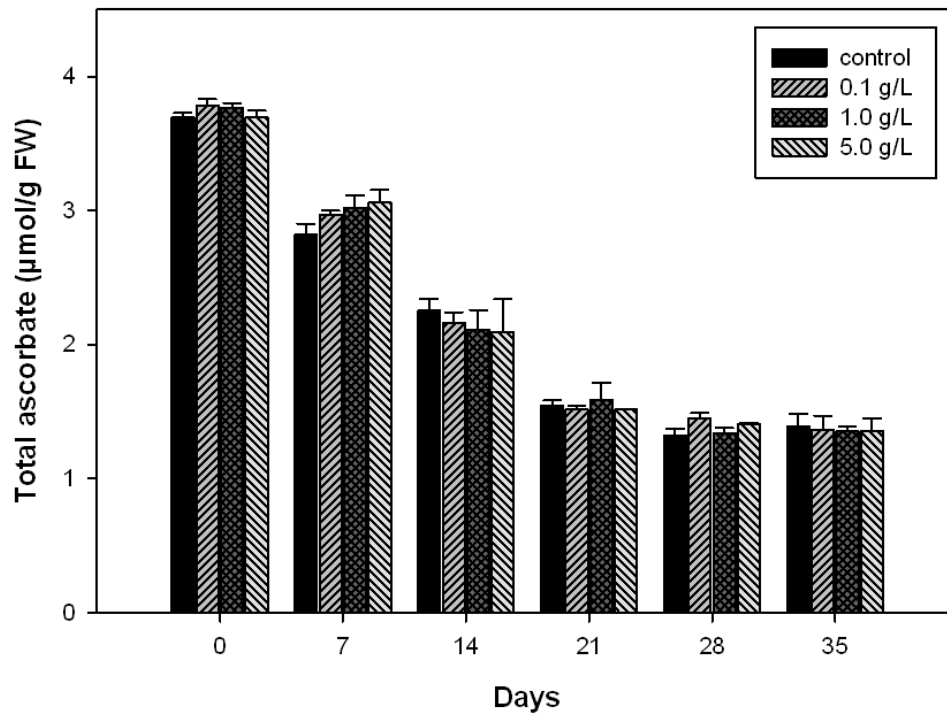
Figure 4.7. The effect of different concentrations of ANE on total ascorbate levels of spinach leaves held over 35 d post-detachment at 10 °C in the dark with RH ≥ 95%. Bars indicate mean ± standard error.

4.4.6 Linear Regression Analysis

Using regression analysis significant correlations were found between the quality of appearance with MDA levels (R^2 = 0.811) and ascorbate content (R^2 = 0.820), respectively, during the 35-day storage period (Fig. 4.8). The higher visual quality of the

spinach leaves correlated with a lower level of lipid peroxidation or a higher concentration of ascorbate.

A

$R^2=0.811$
$P<0.001$

Visual quality (y-axis)
MDA (nmol/g FW) (x-axis)

B

$R^2=0.820$
$P<0.001$

Visual quality (y-axis)
Total ascorbate (µmol/g FW) (x-axis)
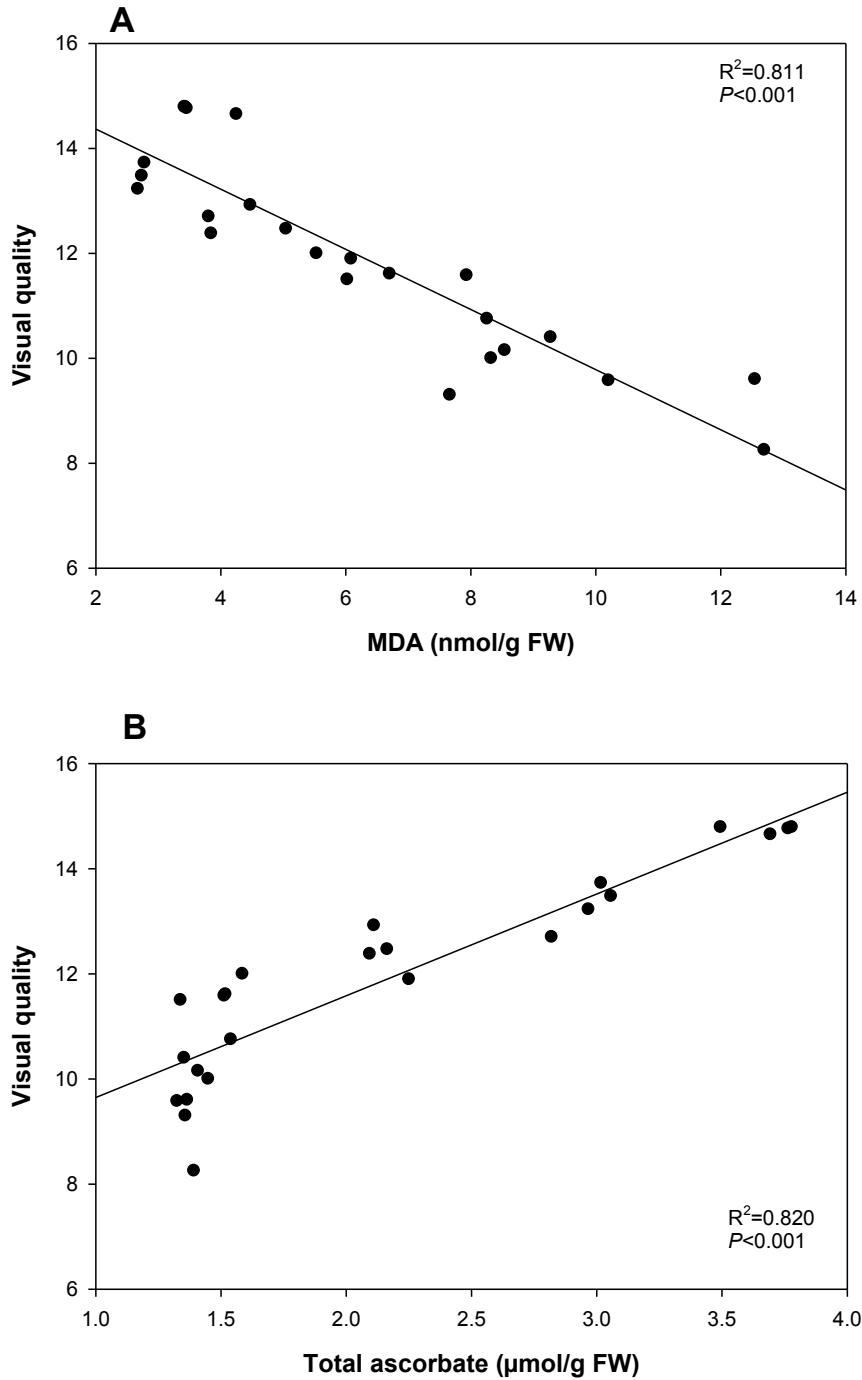
Figure 4.8. Correlation between visual quality and (A) MDA content and (B) total ascorbate content of spinach leaves held over 35 d post-detachment at 10 °C in the dark with RH ≥ 95%.

4.5 Discussion

The results drescribed in this chapter demonstrate that *Ascophyllum nodosum* extract (ANE) treatment can improve the post-harvest storage of spinach. The ANE treatments were applied as root irrigation which resulted in reduced impaired post-harvest quality during extended storage in spinach. This suggests that the ANE treatments may induce certain physiological responses leading to improved post-harvest maintenance in spinach leaves.

Spinach is an important leafy vegetable in regard to nutrients and ranking the third in total antioxidant capacity, just behind garlic (*Allium sativum*) and kale (*Brassica oleracea*) (Cao et al. 1996). The genotype and growth conditions, etc. can significantly impact plant metabolism, which in turn can affect crop quality at harvest and also post-harvest quality (Weston and Barth 1997). Commercially, spinach leaves are stored in polypropylene bags at low temperatures close to 0 °C (Bergquist et al. 2006), but often they are kept in the range of 4 to 10 °C. Unipack 12 is considered as a relative tough cultivar that is not as prone to deteriorate as other spinach cultivars, thus 10 °C was chosen as storage temperature for this study (communication data). Spinach leaves have a high respiration rate (Bergquist et al. 2006) and a short shelf life, the duration of which is highly dependent on cultivars (Kader 2002). Post-harvest changes in spinach may include losses in visual quality and increase in microbial populations concomitant with a reduction in nutrient content (Conte et al. 2008).

Weight loss and visible signs of deterioration are two major commercial concerns for the storage of leafy vegetables. Fresh weight loss was significantly reduced in pre-harvest 1.0 g/L ANE-treated leaves as compared to the control after day 21 during storage. The dry weight (DW) of spinach leaves from all treatments was identical at each removal date. Transpiration is the evaporative loss of water while respiration deprives the leaves of carbohydrates and water. These indicate that pre-harvest ANE-treatment might reduce the transpiration and respiration activities in the spinach leaves during storage. One possibility is that the application of ANE may lead to reduced activities of lipoxygenase and peroxidase which have been shown to accelerate the degradation processes (Pandrangi and Laborode 2004).

Changes in leaf visual quality were analyzed using two parameters, color and turgor. Control leaves turned to yellow and wilted early during storage as compared to the pre-treated leaves. ANE-treated (1.0 g/L) leaves retained their turgor better than the control. The shriveled appearance of spinach leaves during storage correlated with the loss of fresh weight in each of the treatment. In order to understand the extent chlorophyll content may affect visual quality of stored spinach leaves, the changes of total chlorophyll during storage were studied. The total chlorophyll content decreased during storage regardless of the treatment, and degradation rate increased after day 14 of storage, which was in line with the report from Hodges and Forney (2003). This suggests that the changes in chlorophyll levels may not directly correlate with visual quality of stored spinach leaves. One possible reason is that the loss of chlorophyll in leaves tend to be distributed unequally (Bergquist et al. 2006). Interestingly, in this study we observed that ANE did not influence the total chlorophyll whilst applications of ANE to the early growth stages of spinach plants in Chapter 3, where the total chlorophyll increased significantly by 30%, indicating that the timing of application affect total chlorophyll content in spinach.

Changes in color of the harvested leaves have been shown to be related to senescence (Yamauchi and Watada 1991) which is largely regulated by reactive oxygen species (ROS). ROS can lead to degradation of phospholipids which result in accumulation of free fatty acids that may be oxidized and therefore act as radical intermediates, thus exacerbating the senescence process (Hodges and Forney 2003). Increased lipid peroxidation is one of the characters of leaf senescence and malondialdehyde (MDA) is the major by-product of lipid peroxidation. Zhang and Ervin (2008) reported that ANE application significantly reduced lipid peroxidation, accompanied with notable increase in leaf superoxide dismutase activity in creeping bentgrass (*Agrostis stolonifera* L.) under heat stress. A decrease in α-tocopherol content after industrial processing of spinach leaves was accompanied by an increase in MDA level (Murcia et al 1992). In order to assay the degree of oxidative stress in detached spinach leaves during storage, the content of MDA was measured. ANE treatments of plants priorto leaf harvest did not affect the MDA in leaves at the time of harvest. Though the MDA content increased during storage in all treatments, ANE treatments at 1.0 and

5.0 g/L significantly reduced the rate of increase after day 21, suggesting that ANE applications to plants pre-harvest might reduce ROS production through stimulation of antioxidant systems such as enzymes, flavonoids, and carotenoids, which would delay subsequent senescence and deterioration, or may limit ROS production itself. The high level of polyunsaturated fatty acids (PUFA) in spinach has been shown to be associated with high MDA content; hence ANE application to plants pre-harvest may also lead to direct effects on the reduced activities of some enzymes that are associated with phospholipids degredation and subsequent senescence (Barclay and McKersie 1994).

Vitamin C is a key component of the plant defense systems (Hodges and Forney 2003). In this study, ascorbate decreased significantly during post-harvest storage and no difference was observed between ANE-treated leaves and untreated controls. Higher initial ascorbic acid content and initial dry matter content in baby spinach resulted in better visual quality during storage (Bergquist et al. 2006). In this study, the correlation ($R^2 = 0.304$) between DW and visual quality in mature spinach leaves during storage was weak (data not shown). However, strong correlations were found between MDA or ascorbate content with the visual quality of spinach leaves during storage. This may be attributed to the higher antioxidant levels in the leaves and an increased ability to detoxify ROS during post-harvest storage, leading to a retarded senescence rate (Zhang and Ervin 2008).

It must be noted that phenolic content increased significantly in ANE treatment pre-harvest (Fan et al. 2011); hence its effect on post-harvest storage quality of spinach leaves cannot be ignored. It has been shown that vitamin C in some fruit and vegetables contributes <0.4% of the total antioxidant activity (Dewanto et al 2002). Our preliminary experiments showed that total phenolics in spinach leaves increased during storage at 22 °C and ANE treatment pre-harvest showed a higher increasing rate (data not shown). Meanwhile, ANE-treated leaves retained their visual quality to a greater extent as compared to control (data not shown). This may be due to the fact that a high antioxidant content results in a decreased rate of senescence (Hodges and Forney 2003).

The differences in the changes in fresh weight, chlorophyll content and MDA levels between ANE-treated leaves and control during post-harvest storage suggest that pre-harvest treatment of spinach with ANE, especially at 1.0 g/L, may improve the

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storage quality of spinach through regulating the senescence rates of leaves, and probably is independent of ascorbate content.

Chapter 5

Commercial extract of the brown seaweed *Ascophyllum nodosum* enhances the capacity of *Spinacia oleracea* L. extracts to impart protective effects on the model nematode, *Caenorhabditis elegans*, against oxidative and thermal stress

5.1 Abstract

Spinach (*Spinacia oleracea* L.) is one of the most popular vegetables with antioxidative properties. It contains large quantities of bioactive components, including phenolics. In mammals, spinach leaf extracts containing mainly phenolics and flavonoid compounds have been extensively shown to exhibit antioxidative, anti-inflammatory, anti-mutagenic, anti-proliferative and anti-carcinogenic properties. In the present investigation, the *Caenorhabditis elegans* nematode model was used to study the protective effects of spinach extracts against oxidative and thermal stress. The biological effect of commercial *Ascophyllum nodosum* extract (ANE)-enhanced polyphenols was also tested. The extracts of spinach treated with ANE and that of untreated plants increased the life span of *C. elegans*. However, there was no difference between the ANE-treated spinach and the control. In contrast, extracts from ANE-treated spinach significantly improved the survival of the animals under oxidative stress by a magnitude of 50% and increased survival under high temperature stress up to 61% as compared to extracts from untreated plants (0 and 38% respectively). A caloric restriction mimetic was not identified as a possible mode of action. The results suggested that spinach extracts may act as hormetic agents and mimic the effects of mild stresses that elicit defense response(s) and thus result in prolonged life span and increased stress resistance in *C. elegans*. Furthermore, the increased flavonoid content in spinach exerts beneficial effects in *C. elegans* against oxidative and heat stresses probably via different mechanisms. Spinach extract also led to significant increased fat storage by comparison with that of control worms, and down-regulated the proportions of 19:0 and 20:4n6 and

up-regulated the proportions of 15:iso, 17:iso, 18:4n3, 20:3n3, 14:1n9, and 16:1n9T. Taken together, these results suggest that chemical components, primarily polyphenolics in spinach extracts, impart beneficial effects on the life span and thermal and oxidative stress and influence the regulation of fat storage in *C. elegans*. Extracts prepared from ANE-treated plants seem to exert the strongest effects.

5.2 Introduction

Aging is a progressive accumulation of molecular damage and failure of homeo-dynamics that consequently results in age-related diseases and, ultimately, death (Rattan 2008). Accumulation of reactive oxygen species (ROS), which results in an imbalance of pro- and anti-oxidative side in favor of a pro-oxidative state, has been considered as one of the major factors in aging; this is considered as the basis of the free radical theory of aging. This rationale is also the key reason for the recommendation of generous intake of fresh fruits, vegetables and tea since these foods and drink are rich in antioxidants that can protect against ROS. Organisms have evolved regulatory pathways to induce the expression of stress responsive genes to defend against oxidative stress, functional decline, and ultimately, aging (Kenyon 2005). For example, the DAF-2/insulin/IGF-signaling pathway is markedly conserved in *C. elegans*, *Drosophila*, and mammals; reduced or abrogated *daf-2* signaling leads to extended life span and increased stress resistance (Kimura 1997). The reduced *daf-2* leads to the activation of DAF-16/FOXO, a transcription factor and a key regulator of a wide range of genes involved in stress responses, life span and metabolism in *C. elegans* (Baumeister et al. 2006).

Earlier studies suggested that certain extracts of plant origin could be effective in reversing aging (Joseph et al. 1998), extending life span (Peng et al. 2009) and improving stress tolerance (Gruber et al. 2007; Wilson et al. 2006). Phenolic compounds in plants have been shown to stabilize free electrons originating from free radicals because of their high reactivity as electron or hydrogen donors (Podsędek, 2007). Recent studies demonstrated that polyphenols such as blueberry polyphenols (Joseph et al. 1999; Wilson et al. 2006), resveratrol from wine grapes (Gruber et al. 2007; Kang et al. 2002; Valenzano et al. 2006), *Ginkgo biloba* (Kampkötter et al. 2007c; Winter 1998), and epigallocatechin gallate (EGCG) from green tea (Zhang et al. 2009) were able to prolong

the life span and/or increase resistance to stressors in several model systems. Extracts from spinach leaves which primarily contain glucuronic acid derivatives of flavonoids and *p*-coumaric acid derivatives have been shown to be free of estrogenic or anti-estrogenic activity (Lomnitski et al. 2003b), and are highly effective free-radical scavengers with anti-aging, anti-inflammatory properties and anti-neoplastic potential in various species such as rat, mouse and rabbit (Bakshi et al. 2004; Bergman et al. 2001; Joseph et al. 1998; Nyska et al. 2003).

Categories of aging mechanisms

(1)	Cell and molecular damage late in life (A) Oxidative damage (B) Somatic DNA mutation (C) Telomere shortening (D) etc.	**Different between species**
(2)	Mechanisms linked to young adulthood (A) Antagonistic pleiotropy; such as cell senescence (B) Caloric restriction – famine survival in young adulthood and extended lifespan in old age (C) Insulin-signaling pathway – regulates caloric restriction	**Similar between species**
(3)	Unavoidable consequence of old age (A) Inflammation response caused by infection and pathogenic invasion in old age	**Similar between species**

Fig. 5.1. Three categories of aging predicted by the mutation accumulation theory (Kim 2007).

