To the Graduate Council:

I am submitting herewith a dissertation written by Mark Gregory Lefsrud entitled "Environmental Manipulation to Increase the Nutritional Content in Leafy Vegetables." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plants, Soils and Insects.

Dean A. Kopsell
Major Professor

We have read this dissertation
and recommend its acceptance:

Carl E. Sams

Robert M. Augé

James B. Wills Jr.

Accepted for the Council:

Anne Mayhew
Vice Chancellor and Dean of
Graduate Studies

(Original signatures are on file with official student records.)

ENVIRONMENTAL MANIPULATION TO INCREASE THE NUTRITIONAL CONTENT IN LEAFY VEGETABLES

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Mark Gregory Lefsrud

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Acknowledgements

Over the last couple of years I have tried to expand my education from an initial background in Agriculture and Bioresource Engineering to encompass a greater knowledge of plants and horticulture. I have learnt a great deal about plant stress physiology, human health, and scientific research. Through this quest for knowledge I have discovered and honed previously unknown abilities. I would like to thank a number of people who have allowed me to further my knowledge both directly and indirectly. I would first like to thank Dr. Dean Kopsell, who over the last couple of years has been a mentor to me. He has contributed greatly in expanding my knowledge and training me as a scientist. I would like to thank Casey Finn Lefsrud for all of her support and help whenever it was called upon. I would also like to thank my parents Ellen and Edmund Lefsrud, and my siblings, Kevin and Lynette for listening to me. A special thanks to Jorgen Lefsrud for having the patience for the excruciating hours in the laboratory. Thanks to all of my family and friends in Canada. Thank you to everybody at the Plant Biology and Animal and Nutritional Sciences Departments at the University of New Hampshire and the Department of Plant Sciences at the University of Tennessee for helping me with every little thing. Thank you to all of the individuals, in the various laboratories that I have worked including Dr. Joanne Curran-Celantano, Joe Sheehan, Dr. Adam Wenzel, Catherine Gerwick, Jennifer Paris, Brook Gowdy-Johnson, Laura Dukach, Lisa Hunt, and Pam Bishop. In order to have a complete understanding of science, knowledge has to be gained from each step in the process.

Work that occurred in this dissertation is a small part of a whole project. We as a group, tried to understand and improve every step in the process. We began by looking at the nutritional quality of various vegetables and using environmental modification to improve the nutritional content. Improved plants were then harvested, processed, made into consumable food products and feed to test volunteers. Further work was performed to measure the impact of plant based carotenoids on absorption in the human body and deposition in the human eye. Individual researchers were specialized, but through regular meeting and working together on larger sections, each researcher expanded their knowledge across the other areas of the project. The education that I have gained from all of these experiences has greatly expanded my understanding of the complexities of the world and science. Finally, I would like to thank all of my past advisors at UNH, Dr. David Kopsell, Dr. Brent Loy, and Dr. Lee Jahnke and my current dissertation advisors, Dr. Carl Sams, Dr. Bob Augé, Jim Wills and Dr. A. J. Both for their acceptance and support throughout this project. A special thanks to everyone else who helped me in my goals[a].

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Abstract

In plants, carotenoids play critical roles in both light harvesting and energy dissipation for photosynthetic mechanism. In humans, carotenoids have been associated with reduced risk of lung cancer and chronic eye diseases, such as cataracts and age-related macular degeneration. Increasing carotenoid levels in plants, that are commonly consumed in the diet would impart health benefits without changing the dietary habits of individuals.

Kale (*Brassica oleracea* L. var. *acephala* D.C.) ranks highest and spinach (*Spinacia oleracea* L.) ranks second among vegetable crops for the accumulation of the carotenoids, lutein and β-carotene. However, kale has low consumption rates, while spinach has one of the highest rates of consumption among green-leafy vegetables in the United States. Limited research is available on the impact of environment and drying method on the production and destruction of secondary plant compounds. Therefore, the objectives of these studies were to determine how leaf age, light (irradiance, photoperiod, radiation cycle and wavelength), nutrition (nitrogen and selenium), and air temperature influences the accumulation of carotenoids, chlorophylls and dry matter in kale and spinach.

Data from the nitrogen nutrition experiment revealed that increased growth through fertilization resulted in increased lutein and β-carotene when measured on a fresh mass basis for one variety of spinach. However, when carotenoid accumulation was measured on a dry mass basis, both varieties were significantly affected by additional nitrogen. Results from this study showed that to accurately understand

carotenoid accumulation, values need to be analyzed on both a fresh and dry mass basis. Further studies measuring the influences of air temperature, irradiance, leaf age, photoperiod, radiation cycle and wavelength showed that maximum biomass did not always correlate with maximum carotenoid accumulation. A further study demonstrated that selenium fertilization did not affect carotenoid accumulation in kale; therefore kale could be used to provide both selenium and carotenoids in human diets.

When plant pigment concentration were analyzed in relation to % dry matter (%DM) a linear trend was observed. Increases in %DM of the plant resulted in measured increases in fresh mass pigment concentration and decreases in dry mass pigment concentration. Further analysis showed that %DM could be used to explain approximately 40% of the variance in the pigment concentration. A final study was performed to determine the effect of drying method and drying temperature on pigment concentration. Drying method and temperature had no effect on pigment concentration when the drying temperature remained below +25ºC; at temperatures above +25ºC some destruction of the pigments and loss of plant biomass was observed.

Environmental factors can be used to control carotenoids, chlorophylls and %DM in both kale and spinach. Utilizing these cultural practices is important information for growers producing these crops for dry capsule supplements and fresh markets.

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Introduction

Introduction

Carotenoid Chemistry and Biosynthetic Pathway

Carotenoids are lipid soluble yellow, orange, and red pigments that are uniquely synthesized in plants, algae, fungi, and bacteria (Sandmann, 2001). They are secondary plant compounds which are divided into two groups; the oxygenated xanthophylls such as lutein (3R,3'R,6'R β,ε-carotene-3,3'diol) and zeaxanthin (3,3'R-β,β-carotene-3,3'diol) and the hydrocarbon carotenes such as β-carotene (β, β-carotene), α-carotene (6'R, β,ε-carotene), and lycopene (ψ, ψ-carotene) (Zaripheh and Erdman, Jr., 2002).