The nematode *C. elegans*, a simple yet sophisticated multicellular animal, has become an important model organism for the study of biological processes due to its easy culture conditions, short generation time and life span and experimental flexibility (Kaletta and Hengartner 2006). Moreover, *C. elegans* and humans share similar aspects of aging (Herndon et al. 2002), where oxidative stress is considered to be a major limiting factor in life span (Finkel and Holbrook 2000). It is for these reasons that *C. elegans* is highly-valued for identifying compounds, genes and mechanisms that may extend the longevity of humans (Wilson et al. 2006). The nematode responds well to diet intervention. Life span analysis in *C. elegans* has been used as an excellent experimental system for the study of aging. There are many signaling pathways such as insulin/IGF signaling (Kenyon 2005), cell cycle checkpoint signaling (Olsen et al. 2006), rapamycin (TOR) signaling (Vellai et al. 2003), and c-Jin N-terminal kinase (JNK) signaling (Oh et al. 2005), and metabolic functions including mitochondrial electron transport (Anson and

Hansford, 2004) and mRNA translation (Pan et al. 2007) that are involved in the stress response and longevity in *C. elegans*. Pharmacological tests of free-radical theory of aging in *C. elegans* and some other animals are summarized in Table 5.1.

Table 5.1. Pharmacological or dietary supplementations influence life span and stress resistance *in C. elegans* (as compared to other animals)

Name	System	Increased life span	Increased resistance to oxidative/thermal stress	Reference(s)
Vitamin E	*C. elegans*	Yes	nd/nd	Harrington and Harley (1988)
	Mouse	No	nd/nd	Lipman et al. (1998)
	Rat	nd	Yes/nd	Joseph et al. (1998)
Eukaryion-8/ EUK-134	*C. elegans*	Yes or No	Yes/Yes	Melov et al. (2000); Keaney and Gems (2003); Sampayo et al. (2003); Keaney et al. (2004)
	Mouse	Yes	nd/nd	Melov et al. (2001)
Tocotrienols	*C. elegans*	Yes	nd/nd	Adachi and Ishii (2000)
Coenzyme Q_{10}	*C. elegans*	Yes	nd/nd	Ishii et al. (2004)
Ethosuximide	*C. elegans*	Yes	nd/nd	Collins et al. (2008)
Ethosuximide/Trimethadione/ 3,3-diethyl-2-pyrrolidinone	*C. elegans*	Yes	nd/nd	Evason et al. (2005)
Lithium	*C. elegans*	Yes	nd/nd	McColl et al. (2008)

Table 5.1. Cont.

Platinum nanoparticle	*C. elegans*	Yes	nd/Yes	Kim et al. (2008)
Mianserin/Methi othepin	*C. elegans*	Yes or No	nd/nd	Petrascheck et al. (2007); Zarse and Ristow (2008);
Valproic acid	*C. elegans*	Yes	nd/nd	Evason et al. (2008)
Nicotinamide adenine diceotide	*C. elegans*	Yes	Yes/nd	Hashimoto et al. (2010)
Trolox/Lipoic acid/ Propyl gallate/ Taxifolin	*C. elegans*	Yes	Yes/nd	Benedetti et al. (2008)
Kaempferol/ Fisetin	*C. elegans*	Yes	Yes/Yes	Kampkötter et al. (2007a)
Resveratrol	*C. elegans*	Yes	Yes/Yes	Gruber et al. (2007); Kampkötter et al. (2007c)
	Drosophila	Yes	nd/nd	Kang et al. (2002)
	Nothobran chius furzeri	Yes	nd/nd	Valenzano et al. (2006)
Catechin	*C. elegans*	Yes	Yes/Yes	Saul et al. (2009)
Epigallocatechin gallate (EGCG)	*C. elegans*	No	Yes/Yes	Zhang et al. (2009)
Blueberry polyphenols	*C. elegans*	Yes	No/Yes	Wilson et al. (2006)
Blueberry water extract	Rat	nd	Yes/nd	Joseph et al. (1999)

Table 5.1. Cont.

Deuterohemin-AlaHisThrValGluLys(DhHP-6)	*C. elegans*	Yes	Yes/Yes	Guan et al. (2010)
Eleutherococcus senticosus extract	*C. elegans*	Yes	Yes/Yes	Wiegant et al. (2009)
Ginko biloba extract (Eb 761)	*C. elegans*	Yes	Yes/Yes	Wu et al. (2002); Kampkötter et al. (2007b)
	Rat	Yes	nd/nd	Winter (1998)
Rhodiola rosea extract	*C. elegans*	Yes	Yes/Yes	Wiegant et al. (2009)
	Drosophila	Yes	nd/nd	Jafari et al. (2007)
Allyl isothiocyanate (AITC)	*C. elegans*	No	Yes/nd	Hasegawa et al. (2010)
Psralea corylifolia L./ Pine bark extract	*C. elegans*	Yes	Yes/nd	Pun et al. (2010)
Cinnamomum cassia extract	*C. elegans*	Yes	Yes/nd	Yu et al. (2010)
	Rat	nd	Yes/nd	Amin et al. (2009)
Panax ginseng extract	*C. elegans*	Yes	Yes/nd	Yu et al. (2010)
Spinach 70% methanol extract	*C. elegans*	Yes	Yes/Yes	Fan et al. (2011)
Spinach water extract	Rat	nd	No/nd	Joseph et al. (1999)

Table 5.1. Cont.

Reserpine	*C. elegans*	Yes		Srivastava et al. (2008)
Wisconsin ginseng	*C. elegans*	No	nd/nd	Cao et al. (2007)
α-lipoic acid	*C. elegans*	Yes	nd/nd	Brown et al. (2006)
Ascophyllum nodusum (Tasco®)	*C. elegans*	Yes	No/Yes	unpublished
	wether lamb	nd	nd/Yes	Saker et al. (2004)

nd, not determined.

It is universal that fat stores energy to allow organisms, such as *C. elegans* and humans, to continue life in abstinence of food or starvation. Fat storage is specially regulated and any of its abnormalities may lead to pathological conditions which may be related to a disease state. *C. elegans* obtain fats from diet and *de novo* synthesis and store them mainly in epidermal and intestinal cells. Fat storage and metabolism in *C. elegans* is regulated by a complex of regulatory pathways and factors, such as *daf-2/daf-16* pathway (Rea and Johnson 2003), TUBBY proteins which are independent of DAF-16 (Mukhopadhyay et al. 2005), the *nhr-49* regulatory network (Gilst et al. 2005), a proton-coupled di- and tripeptide transporter PEPT-1 (Spanier et al. 2009), and Krüppel-like transcription factors (KLFs) (Zhang et al. 2009). The key fat-regulatory genes and pathways such as those involved in fat making, consuming and transportation in *C. elegans* have been shown to have orthology in mammals (Jones and Ashrafi 2009). Thus it would be very informative to study lipid storage and the effects of chemical interventions on the regulation of fat storage and metabolism in *C. elegans*. Research has shown that altered diet caused changes in fat composition in *C. elegans* (Brock et al. 2007). The effect of spinach extracts on lipid metabolism has been studied in rabbit as pre-clinical trails. The levels of serum triglycerides were notably up-regulated in animals

injected with spinach extract but whether this holds any biological significance is unknown (Lomnitski et al. 2003a).

Recently, it was observed that ANE treatments regulate physiological systems leading to increased antioxidant capacities and polyphenolic compound in spinach leaves (Fan et al. 2011). The question was raised whether extracts prepared from spinach can prolong life span and improve stress resistance in whole organisms, and furthermore, if there are differences in the effects on parameters related to aging, between extracts prepared from *Ascophyllum nodosum* extract (ANE)-treated spinach leaves and the non-treated control. The aims, in the context of this chapter, are to characterize the effects of spinach extracts from plants treated with or without ANE on *C. elegans* in terms of their life span, stress resistance and fat metabolism.

5.3 Materials and Methods

All chemicals and reagents were of analytical grade and were purchased from Sigma Aldrich, Oakville, Ontario, Canada, unless otherwise stated. Soluble powder of *A. nodosum* alkaline extract (Acadian[®]) was obtained from Acadian Seaplants Limited (Dartmouth, NS, Canada). The wild type *Caenorhabditis elegans* strain N2 (var. Bristol) and the OP50 *Escherichia coli* strain were obtained from the Caenorhabditis Genetics Center, University of Minnesota, USA. Cultures of OP50 *E. coli* were grown overnight in LB broth and concentrated 10× by centrifuging at 3,500×g for 10 min. The *C. elegans* population was maintained at 20 °C on 3.5-cm nematode growth medium (NGM) agar plates seeded with live OP50 *E. coli* (50 μL of *E. coli* stock solution per plate) as the food source.

5.3.1 Preparation of Spinach Extracts

The freeze-dried spinach leaf tissue from control plants or those treated with 1.0 g/L ANE (as described in section 2.3.1.) was extracted in 70% methanol (MeOH) at 40 °C for 2 h. The mixture was centrifuged at 10,000×g for 10 min at 4 °C. The extraction was repeated and the supernatants combined. The resulting spinach solution was then dried under nitrogen (N_2) gas, dissolved in 70% ethanol (EtOH), and stored at −20 °C as a stock solution with a concentration of 10 mg/mL.

5.3.2 Life Span Assay

For assaying life span, age-synchronized worms were prepared by the method described by Prithiviraj et al. (2005). Briefly, reproductive N2 worms were transferred to NGM plates and allowed to lay eggs for 6 h. Eggs were permitted to hatch at 20 °C and develop into a uniform adult population 3 days later. To prepare plates supplemented with the spinach extracts, the stock solution of extract was added to freshly autoclaved NGM (55 °C) to give various concentrations (0, 5, 10, 50, 100, or 200 μg/mL). Age-synchronous worms were placed onto treatment plates with heat-killed OP50 (65 °C for 30 min) as nematode food and 400 μM 5-fluorodeoxyuridine (FUdR) which was used to prevent progeny development (Hosono 1978). Nematodes were transferred to fresh treatment plates every 3 days for the first 9 days of the life span assay. Survival of the worms was evaluated each day and animals were scored as dead if they failed to respond to gentle repeated touches with a platinum pick. The first day of adulthood was considered as day 1. Worms that crawled off the walls of the plates and died from dessication were excluded from analysis.

5.3.3 Oxidative Stress Resistance Assay

The oxidative stress resistance assay was performed at 20 °C with hermaphrodites on adult day 3. To induce oxidative stress, juglone, a redox quinine that is capable of generating a superoxide anion (O_2^-) from oxygen (O_2) intracellularly in *C. elegans* was used (Zhang et al. 2009). A fresh 25 mM juglone solution was made by dissolving 44 mg of juglone in 10 mL 100% EtOH. This solution was mixed with liquefied NGM (55 °C) to get a final juglone concentration of 500 μM. The mixture was immediately poured into 3.5-cm Petri plates. After 30 min, each solidified plate was spotted with 50 μL of OP50 *E. coli* (at 10^{10} cells/mL) and allowed to dry for 30 min on a laminar hood bench. The worms, treated on plates containing spinach extracts (0, 5, 10, 50, 100, or 200 μg/mL) for 48 h were then transferred to freshly prepared juglone plates. Viability was scored every 2 h until all of the control worms had died.

5.3.4 Thermotolerance Assay

For the thermal tolerance assay, synchronized young adult worms were incubated on treatment plates containing spinach extracts (0, 5, 10, 50, 100, or 200 µg/mL) for 48 h at 20 °C and then exposed to heat shock (35 °C) for a period of 16 h. The number of dead worms was counted every hour (Lithgow et al. 1995).

5.3.5 Feeding Rate Assay

Age-synchronized worms (day 1) were placed on treatment plates spotted with heat-killed OP50, raised at 20 °C, and transferred to fresh plates with equal food/spinach extract treatments every 2 days. The feeding rate was scored by counting pharyngeal bulb contractions over a 20 s period under a Leica *DM 1000* fluorescence microscope (Leica Microsystems (Canada) Inc. Richmond Hill, ON) at room temperature (22 °C); (Raizen et al. 1995). The pharynx pumping rate was counted every two days for the first 10 days of adulthood on at least 10 worms per treatment.

5.3.6 Nile Red Fat-Staining Assay

The dye Nile Red (5H-benzo[α]phenoxazine-5-one, 9-diethylamino) was applied as a fluorescent vital stain to allow the visualization of lipid droplets in living nematodes (Ashrafi et al. 2003). Nile Red powder was dissolved in acetone at 0.5 mg/mL and stored at –20 °C. Right before use, the solution was diluted to 2 µg/mL in 1× phosphate buffered saline (PBS) and added on top of NGM treatment plates containing 5 or 200 µg/mL Spi or Spi 1.0 seeded with OP50, to a final concentration of 0.05 µg/mL. The worms which had already maintained on treatment plates for 1.5 days were placed onto these plates and incubated overnight at 20 °C. Nematodes were mounted on 2% agar pads (in M9 saline buffer) and paralyzed with 5 µL of 20 mM sodium azide (NaN_3) (in S basal medium). Fat content was monitored using a Leica *DM 1000* fluorescence microscope (Leica Microsystems (Canada) Inc. Richmond Hill, ON) under fluorescent filter attached with a Leica DFC camera (Leica Microsystems (Canada) Inc. Richmond Hill, ON). All Nile Red images were taken using identical magnification (20×) and exposure time. Nile Red intensity was measured using the free Java image processing program ImageJ (http://rsb.info.nih.gov/ij) (U. S. National Institute of Health, Bethesda, Maryland, USA).

5.3.7 Fatty Acid and Lipid Analysis

Cultures of *C. elegans* to be used for lipid analysis were obtained as described by Tanaka et al. (1996) with modifications. L4 synchronized worms were grown on 6-cm Petri plates containing spinach extracts (0, 5, or 200 µg/mL), spread with heat-killed concentrated OP50 as food source, at 20 °C for 3 days. Each sample represented the combined cultures from 10 separate plates of the same treatment grown simultaneously. The worms were washed with abundant deionized water, placed into 50 mL glass tubes and cooled on ice for 10 min to settle before being centrifuged at $1,000 \times g$ for 3 min at 4 °C. Most of the water was discarded with a glass pipette and the soft pellets were rinsed twice with ice-cold deionized water. Bacteria, debris and dead worms were sedimented by adding 5 mL ice-cold 70% sucrose to 5 mL suspended worm pellets and spinning at $1,000 \times g$ for 6 min at 4 °C. The light brown layers of live worms from the top of fluid were immediately aspirated into 50 mL glass tubes containing 15 mL ice-cold deionized water. Washing with deionized water was repeated twice to get rid of most sucrose. The worm pellets were frozen in liquid N_2 and freeze-dried.

Fat was extracted as described by Bligh and Dyer (1959), with modifications. To each lyophilized sample (100 mg worms, 100 mg spinach leaf tissue, 1 g OP50 *E. coli*, or 20 mg spinach extract), 3 mL MeOH plus 0.01% (w/v) butylated hydroxytoluene (BHT) were added. After incubating the tubes in the dark at 4 °C overnight, 6 mL of chloroform was added to each tube, vortexed and then sonicated in ice-cold water for 1 h. Three mL of 0.7% NaCl was added and lipids were recovered in the chloroform phase, dried under N_2, and dissolved in 1.5 mL dichloromethane (DCM) containing 0.01% BHT. Five µg of the internal standard (15:0) was added to the tubes prior to lipid extraction.

The fatty acid profile of the lipid extracted from nematodes was determined as fatty acid methyl esters (FAMEs), according to the method of Shehata et al. (1979). To generate FAMEs, 3 mL freshly-made Hilditch reagent (0.5 N methanolic sulfuric acid) was added and incubated at 100 °C for 1 h. After cooling to room temperature, the mixture was extracted by adding 3 mL hexane and 1 mL chloroform extracted water. The lower layer was extracted twice with 1.5 mL hexane and the hexane extracts were pooled and dried under N_2. Hexane was added to the FAME to a final concentration of 5 mg/mL. The samples of spinach leaf, spinach extract and OP50 *E. coli* were loaded onto the thin

layer chromatography plates which were activated in a drying oven for 1 h at 110 °C. The corresponding band for each sample was scraped into individual tubes and recovered.

The FAME were analyzed by gas chromatography (GC) and identified by comparing peak retention times of fatty acid standards (Kniazeva et al. 2003). The internal control (15:0) was used in the quantitative analysis and the percentage of each fatty acid of the total was calculated.

5.3.8 Statistical Analysis

Unless specified otherwise, for each experiment three independent trials were carried out. The experimental data were analyzed by ANOVA and differences were considered statistically significant at $P \leq 0.05$ using Tukeys HSD test of the COSTAT® statistical software unless otherwise mentioned. Results from life span and stress resistance experiments were processed using the Kaplan-Meier survival analysis of the SPSS 15.0 and compared amongst the control and the spinach extract treated groups for significance by means of a log-rank pairwise comparison test. Nematodes grown on NGM plates without spinach extract were considered as the controls.

5.4 Results

5.4.1 Life Span Assay

In order to determine the lifespan-extending effects of spinach extract, *C. elegans* were grown on NGM plates implemented with 0, 5, 10, 50, 100, or 200 μg/mL extracts from control plants (Spi) or those treated with 1.0 g/L ANE (Spi 1.0). There were no differences in the life span of the nematodes between the 2% ethanol solvent treated and water controls, thus distilled water controls were used in all experiments. A concentration dependent effect on longevity was observed. There were no differences between Spi and Spi 1.0 (Fig. 5.2). A low concentration (5 μg/mL) did not affect longevity notably (Table 5.1.), while all the other treatments led to a higher percentage of living worms in the population than that of the control treatment, after 12 days of adulthood until the end of the experiment (Fig. 5.2 and Table 5.2.). The most effective beneficial concentrations of extracts (50, 100, and 200 μg/mL) from both control and treated spinach, not only extended the mean life span of the nematodes by roughly 8 days (which is an extension of

the mean life span obyapproximately 35 %), but also increased the maximum life span by approximately 36% (12 days).

Spinach extract treatments (100 and 200 μg/mL) almost doubled the time before the first dead worm was observed (from day 10 to day 20) but did not significantly affect the rate of mortality after the onset of death. That is, $T^{1/2}$ was almost the same (12 days). The half time $T^{1/2}$ means the time gap between the outset of death and the time at which 50% of the original population was still living (Gruber et al. 2007).
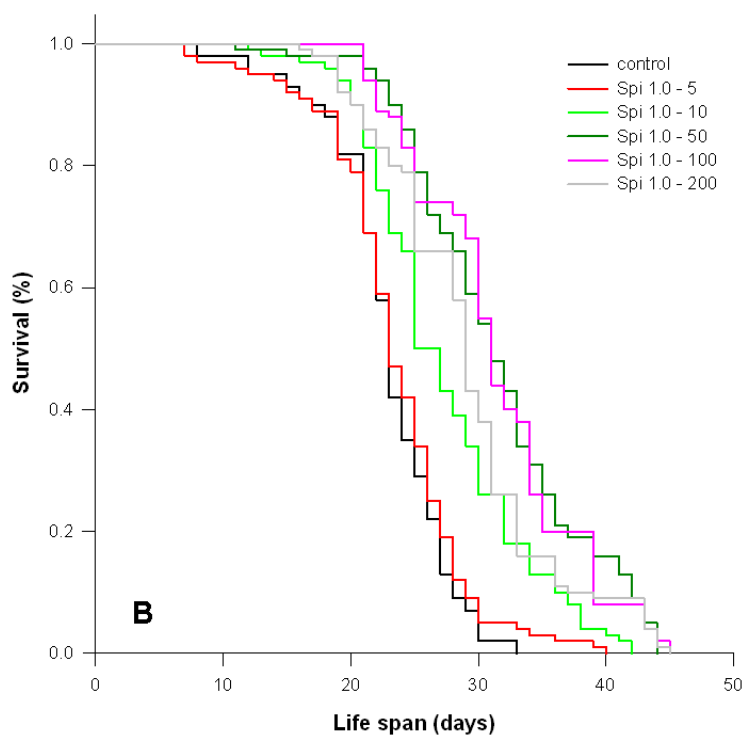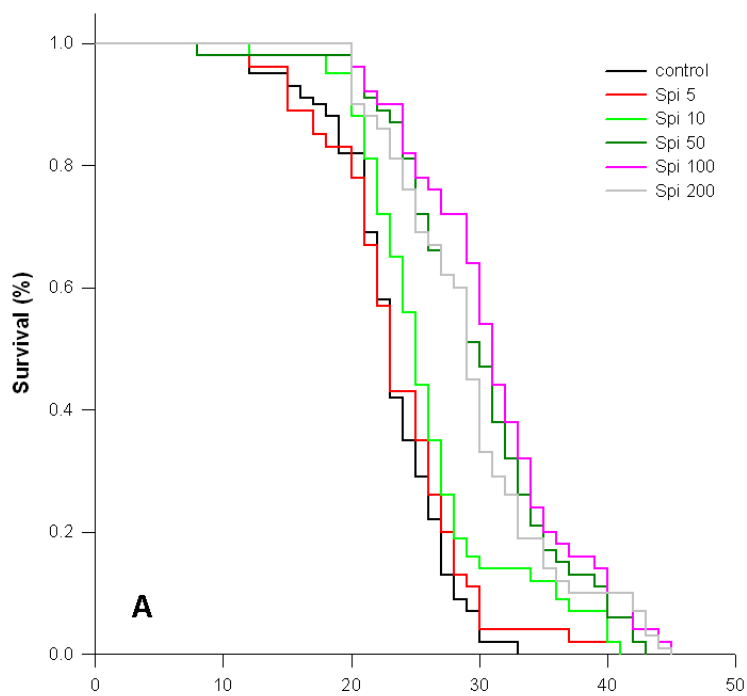
Figure 5.2. Spinach extracts from (A) control plants and (B) 1.0 g/L ANE-treated plants extend the life span of wild-type *C. elegans* N2 at 20 °C.

Table 5.2. Effect of spinach extract from control and ANE-treated plants on wild-type *C. elegans* N2 feeding on heat-killed *E. coli* OP50

Treatment (20 °C)	Total (N)	Censored (N)	Adult life span, days (Mean, S.E.)	*P* vs. control (log-rank)
Control	241	0	22.9, 0.47	
Spi 5	189	0	23.4, 0.57	0.292
Spi 10	265	0	25.9, 0.59	0.0003
Spi 50	202	0	29.8, 0.67	< 0.0001
Spi 100	198	0	31.2, 0.63	< 0.0001
Spi 200	230	0	29.4, 0.64	< 0.0001
Spi 1.0 - 5	199	0	23.4, 0.56	0.297
Spi 1.0 - 10	255	0	27.2, 0.62	< 0.0001
Spi 1.0 - 50	262	0	31.5, 0.69	< 0.0001
Spi 1.0 - 100	218	0	31.4, 0.63	< 0.0001
Spi 1.0 - 200	254	0	29.1, 0.66	< 0.0001

S.E., standard error.

5.4.2 Stress Resistance Assay

      To investigate whether spinach extracts were protective against thermal stress and oxidative stress in *C. elegans*, young adults were pre-treated with Spi or Spi 1.0 for 48 h before being exposed to 35 °C or 500 μM juglone, a pro-oxidant that is able to generate intracellular oxidative stress. Pre-treatment enhanced the worms' resistance to heat shock and juglone, and consequently increased survival rates.

      After the first 6 h of heat stress, the worms started to die but the rate of death in the population treated with spinach extract, at different concentrations (except Spi 200 μg/mL), was clearly lower than in the control (Fig. 5.3). This demonstrated an increased resistance against thermal stress. The Spi 1.0 5 μg/mL treatment was more efficient at reducing nematode mortality under thermal stress than Spi 5 μg/mL (Table 5.3.). A 6 h period of thermal stress was survived by almost all nematodes from all populations but after 12 h the survival rate of the worms treated with Spi 1.0 5 μg/mL (>70%) was strikingly higher than that of the control worms (40%). When all control worms died, there were still 63% and 51% alive in the Spi 1.0 5 μg/mL and Spi 10 μg/mL groups, respectively (Fig. 5.3). The mean survival rate was significantly increased by 29% at Spi 1.0 5 μg/mL, and 27% at Spi 10 μg/mL versus the control (Table 5.3.).

      As for oxidative stress, the slopes of the curves of all designated concentrations (except Spi 50 μg/mL) were much smaller than that of the control. This showed strong protective effects of treatments for resistance to oxidative stress induced by juglone (Fig. 5.4). Though the optimum effective concentration from both Spi and Spi 1.0 extracts was the same (200 μg/ml), Spi 1.0 200 μg/ml showed a much higher mean survival rate (Fig. 5.4 and Table 5.4.).
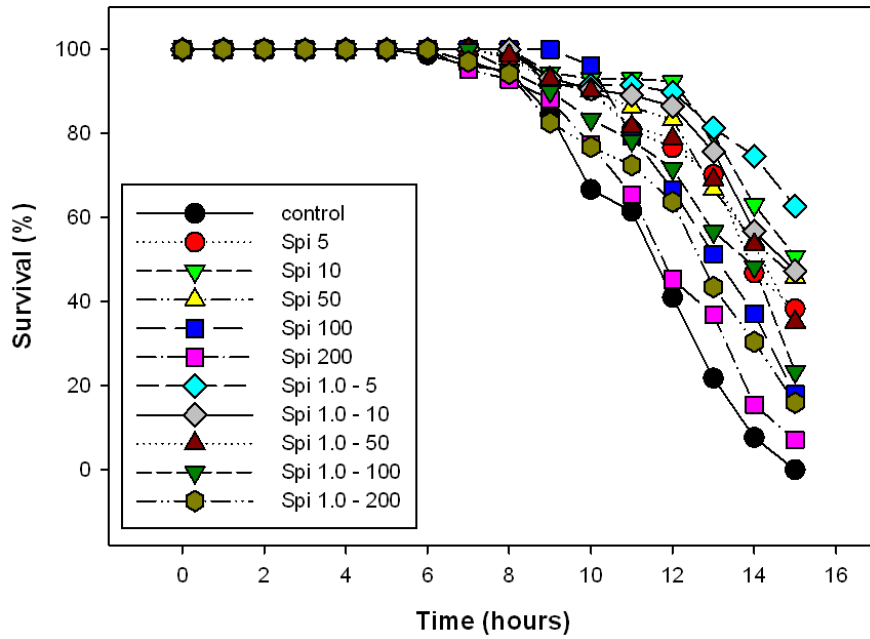
Figure 5.3. Protective effects of spinach extracts on *C. elegans* N2 under heat stress (35 °C).
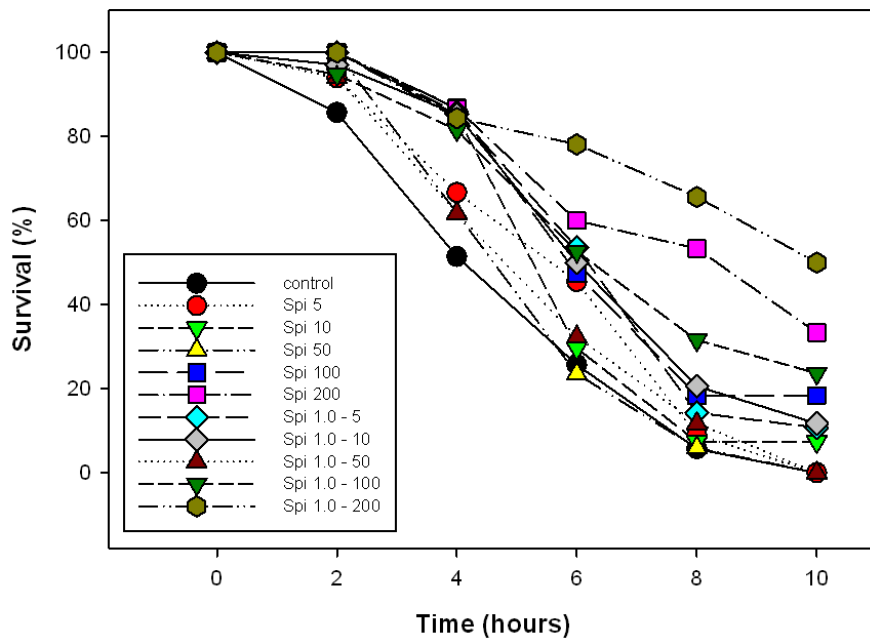
Figure 5.4. Protective effects of spinach extracts on *C. elegans* N2 under oxidative stress.

Table 5.3. Protective effect of spinach extracts on wild-type *C. elegans* N2 feeding on heat-killed *E. coli* OP50 under heat stress

Treatment (35 °C)	Total (N)	Censored (N)	Survival time, hours (Mean, S.E.)	*P vs. control* (log-rank)
Control	156	0	10.8, 0.21	
Spi 5	192	73	12.9, 0.23	< 0.0001
Spi 1.0 - 5	144	91	13.9, 0.20	< 0.0001
Spi 10	156	80	13.7, 0.19	< 0.0001
Spi 1.0 - 10	186	87	13.4, 0.21	< 0.0001
Spi 50	234	108	13.4, 0.74	< 0.0001
Spi 1.0 - 50	262	92	13.0, 0.22	< 0.0001
Spi 100	242	44	12.5, 0.19	< 0.0001
Spi 1.0 - 100	231	53	12.5, 0.24	< 0.0001
Spi 200	194	14	11.2, 0.23	0.03
Spi 1.0 - 200	258	15	11.7, 0.24	< 0.0001

S.E., standard error.

Table 5.4. Protective effect of spinach extracts on wild-type *C. elegans* N2 feeding on heat-killed *E. coli* OP50 under oxidative stress induced by juglone

Treatment (20 °C)	Total (N)	Censored (N)	Survival time, hours (Mean, S.E.)	*P* vs. control (log-rank)
Control	141	0	3.4, 0.23	
Spi 5	225	0	4.3, 0.22	0.009
Spi 1.0 - 5	269	30	5.3, 0.22	< 0.0001
Spi 10	149	10	4.6, 0.20	< 0.0001
Spi 1.0 - 10	165	20	5.3, 0.25	< 0.0001
Spi 50	214	0	3.8, 0.18	0.314
Spi 1.0 - 50	259	31	4.0, 0.22	0.009
Spi 100	192	35	5.4, 0.25	< 0.0001
Spi 1.0 - 100	243	58	5.7, 0.30	< 0.0001
Spi 200	112	37	6.7, 0.30	< 0.0001
Spi 1.0 - 200	199	100	7.6, 0.30	< 0.0001

S.E., standard error.

5.4.3 Feeding Rate Assay

C. elegans use the pharynx to pump in the bacteria upon which it feeds and transport them to the intestine. The speed of pharyngeal contraction declines with age and the reduction in pumping rate has been applied as one of the indices of the decline of overall physiological functions (Huang et al. 2004). The pharynx pumping rate was scored every two days during the first 10 days of adult life. For the first 4 days, the pumping rates decreased slightly and were identical for treated and untreated worms. From day 6-10, pumping declined gradually, from about 220 to 100 contractions per minute, with increased age. However, most treated worms showed an apparently higher

pumping rate, from 220–240 contractions per minute to 140 times per minutes, by comparison to that of the controls ($P < 0.05$) (Fig. 5.5).
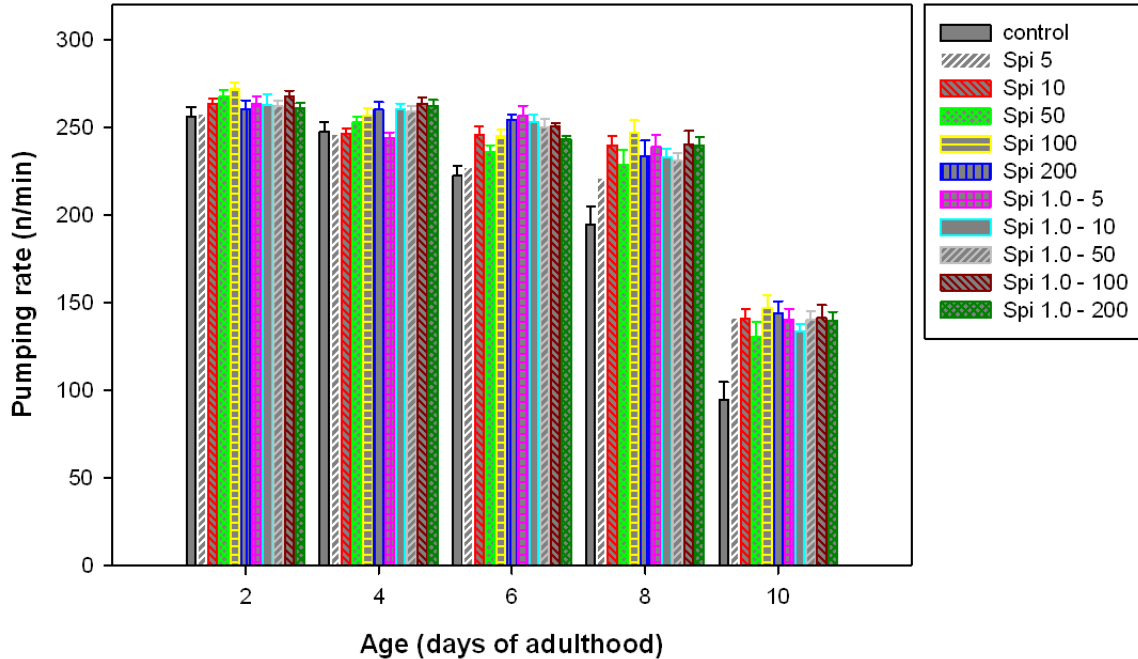
Figure 5.5. The decline in pharyngeal function for spinach extract-treated and untreated control *C. elegans* N2. Bars indicate mean ± standard error.

5.4.4 Nile Red Fat-Staining Assay

In order to investigate the effect of spinach extracts on the levels of fat deposition in *C. elegans*, worms were treated for 36 h and then cultured for 12 h on Nile Red-containing NGM treatment plates. The fluorescent, lipophilic vital dye Nile Red, which has been shown to not affect the growth, feeding or life span of *C. elegans*, was used for the visualization of lipid droplets in living individuals. As shown in Fig. 5.6A, treatments at 5 and 200 µg/mL from both ANE-treated and control plants caused a dramatic increase of fat storage in the intestine of animals since the treated worms showed brighter fluorescence. Quantitative analysis showed significant differences among control, 5 µg/mL and 200 µg/mL (Fig. 5.6B).

A

B
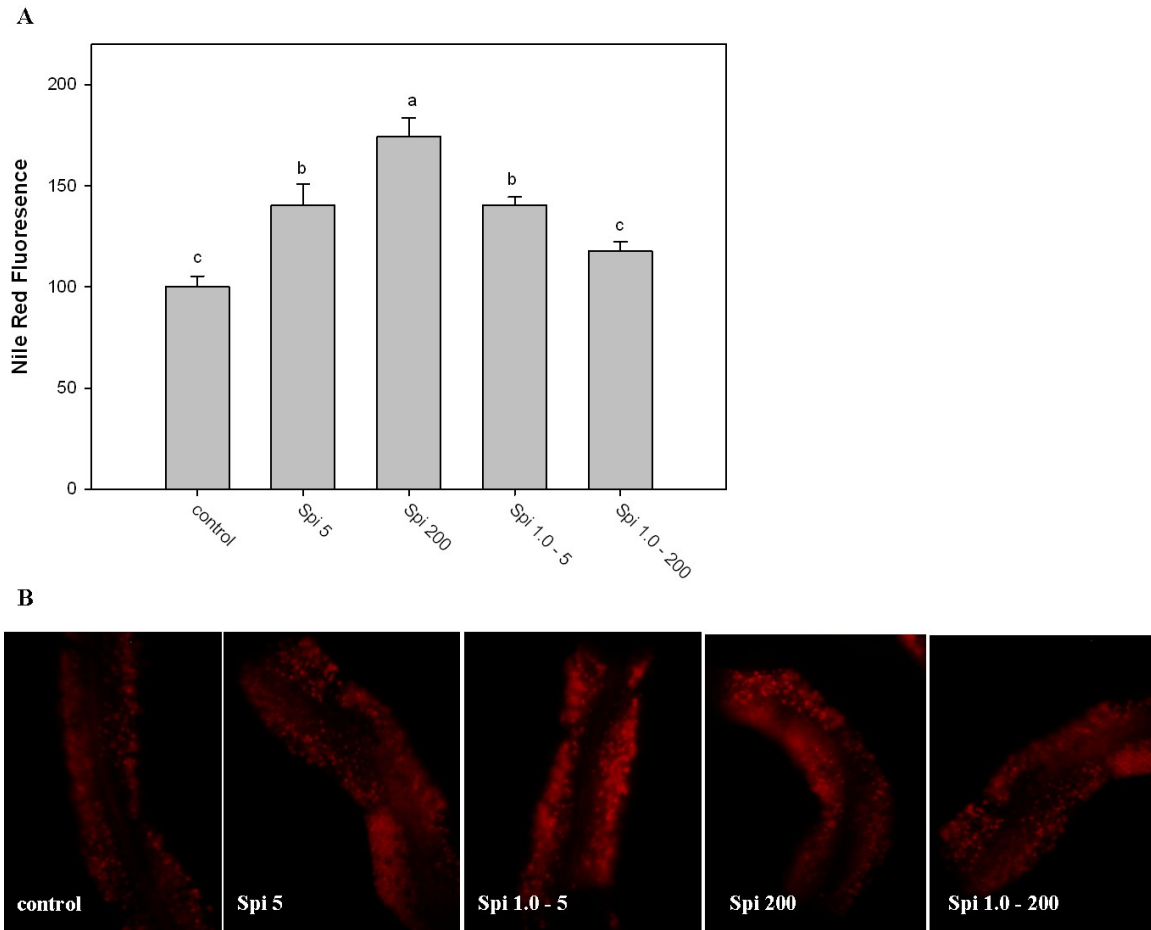
Figure 5.6. Spinach extracts increase fat deposition in *C. elegans* N2. (A) Quantification of Nile red fluorescence. Data are expressed as a percentage of control animals ± standard error of the mean (SEM) ($P < 0.05$) (B) Images of Nile red-stained wild-type animals treated with spinach extract or that from ANE-treated plants.