Carotenoid C_{40} biosynthesis is a branch of the isoprenoid pathway. To begin the process of biosynthesis, isoprene (2-methyl-1,3-butadiene; C_5) is converted into isopentenyl diphosphate (IPP; C_5) which then in turn is converted into dimethylallyl diphosphate (DMAPP; C_5) (Figure I.1[a]). Combining 4 DMAPP molecule together results in the formation of geranylgeranyl pyrophosphate (GGPP). The first step considered as part of carotenoid biosynthesis is the condensation of two molecules of the C_{20} GGPP to form the first C_{40} carotenoid, phytoene. Desaturation of pytoene then produce in sequence 4 acyclic compounds phytofluene, ζ-carotene, neurosporene and lycopene. Cyclization of lycopene can occur on one end producing monocyclic γ-carotene or δ-carotene, or to both ends producing dicyclic α-carotene or β-carotene. Further modification of the pathway can occur with the addition of oxygen functions in the form of hydroxyl, epoxide or keto groups resulting in the xanthophylls. The

[a] All tables and figures are located in the appendix at the end of this part.

carotenoid pathway can be further modified with other structural end groups, such as esterification. Approximately 700 different types of carotenoids have been discovered and characterized (Baranski et al., 2005; Beyer et al., 2002; Hornero-Mendez and Britton, 2002; Niyogi et al., 2001).

Carotenoid Function

Carotenoids function to help harvest light energy during photosynthesis and to dissipate excess energy before damage occurs. Within the thylakoid membranes of chloroplast organelles, carotenoids are found bound to specific protein complexes of photosystem I (PS I) and photosystem II (PSII). The concentrations of carotenoids in the two photosystems are not uniform. The carotenoids in PS I are predominantly β-carotene with lower concentrations of lutein, while the carotenoids in PS II are predominantly lutein with lower concentrations of β-carotene (Demmig-Adams et al., 1996; Thayer and Bjorkman, 1992). Within each photosystem, carotenoids are located in both the antenna pigments and the photosynthetic reaction center (Peng and Gilmore, 2003; Taiz and Zeiger, 1998). In the PSII complex, β-carotene is highly concentrated close to the reaction center, while lutein is present in several light-harvesting antennae components (Demmig-Adams et al., 1996; Niyogi et al., 1997). When the absorption of light radiation exceeds the capacity of photosynthesis, excess excitation energy can result in the formation of triplet excited chlorophyll (^{3}Chl) and reactive singlet oxygen ($^1O_2$). Carotenoid pigments protect photosynthetic structures by quenching excited ^{3}Chl to dissipate excess energy (Frank and Cogdell, 1996; Kühlbrandt et al., 1994; Tracewell et al., 2001) and binding $^1O_2$ to inhibit oxidative

damage (Demmig-Adams et al., 1996; Tracewell et al., 2001). The carotenoid molecule then slowly releases this excess energy as heat and inhibits further oxidative damage. Carotenoids are also integral constituents of membranes (Peng and Gilmore, 2003; Taiz and Zeiger, 1998) and may be involved in structural stabilization of membranes and reduction of lipid peroxidation (Frank and Codgell, 1996).

Antioxidant Properties of Plants

Cruciferous vegetables, including subspecies of *Brassica oleracea* L., are relatively abundant sources of potential anticarcinogenic activity (Kurilich et al., 1999). In the human body, oxidants produced during normal metabolism and immune defense against infectious and chemical agents are responsible for damage to DNA, proteins, and cellular tissues (Ames et al., 1993; Mortensen, et al., 2001). This harmful oxidative damage is considered the major cause of aging and degenerative diseases such as cancer, cardiovascular disease, immune-system decline, and cataract (Ames et al., 1993). Compounds such as ascorbate, α-tocopherol, and carotenoids are examples of antioxidants that have the ability to quench reactive oxygen species (Ames et al., 1993). In fact, carotenoids are the most potent biological quenchers of reactive oxygen species (DiMascio et al., 1989). Cao et al. (1996) ranked kale second only to garlic (*Allium sativum* L.) in antioxidant activity using an oxygen radical absorbance capacity (ORAC) assay against peroxyl radicals and highest against hydroxyl radicals among 22 common vegetables. The antioxidant activity of carotenoids comes from the susceptibility of 5,6 and 5',6' double bonds in their cyclic end groups to undergo epoxidation with $^1O_2$ (Figure I.2; Mortensen et al.,

2001). Antioxidant activity *in vivo* is determined by carotenoid structure and concentration and the nature and concentration of the reactive oxygen species (Young and Lowe, 2001). Although these associative relationships indicate carotenoids may serve photoprotective and antioxidant functions in humans, as they do in plants, direct evidence of these actions is still lacking (Krinsky, 2002; Miki, 1991; Olsen, 1999). This is likely due to the complexity of measuring *in vivo* antioxidant behavior or the variability associated with carotenoid content of vegetables used in human nutrition studies.

Plants

Kale (*Brassica oleracea* L. var. *acephala* D.C.) was chosen as our primary test plant because of the large rate of carotenoid accumulation within the leaf tissue. Spinach (*Spinacia oleracea* L.) has been used as a model plant in a number of basic plant development studies (Pascal et al., 1999; Santabarbara et al., 2005). In our experiment, spinach was grown to confirm results from related experiments and provide multiple species evaluation. The kale variety grown was 'Winterbor' and spinach varieties grown were 'Melody' and 'Springer', all are popular commercial varieties.

Brassica

The *Brassica* genus encompasses a diverse group of plants that grow all over the world (Rubatzky and Yamaguchi, 1983). Brassicaceae (Cruciferae) are defined by their sulfur containing plant compounds called glucosinolates (Judd et al., 1999).

This group is comprised of a number of common plants such as cabbage, cauliflower, collards, kale, mustard, and rapeseed. The brassica genera consist of 6 species: *B. oleracea, B. nigra, B. campestris*, *B. juncea*, *B. napus* and *B. carinata*. These six genera can be separated into 3 elementary species and by crossing the elementary species produce 3 hybrid species. The large diversity in the *Brassica* family is due to this hybridization, natural mutations, natural selection and has been further modified by human selection. The result of these two forces on Brassicaceae has resulted in plants that are grown for their stems, leaves, roots, terminal and auxiliary buds, seeds and floral tissues.