5.4.5 Fatty Acid and Lipid Analysis

The major macronutrient in *E. coli* OP50 is protein, constituting about 40% of the dry weight (DW) of the cell (Brooks et al. 2009). The fatty acid levels in OP50 is about 3% of cell DW (Brooks et al. 2009) and the profile of the fatty acid composition of total lipid of OP50 is shown in Table 5.5. OP50 contains about 30% palmitic, 19% hexadecatrienoic, 3% linoleic, 8% linolenic, 3 % palmitoleic and 18% oleic acids.

The lipid content in spinach is about 5% on a dry mass basis (Lee et al. 2002). The fatty acid profile of lipid extracted from spinach leaves and their extracts is presented in Table 5.6. The lipid in spinach and its extract contains about 8 and 6% palmitic, 8 and 16% hexadecatrienoic, 8 and 4% linoleic, 54 and 53% linolenic, 2 and 4 % palmitoleic and 3 and 1% oleic acids, respectively. Though a minor increase in the ratio of the contents of unsaturated fatty acids (UFA) to saturated fatty acids (SFA) between spinach leaves and leaf extracts from 8% to 10%, was observed, no significant differences in fatty acid composition, SFA, monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) could be detected between control and ANE-treated spinach plants or between leaf extracts from control and ANE-treated plants.

To confirm the semi-quantitative result from morphological analysis of lipid storage and to determine if spinach extracts regulate fatty acid compositions of *C. elegans*, young adult worms were grown on treatment plates for 3 days and fatty acid analysis was performed by GC. Though OP50 and spinach extracts lack many long-chain and PUFAs as well as monomethyl branched-chain fatty acids (mmBCFAs) (Table 5.5. and 5.6.), *C. elegans* can synthesize all of its necessary fatty acids *de novo* up to and including 20:5n3 (eicosapentaenoic acid, EPA) (Table 5.7.).

There are significant differences in the lipid content of *C. elegans* N2 expressed as % of worm DW among control and treated-worms. Spi 1.0 5 μg/mL treatment resulted in the highest lipid content (34%), followed by Spi 200 μg/mL (27%), Spi 5 μg/mL (25%), Spi 1.0 200 μg/mL (23%) and control (21%). These results are in concordance with Nile Red staining. Total lipid analysis showed that supplementing spinach extract significantly lowered the proportions of 19:0 and 20:4n6, whereas it strikingly increased the proportions of 15:iso, 17:iso, 14:1n9, 16:1n9T, 18:4n3, and 20:3n3 (Table 5.6.). The proportion of 14:1n9 was only significantly increased in Spi 200 μg/mL and 16:1n9T in

Spi 1.0 200 µg/mL. No other significant changes in individual fatty acids, SFA, MUFA, or PUFA were found.

Table 5.5. Fatty acid composition of *E. coli* OP50 at 22 °C [a]

fatty acid	OP50
14:0	3.338
15:0	2.677
16:0	30.772
17:0	0.783
Ci17:0	0.207
18:0	0.763
DMA18:0	0.499
SFA	39.039
14:1n9	0.118
16:1n11	1.043
16:1n9	0.329
16:1n7	2.359
16:1n5	0.231
18:1n13	0.688
18:1n9	0.372
18:1n7	16.849
MUFA	21.989
16:2n6	0.413
16:2n4	0.588
16:3n4	0.188
16:3n3	19.084
18:2n6	2.527
18:2n4	0.508
18:3n6	0.162
18:3n4	0.233
18:3n3	8.297
18:4n3	0.374
18:4n1	0.453
20:2n6	0.665
20:4n3	0.078
PUFA	32

[a] Data are weight percentage of total OP50 fatty acids. 20:4n3, fatty acid with 20 carbons and 4 methylene-interrupted double bonds, the first occurring at the n-3 position.

Table 5.6. Fatty acid composition of spinach [a]

fatty acid	treatment			
	control leaf	leaf 1.0	control extract	extract 1.0
13:0	0.147	0.157	0.109	0.103
14:0	0.153	0.147	0.169	0.265
15:0	0.095	0.093	0.101	0.167
16:0	8.325	8.141	5.731	6.520
Cai17:0	0.077	0.077	–	–
17:0	0.063	0.059	0.042	0.000
18:0	0.762	0.735	1.121	0.954
20:0	0.178	0.173	0.262	0.199
22:0	0.122	0.066	–	–
SFA	9.922	9.648	7.535	8.208
14:1n9	0.042	0.041	0.143	0.104
14:1n5	0.045	0.048	–	–
16:1n9T	0.200	0.251	0.162	0.168
16:1n9	0.120	0.120	0.111	0.094
16:1n7	1.883	1.835	4.400	4.140
18:1n9	2.512	2.261	0.843	0.712
18:1n7	1.238	1.168	0.149	0.349
20:1n11	0.047	0.042	–	–
20:1n9	0.238	0.193	–	–
20:1n7	0.023	0.000	–	–
MUFA	6.348	5.959	5.808	5.567
16:3n3	8.484	8.380	16.333	15.428
16:4n3	0.160	0.129	–	–
18:2n6	8.580	8.360	3.698	4.091
18:2n4	0.061	0.056	–	–
18:3n4	0.073	0.072	0.087	0.000
18:3n1	54.214	54.728	52.901	53.422
18:4n3	0.055	0.046	–	–
18:4n1	0.110	0.114	0.326	0.272
20:2n6	0.084	0.081	–	–
20:3n3	0.122	0.110	–	–
PUFA	71.943	72.076	73.345	73.213

[a] Data are weight percentage of total leaf fatty acids. Leaf 1.0, leaf from plants treated with 1.0 g/L ANE. extract 1.0, extract of leaves from 1.0 g/L ANE-treated spinach.

Table 5.7. Fatty acid composition of wild-type *C. elegans* N2 grown at 20 °C [a]

fatty acid	control	Spi 5	Spi 1.0 - 5	Spi 200	Spi 1.0 - 200
			treatment		
14:0	1.217	1.284	1.521	1.378	1.205
15:iso	2.488 b	2.633 b	3.453 a	3.408 a	3.358 a
16:0	4.516	3.966	4.372	3.914	3.773
Cai17:0	0.105	0.188	0.219	0.053	0.000
17:iso	3.931 b	4.219 b	4.959 a	5.368 a	4.974 a
17:0	0.551	0.506	0.546	0.496	0.461
18:0	4.445	4.122	4.287	4.006	3.998
19:0	0.120 a	0.043 ab	0.026 b	0.017 b	0.000 b
20:0	0.277	0.227	0.235	0.233	0.170
SFA	17.65	17.168	19.536	18.87	17.616
14:1n9	0.603 b	0.765 ab	0.814 ab	0.883 a	0.691 ab
16:1n11	0.438	0.406	0.433	0.453	0.392
16:1n9T	0.879 b	0.993 ab	0.914 b	0.904 b	1.094 a
16:1n7	2.376	2.495	2.698	2.611	2.248
16:1n5	0.000	0.094	0.118	0.103	0.049
18:1n13	0.378	0.401	0.352	0.395	0.357
18:1n9	3.056	3.317	3.387	3.467	3.046
18:1n7	22.185	22.318	21.844	23.126	21.701
18:1n5	0.292	0.296	0.322	0.367	0.346
20:1n9	0.138	0.158	0.110	0.218	0.022
20:1n7	0.630	0.661	0.459	0.558	0.558
MUFA	30.975	31.904	31.451	33.085	30.504
16:2n6	0.142	0.091	0.158	0.145	0.101
16:2n4	0.439	0.415	0.449	0.484	0.460
16:3n4	0.252	0.216	0.229	0.155	0.220
16:3n3	8.692	8.210	9.054	8.864	10.143
18:2n6	8.853	9.630	9.712	10.354	9.690
18:2n4	0.743	0.578	0.567	0.579	0.562
18:3n6	0.912	0.821	0.833	0.805	0.983
18:3n4	0.596	0.524	0.507	0.393	0.435
18:3n3	4.430	4.611	4.082	4.909	5.352
18:4n3	0.241 b	0.267 b	0.299 a	0.348 a	0.390 a
18:4n1	0.475	0.450	0.406	0.436	0.375
20:2n6	0.959	1.205	0.843	0.972	1.006
20:3n6	2.109	1.940	1.466	1.223	1.539
20:4n6	0.775 a	0.781 a	0.588 b	0.457 b	0.551 b
20:3n3	0.169 b	0.219 ab	0.251 a	0.282 a	0.314 a
20:4n3	3.263	3.030	2.271	2.212	2.734
20:5n3	8.518	8.794	6.711	6.888	7.892
PUFA	41.598	41.812	38.349	39.488	42.747

[a] Data are weight percentage of total worm fatty acids. Values in the same row with the same letters are not significantly different ($P < 0.05$).

5.5 Discussion

Spinach extracts from both control (Spi) and ANE-treated plants (Spi 1.0) did not only significantly prolong the life span of *C. elegans* but also elevated the worm's resistance to oxidative and thermal stress. Moreover, spinach extracts led to increased body fat in *C. elegans* and proportions of certain fatty acids were notably changed.

The free-living soil nematode *C. elegans* is a powerful model system for aging research and drug screening (Kaletta and Hengartner 2006; Wilson et al. 2006; Yu et al. 2010) because of the strong conservation in biochemical pathways and the shared similar aspects of aging between *C. elegans* and mammals (Herndon et al. 2002). In this study, nematodes were fed with live OP50 for propagation while heat-inactivated OP50 was used for treatment plates to prevent any possible effect of bio-transformation of spinach extract by live bacteria (Collins et al. 2006).

Spinach leaves have been used as a dietary supplement for health maintenance and in herbal medicine for human ailments, fatigue and for promoting convalescence (Lomnitski et al. 2003a). Spinach extracts have been studied in both *in vitro* and *in vivo* systems, and are widely shown to possess anti-inflammatory effects, anti-mutagenic potential, anti-neoplastic effects, as well as chemo-preventive activities (Boivin et al. 2009; Hait-Darshan et al. 2009; Lomnitski et al. 2003a), all of which were found to be superior to those of the well-documented antioxidants such as EGCG, ascorbic acid, and vitamin E (Lomnitski et al. 2003a). This is probably due to the broad free-radical scavenging capacity of antioxidants, especially the novel antioxidant glucurinated flavonoids (Bergman et al. 2003; Cho et al. 2008) in spinach. For instance, Joseph et al. (1998, 1999) reported that feeding young (6-month-old) or old (19-month-old) rats with diet supplemented with spinach significantly delayed or prevented the age-related deterioration and cognitive behavior, with some dysfunctions reversed, as compared with blueberry, strawberry, or vitamin E supplementation. Our study showed that spinach extracts are capable of modulating the normal life span of wild type *C. elegans* Bristol N2. Exposure of *C. elegans* to spinach extracts resulted in a dose-dependent increase in their longevity. In addition, significant increases in survival of *C. elegans* under heat and oxidative stress were observed when they were pre-incubated in the presence of spinach extracts.

The free-radical theory of aging hypothesizes that accumulation of free radicals may lead to deterioration of an organism (Kregel and Zhang 2007). It is often postulated that aging and mortality in a population is partly associated with environmental stresses and the subsequent elevated concentration of free-radicals. The results shown here demonstrated the effective free-radical scavenging ability and the anti-aging potential of spinach extract. Spinach is rich in antioxidant constituents, especially flavonoids and other polyphenolic ingredients acting synergistically as antioxidative and anti-cancer agents. In this respect, a high occurrence of polyphenols in spinach may exert an anti-oxidative effect of capturing and stabilizing free radicals that helps prolong the life span in *C. elegans*. We observed that extract from Spi 1.0 exerted identical effects on nematode longevity as compared to that from control plants (Fig. 5.1 and Table 5.1.). It should be noted that antioxidant capacities of plant extracts may not be always positively related to their effect on life span. Pun et al. (2010) found that although Pine bark exhibited strong antioxidant activity, its impact on life expectancy is less than that of extract of *Psoralea corylifolia* L. (fruit, PC), a very weak antioxidant *in vitro*. Moreover, all the other extracts showing intermediate *in vitro* and *in vivo* antioxidant capacity failed to extend the life span in *C. elegans* (Pun et al. 2010). In the current study, though spinach extract showed a concentration-dependent increase in life span of *C. elegans*, when at the same concentrations, the Spi 1.0 treatment, even with a higher polyphenolic content, showed identical life span extension effect by comparison with Spi treatment. Higher concentrations of spinach extract (which means higher doses of polyphenols in the treatment), conferred an increase in oxidative stress resistance while lower concentrations (lower doses of polyphenols), exerted higher survival capacity under thermal stress. It is interesting to note that the optimum concentration of Spi 1.0 5 μg/mL for heat stress did not affect the life span. Moreover, Spi and Spi 1.0 200 μg/mL treatments showed identical life span extension effect but Spi 1.0 200 μg/mL apparently exerted stronger oxidative resistance in *C. elegans* than did Spi 200 μg/mL treatment.

It has been recognized that caloric restriction (CR) can lead to a significant extension of life span in *C. elegans* (Sutphin and Kaeberlein 2008) as well as in rat and mouse (Samaras et al. 2003). Schulz et al. (2007) found that glucose restriction, acting as a nutritive CR, extended the life span of *C. elegans* and triggered increased resistance to

further oxidative stress. In this study, there was no significant difference among untreated and treated worms in pumping rates during the first 4 days of adulthood. The decline of the age-related pumping rate was slowed down in all treated worms from day 4-10. This indicated that spinach extract did not cause defective feeding and thus was not a CR mimic, and more importantly, it could ameliorate the age-dependent decline in *C. elegans*. Moreover, from the kinetic analysis of the survival curves of life span we found that spinach extract markedly delayed the onset of worm death, but did not significantly affect the $T^{1/2}$. This suggested that once the dying phase was initiated, spinach treatment did not affect the metabolism significantly.

Wilson et al. (2006) reported that blueberry polyphenols could prolong the life span and increase thermotolerance, but not oxidative stress resistance in *C. elegans*. Gruber et al. (2007) found that life span extension was associated with an increased resistance to oxidative damage but not to thermal stress in resveratrol-treated worms. In this study, it observed that *C. elegans* treated with spinach-extract exhibited not only extended longevity but also increased resistance to both thermal and oxidative stresses. Treatment with *Rhodiola rosea* extract can increase the life span of *C. elegans* (Wiegant et al. 2009) through affecting the localization of DAF-16, while quercetin treatment (Pietsch et al. 2009) can extend the life span of nematodes lacking functional DAF-16. Blueberry polyphenols exerted beneficial effects on longevity through interactions with the osr-1/unc-43/sek-1 pathway that mediates osmotic stress resistance (Wilson et al. 2006). Therefore, it may be postulated that spinach extract exhibits beneficial effects on life span, heat and oxidative stress resistance in *C. elegans* through different mechanisms or by a combination of them.

Mild stresses, including heat shock, irradiation, pro-oxidants, alcohols, and exercise, have been shown to delay aging and prolong lifespan in several biological systems such as *Drosophila*, *C. elegans*, rats, and human cells (Rattan 2004). Increased ROS levels within the mitochondria from restriction of glucose metabolism (Schulz et al. 2007) and mild heat shock or juglone treatment (Cypser and Johnson, 2002) could induce hormesis in *C. elegans*, resulting in subsequent strengthened stress resistance and extended longevity. Phenolic compounds have also been shown to display pro-oxidant capacities and some of the flavonoids were demonstrated to exhibit a kind of stress in *C.*

elegans by increasing ROS formation in the mitochondria. Thus, apart from the possible direct roles as radical scavengers, spinach extracts may act as moderate stimuli in *C. elegans* to trigger adaptive stress responses or cellular defense mechanisms such as antioxidant defense systems and signaling pathways, resulting in an increased resistance to the subsequent biotic or abiotic stresses (Wiegant et al. 2009).

Fatty acids are building blocks in lipids and play important roles in numerous cellular processes such as cell signaling, membrane function, and energy storage. Researchers have shown that seaweed extracts from *Ascophyllm nodosum* influenced the MUFAs accumulation in differentiating 3T3-L1 adipocytes (He et al. 2009) and lipid metabolism in wether lambs (Fike et al. 2005). EGb 761 treatment in rat significantly increased the PUFAs and decreased the saturation index, all of which were considered to be responsible in part for some of the therapeutic effects of the *Ginkgo biloba* extracts (Drieu et al. 2000). We found that Spi 1.0 5 µg/mL treatment induced the highest increase in lipid content in *C. elegans* in comparison with controls. It is interesting to note that treatments with Spi 200 µg/mL and Spi 1.0 5 and 200 µg/mL markedly reduced the proportions of 19:0 and 20:4n6 while strikingly increased the proportions of 15:iso, 17:iso, 18:4n3, and 20:3n3. The saturated tetradecanoic and hexadecanoic fatty acids 15:iso and 17:iso are ubiquitous in bacteria, fungi, plants and animals. These mmBCFAs contribute to membrane fluidity and proton permeability in bacteria, and are crucial for growth and development of *C. elegans* (Kniazeva et al. 2004); they are also associated with human health (Hradec and Dufek, 1994; Yang et al. 2000). The elevated amount of mmBCFAs indicated that spinach extract treatments, especially those with higher doses of polyphenols, may activate ELO-5 and ELO-6, the long-chain elongation enzymes (Kniazeva et al. 2004) or LET-767, a family of short-chain dehydrogenases/reductases (Entchev et al. 2008), to stimulate the elongation of 15:iso and 17:iso. The observed negative correlation between the levels of 18:4n3 and 20:3n3 and 19:0 suggested that spinach extract may up-regulate LPD-1, a protein homologous to mammalian Ic isoform of sterol regulatory element binding protein (SREBP-1c that regulates the first step of fatty acid biosynthesis), or desaturases, to desaturate straight-chain fatty acids (Kniazeva et al. 2004). The biological influences of the down-regulated levels of arachidonic acid (20:4n6), an important metabolite of linoleic acid which plays an essential role in

regulating membrane properties, as well as all the other influenced fatty acids, is not clear yet. The content of SFA, MUFA, and PUFA remained unchanged in all treatments.

It has been reported that disruption of *daf-2* led to increased *C. elegans* body fat as well as prolonged life span, CR has been shown to extend life span of *C. elegans* but decrease fat content (Schulz et al. 2007), while drug interventions such as anti-depressants mianserin and methiothepin resulted in shortened life span in *C. elegans*, accompanied by increased accumulation of body fat (Zarse and Ristow, 2008). We observed that *C. elegans* fed spinach extract contained significantly higher total lipid by comparison with control worms but it should be noted that Spi 1.0 5 μg/mL treatment, which exerted the highest fat storage, did not significantly extend life span but notably increased heat-stress resistance in *C. elegans*. Spinach extract alters the total fat storage as well as the levels of fatty acids but whether this is correlated with the beneficial effects exerted on *C. elegans* is waiting to be explored.

Chapter 6

General Discussion

Fruit and vegetables are rich in health-promoting phytochemicals such as antioxidants, or agents with antibacterial, antifungal, antiviral, cholesterol-lowering or anti-inflammatory effects (Schreiner and Huyskens-Keil 2006). Fruit and vegetable consumption has been associated with reduced incidence of numerous diseases such as obesity, hypertension, cardiovascular diseases and diabetes. The average intake of fresh fruit and vegetables is below optimal levels in many populations (Guenther et al. 2006; Kanungsukkasem et al. 2009). Therefor enhancing nutritional quality of fruit and vegetables will be beneficial to human population.

A number of chemical inputs like fertilizers, fungicides, herbicides, pesticides, plant growth regulators (e.g. synthesized plant hormones such as cytokinins, auxins, and gibberellins), are currently used to improve nutritional quality of fruit and vegetables. Genetic engineering of plants has also been used along side conventional plant breeding to improve nutritional content of fruit and vegetables (Jauhar 2006). However, there are wide-ranging public concerns regarding the potential adverse impacts of fertilizers, herbicides, pesticides and genetic engineering on human health and the well-being of ecosystems (Ubalua 2009). In this respect, some naturally occurring products such as biostimulants (e.g. humic substances, natural hormone containing products and amino acid containing products) appear to be better choice since they may be economically viable, and most importantly, are easily biodegradable, and are less or non-phytotoxic.

It must be noted that some fruit and vegetables, especially leafy vegetables, do not store well. A number of nutrients and phytochemicals in fruit and vegetables often degrade during the post-harvest handling and during storage period (Lampe 1999). Numerous practices have been reported to enhance storability and maintenance of nutritional values of fresh crop products, e.g. coatings, which provide physical protection to the surface and post-harvest treatments (e.g. low temperatures and atmospheric treatments), all of which enhance tolerance to oxidative stress (Toivonen 2003). Furthermore, it is important to maintain the high concentrations of bioactive compounds in fruit and vegetables during post-harvest storage.

Seaweeds (marine macroalgae) which dates back to 300 B.C. in China as a food and has been wildly consumed as sea vegetables in East Asia, is an excellent source of minerals, fiber, and phytonutrients (MacArtain et al. 2007). Certain seaweeds are considered to be healthy foods and are used in pharmaceutical and cosmetic industries, and fish and poultry farming. There is an increasing interest in the brown alga *Ascophyllum nodosum* extract (ANE) as an organic fertilizer to improve plant growth (Khan et al. 2009). Several studies have demonstrated that ANE, a plant biostimulant, contributes to enhanced growth, increased nutrient uptake, a better resistance to biotic and abiotic stresses, higher yield, as well as prolonged shelf life in a wide range of crops (Khan et al. 2009; Norrie and Hiltz 1999). The mechanisms by which ANE affects plant metabolism are mainly due to minerals, amino acids, vitamins, and hormone-like growth substances present in ANE (Allen et al. 2001; Fike et al. 2001). A 12-15% increase in total fresh matter production of spinach (*Spinacia oleracea* L. cv. Monstrueux De Viroflay and cv. Polka) leaves was obtained following ANE treatment (Cassan et al. 1992). Both foliar and soil application of ANE increased the endogenous antioxidative levels in turf and forage grasses (Allen et al. 2001), but there are very few reports showing the antioxidant-altering properties in fruit and vegetable in response to ANE treatment. Zodape et al. (2008) reported that foliar spray on okra (*Abelmoschus esculentus* L.) with a liquid seaweed fertilizer tended to enhance ascorbic acid content. There are some reports that post-harvest treatment of ANE increased shelf life in fruit and vegetables, such as capsicums and limes (Blunden et al. 1979). Pre-harvest application has been shown to delay pear (*Pyrus leconte*, Rehd) fruit decolouration, thus prolonging shelf life (Abdel-Hafeez 2005).

Extracts from spinach leaves, which mainly contain phenolic compounds, have been shown to be effective free radical scavengers with strong anti-inflammatory properties and anti-mutagenic potentials in several biological systems (Bakshi et al. 2004; Bergman et al. 2001; Nyska et al. 2003). Their role in health promotion and disease prevention in humans has received significant attention. The effects of spinach extract on prolonging life span and improving stress resistance in whole organisms, and whether there are differences in the effects on parameters related to aging between extracts prepared from ANE-treated spinach leaves and non-treated control are not known yet.

The present studies were carried out to investigate the effect of pre-harvest application of ANE in enhancing the nutritional quality and post-harvest storability in spinach (*Spinacia oleracea* L., var. Unipack 12). Moreover, the effect of spinach extracts (with and without ANE-treatment) on life span, stress tolerance and fat metabolism of the model animal system, *Caenorhabditis elegans*, was also studied. The results of this thesis demonstrated that applications of ANE impart significant increases in nutritional quality and storability of spinach, and that extracts from ANE-treated spinach exert stronger protective effects in *C. elegans* against oxidative and thermal stress.

The alkaline extract of *A. nodosum* (ANE) significantly improved the quality of spinach in *in-vivo* bioassay. ANE treatment did not significantly change the whole metabolite profile of spinach leaves. The total antioxidant capacity, total phenolics and total flavonoids were all increased by 20-50% in ANE-treated spinach leaves as compared to water controls. It was observed that phenolic content contribute to the total antioxidant activity of ANE-treated spinach. A strong correlation (R^2 = 0.933) between total phenolics and DPPH measurements was observed. There were 9 major flavonoids identified via ^{1}H NMR and LC-MS analyses, showing a 36-51% increase as compared to untreated controls. The content of total soluble protein and minerals (potassium and iron) were significantly increased in 1.0 g/L ANE-treated plants as compared to water controls, indicating that ANE may induce a better efficiency of nutrient uptake and an enhanced ability of protein synthesis in spinach. The concentration of ascorbic acid and oxalate was not influenced by ANE.

In the *in-vitro* assay, the total antioxidant capacity, total phenolics and total flavonoids were also significantly increased in 0.1 g/L ANE-treated spinach leaves as compared to water controls. The dry weight and total chlorophyll of spinach leaves were significantly enhanced in treated spinach leaves in the *in vitro* bioassay, while their contents were not affected by ANE treatment in the *in vivo* assa. This suggests the effect of ANE is partly dependent on the timing and duration of application. Only applications of medium concentration (1.0 g/L or 0.1 g/L) exerted significantly positive effects suggesting that high concentration (5.0 g/L or 0.5 g/L) of ANE may negatively affect plant growth probably due to the high salt content.

The ANE treatment was applied as root irrigation but resulted in an impact on the increased nutritional level of the leaves. In an effort to partially characterize the effect of ANE applications at the enzymatic and transcriptional levels, a combination of enzymology and molecular techniques were used.

ANE treatment had no effect on the activity of phenylalanine ammonia lyase (PAL, EC 4.3.1.5) but significantly increased the activity of chalcone isomerase (CHI, EC 5.5.1.6). The latter is one of the key enzymes for the biosynthesis of flavanone precursors and phenylpropanoid plant defense compounds. This suggests that ANE-treatment through root drench may induce specific systemic physiological responses, including eliciting the phenylpropanoid and flavonoid pathways, thus leading to promotion of anti-radical capacity in spinach leaves. Previous reports have shown that a number of gene products, such as sucrose phosphate synthase (*SPS*) (Park et al. 2008), cytosolic glutamine synthetase (*GS1*) (Oliveira et al. 2002), and *NR* (Durand et al. 2003) are involved in plant growth and yield. The gene expression studies using semi-quantitative RT-PCR revealed that ANE-treatment affected the expression of some growth-related genes. GS1 transcript was higher in 0.1 g/L ANE-treated spinach than that in untreated ones while the transcripts of SPS and plastid glutamine synthetase (GS2) were not affected. Additionally, ANE treatment induced increases in the expression of betaine aldehyde dehydrogenase (BADH) and choline monooxygenase (CMO) in spinach leaves. These two genes are involved in the endogenous production of betaines, indicating that ANE might increase glycine betaine synthesis in spinach leaves which may also aid in post-harvest storage. Furthermore, ANE treatment resulted in up-regulation of glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and thylakoid-bound ascorbate peroxidase (tAPX) while did not affect the mRNA levels of stromal APX (sAPX) and dehydroascorbate reductase (DHAR). These genes are involved in ascorbate-glutathione cycle in plants. Since no significant difference was found in terms of total ascorbate between ANE-treated and untreated plants, the up-regulated GR and MDHAR might be compensated for the increased transcription of tAPX.

The biochemical analysis of spinach leaves during post-harvest storage revealed that 1.0 g/L ANE applied pre-harvest imparted significant enhancement to the storability

of spinach leaves at 10 °C with a relative humidity $\geq$ 95% for a 35-day storage period. ANE-treatment significantly improved the visual quality and reduced the fresh weight loss during storage, but had no effect on total ascorbate and chlorophyll content. Lipid peroxidation in leaves was significantly reduced in 1.0 g/L ANE-treated leaves as compared to the control. A clear link was observed between lipid peroxidation and visual quality. When lipid peroxidation was reduced in appearance the quality increased. Since reactive oxidative species (ROS) accumulate during post-harvest senescence and the total antioxidant activity was positively associated with the total phenolics content in spinach, it is plausible to propose that ANE-enhancement of the endogenous antioxidant status of spinach may contribute to improved post-harvest tolerance by quenching ROS and protecting membrane integrity, leading to better shelf life of spinach leaves.

Pharmacological studies have shown in animals and humans that extended lifespan could delay age-related degenerations and age-related illnesses such as cardiovascular disease and Alzheimer's disease. The transparent, free-living soil nematode *C. elegans* has been an essential model system for studying aging and genetic and pharmacological influences on aging. Our diet intervention studies revealed that when applied at the optimum concentration, spinach extract, containing bioactive molecules, mainly phenolic compounds, either from ANE-treated or untreated plants, could prolong the life span of *C. elegans* by approximately 45% as compared to control nematodes. Spinach extracts increased the resistance of *C. elegans* to heat stress (35 °C); the same held true for oxidative stress (500 µM juglone) resistance. Extract from ANE-treated spinach exhibited even stronger protective effects against stresses in *C. elegans*. These results suggested that spinach extract might reveal their effect via radical scavenging activities since ROS accumulates with aging, or via the activation of endogenous antioxidant defense systems and signaling pathways so as to act indirectly to delay oxidative damage and aging. Spinach extract from either ANE-treated or untreated plants influenced the proportions of some fatty acids but whether there were correlated with the beneficial effects exerted by extracts is not clear.

In conclusion, I found that ANE-modulated specific metabolic pathways in spinach resulted in increased nutritional quality at harvest and enhanced post-harvest storability, which provides some important pieces of information, adding to the

knowledge on how ANE affects the quality of spinach at harvest and during storage. In addition, induced polyphenols in extract of ANE-treated spinach exerted stronger effects in *C. elegans* in terms of stress resistances and fat metabolism. The results reported in this thesis suggest that application of ANE or ANE-treated spinach extract elicits responses reminiscent of a hormetic effect in spinach or *C. elegans*, respectively.

The effect of ANE on spinach seems to be via synergistic affects exerted by its numerous components such as mineral nutrients and plant hormone-like growth regulators. The use of natural, environmentally friendly and safe extracts to increase antioxidant levels of vegetables should be ideal, thus the investigations into the mechanism(s) by which ANE treatment may exert such beneficial effects on spinach have to be further extended. The analysis of the effect of ANE on the levels of carotenoids and tocopherols, and maybe folate, the other three major nutrient contents in spinach, as well as their change patterns during post-harvest storage, should be further studied. Since spinach is prized for its high content of bioactive compounds, yet it is prone to deterioration during storage, enhancing nutritional values at harvest and improving storability with retention of appearance and maintenance of nutrients via ANE-treatments, is very promising.

Further detailed studies are needed to delineate the underlying mechanisms of how spinach extracts prolong life span of C. *elegans* under normal and stress conditions. It seems that spinach extract did not prolong the life span in *C. elegans* through caloric restriction (CR). Other experiments such as a reproduction assay are required to elucidate whether spinach-mediated life span elongation alter other fitness parameters (e.g. fecundity, onset of egg laying and body size) in order to further confirm the hypothesis that the spinach extract is not a CR mimetic. Furthermore, analysis of ROS accumulation during aging and stress and studies of genetic background on spinach extract-mediated longevity, using mutant strains and studies of defense gene expression, would also be very useful. The physiological and molecular signaling mechanisms of spinach extract on elongated life span and increased stress resistance in *C. elegans* should be thoroughly investigated. Synergistic effects amongst individual compounds of spinach extract require elucidation (Bergman et al., 2003). Our results provide the first tantalizing insight of the manifold functions of spinach extract in the model nematode *C. elegans*.

It must be noticed that the spinach extract used for animal experiments was obtained from a 70% aqueous MeOH extract of dry leaf tissue. Though most of the active compounds were flavonoids and other polyphenols, I admit that a certain amount of chlorophylls, carotenoids and tocopherols was not evaluated as they remained in the lipophilic phase. Vitamin E has been shown to prolong the lifespan of *C. elegans* (Harrington and Harley, 1988) but its effects on heat and oxidative stresses are unknown. There was no significant difference in the amount of total chlorophyll between control and ANE-treated spinach, but the effect of ANE application pre-harvest on carotenoid and tocopherol content in spinach has to be elucidated in order to further confirm that it is primarily the phenolic components in spinach extracts that contribute to the protective effects imparted on *C. elegans* against stress conditions.

References

Abetz P (1980) Seaweed extracts: Have they a place in Australian agriculture or horticulture? J Aust Inst Agri Sci 46: 23-29.

Abetz P, Young CL (1983) The effect of seaweed extract sprays derived from *Ascophyllum nodosum* on lettuce and cauliflower crops. Bot Mar XXVI: 487-492.

Abdel-Hafeez A A (2005) Effect of pre-harvest spraying with seaweed extract "Acadian" and active dry yeast on "Le Conte" pear (*Pyrus leconte*, Rehd) fruit quality and cold storability. Ann Agric Sci Moshtohor 43: 1915-1935.

Adachi H, Ishii N (2000) Effects of tocotrienol on lifepan and protein carbonylation in *Caenorhabditis elegans*. J Gerontol A Biol Sci Md Sci 55: B280-285.

Allen VG, Pond KR, Saker KE, Fontenot JP, Bagley CP, Ivy RL, Evans RR, Schmidt RE, Fike JH, Zhang X, Ayad JY, Brown P, Miller MF, Montogmery JL, Mahan J, Wester DB, Melton C (2001) Tasco: Influence of a brown seaweed on antioxidants in forages and livestock-A review. J Anim Sci 79: E21-E31.

Ames BM, Shigenaga MK, Hagen TM (1993) Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci 90: 7915-7922.

Amin KA, Abd El-Twab TM (2009) Oxidative markers, nitric oxid and homocysteine alteration in hypercholesterolimic rats: role of atrocastatine cinnamon. Int J Clin Exp Med 2: 254-265.

Anson RM, Hansford RG (2004) Mitochondrial influence on aging rate in *Caenorhabditis elegans*. Aging Cell 3: 29-34.

Arts ICW, Hollman PCH (2005) Polypenols and disease risk in epidemiologic studies. Am J Clin Nutr 81: 317S-325S.

Ashrafi K, Chang FY, Watts JL, Fraser AG, Kamath RS, Ahringer J, Ruvkum G (2003) Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. Nature 421: 268-272.

Bakshi S, Bergam M, Dovrat S, Grossman S (2004) Unique natural antioxidants (NAOs) and derived purified components inhibit cell cycle progression by downregulation of ppRb and E2F in human PC3 prostate cancer cells. FEBS Lett 573: 31-37.

Barclay KD, McKersie BD (1994) Peroxidation reactions in plant membranes: effects of free fatty acids. Lipids 29: 877-882.

Barzegar M, Erfani F, Jabbari A, Hassandokit MR (2007) Chemical composition of 15 spinach (*Spinacea oleracea* L.) cultivars grown in Iran. Ital J Food Sci 19: 309-318.

Bazzano LA, Li TY, Joshipura KJ, Hu FB (2008) Intake of fruit, vegetables, and fruit juices and risk of diabetes in women. Diabetes Care 31:1311-1317.

Benedetti G Foster AL, Vantipalli MC, White MP, Sampayo JN, Gill MS, Olsen A, Lithgow GJ (2008) Compounds that confer thermal stress resistance and extended lifespan. Exp Gerontol 43: 882-891.