Brassica is thought to of originated during the Miocenic period (Gomez-Campo and Tortosa, 1974) and has been mentioned since ancient times. The most ancient reference was in Sanskrit literature from around 1500 B.C., with other references in Greek, Roman, Indian and Chinese literature (Prakash and Hinata. 1980). Based on the genetic chromosome number, the original line diverged into three separate elementary plant species and three tetraploid species that are tetraploids. The three elementary species are *B. oleracea*, *B. nigra* and *B. campestris* and the three tetrapoids include *B. juncea*, *B. napus* and *B. carinata*. Morinaga (1928) assigned letters to each of the elementary species, using the letter A for *B. campestris*, the letter B for *B. nigra*, and the letter C for *B. oleracea*. Utilizing information from interspecific hybridization of the three elementary species, Morinaga (1928) was able to assume that the remaining three species where hybrids of the original three. He then assigned AB to *B. juncea*, to show that it was a hybrid of *B. campestris* and *B. nigra*. *Brassica napus* was given the letters AC and *B.*

carinata was given the letters BC. The chromosome number for *B. nigra* is 16, *B. oleracea* is 18 and *B. campestris* is 20. This results in a chromosome number of 34 for *B. carnata*, 36 for *B. juncea* and 38 for *B. napus*. A triangular graph proposed by U (1935) is shown in Figure I.3.

Brassica carinata is also known as Abyssinian mustard. It is only cultivated on the Ethiopian Plateau where it is grown for its seed oil content and as a vegetable (Martin et al., 1975). *Brassica juncea* is commonly known as mustard. It is grown for both its seed oil and as a vegetable (Magness et al., 1971). It is of economic importance with roughly 20 thousand metric tons of mustard produced in the United States per year (Downey and Rakow, 1987, Martin et al., 1975). *Brassica napus* is also known as rape or rutabagas. It is grown for its root and leaves in rutabagas and swedes and for the seed oil in rape (Martin et al., 1975; Magness et al., 1971). Rape oil accounts for more than 12% of the world's edible oil (Downey and Rakow, 1987), with a worldwide production of 7.3 million metric tons (Martin et al., 1975). The plants as well as their seed meal are used as animal feed. *Brassica nigra* is commonly known as black mustard. Black mustard for the most part is a weed but is in limited cultivation and the young leaves are sometimes gathered as an herb (Magness et al., 1971). Some mustard condiments also use black mustard. *Brassica campestris* is commonly known as the turnip rape, Chinese cabbage or Sarson. Vavilov (1949) and Sinskaia (1928) suggested that the oleiferous forms originated in India and were grown as a source of edible oil.

Brassica oleracea is commonly known as the cabbage group. This species represents a large number of vegetable crops that are part of human diets. The

cabbage group has been selected to produce a wide range of crop types including: broccoli, Brussels sprouts, cabbage, cauliflower, kales, kohl-rabi, savoy, and swede. Cabbage is the 8th most commonly consumed fruit or vegetable in the United States, followed by broccoli at 21st, cauiliflower at 30th and Brussels sprouts at 34th (Lucier and Plummer, 2003; Magness et al., 1971; Peirce, 1987). Rubatzky and Yamaguchi (1983) separated *B. oleracea* into 7 botanical groups: *acephala* (kale and collards), *alboglabra* (Chinese kale), *botrytis* (cauliflower), *capitata* (cabbage), *gemmiferae* (Brussels sprouts), *gongylodes* (kohl-rabi) and *italica* (sprouting broccoli). A number of wild forms are found throughout most of Europe and the Mediterranean. A number of hypotheses attribute the origin of the different varieties of *B. oleracea* to a wild kale, thousand head kale possibly (Peirce, 1987; Prakash and Hinata, 1980).

Kale and Spinach Taxonomy

The diversity of nomenclature within the *Brassica* family is very broad. The majority and commonly known types of kales are in *B. oleracea* (Slunkhe and Deshpande, 1991). These include thousand head kale, marrow stem kale and common kales, borecole and collards (a cross between cabbage and kale) (Decoteau, 2000). According to Magness et al. (1971) the name kale has been given to other species were the leaves are eaten. Within *B. campestris*, seven top and Italian kales are grown primarily for their leaves. *B. napus* has a number of kale crops including Hanover kale, Spring kale and Siberian kale. This diversity in the naming of kale can be expected due to the similarity of the species with the *Brassica* family.

Spinacia oleracea L. belongs to the family Amaranthaceae, which also contains beets (*Beta vulgaris* L.) and quinoa (*Chenopodium quinoa* Wild.). It was first cultivated in southwest Asia, perhaps in Persia. Ancient literature first referred to the plant in 647 B.C.. Spinach subsequently spread through Europe and around the World (Martin et al., 1975; Swiader and Ware, 2002).

Nutritional Value and Consumption of Kale and Spinach

Analyses of a wide range of fruits and vegetables showed kale (*Brassica oleracea* L. var. *acephala* D.C.) to contain the highest levels of lutein/zeaxanthin and β-carotene, while spinach (*Spinacia oleracea* L.) ranked second among vegetable crops (Holden et al., 1999). Although lutein has been reported as the most abundant carotenoid in all photosynthetic plant tissues (Pogson et al., 1998), lutein and zeaxanthin are usually reported together in food composition tables because of their structural similarity (Holden et al., 1999). Combined lutein/zeaxanthin levels in the leaves of kale were reported to range from 14,700 to 39,500 μg $100g^{-1}$ fresh tissue, while levels of β-carotene ranged from 2,800 to 14,500 μg $100g^{-1}$ fresh tissue (Holden et al., 1999; Khachik et al., 1986; Mangels et al., 1993; Sommerburg et al., 1998). The wide ranges reported in lutein/zeaxanthin and β-carotene for kale and other vegetables may be the product of sampling technique, variation associated with plant genetics and environmental factors, or differences in quantification methodology.

Kale and spinach have been reported as excellent sources of calcium (Ca), magnesium (Mg), and potassium (K) (Holden et al., 1999; Mills and Jones, 1996; U.S. Dept. Agr., 2002). However, kale has had low consumption rates with per capita fresh intake at less than 0.33 kg $year^{-1}$ (Lucier and Plummer, 2003), but spinach has had the highest rate of consumption among green-leafy vegetables in the United States. Spinach is high in iron (Fe), but is not highly bioavailable in human diets (Miller, 1987). Spinach has had a per capita intake of 0.73, 0.09, and 0.36 kg $year^{-1}$ for fresh, canned, and frozen markets, respectively (Lucier and Plummer, 2003).

Nutrition and Human Health

Current U.S. Dept. Agr. dietary guidelines recommend eating 7-9 servings of fruits and vegetables per day (U.S. Dept. Agr., 2005). However, average adult consumption in the United States in 1994 was only 4.4 servings per day with 42% of Americans eating less than 2 servings a day (Kurtzweil, 1997). Research into the vitamin and carotenoid enhancement of vegetable crops to benefit human health has paralleled the effort to increase consumption of fruits and vegetables in the diet.