Bergman M, Perelman A, Dubinsky Z, Grossman S (2003) Scavenging of reactive oxygen species by a novel glucurinated flavonoid antioxidant isolated and purified from spinach. Phytochem 62: 753-762.

Bergman M, Varshavsky L, Gottlieb HE, Grossman S (2001) The antioxidant activity of aqueous spinach extract: chemical identification of active fractions. Phytochem 58: 143-152.

Bergquist SÅM, Gertsson UE, Knuthsen P, Olsson ME (2005) Flavonoids in baby spinach (*Spinacia oleracea* L.): Changes during plant growth and storage. J Agric Food Chem 53: 9459-9464.

Bergquist SÅM, Gertsson UE, Nordmark LYG, Olsson ME (2007a) Effects of shade nettings, sowing time and storage on baby spinach flavonoids. J Sci Food Agric 87: 2464-2471.

Bergquist SÅM, Gertsson UE, Nordmark LYG, Olsson ME (2007b) Ascorbic acid, carotenoids, and visual quality of baby spinach as affected by shade netting and postharvest storage. J Agric Food Chem 55: 8444-8451.

Bergquist SÅM, Gertsson UE, Olsson ME (2006) Influence of growth stage and postharvest storage on ascorbic acid and carotenoid content and visual quality of baby spinach (*Spinacia oleracea* L.). J Sci Food Agric 86: 346-355.

Black RE (2003) Zinc deficiency, infectious disease and mortality in the developing world. J Nutr 133: 1485S-1489S.

Bligh EG, Dyer WJ (1959) A rapid method for total lipid extraction and purification. Can J Biochem Physiol 37: 911-917.

Blunden G, Enkins T, Liu YW (1997) Enhanced leaf chlorophyll levels in plants treated with seaweed extract. J Appl Phycol 8: 535-543.

Blunden G, Jones EM, Passam HC (1978) Effects of post-harvest treatment of fruit and vegetables with cytokinin-active seaweed extracts and kinetin solutions. Bot Mar XXI: 237-240.

Blunden G, Wildgoose PB, Nicholson FE (1979) The effects of aqueous seaweed extract on sugar beet. Bot Mar XXII: 539-541.

Boivin D, Lamy S, Lord-Dufour S, Jackson J, Beaulieu E, Côté M, Moghrabi A, Barret S, Gingras D, Béliveau R (2009) Antiproliferative and antioxidant activities of common vegetables: A comparative study. Food Chem 112: 374-380.

Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. LWT-Food Sci Technol 28: 25-30.

Brock TJ, Browse J, Watts JL (2007) Fatty acid desaturation and the regulation of adiposity in *Caenorhabditis elegans*. Genetics 176: 865-875.

Brooks KK, Liang B, Watts JL (2009) The influence of bacterial diet on fat storage in *C. elegans*. PLoS One 4: e7545. doi:10.1371/journal.pone.0007545

Brown MK, Evans JL, Luo Y (2006) Beneficial effects of natural antioxidants EGCG and alpha-lipoic acid on life span and age-dependent behavioral declines in *Caenorhabditis elegans*. Pharmacol Biochem Behave 85: 620-628.

Buescher RW, Howard LR, Dexter P (1999) Postharvest enhancement of fruits and vegetables for improved human health. Hortscience 34: 12.

Burri BJ (1997) Beta-carotene and human health: A review of current research. Nutr Res 17: 547-580.

Butelli E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen EG, Hall RD, Bovy AG, Luo J, Martin C (2008) Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat Biotechnol 26: 1301-1308.

Butler T, Hunter A (2006) Impact of seaweed extract on turfgrass growth and nutrition on a golf green to USGA Specification. Acta Hort (ISHS) 762: 81-90.

Cao G, Sofic E, Prior RL (1996) Antioxidant capacity of tea and common vegetables. J Agric Food Chem 44: 3426-3431.

Cao Z, Wu Y, Curry K, Wu Z, Christen Y, Luo Y (2007) Ginkgo biloba extract Egb 761 and Wisconsin Ginseng delay sarcopenia in *Caenorhabditis elegans*. J Gerontol A Biol Sci Med Sci 62: 1337-1345.

Capocasa F, Diamanti J, Tulipani S, Battino M, Mezzetti B (2008) Breeding strawberry (*Fragaria × ananassa* Duch) to increase fruit nutritional quality. BioFactors 34: 67-72.

Cardozo K, Guaratini T, Barros MP, Falcão VR, Tonon AP, Lopes NP, Campos S, Torres MA, Souza AO, Colepicolo P, Pinto E (2007) Metabolites from algae with economical impact. Comp Biochem Physiol, Part C 146: 60-78.

Cassan L, Jeannin I, Lamaze T, Morot-Gaudry JF (1992) The effect of the *Ascophyllum nodosum* extract Goëmar GBA 14 on the growth of Spinach. Bot Mar 35: 437-439.

Champagne CM (2008) Magnesium in hypertension, cardiovascular disease, metabolic syndrome, and other conditions: a review. Nutr Clin Pract 23: 142-151.

Cho MJ, Howard LR, Prior RL, Morelock T (2008) Flavonoid content and antioxidant capacity of spinach genotypes determined by high-performance liquid chromatography/mass spectrometry. J Sci Food Agric 88: 1099-1106.

Chouliaras V, Gerascapoulos D, Lionakis S (1997) Effects of seaweed extract on fruit grown, weight and maturation of 'Hayward' kiwifruit. Acta Hort 444: 485-489.

Chouliaras V, Tasioula M, Chatzissavvidis C, Therios I, Tsabolatidou E (2009) The effects of a seaweed extract in addition to nitrogen and boron fertilization on productivity, fruit maturation, leaf nutritional status and oil quality of the olive (*Olea europaea* L.) cultivar Koroneiki. J Sci Food Agric 89: 984-988.

Christen S, Woodall AA, Shigenaga MK, Southwell-Keely PT, Duncan MW, Ames BN (1997) γ-Tocopherol traps mutagenic electrophiles such as NOx and compliments α-tocopherol: physiological implications. Proc Natl Acad Sci 94: 3217-3222.

Collins JJ, Evason K, Kornfeld K (2006) Pharmacology of delayed aging and extended lifespan of *Caenorhabditis elegans*. Exp Gerontol 41: 1032-1039.

Collins JJ, Evason K, Pickett CL, Schneider DL, Kornfeld K (2008) The anticonvulsant ethosuximide disrupts sensory function to extend *C. elegans* lifespan. PLoS One 4: e1000230. doi:10.1371/journal.pgen.1000230

Conte A, Conversa G, Scrocco C, Brescia I, Laverse J, Elia A, Del Nobile MA (2008) Influence of growing periods on the quality of baby spinach leaves at harvest and during storage as minimally processed produce. Postharvest Biol Tech 50: 190-196.

Cypser JR, Johnson TE (2002) Multiple stressors in *Caenorhabditis elegans* induce stress hormesis and extended longevity. Gerontol A Biol Sci 57: B109-B114.

Dalal M, Dani RG, Kumar PA (2006) Current trends in the genetic engineering of vegetable crops. Scientia Horticul 107: 215-225.

Davey MW, Van Montagu M, Inze D, Sanmartin M, Kanellis A, Smirnoff N, Benzie, IJJ, Strain JJ, Favell D, Fletcher J (2000) Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. J Sci Food Agric 80: 825-860.

Debolt S, Melino V, Ford CM (2007) Ascorbate as a biosynthetic precursor in plants. Ann of Bot 99: 3-8.

Dewanto V, Wu XZ, Adom KK, Liu RH (2002) Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agric Food Chem 50: 3010-3014.

Dowd P, Zheng ZB (1995) On the mechanism of the anticlotting action of vitamin E quinine. Proc Natl Acad Sci USA 92: 8171-8175.

Drieu K, Vranckx, R, Benassayad C, Haourigi M, Hassid J, Yoa RG, Rapin JR, Nunez EA (2000) Effect of the extract of *Ginkgo biloba* (EGb 761) on the circulating and cellular profiles polyunsaturated fatty acids: correlation with the anti-oxidant properties of the extract. Prostaglandins Leukot Essent Fatty Acids 63: 293-300.

Durand N, Brand X, Meyer C (2003) The effect of marine bioactive substances (N PRO) and exogenous cytokinins on nitrate reductase activity in *Arabidopsis thaliana*. Physiol Plant 119: 489-493.

Edenharder R, Keller G, Platt KL, Unger KK (2001) Isolation and characterization of structurally novel antimutagenic flavonoids from spinach (*Spinacia oleracea*). J Agric Food Chem 49: 2767-2773.

Entchev EV, Schwudke D, Zagoriy V, Matyash V, Bogdanova A, Habermann B, Zhu L, Shevchenko A, Kurzchalia TV (2008) LET-767 is required for the production of branched chain and long chain fatty. J Biol Chem 283: 17550-17560.

Evason K, Collins JJ, Huang C, Hughes S, Kornfeld K (2008) Valproic acid extends *Caenorhabditis elegans* lifespan. Aging Cell 7: 305-317.

Fan D, Hodges DM, Zhang JZ, Kirby CW, Ji XH, Locke SJ, Critchley AT, Prithiviraj B (2011) Commercial extract of the brown seaweed *Ascophyllum nodosum* enhances phenolic antioxidant content of spinach (*Spinacia oleracea* L.) which protects *Caenorhabditis elegans* against oxidative and thermal stress. Food Chem 124:195-202.

Featonby-Smith BC, van-Staden J (1983) The effect of seaweed concentrate on the growth of tomatoes in nematode infested soil. Sci Hort 20: 137-146.

Ferguson I, Volz R, Woolf A (1999) Preharvest factors affecting physiological disorders of fruit. Postharvest Biol Tec 15: 255-262.

Fike JH, Allen VG, Schmidt RE, Zhang X, Fontenot JP, Bagley CP, Ivy RL, Evans RR, Coelho RW, Wester DB (2001) Tasco-Forage: I. Influence of a seaweed extract on antioxidant activity in tall fescue and in ruminants. J Anim Sci 79: 1011-1021.

Fike JH, Saker KE, O'Keefe SF, Marriott NG, Ward DL, Fontenot JP, Veit HP (2005) Effects of Tasco (a seaweed extract) and heat stress on N metabolism and meat fatty acids in wether lambs fed hays containing endophye-infected fescue. Small Rumin Res 60; 237-245.

Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408: 239-247.

Fornes F, Sánchez-Perales M, Guardiola JL (2002) Effect of a seaweed extract on the productivity of 'de Nules' clementine mandarin and navelina orange. Bot Mar 45: 486-489.

Fuentes SI, Allen DJM Ortiz-Lopez A, Hernández G (2001) Over-expression of cytosolic glutamine synthetase increases photosynthesis and growth at low nitrogen concentrations. J Exp Bot 52: 1071-1081.

Gadzovska S, Maury S, Delaunay A, Spasenoski M, Joseph C, Hagège D (2007) Jasmonic acid elicitation of *Hypericum perforatum* L. cell suspensions and effects on the production of phenylpropanoids and naphtodianthrones. Plant Cell Tiss Organ Cult 89: 1-13.

Gil MI, Ferreres F, Tomas-Barberan FA (1999) Effect of postharvest storage and processing on the antioxidant constituents (flavonoids and vitamin C) of fresh-cut spinach. J Agric Food Chem 47: 2213-2217.

Gilst MRV, Hadjivassiliou H, Jolly A, Yamamoto KR (2005) Nuclear hormone receptor NHR-49 controls fat consumption and fatty acid composition in *C. elegans*. PLoS Biol 3: e53.

Golden TR, Hinerfeld DA, Melov S (2002) Oxidative stress and aging: beyond correlation. Aging Cell 1: 117-123.

González MJ, Miranda-Massari JR, Mora EM, Guzmán A, Riordan NH, Riordan HD, Casciari JJ, Jackson JA, Román-Franco A (2005) Orthomolecular Oncology Review: Ascorbic Acid and Cancer 25 Years Later. Integr Cancer Ther 4: 32-44.

Goto F, Yoshihara T, Shigemoto N, Toki S, Takaiwa F (1999) Iron fortification of rice seed by the soybean ferritin geng. Nat Biotechnol 17: 282-286.

Grof MJCPL, Brownell PF (1984) Effect of sodium nutrition on chlorophyll a/b ratios in C4 plants. Aust J Plant Physiol 11:325-332.

Gruber J, Tang SY, Halliwell B (2007) Evidence for a trade-off between survival and fitness caused by resveratrol treatment of *Caenorhabditis elegans*. Ann N Y Acad Sci 1100: 530-542.

Guan SW, Li PF, Luo J, Li YY, Huang L, Wang G, Zhu LM, Fan HK, Li W, Wang LP (2010) A deuterohemin peptide extends lifespan and increases stress resistance in *Caenorhabditis elegans*. Free Radic Res 44: 813-820.

Guenther PM, Dodd KW, Reedy J, Krebs-Smith SM (2006) Most Americans eat much less than recommended amount of fruits and vegetables. J Am Diet Assoc 106: 1371-1379.

Gutteridge JMC, Halliwell B (2000) Free radicals and antioxidants in the year 2000: A historical look to the future. Ann N Y Acad Sci 899: 136-147.

Hait-Darshan R, Grossman S, Bergman M, Deutsch M, Zurgil N (2009) Synergistic activity between a spinach-derived natural antioxidant (NAO) and commercial antioxidants in a variety of oxidation systems. Food Res Int 42: 246-253.

Harrington LA, Harley CB (1988) Effect of vitamin E on lifespan and reproduction in *Caenorhabditis elegans*. Mech Ageing Dev 43: 71-78.

Hasegawa K, Miwa S, Tsutsumiuchi K, Miwa J (2010) Allyl isothiocyanate that induces GST and UGT expression confers oxidative stress resistance on *C. elegan*s, as demonstrated by nematode biosensor. PLoS ONE 5: e9267. doi:10.1371/journal.pone.0009267

Hashimoto T, Horkwa M, Nomura T, Sakamoto K (2010) Nicotinamide adenine diceotide extends the lifespan of *Caenorhabditis elegans* mediated by *sir-2.1* and *daf-16*. Biogerontology 11: 31-43.

He ML, Wang Y, You JS, Mir PS, McAllister TA (2009) Effect of a seaweed extract on fatty acid accumulation and glycerol-3-phosphate dehydeogenase activity in 3T3L1 adipocytes. Lipids 44: 125-132.

Heimler D, Isolani L, Vignolini P, Tombelli S, Romani A (2007) Polyphenol content and antioxidative activity in some species of freshly consumed salads. Food Chem 55: 1724-1729.

Herndon LA, Schmeissner PJ, Dudaronek JM, Brown PA, Listner KM, Sakano Y, Paupard MC, Hall DH, Driscoll M (2002) Stochastic and genetic factors influence tissue-specific decline in ageing *C. elegans*. Nature 419: 808-814.

Hodges DM (2003) Overview: Oxidative stess and postharvest produce. In: Hodges DM (Ed.) Postharvest oxidative stress in horticultural crops. Food Products Press, New York, pp 1-12.

Hodges DM, DeLong JM (2007) The relationship between antioxidants and postharvest storage quality of fruits and vegetables. Stewart Postharvest Review 3:12.

Hodges DM, DeLong JN, Forney CF, Prange RK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207: 604-611.

Hodges DM, Forney CF (2003) Postharvest ascorbate metabolism in two cultivars of spinach differing in their senescence rates. J Amer Soc Hort Sci 128: 930-935.

Hodges DM, Kalt W (2003) Health functionality of small fruit. Acta Horticulturae (ISHS) 616: 17-23.

Hodges DM, Lester GE (2006) Comparisons between orange- and green-fleshed non-netted and orange-fleshed netted muskmelons: antioxidant changes following different harvest and storage periods. J Amer Soc Hort Sci 131: 110–117.

Hodges DM, Lester GE, Munro KD, Toivonen PMA (2004) Oxidative stress: Importance for postharvest quality. HortScience 39: 924-929.

Holick CN, Michaud DS, Stolzenberg-Solomon R, Mayne ST, Pietinen P, Taylor P R, Virtamo J, Albanes D (2002) Dietary carotenoids, serum beta-carotene, and retinol and risk of lung cancer in the alpha-tocopherol, beta-carotene cohort study. Am J Epidemiol 156: 536-547.

Hosono R (1978) Sterilization and growth inhibition of *Caenorhabditis elegans* by 5-fluorodeoxyuridine (FdUrd). Exp Geron 13: 369-374.

Houstis N, Rosen E Lander ES (2006) Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 440: 944-948.

Howard LR, Pandjaitan N, Morelock T, Gil MI (2002) Antioxidant capacity and phenolic content of spinach as affected by genetics and growing season. J Agric Food Chem 50: 5891-5896.

Hradec J, Dufek P (1994) Determination of cholesteryl 14-methylhexadecanoate in bllod serum by reversed-phase high-performance liquid chromatography. J Chromatogr B Biomed Appl 660: 386-389.

Hung H-C, Joshipura KJ, Jiang R, Hu FB, Hunter D, Smith-Warner SA, olditz GA, Rosner B, Spiegelman D, Willett WC (2004) Fruit and vegetables intake and risk of major chronic disease. J Natl Cancer Inst 96: 1577-1584.

Huntley AL (2007) Grape flavonoids and menopausal health. Menopause Int 13: 165-169.

Ishii N, Senoo-Matsuda N, Miyake Kasuda K, Ishii T, Hartman PS, Furukawa S (2004) Coenzyme Q 10 can prolong *C. elegans* lifespan by lowering oxidative stress. Mech Ageing Dev 125: 41-46.

Jafari M, Felgner JS, Bussel II, Hutchili T, Khodayari B, Rose MR, Vince-Cruz C, Mueller LD (2007) Rhodiola: a promising anti-aging Chinese herb. Rejuvenation Res 10: 587-602.

Jauhar PP (2006) Modern biotechnology as an integral supplement to conventional plant breeding: The prospects and challenges. Crop Sci 46: 1841-1859.

Jeannin I, Lescure J-C, Morot-Gaudry J-F (1991) The effects of aqueous seaweed sprays on the growth of maize. Bot Mar 34: 469-473.

Jones KT, Ashrafi K (2009) *Caenorhabditis elegans* as an emerging model for studying the basic biology of obesity. Dis Model Mech 2: 224-229.