Human Use and Location

The nutritional and medicinal importance of the dietary carotenoids is being established (Balentine et al., 1999; Grusak and DellaPenna, 1999). Mammals cannot produce carotenoids; so consuming plants is one option for mammals to acquire these molecules. Fruits and vegetables are a primary sources of carotenoids in the human

diet (Muller et al., 1999). Diets high in carotenoids have a reduced risk of lung cancer, prostate cancer, cardiovascular disease, and chronic eye diseases including cataracts, and age-related macular degeneration (Ames et al., 1995; Canene-Adams et al., 2005; Johnson et al., 2000; Landrum and Bone, 2001; Le Marchand et al., 1993; Semba and Dagnelie, 2003; van het Hof et al., 1999). The two most important carotenoids for human heath are lutein and β-carotene. Lutein has been postulated to protect the eye against short-wavelength light, while β-carotene is most important as a precursor of vitamin A (Garrett et al., 1999).

Macular Pigment

Unlike many carotenoids in the human diet, lutein, β-carotene and zeaxanthin are absorbed in the small intestine and are regularly found in blood (Burke et al., 2005), adipose tissue (El-Sohemy et al., 2002), and various organs (Kaplan et al., 1990). Lutein and zeaxanthin, in particular, can move across the blood-retina-barrier and accumulate in the retina (Bone et al., 1997). The retina is a multi-layered sensory tissue that is located at the back of the eye. These carotenoids are found throughout cells in the retina (Handelman et al., 1988), and are chiefly responsible for the yellow pigmentation. This pigmentation is commonly known as macular pigment because they accumulate in cells in the macula region of the eye. The macula is located roughly in the center of the retina, near the optic nerve. This region of the eye is small and is a highly sensitive part of the retina which is responsible for detailed central vision. The center of the macula region is the fovea, which is made up almost entirely of cone photoreceptors used in color detection. Rod photoreceptors are

limited within the fovea but are distributed throughout the rest of the retina and are insensitive to color (Curcio, 2001).

Eye Health

Macular pigment absorbs short-wavelength light before it reaches the photopigments and tissue at the back of the eye. Consequently, a high macular pigment optical density (MPOD) may offer greater retinal protection from light-induced damage than a low MPOD (Mares-Perlman and Klein, 1999; Wooten et al., 1999). Age-related macular degeneration (AMD) results in the loss of vision in the central fovea region of the sight pattern (Landrum and Bone, 2001). Bone et al. (2001) reported that when eyes with AMD were compared to age-matched controls, AMD patients had significantly lower concentrations of macular carotenoids. Macular degeneration is the third leading cause of blindness worldwide (Resnikoff et al., 2004), but retinal concentrations of carotenoids vary widely between individuals.

Factors that increase macular pigment include high fruit and vegetable consumption, increased antioxidant ingestion, high dietary carotenoid intake, elevated serum carotenoid concentration, along with normal body mass index, and no tobacco use history. Many of these same factors are also associated with a decreased risk of developing AMD and suggest there may be a causal relationship (Hammond et al., 1997; Hammond et al., 1998; Schalch et al., 1999). In general, individuals who consume diets high in lutein and zeaxanthin tend to have high MPOD, whereas individuals who consume low carotenoid diets tend to have low MPOD (Burke et al.,

2005; Curran-Celentano et al., 2001). Individuals, who consume diets low in carotenoids, may be at greater risk for developing retinal diseases such as AMD.

However, epidemiological evidence is inconsistent regarding the potential relationships among diet, genetics, and environment and the development of AMD. Studies have indicated that a high intake of a variety of vegetables providing a mixture of carotenoids was more strongly associated with reduced cancer and eye disease risk than intake of individual carotenoid supplements (Cardinault et al., 2005; Das et al., 2005; Johnson et al., 2000; Le Manchand et al., 1993). Research by Seddon et al. (1994) reported a significant relationship between low dietary intake of lutein and zeaxanthin and increased risk for AMD. Cardinault et al. (2005) showed that increased levels of the carotenoid lycopene in the blood serum combined with lutein and zeaxanthin lead to a reduced risk of AMD. Although several studies did not produce a direct relationship between dietary lutein and zeaxanthin and retinal disease, lutein supplementation has been shown to increase both MPOD and visual function in AMD patients (Landrum and Bone, 2001; Mares-Perlman and Klein, 1999; Richer et al., 2004).

Environment Stress

Environmental stresses can have a dramatic effect on the growth and development of plants. Plants are immobile organisms that have adapted both physical and chemical mechanisms to protect themselves against stressful environmental conditions (Dat et al., 2000; Mahan et al., 1995). These mechanisms include specialized tissue, cells and layers such as epidermal cells, guard cells and

components such as walls, waxes, suberin, and cutin (Jones et al., 2005; Mahan et al., 1995). The chemical mechanisms that plants use to protect themselves are chemical compounds such as alkaloids, anthocyanins, cardenolides, flavones, glucosinolates, isoflavones, phenolics, tannins, terpenes, and carotenoids (Agrawal, 2004; Charron and Sams, 2004; Eyles et al., 2003; Sullivan and Hagen, 2002; Tomas-Barberan and Espin, 2001).

Temperature

Changes in air temperature can limit plant growth at both low and high temperature extremes. However, plants have become adapted to temperature variations and can usually adjust to conditions above and below optimum. Plants grown well below optimum temperature can experience damage to the photosynthetic apparatus, inhibition of the synthesis and/or degradation of proteins, damage to the thylakoid membrane, and reduction of the electron transfer capacity of the plant (Guy et al., 1985; Holaday et al., 1991). To adapt to cold air temperatures plants increase the percentage of unsaturated fatty acid chains in membranes, specifically thylakoid membranes, and increase abscisic acid concentrations (Jun et al., 2001; Shewfelt, 1992). Plants grown above optimum temperature can experience loss of membrane integrity, damage to the thylakoid membranes, along with reduction in the rates of photosynthesis and respiration (Arvidsson et al., 1997; Maevskaya et al., 2003; Rokka et al., 2000). Plants adapt to elevated air temperatures through increased leaf wax, leaf rolling, change in leaf orientation, change in leaf size and the production of heat shock proteins (Mahan et al., 1995). Carotenoid conversion and levels of lutein

increased as air temperature increased for spinach (Arvidsson et al., 1997). Ogawa et al. (2005) reported that temperature was a factor for β-carotene accumulation in mango (*Mangifera idica* L.).