Kader AA (2002) *Postharvest Technology of Horticultural Crops* (3th Edition). UC Publication 3311. University of California, Division of Agriculture and Natural Resources, Oakland, California, pp. 5-37, 163-149, 279-284, 423-433.

Kaletta T, Hengartner MO (2006) Finding function in novel targets: *C. elegans* as a model organism. Nat Rev Drug Discovery 5: 387-399.

Kalt W (2005) Effects of production and processing factors on major fruit and vegetable antioxidants. J Food Sci 70: 11-19.

Kampkötter A, Gombitang Nkwonkam C, Zurawski RF, Timpel C, Chovolou Y, Wätjen W, Kahl R (2007a) Effects of the flavonoids kaempferol and fisetin on thermotolerance, oxidative stress and FoxO transcription factor DAF-16 in the model organism *Caenorhabditis elegans*. Arch Toxicol 81:849-858.

Kampkötter A, Pielarski T, Rohrig R, Timpel C, Chovolou Y, Wätjen W (2007b) The *Ginkgo biloba* extract EGb761 reduces stress sensitivity, ROS accumulation and expression of catalase and glutathione S-transferase 4 in *Caenorhabditis elegans*. Pharmacological Research 55: 139-147.

Kampkötter A, Timpel C, Zurawski RF, Ruhl S, Chovolou Y, Proksch P, Wätjen W (2007c) Increase of stress resistance and lifespan of *Caenorhabditis elegans* by quercetin. Comp Biochem Physiol B Biochem Mol Biol 149: 314-323.

Kang HL, Benzer S, Min KT (2002) Life extension in *Drosophila* by feeding drug. Proc Natl Acad Sci USA 99: 838-843.

Kang JH, Ascherio A, Grodstein F (2005) Fruit and vegetable consumption and cognitive decline in aging women. Ann Neurol 57: 713-720.

Kanungsukkasem U, Ng N, Minh HV, Razzaque A, Ashraf A, Juvekar S, Ahmed SM, Bich TH (2009) Fruit and vegetable consumption in rural adults population in INDEPTH HDSS sites in Asia. Glob Health Action 2: 35-43.

Kevers C, Falkowski M, Tabart J, Defraigne JO, Dommes J, Pincemail J (2007) Evolution of antioxidant capacity during storage of selected fruits and vegetables. J Agric Food Chem 55: 8596-8603.

Kenyon C (2005) The plasticity of aging: insights from long-lived mutants. Cell 120: 449-460.

Khan W, Rayirath UP, Subramanian S, Jithesh MN, Rayorath P, Hodges DM, Critchley AT, Craigie JS, Norrie J, Prithiviraj B (2009). Seaweed extracts as biostimulants of plant growth and development. J Plant Growth Regul 28: 386-399.

Kim J, Takahashi M, Shimizu T, Shirasawa T, Kajita M, Kanayama A, Miyamoto Y (2008) Effects of a potent antioxidant, platinum nanoparticle, on the lifespan of *Caenorhabditis elegans*. Merh Ageing Dev 9: 322-331.

Kim SK (2007) Common aging pathways in worms, flies, mice and humans. J Exp Biol 210: 1607-1612.

Kimura KD, Tissenbaum ha, Liu Y, Ruvkun G (1997) daf-2, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. Science 277: 942-946.

Kniazeva M, Crawford QT, Seiber M, Wang CY, Han M (2004) Monomethyl branched-chain fatty acids play an essential role in *Caenorhabditis elegans* development. PLoS Biol 2: e257.

Kniazeva M, Sieber M, McCauley S, Zhang K, Watts JL, Han M (2003) Suppression of the ELO-2 FA elongation activity results in alterations of the fatty acid composition and multiple physiological defects, including abnormal ultradian rhythms, in *Caenorhabditis elegans*. Genetics 163: 159-169.

Kregel KC, Zhang HJ (2007) An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. Am J Physiol 292: R18-R36.

Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD (2002) Bioactive compounds in food: their role in the prevention of cardiovascular disease and cancer. Am J Med 113: 71S-88S.

Kritchevsky SB (1999) Beta-carotene, carotenoids and the prevention of coronary heart disease. J Nutr129: 5-8.

Kurilich AC, Juvik JA (1999) Quantification of carotenoid and tocopherol antioxidants in Zea mays. J Agric Food Chem 47: 1948-1955.

Lampe JW (1999) Health effects of vegetables and fruit: assessing mechanisms of action in human experimental studies. Am J Clin Nutr 70: 475S-490S.

Lee J, Lee S, Lee H, Park K, Choe E (2002) Spinach (*Spinacia olerea*) powder as a natural food-grade antioxidant in deep-fat-fried products. J Agric Foo Chem 50: 5664-5669.

Lee J, Park K, Lee S, Choe E (2000) Lipid changes of freeze-dried spinach by various kinds of oxidation. J Food Sci 65: 1290-1295.

Lefsrud MG, Kopsell DA, Kopesll DE, Curran-Celentano J (2005) Air temperature affects biomass and carotenoid pigment accumulation in kale and spinach grown in a controlled environment. Hortscience 40: 2026-2030.

Li M, Mang F, Shang P, Zhang M, Hou C, LInag D (2009) Influence of light on ascorbate formation and metabolism in apple fruits. Planta 230: 39-51.

Lin JY, Tang CY (2007) Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. Food Chem 101: 140-147.

Lipman RD, Bronson RT, Wu D, Smith DE, Prior R, Cao G, Han SN, Martin KR, Meydani SN, Meydani M (1998) Disease incidence and longevity are unaltered by dietary antioxidant supplementation initiated during middle age in C57BL/6 mice. Mech Ageing Dev 103: 269-284.

Lithgow GJ, Wgute TM, Mekov S, Johnson TE (1995) Thermotolerance and extended life-span conferred by single-gene mutantions and induced by thermal stress. Proc Natl Acad Sci USA 92: 7540-7544.

Liu B, Zhu Y (2007) Extraction of flavonoids from flavonoid-rich parts in tartay buckwheat and identification of the main flavonoids. J Food Eng 78: 584-587.

Lomnitski L, Bergman M, Nyska A, Ben-Shaul V, Grossman S (2003a) Composition, efficacy, and safety of spinach extracts. Nutr Cancer 46: 222-231.

Lomnitski L, Padilla-Bnaks E, Wendy NJ, Nyska A, Grossman S, Newbold RR (2003b) A natural antioxidant mixture from spinach does not have estrogenic or antiestrogenic activity in immature CD-1 mice. J Nutr 133: 3584-3587.

Lurie S (2003) Antioxidants. In: Hodges DM (Ed.) Postharvest Oxidative Stress in Horticultural Crops. Food Products Press, New York, pp 131-150.

MacArtain P, Gill CIR, Brooks M, Campbell R, Rowland IR (2007) Nutritional value of edible seaweeds. Nutr Rev65: 535-543.

McColl G, Killilea DW, Hubbard AE, Vantipalli MC, Melov S, Lithgow GJ (2008) Pharmacogenetic analysis of lithium-induced delayed aging in *Caenorhabditis elegans*. J Biol Chem 283: 350–357.

Meng XH, Li BQ, Liu J, Tian SP (2008) Physiological responses and quality attributes of table grape fruit to chitosan preharvest spray and postharvest coating during storage. Food Chem 106: 501-508.

Metting B, Zimmerman WJ, Crouch T Van-Staden J (1990) Agronomic uses of seaweed and microalgae. In: Akatsuka, I. (Eds.) Introduction of Applied Phycology. SPB Academic Publishing, Hague, Netherlands. pp. 589-627.

Meydani SN (1995) Vitamin E enhancement of T cell-mediated function in healthy elderly: mechanisms of action. Nutr Rev 53: S52.

Morck C, Pilon M (2006) *C. elegans* feeding defective mutants have shorter body lengths and increased autophagy. BMC Dev Bio 6: 39.

Mukhopadhyay A, Deplancke B, Walhour AJ, Tissenbaum HA (2005) *C. elegans tubby* regulates life span and fat storage by two independent mechanisms. Cell Metab 2: 35-42.

Mullaney BC, Ashrafi K (2009) *C. elegans* fat storage and metabolic regulation. Biochim Biophys Acta 1791: 474-478.

Murcia MA, Vera A, García-Carmona F (1992) Determination by HPLC of changes in tocopherol levels in spinach after industrial processing. J Sci Food Agri 60: 81-84.

Neuhouser ML (2004) Dietary flavonoids and cancer risk: Evidence from human population studies. Nutr Cancer Int J 50: 1-7.

Niki E, Noguchi N, Tsuchihashi H, Gotoh N (1995) Interaction among vitamin-C, vitamin-E, and β-carotene. Amer J Cli Nutr 62: S1322-S1326.

Nishikawa F, Kato M, Hyodo H, Ikoma Y, Sugiura M, and Yano M (2003) Ascorbate metabolism in harvested broccoli. J Exp Bot 54: 2439-2448.

Norrie J, Hiltz DA (1999) Seaweed extract research and applications in agriculture. Agro Food Industry Hi-tech 10: 15-18.

Nyska A, Suttie A, Bakshi S, Lomnitski L, Grossman S, Bergan M (2003) Slowing tumorigenic progression in TRAMP mice and prostatic carcinoma cell lines using natural anti-oxidant from spinach, NAO–a comparative study of three anti-oxidants. Toxicol Pathol 1: 39-51.

Oh SW, Mukhopadhyay A, Surzikapa N, Jiang F, Davis RJ, Tissenbaum HA (2005) JNK regulates lifespan in *Caenorhabditis elegans* by modulating nuclear translocation of forkhead transcription factor/DAF-16. Proc Natl Acad Sci USA 102: 4494-4499.

Oliveira IC, Brears T, Knight TJ, Clark A, Coruzzi GM (2002) Overexpression of cytosolic glutamine synthetase. Relation to nitrogen, light, and photorespiration. Plant Physiol 129: 1170-1180.

Olsen A, Vantipalli MC, Lithgow GJ (2006) Checkpoint proteins control survival of the postmitotic cells in *Caenorhabditis elegans*. Science 312: 1381-1385.

Paiva S, Russell RM (1999) β-carotene and other carotenoids as antioxidants. J Am Coll Nutr 18: 426-433.

Pan KZ, Palter JE, Rogers AN, Olsen A, Chen D, Lithgow GJ, Kapahi P (2007) Inhibition of mRNA translation extends lifespan in *Caenorhabditis elegans*. Aging Cell 6: 111-119.

Pandjaitan N, Howard LR, Morelock T, Gil MI (2005) Antioxidant capacity and phenolic content of spinach as affected by genetics and maturation. J Agric Food Chem 53: 8618-8623.

Pandrangi S, Laaborde LF (2004) Retention of folate, carotenoids, and other quality characteristics in commercially packaged fresh spinach. J Food Sci 69: C702-707.

Park JY, Canam T, Kang KY, Ellis DD, Mansfield SD (2008) Over-expression of an arabidopsis family A sucrose phosphate synthase (SPS) gene alters plant growth and fibre development. Transgenic Res 17: 181-192.

Patier P, Yvin JC, Kloareg B, Lienart Y, Rochas C (1993) Seaweed liquid fertilizer from *Ascophyllum nodosum* contains elicitors of plant D-glycanases. J Appl Phycol 5: 343-349.

Petrascheck M, Ye X, Buck LB (2007) An antidepressant that extends lifespan in adult *Caenorhabditis elegans*. Nature 450: 553-556.

Pietsch K, Saul N, Menzel R, Stürzenbaum SR, Steinberg CE (2009) Quercetin mediated lifespan extension in *Caenorhabditis elegans* is modulated by *age-1*, *daf-2*, *sek-1* and *unc-43*. Biogerontology 10: 565-578.

Podsędek A (2007) Natural antioxidants and antioxidant capacity of Brassica vegetables: A review. LWT-Food Sci Technol 40: 1-11.

Prithiviraj B, Bais HP, Weir P, Suresh B, Najarro EH, Dayakar BV, Schweizer HP, Vivanco JM (2005) Down regulation of virulence factors of *Pseudomonas aeruginosa* by salicylic acid attenuates its virulence on *Arabidopsis thaliana* and *Caenorhabditis elegans*. Infect Immun 73: 5319-5328.

Pun PBL, Gruber J, Tang SY, Schaffer S, Ong RLS, Fong S, Ng LF, Cheah I, Halliwell B (2010) Ageing in nematode: do antioxidants extend lifespan in *Caenorhabditis elegans*? Biogerontology 11: 17-30.

Raizen DM, Lee RYN, Avery L (1995) Interacting genes required for pharyngeal excitation by motor neuron MC in *Caenorhabditis elegans*. Genetics 141: 1365-1382.

Rattan SIS (2004) Aging intervention, prevention, and therapy through hormesis. J Gerontol Biol Sci 59A: 705-709.

Rattan SIS (2008) Increased molecular damage and heterogeneity as the basis of aging. Biol Chem 389: 267-272.

Rayorath P. Jithesh MN, Farid A, Khan W, Palanisamy R, Hankins SD, Critchley AT, Prithiviraj B (2008) Rapid bioassays to evaluate the plant growth promoting activity of *Ascophyllum nodosum* (L.) Le Jol. using a model plant, *Arabidopsis thaliana* (L.) Heynh. J Appl Phycol 20: 423-429.

Rayorath P (2009) Molecular characterization of *Ascophyllum nodosum* extract-mediated enhanced freezing tolerance in *Arabidopsis thaliana*. M.Sc. dissertation. Nova Scotia Agricultural College & Dalhousie Univ., Truro.

Rea S, Johnson TE (2003) A metabolic model for life span determination in *Caenorhabditis elegans*. Dev Cell 5: 197-203.

Retamales J, Defilippi BG, Arias M, Castillo P, Manríquez D (2003) High-CO_2 controlled atmospheres reduce decay incidence in Thompson Seedless and Red Globe table grapes. Postharvest Biol Tec 29: 177-182.

Reyes LF, Villarreal JE, Cisneros-Zevallos L (2007) The increase in antioxidant capacity after wounding depends on the type of fruit or vegetable tissue. Food Chem 101: 1254-1262.

Ribaya-Mercado JD, Blumberg JB (2004) Lutein and zeaxanthin and their potential roles in disease prevention. J Am Coll Nutr 23: 567S-587S.

Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Rad Biol Med 20: 933-956.

Ritchie RJ (2008) Universal chlorophyll equations for estimating chlorophylls a, b, c, and d and total chlorophylls in natural assemblages of photosynthetic organism using acetone, methanol, or ethanol solvents. Photosynthetica 46: 115-126.

Rupérez P (2002) Mineral content of edible marine seaweeds. Food Chem 79: 23-26.

Russell BL, Rathinasabapathi B, Hanson AD (1998) Osmotic stress induces expression of choline monooxygenase in sugar beet and amaranth. Plant Physiol 116: 859-865.

Saker KE, Fike JH, Verr H, Ward DL (2004) Brown seaweed-(TascoTM) treated conserved forage enhances antioxidant status and immune function in heat-stressed wether lambs. J Anim Physiol a Anim Nutr 88: 122-130.

Samaras TT, Elrick H, Storms LH (2003) Is height related to longevity? Life Sci 72: 1781-1802.

Saul N, Pietsch K, Menzel R, Stürzenbaum SR, Steinberg CEW (2009) Catechin induced longevity in *C. elegans*: From key regulator genes to disposable soma. Mech Ageing Dev 130: 477-486.

Savage GP, Vanhanen L, Mason SM, Ross AB (2000) Effect of cooking on the soluble and insoluble oxalate content of some New Zealand foods. J Food Comp Ana 13: 201-206.

Schreiner M, Huyskens-Keil S (2006) Phytochemicals in fruit and vegetables: Health promotion and postharvet elicitors. Crit Rev Plant Sci 25: 267-278.

Schulz TJ, Zarse K, Voigt A, Urban N, Birringer M, Ristow M (2007) Glucose restriction extends *Caenorhabitis elegans* life span by inducing mitochondrial respiration and increasing oxidative stress. Cell Metab 6: 280-293.

Shehata AY, De Man JM, Alexander JG (1979) A simple and rapid method for the preparation of methyl esters of fats in milligram amounts for gas chromatography. J Can Inst Food Technol 3: 85-89.

Singh RP, Muthy KNC, Jayaprakasha GK (2002) Studies on the antioxidant activity of pomegranate (*Pinica granatum*) peel and seed extracts using *in vitro* models. J Agri Food Chem 50: 81-86.

Smirnoff N (1996) The function and metabolism of ascorbic acid in plants. Ann Bot 78: 661-669.

Spamoer B, Lasch K, Marsch S, Jacqueline B, Liao WJ, Hu H, Kienberger H, Eisenreich W, Daniel H (2009) How the intestinal peptide transporter PEPT-1 contributes to an obesity phenotype in *Caenorhabditis elegans*. PLoS One 4: e6279. doi:10.1371/journal.pone.0006279

Srivastava D, Arya U, SoundaraRajan T, Dwivedi H, Kumar S, Subramaniam JR (2008) Reserpine can confer stres tolerance and lifespan extension in the nematode *C. elegans*. Biogerontology 9: 309-316.

Sutphin GL, Kaeberlein M (2008) Dietary restriction by bacterial deprivation increases lifespan in wild-derived nematodes. Exp Gerontol 43: 130-135.

Tanaka T, Ikita K, Ashida T, Motoyama Y, Yamaguchi Y, Satouchi K (1996) Effects of growth temperature on the fatty acid composition of the free-living nematode *Caenorhabditis elegan*. Lipids 31: 1173-1178.

Tasinato A, Boscoboinik D, Bartoli GM, Maroni P, Azzi A (1995) D-α-Tocopherol inhibition of vascular smooth muscle cell proliferation occurs at physiological concentrations, correlates with protein kinase C inhibition, and is independent of its antioxidant properties. Proc Natl Acad Sci USA 92: 12190-12194.

Terai Y, Fujii I, Soon-He B, Nakajima O, Hakamatsuka T, Ebizuka Y, Sankawa U (1996) Cloning of chalcone-flavanone isomerase cDNA from *Pueraria Iobata* and its overexpression in *Escherichia coli*. Protein Expr Purif 8: 183-190.

Toivonen PMA (2003) Postharvest treatments to control oxidative stress in fruits and vegetables, in Postharvest Oxidative Stress in Horticultural Crops, ed. by Hodges DM. Food Products Press, New York, pp. 225-246.

Toledo MEA, Ueda Y, Imahori Y, Ayaki M (2003) L-ascorbic acid metabolism in spinach (*Spinacia oleraces* L.) during post-harvest storage in light and dark. Postharvest Biol Technol 28: 47-57.