Light

Light is a complex group of stresses that can affect plant growth and development. Light can be broken down into both quantity and quality. Light quantity can be further broken down into light intensity (irradiance), ratio of light to darkness in a given day (photoperiod) and length of each day (radiation cycle). The quality of light is determined by the wavelengths and the intensity of each of the wavelengths. As light strikes the surface of plant, photons are absorbed by antenna pigments which funnel this energy to the photosynthetic reaction center. In the reaction center, chlorophylls, pheophytins, and quinones molecules convert light energy into chemical energy (Frank and Cogdell, 1996). Irradiance level directly influences the photosynthetic rate of plants, resulting in increased production of carbohydrates and total biomass (Mills and Jones, 1996). In shade leaves, lutein and β-carotene content is less than in sun leaves (Demmig-Adam et al., 1996). Similarly, summer-grown kale has higher lutein and β-carotene concentrations than kale grown during winter months, when light levels and photoperiod are reduced (Azevedo and Rodriguez-Amaya, 2005).

The length of the photoperiod has been shown to control a number of plant physiological factors including biomass production, bud formation, flowering, germination, leaf elongation, and leaf emergence (Degli Agosti et al., 1990;

Densmore, 1997; Drozdova et al., 2004; Gottdenker et al., 2000; Junttila et al., 1997; Koontz and Prince, 1986; Masuda and Murage, 1998; Murage et al., 1997; Riihimaki and Savolainen, 2004; Taylor et al., 1994). Increasing the photoperiod has consistently resulted in increased plant biomass production (Garner and Allard, 1931; Koontz and Prince, 1986; Masuda and Murage, 1998; Ohler and Mitchell, 1996), but under continuous irradiance, some plants have experienced decreases in biomass with the potential of interveinal chlorosis and necrosis (Bradley and Janes, 1985; Murage et al., 1997; Ohler and Mitchell, 1996; Stutte et al., 1996). Arizmendi-Maldonado et al. (2003) reported that adding supplemental lighting to extend the standard day to a 15 hr photoperiod, did not affect the accumulation of β-carotene in bermudagrass (*Cynodon dactylon* (L.) Pers.) or stargrass (*Cynodon nlemfuensis* Vanderyst.).

Previous research has shown that changes in radiation cycles can result in decreased fresh mass, dry mass, chlorophyll content, stem elongation and leaf area (Berry et al. 1986; Bonde 1955; Bonde 1956; Garner and Allard 1931; Morrow et al. 1987; Takano et al. 1987). Further research points to a possible increase in plant growth and pigment accumulation under changes in radiations cycles (Bonde, 1955; Morrow et al., 1987).

Plants pigments have specific wavelength absorption patterns known as absorption spectra (Table I.1). The absorption of specific wavelengths of light required for chlorophyll and carotenoid biosynthesis is known as the action spectrum. Chlorophyll absorbs wavelengths of light strongly in the red and blue region, with less absorbance occurring in the green wavelengths. In acetone, chlorophyll *a* has peak absorbance at 430 and 663 nm while chlorophyll *b* peaks at 453 and 642 nm.

The pigments β-carotene and lutein in acetone absorb strongly in the blue region of light with a maximum peak occurring at 454 and 448 nm, respectively (Davies and Köst, 1988; Hopkins and Huner, 2004). However, peak absorption in a plant can shifts up to 38 nm and is dependent on the specific environment surrounding the chloroplasts (Heber and Shuvalov, 2005; Jouni and Wells 1996). The absorption of these wavelengths of light does not always directly correlate into biosynthesis of chlorophylls and carotenoids. Wheat's (*Triticum aestivum* L.) action spectrum for the chlorophylls and β-carotene is maximum at 447 and 646 nm, respectively (Ogawa et al., 1973), while corn's (*Zea mays* L.) action spectrum has peaks at 445 and 650 nm (Koski et al., 1951). Wavelengths of light at 500 nm and levels greater than 700 nm result in very little biosynthesis of chlorophyll (Koski et al., 1951; Ogawa et al., 1973).

Fertilization

Fertilization has been used to improve plant growth and development since the beginning of agriculture. Plant growth requires six macro nutrients (N, P, K, S, Ca and Mg), eight micro nutrients (Fe, Cu, B, Mn, Zn, Mo, Cl and Ni) and some plants have improved growth with three beneficial nutrients (Si, Co, Na) (Marschner, 1997). Specific research on the effect of nutrition in carotenoid accumulation is varied. Carotenoid accumulation increased with increased nitrogen fertilization in parsley (*Petroselinum crispum* Nym.; Chenard et al., 2005), increased zinc fertilization in the herb *Mentha pulegium* L.(Candan and Tarhan, 2003), and increased iron fertilization in pepper leaves (*Capsicum annuum* L.) and green fruit

(Anchondo et al., 2001). Trudel and Ozbun (1971) reported that increased potassium fertilization resulted in increased lycopene but decreased β-carotene accumulation in tomato fruit, while Fontes et al, (2000) found that potassium fertilization rate had no effect on the accumulation of carotenoids in tomato fruits. Likewise, Kopsell et al. (2003) found that sulfur fertilization did not affect carotenoid accumulation in kale. Carotenoid accumulation decreased with increased phosphorous fertilization in Scots pine (*Pinus sylvestris* L.; Utriainen and Holopainen, 2002), and increased cobalt fertility in tomato leaves (Perez-Espinosa et al., 2002). Carotenoid accumulation decreased with increasing levels of calcium in tomato (Paiva et al., 1998) and the herb *Mentha pulegium* L. (Candan and Tarhan, 2005). Changing fertility will result in changes of biomass and pigment accumulation in plants with changes in mineral nutrition resulting in some increases and some decreases. Deficiency of a specific element can be just as detrimental to plant health as toxicity of the same element. Providing an optimized growing environment for one measured component of plant health does not necessarily result in optimum conditions for all factors.

Genetic

Carotenoid accumulation appears to be shaped by a plant species' physiological, genetic, and biochemical attributes, as well as environmental growth factors such as light, temperature, and fertility (Goldman et al., 1999; Kopsell et al., 2004; Kurilich et al., 1999; Lefsrud et al., 2005; Lefsrud et al., 2006). Kopsell et al. (2004) reported variation in the carotenoid accumulation of lutein and β-carotene in 22 varieties of kale. Klein and Perry (1982) reported differences in total carotene

content in five different vegetables [carrot (*Daucus carrota* L.), celery (*Apium graveolens* L. var. *dulce* Mill.), tomato (*Solanum lycopersicum* L.), cabbage, and corn (*Zea mays* L.)] sampled from six different United States locations, but could not conclude if differences were due to cultivar, growing conditions, or postharvest handling procedures. Mercadante and Rodriguez-Amaya (1991) have reported genetic differences in β-carotene, lutein + violaxanthin, and total carotenoid content between field grown kale cultivars in Brazil. Lycopene content varied among seven plum and cherry tomato cultivars (Muratore et al., 2005). Kurilich et al. (1999) reported genotypic differences among subspecies of *Brassica oleracea* L. accounted for 79% of the variance of β-carotene concentration, 82% of the variance of α-tocopherol (Vitamin E), and 55% of the variance of ascorbate (Vitamin C). Therefore, it is critical to consider both genetic and environmental influences when determining vegetable carotenoid accumulation.

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Appendix

Table I.1. Local maximum absorption in acetone of plant pigments (Hopkins and Huner, 2004).

Pigments	Peak Absorption in acetone (nm)	Local Peak Absorption in acetone (nm)
β-carotene	454	477
Chlorophyll *a*	663	430
Chlorophyll *b*	642	453
Lutein	448	422, 474

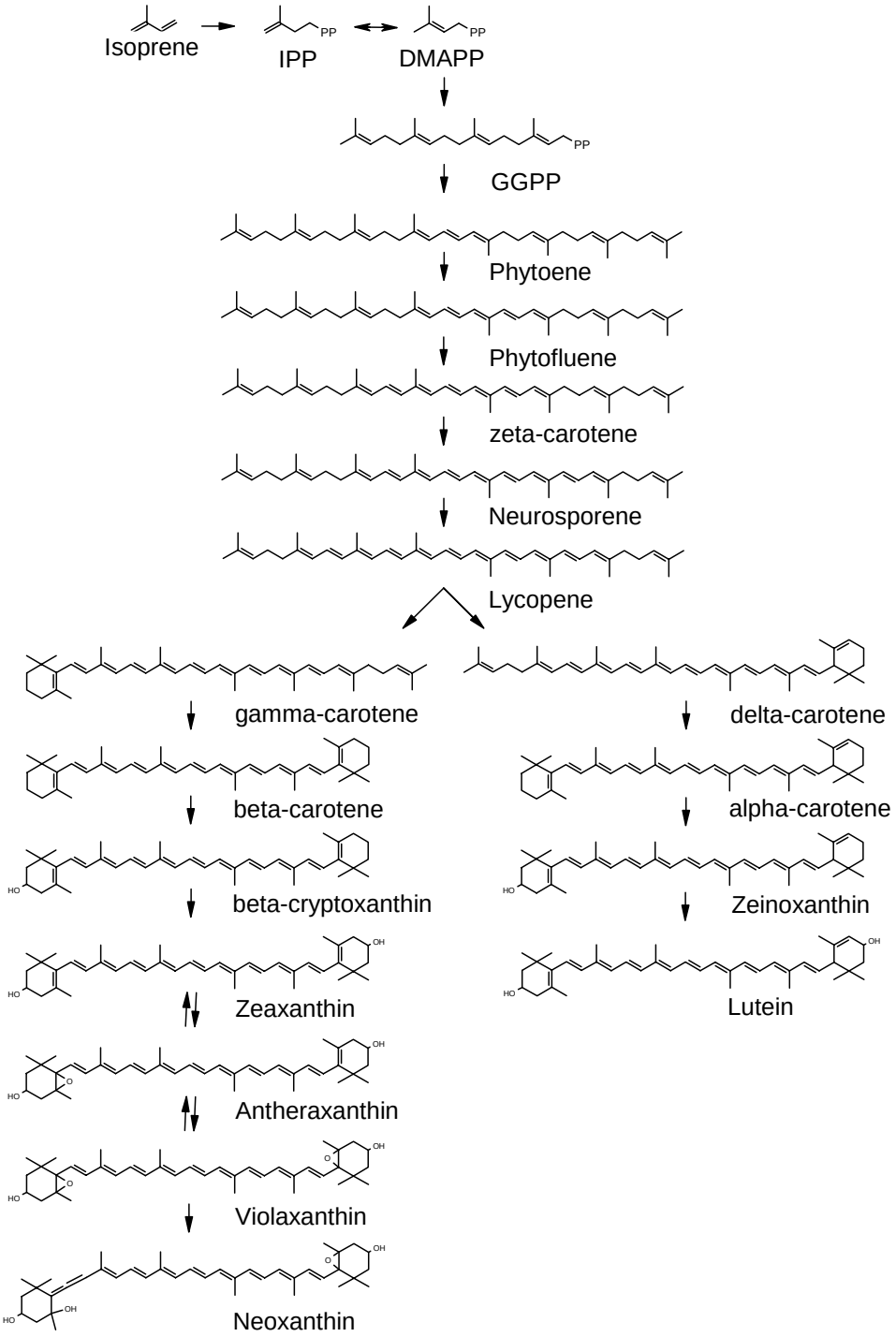
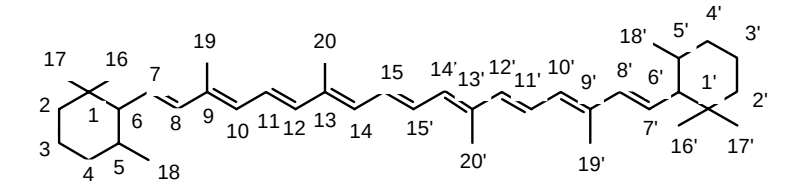
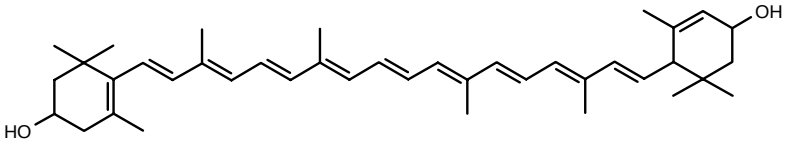


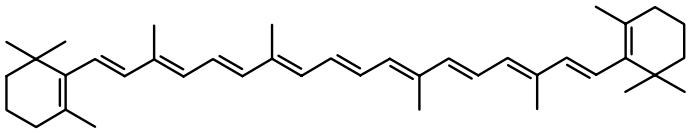
Figure I.1. Simplified version of the carotenoid biosynthetic pathway in plants. IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl pyrophosphate.



Carotenoid Carbon Position



Lutien



beta-carotene

Figure I.2. Carotenoid carbon positions and structure of lutein and β-carotene. Conjugated double bonds at 5,6 and 5',6' positions are highly effective at quenching singlet oxygen.