Tripathi P, Dubey NK (2004) Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. Postharvest Biol Tec 32: 235-245.

Turan M, Köse C (2004) Seaweed extracts improve copper uptake of grapevine. Acta Agric Scand B: Plant Soil Sci 54: 213-220.

Ubalua AO (2009) Transgenic plants: Successes and controversies. Biotechnol Mol Biol Rev 4: 118-127.

Valenzano DR, Terzibasi E, Genade T, Cattaneo A, Domenici L, Cellerino A (2006) Resveratrol prolongs lofespan and retards the onset of age-related markers in a shor-lived vertebrate. Curr Biol 16: 296-300.

Valpuesta V, Botella MA (2004) Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant. Trends Plant Sci 9: 573-577.

Veit M, Bilger T, Muhlbauer T, Brummet W, Winter K (1996) Diurnal changes in flavonoids. J Plant Physiol 148: 478-482.

Vellai T, Takacs-Vellai K, Zhang Y, Kovacs A, Orosz L, Muller F (2003) Influence of TOR kinase on lifespa in *C. elegans*. Nature 426: 620.

Walker G, Houthoofd K, Vanfleteren JR, David G (2005) Dietary restriction in *C. elegans*: From rate-of-living effects to nutrient sensing pathways. Mech Ageing Dev 126: 929-937.

Weston LA, Barth MM (1997) Preharvest factors affecting postharvest quality of vegetables. HortScience 32: 812-816.

Whapham CA, Blunden G, Jenkins T, Hankins SD (1993) Significance of betaines in the increased chlorophyll content of plants treated with seaweed extract. J Appl Phycol 5: 231-234.

Wiegant FAC, Surinova S, Ytsma E, Langelaar-Makkinje M, Wikman G, Post JA (2009) Plant adaptogens increase lifespan and stress resistance in *C. elegans*. Biogerontology 10: 27-42.

Williams RJ, Spencer JP, Rice-Evans C (2004) Flavonoids: antioxidants or signaling molecules? Free Radic Bio Med 36: 834-849.

Wilson MA, Shukit-Hale B, Kalt W, Ingram DK, Joseph JA, Wolkow CA (2006) Blueberry polyphenols increase lifespan and thermotolerance in *Caenorhabditis elegans*. Aging Cell 5: 59-68.

Winkel-Shirley B (2002) Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol 5: 218-223.

Winter JC (1998) The effects of an extract of *Ginkgo biloba*, EGb 761, on cognitive behavior and longevity in the rat. Physiol Behav 63: 425-433.

Wu WS (2006) The signaling mechanism of ROS in tumor progression. Cancer and Metastasis Rev 25: 695-705.

Wu Z, Smith JV, Paramsivam V, Butko P, Khan I, Cypser JR, Luo Y (2002) *Ginkgo biloba* extract EGb 761 increases stress resistance and extends life span of *Caenorhabditis elegans*. Cell Mol Biol (Noisy-le-gand) 48: 725-731.

Wurr DCE, Hambidge AJ, Fellows JR, Lynn JR, Pink DAC (2002) The influence of water stress during crop growth on the postharvest quality of broccoli. Postharvest Biol Tec 25: 193-198.

Xu XQ, Zhang ZQ (2000) Kinetic spectrophotometric determination of oxalic acid based on the catalytic oxidation of bromophenol blue by dichromate. Mikrochim Acta 135: 169-172.

Yamauchi N, Watada AE (1991) Regulated chlorophyll degradation in spinach leaves during storage. J Amer Soc Hort Sci 116: 58-62.

Yang Z, Liu S, Chen X, Chen H, Huang M, Zheng JP (2000) Induction of apoptotic cell death and *in vivo* growth inhibition of human cancer cels by a saturated branched-chain fatty acid, 13-methyltetradecanoic acid. Cancer Res 60: 505-509.

Yu YB, Dosanjh L, Lao LX, Tan M, Shim BS, Luo Y (2010) *Cinnamomum cassia* bark in two herbal formulas increases life span in *Caenorhabditis elegans* via insulin signaling and stress response pathways. PLoS One 5: e9339. doi:10.1371/journal.pone.0009339

Zarse K, Ristow M (2008) Antidepressant of the serotonin-antagonist type increase body fat and decrease lifespan of adult *Caenorhabditis elegans*. PLoS One 3: e4062. doi:10.1371/journal.pone.0004062

Zhang X, Schmidt RE (1999) Antioxidant response to harmone-containing product in Kentucky bluegrass subjected to drought. Crop Sci 39: 545-551.

Zhang X, Schmidt RE (2000) Hormone-containing products' impact on antioxidant status of tall fescue and creeping bentgrass subjected to drought. Crop Sci 40: 1344-1349.

Zhang J, Yang C, Brey C, Rodriguez M, Oksov Y, Gaugler R, Dickstein E, Huang CH, Hashmi S (2009) Mutation in *Caenorhabditis elegans* Krüppel-like factor, KLF-3 results in fat accumulation and alters fatty acid composition. Exp Cell Res 315: 2568-2580.

Zhang LZ, Jie GL, Zhang JJ, Zhao BL (2009) Significant longevity-extending effects of EGCG on *Caenorhabditis elegnas* under stress. Free Rad Biol Med 46: 414-421.

Zhang X, Ervin EH (2004) Cytokinin-containing seaweed and humic acid extracts associated with creeping bentgrass leaf cytokinins and drought resistance. Crop Sci 44: 1737-1745.

Zhang X, Ervin EH (2008) Impact of seaweed extract-based cytokinins and zeatin riboside on creeping bentgrass heat tolerance. Crop Sci 48: 364-370.

Zhang X, Ervin EH, Schmidt RE (2003) Plant growth regulators can enhance the recovery of Kentucky bluegrass sod from heat injury. Crop Sci 43: 952-956.

Zhang X, Schmidt RE (2000) Hormone-containing products' impact on antioxidant status of tall fescue and creeping bentgrass subjected to drought. Crop Sci 40: 1344-1349.

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53: 247-273.

Zodape ST, Kawarkhe VJ, Patolia JS, Warade AD (2008) Effect of liquid seaweed fertilizer on yield and quality of okra (*Abelmoschus esculentus* L.). J Sci Ind Res 67: 1115-1117.
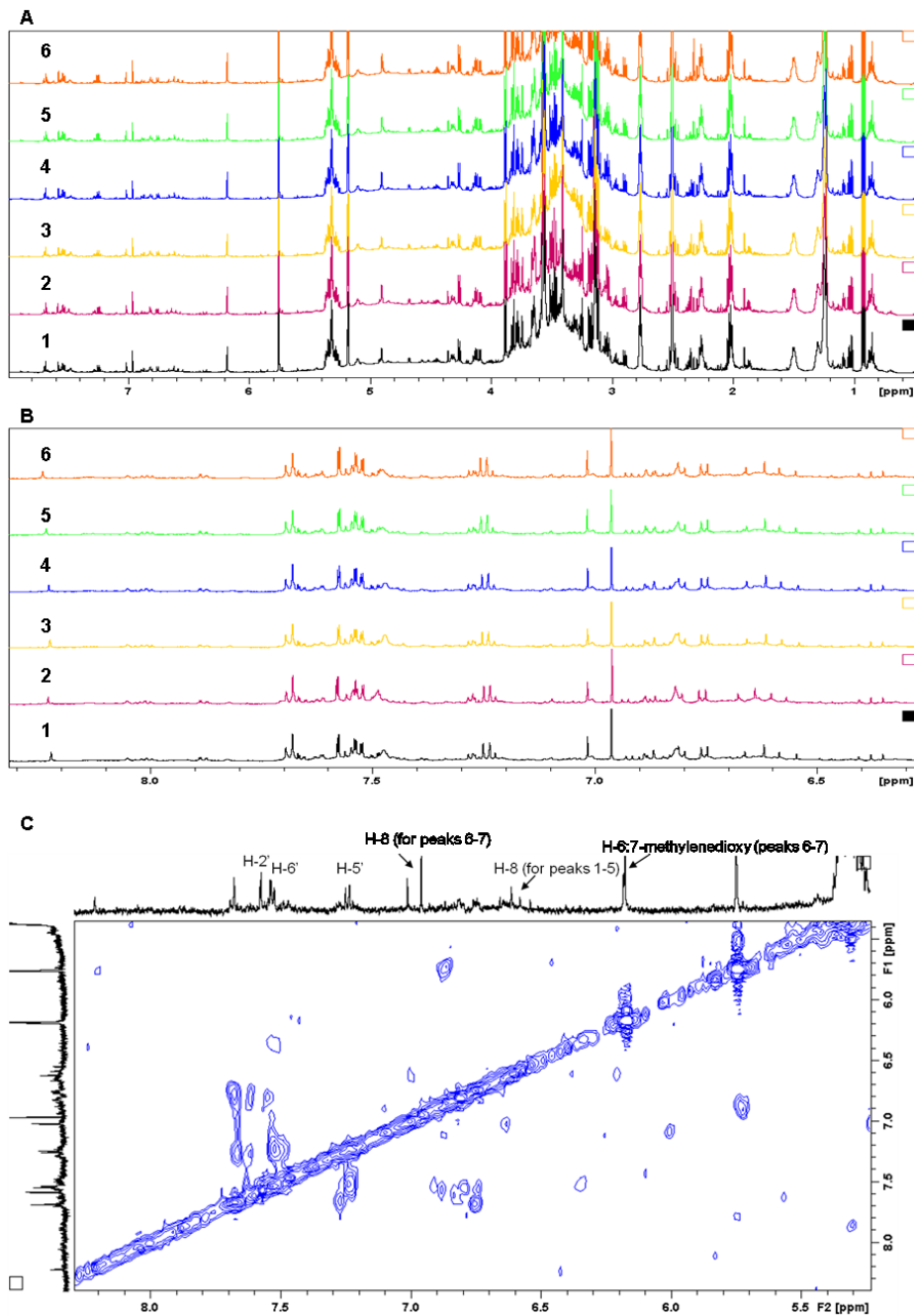
^{1}H-NMR analysis of extracts from ANE-treated and untreated spinach (*Spinacia oleracea* L.): (A) ^{1}H-NMR metabolite profile spectra of extracts from control (1-3) and 1.0 g/L ANE-treated (4-6) plants, (B) ^{1}H-NMR spectra (region of phenolic signals) of extracts from control (1-3) and 1.0 g/L ANE-treated (4-6) plants, and (C) 2D NMR ^{1}H-^{1}H COSY spectrum of extract from 1.0 g/L ANE-treated plants.
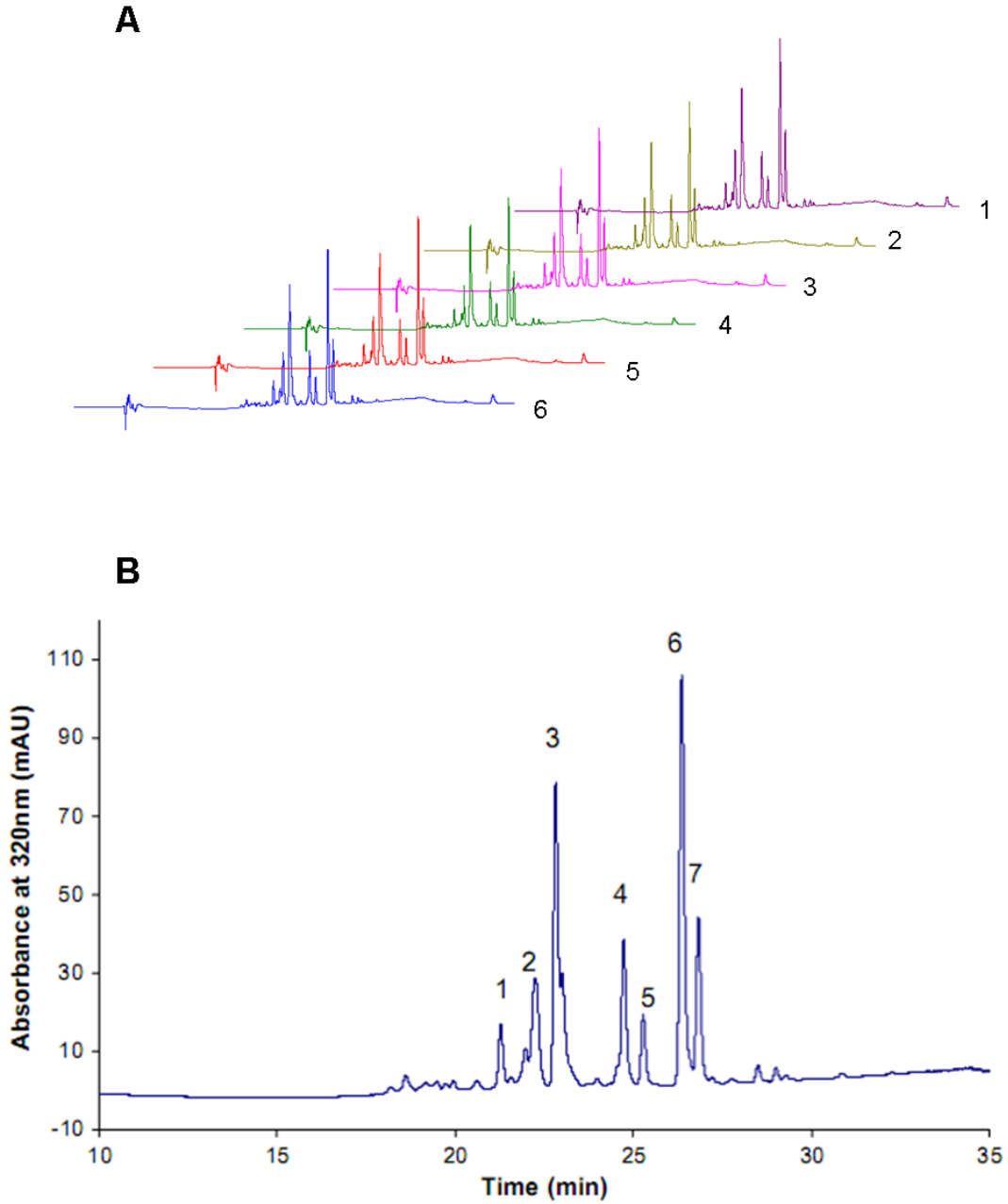
High Pressure Liquid Chromatography (HPLC) analysis of spinach (*Spinacia oleracea* L.) extracts: (A) HPLC profile comparison of spinach extracts from control (1-3) and 1.0 g/L ANE-treated (4-6) plants and (B) HPLC chromatogram of flavonoids from 1.0 g/L ANE-treated spinach at 320 nm. See Appendix C for peak identification.

Appendix C. Peak assignment, retention time (RT), UV spectra, mass spectral data, and the changes of peak area of flavonoids detected in spinach

Peak	RT(min)	Identification	Spectral characterization (nm)	m/z	Fragments	Increase (compared with control)		
						0.1 g/L	1.0 g/L	5.0 g/L
1	21.277	Patuletin-3-glucosyl-(1→6)[apiosyl(1→2)]-glucoside	353, 257	789 ([M+H]+): 811 ([M+Na]+) 787 ([M-H]-)	657 ([M-apiose+H]$^+$), 495 ([M-apiose-glucose+H]$^+$), 332 (patuletin)	–	38%	–
2	22.247	Spinacetin-3- glucosyl-(1→6)[apiosyl(1→2)]-glucoside	345, 245	803 ([M+H]+): 825 ([M+Na]+) 801 ([M-H]-)	693 ([M-apiose+Na]$^+$), 347 ([spinacetin+H]$^+$)	-12%	51%	–
		and						
3	22.826	Patuletin-3-(2''-feruloylglucosyl)-(1→6)[apiosyl(1→2)]-glucoside Spinacetin-3- (2''-feruloylglucosyl)-(1→6)[apiosyl(1→2)]-glucoside	313, 242, 230	965 ([M+H]+): 987 ([M+Na]+) 963 ([M-H]-) 979 ([M+H]+): 1001 ([M+Na]+) 977 ([M-H]-)	333 ([patuletin+H]+) 869 ([M-apiose+Na]$^+$), 531 ([M-apiose-glucose-feruloyl+Na]$^+$), 346 (apinacetin)	–	47%	–
		and						
		Spinacetin-3- (2''-coumaroylglucosyl)-(1→6)[apiosyl(1→2)]-glucoside		949 ([M+H]+): 971 ([M+Na]+) 947 ([M-H]-)				

4	24.737	Spinatoside	341, 271, 252	523 ([M+H]+) 521 ([M-H]-)	346 ([M-glucuronide])	-22%	36%	–
5	25.282	Jaceidin-4'- glucuronide	344, 272, 246	537 ([M+H]+) 535 ([M-H]-)	361 ([M-glucuronide+H]$^+$)	-15%	47%	–
6	26.370	5,3',4'-Trihydroxy-3-methoxy-6:7-methylendioxyflavone-4'-glucuronide	341, 277, 250	521 ([M+H]+): 543 ([M+Na]+) 519 ([M-H]-)	344 ([M-glucuronide])	–	44%	–
7	26.831	5,4'-Dihydroxy-3,3'-dimethoxy-6:7-methylendioxyflavone-4'-glucuronide	342, 279	535 ([M+H]+): 557 ([M+Na]+) 533 ([M-H]-)	359 ([M-glucuronide+H]$^+$)	–	44%	–

APPENDIX D

Quality assessment scores for senescence in spinach leaves (Property of Agriculture and Agri-Food Canada, reproduced with permission)

	Color			**Firmness**	
10	vibrant green (excellent quality)		**5**	firm	
9	dark dull green–½ of leaf or vibrant green–yellow corner/tip		**4**	lack of turgor	
8	dark dull green–whole leaf or green–yellow or dry edge/tip		**3**	shriveled/limp/ rolling/folding	
7	green–yellowing on ½ of leaf		**2**	partly wet and slimy	
6	mostly yellow with lt green		**1**	no form–wet and slimy	
5	all yellow/some brown spots/slight green hint				
4	yellow/brown/lt green or dark dull green –10% black tip/edge				
3	yellow/brown/lt green or dark dull green–50 % black				
2	yellow/brown/lt green or dark dull green–75 % black				
1	100% black (total senescence)				

Total quality assessment = Color score + Firmness score
Score for pitted/shiny areas - reduce 1/4 point for a few, ½ for several and 1 for many (½ leaf affected)