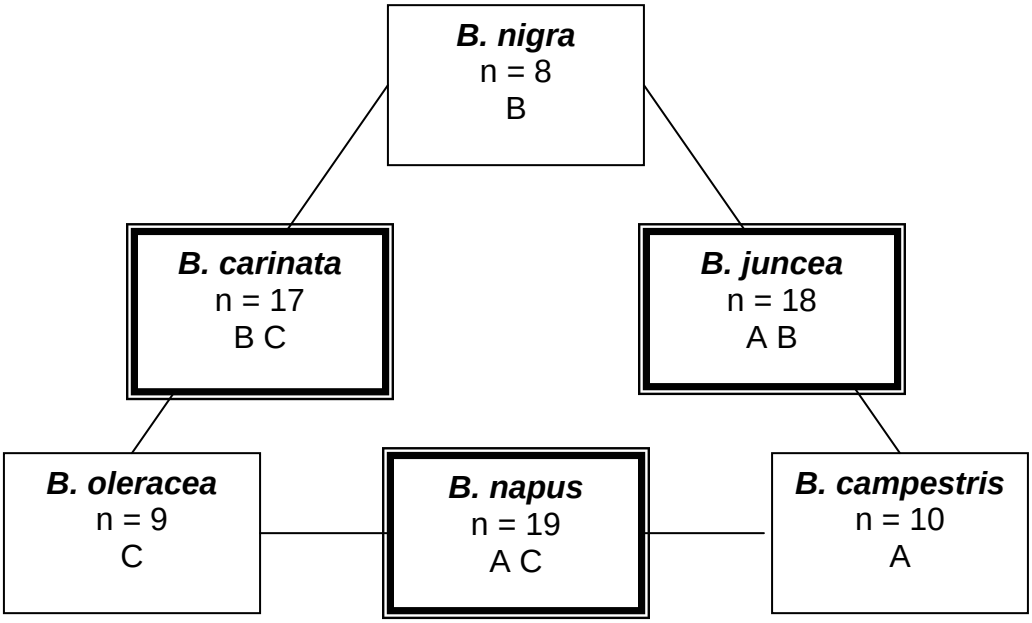


Figure I.3. U's Triangle of the 6 species of *Brassica*. Ancestral genome represented by letters and n denotes the number of chromosomes.

Part 1

Nitrogen Levels Influence Biomass, Elemental Accumulations, and Pigment Concentrations in Spinach

Nitrogen Levels Influence Biomass, Elemental Accumulations, and Pigment Concentrations in Spinach

This part is a lightly revised version of a paper by the same name to be submitted to the Journal of Plant Nutrition by Mark G Lefsrud, Dean A Kopsell and David E. Kopsell:

Lefsrud, M.G., D.A. Kopsell, and D.E. Kopsell. Nitrogen levels influence biomass, elemental accumulation, and pigment concentration in spinach.

My use of "we" in this part refers to my co-authors and myself. My primary contributions to this paper include (1) selection of the topic and development of the problem into a work relevant to my study of nitrogen fertilization on spinach, (2) determination of species, variety and nitrogen levels, (3) plant propagation and nutrient control, (4) sampling and analysis, (5) most of the gathering and interpretation of the literature, (6) compiling the information into a single paper, and (7) most of the writing and editing.

Abstract

Spinach (*Spinacia oleracea* L.) has one of the highest United States per capita consumption rates among leafy vegetable crops, and also ranks second for lutein and β-carotene carotenoid concentration. The objectives of this study were to determine the effects of N concentration on elemental and pigment accumulation in spinach. Two spinach cultivars ('Melody' and 'Springer') were greenhouse grown in nutrient solution culture under N treatments of 13, 26, 52, and 105 mg L^{-1}. Leaf tissue biomass increased from 45.6 to 273.2 g $plant^{-1}$ and from 127.0 to 438.6 g $plant^{-1}$ as N increased from 13 to 105 mg L^{-1} for 'Springer' and 'Melody', respectively. Leaf tissue N, P, Ca, Mg, Cu and Zn responded to N treatments. Lutein accumulations, expressed on a fresh mass basis, responded quadratically to increasing N treatments for 'Springer'. Maximum lutein values were 110 and 76 μg g^{-1} on a fresh mass basis, and maximum β-carotene values were 85 and 57 μg g^{-1} on a fresh mass basis for

'Springer' and 'Melody', respectively. Interestingly, N levels had a significant effect on carotenoid accumulation in both 'Springer' and 'Melody' when the pigments were expressed on a dry mass basis. Leaf tissue lutein increased from 0.59 to 1.06 mg g^{-1} and from 0.59 to 0.90 mg g^{-1} on a dry mass basis with increasing N treatments for 'Springer' and 'Melody', respectively. Reporting lutein and β-carotene on both a fresh and dry mass basis may be the most accurate way to express the carotenoid values of spinach.

Introduction

Spinach (*Spinacia oleracea* L.) has a high rate of consumption among green-leafy vegetables in the United States, with per capita intakes of 0.73, 0.09, and 0.36 kg $year^{-1}$ for fresh, canned, and frozen markets, respectively (Lucier and Plummer, 2003). Spinach is high in magnesium (Mg), potassium (K), calcium (Ca), and iron (Fe), which are bioavailable in human diets (Mills and Jones, 1996; U.S. Dept. Agr., 2002; Zhang et al., 1989). The United States Department of Agriculture food composition database reports that spinach also ranks highest for β-carotene, and ranks second only to kale (*Brassica oleracea* L. var. *acephala* D.C.) for lutein, two nutritionally important dietary carotenoids (Holden et al., 1999). Understanding environmental and genetic factors that may contribute to the nutritional value of spinach and other green-leafy vegetables will therefore be important when making cultural management decisions.

Nitrogen (N) is critical in plant growth and development and is an essential component of amino acids, proteins, nucleic acids and many enzymes. Plants grown

under limited N levels have reduced chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) pigments, resulting in stunted plants and characteristic leaf chlorosis (Marschner, 1997). Increased additions of N usually result in increased yield of crop plants (Greenwood et al., 1980; Hochmuth et al., 1999; Mills and Jones, 1996; Szwonek, 1986). However, toxicity from excessively high N concentrations is possible. As an example, severe yield depression was reported when cabbage (*Brassica oleracea* L. var. *capitata* L.) was grown at elevated N rates (602 mg N L^{-1}; Huett, 1989). Therefore, proper N management is critical for optimum plant performance.

Nitrogen is taken up by plant roots as nitrate-N (NO_3^-) and ammonium-N (NH_4^+). Nitrate is the principle form of N acquired by plants when present in adequate amounts, and it can be both actively and passively absorbed (Taiz and Zeiger, 1998). Plants reduce NO_3^- to nitrite in the cytosol via the light-dependent enzyme nitrate reductase. Nitrite can then be stored in the vacuole or converted via NH_4^+ into organic molecules. Soil NH_4^+ passively diffuses across plant membranes, and is then converted directly into organic compounds (Taiz and Zeiger, 1998). However, NH_4^+ must be rapidly converted into organic molecules, since free NH_4^+ can damage redox reactions in the photosynthetic pathway. Plants tolerate much higher levels of substrate NO_3^- than NH_4^+ (Marschner, 1997). The type, as well as the amount, of N present can therefore have significant impacts on plant growth.

Carotenoids are lipid soluble yellow, orange, and red pigments synthesized in higher plants, fungi, algae, and bacteria. In higher plants, carotenoids function in photoprotection as light harvesting antennae pigments and free radical scavengers (Miki, 1991; Tracewell et al., 2001). Two important dietary carotenoids in human

health maintenance are lutein and β-carotene. Increased intake of lutein and β-carotene has been associated with reduced risk of lung cancer and chronic eye diseases, such as cataracts and age-related macular degeneration (Ames et al., 1995; Landrum and Bone, 2001; Le Marchand et al., 1993; Semba and Dagnelie, 2003). Increasing the lutein and β-carotene concentrations in vegetable crops through cultural management techniques would be beneficial to the health status of consumers.

Spinach is the most consumed green-leafy vegetable crop in the United States, and research has shown spinach to be high in lutein and β-carotene carotenoids. Nitrogen is critical in plant metabolism and can be easily controlled by producers. What remains unclear, however, is the effect of N on secondary plant compounds, such as carotenoids. Therefore, the goal of this study was to investigate the influence of N levels on plant biomass production, tissue elemental concentrations, and accumulation patterns of lutein and β-carotene in the leaf tissues of spinach. Two spinach cultivars were utilized to measure possible genetic differences in the response to changes in N treatment levels.

Material and Methods

Plant Culture

'Melody' and 'Springer' spinach (Johnny's Selected Seed, Winslow, Maine) were seeded on 2 April 2002 into rockwool growing cubes (Grodan A/S, Dk-2640, Hedehusene, Denmark). The seeds were germinated and grown in a greenhouse (22

°C day/14 °C night) under natural lighting conditions (Durham, N.H., Lat. 43° 09' N). Peter's 20N-6.9P-16.6K water-soluble fertilizer (Scotts, Marysville, Ohio) was applied every five days at a rate of 200 mg L^{-1}. After four weeks the plants were transferred to 38 L plastic containers (Rubbermaid Inc., Wooster, Ohio). Six plants of each cultivar were placed into 2 cm round holes set at 10.6 x 9.5 cm spacing on each container lid. The plants were grown in 30 L of a nutrient solution (Hoagland and Arnon, 1950), with elemental concentrations of (mg L^{-1}): K (117.3), Ca (80.2), Mg (24.6), S (32.0), Fe (0.5), B (0.25), Mo (0.005), Cu (0.01), Mn (0.25), and Zn (0.025). The EC and pH of the starting nutrient solution were 0.7 mS cm^{-1} and 5.6, respectively. Plants were grown under increasing N treatment (T) levels of 13, 26, 52, and 105 mg L^{-1}. The ratio of NO_3-N to NH_4-N was kept constant at 3:1. The P level varied slightly with the N rate, changing from 91.5, 89.9, 86.8, and 80.6 mg L^{-1} for the N treatment levels of 13, 26, 52, and 105 mg L^{-1}, respectively. The experimental design was a split-plot, with N treatment levels as the main plots and spinach cultivars as the subplots. Nitrogen treatments were randomized in each of four replications. Solutions were aerated with an air blower (Model VB-007S, Sweetwater, Ft. Collins, Col.) connected to an air stone in each container. Nutrient solutions were replaced every two weeks throughout the experiment to refresh the solution to the initial nutrient concentrations.

Plants were harvested on 21 May 2002. At harvest, shoot and root tissues were separated and five plants in each treatment/cultivar combination were bulked and weighed. Shoot tissues were washed with soap (Aquet, Bel-art Products, Pequannock, N.J.), rinsed, and blotted dry with paper towels. Spinach shoot samples

were randomly separated into two groups. One shoot group was stored at –80 °C prior to lyophilization. The other shoot group was dried at 60 °C for no less than 72 hr, at which time shoot dry mass was calculated.

Elemental Determination

Dried tissues was ground to pass a 0.5 mm screen (Model 1093, Cyclotec-Tector, Höganäs, Sweden), and 0.300 g tissue sample was mixed with 10.0 mL of 70% concentrated nitric acid (HNO_3) and digested in a microwave accelerated reaction system (MARS5, CEM Corp., Matthews, N.C.). The digested solution was cooled to room temperature and deionized water was added to achieve a final volume of 40 mL. Elemental analysis was determined by Inductively Coupled Argon Plasma Atomic Emission Spectrometry (ICP-AES model Vista AX, Varian, Inc., Palo Alto, Calif.). Nitrogen tissue levels were measured using a nitrogen analyzer (Model 2410 Series II, Perkin Elmer, Norwalk, Conn.).

Carotenoid and Chlorophyll Determination

The frozen spinach samples were lyophilized for a minimum of 72 hr (Model 6 L FreeZone, LabConCo, Kansas City, Mo.). The dried tissues samples were ground with dry ice in a kitchen grinder (Handy Chopper Plus, HC 3000, Household Products Inc., Shelton, Conn.). Pigments were extracted and separated according to Kopsell et al. (2004), which is based on the method of Khachik et al. (1986). A 0.100 g sub-sample was placed into a Potter-Elvehjem tissue grinder tube (Kontes, Vineland, N.J.) and hydrated with 0.80 mL of deionized water. The sample was

placed in a water bath at 40 °C for 20 min. After hydration, 0.80 mL of the internal standard, ethyl-β-apo-8'-carotenoate (Sigma Chemical Co., St. Louis, Mo.) and 2.50 mL of tetrahydrofuran (THF) stabilized with 25 mg L^{-1} 2,6-Di-*tert*-butyl-4-methoxyphenol (BHT) were added. The sample was homogenized in the tube with 25 insertions of the grinder pestle attached to a drill press (Model Craftsman 15 inch Drill Press, Sears Co., Hoffman Estates, Ill.) at 540 rpm while immersed in ice. The tube was placed into a clinical centrifuge for 3 min at 500 g_n. The supernatant was removed with a Pasteur pipet, placed into a conical 15 ml test tube, capped and held on ice. The sediment was resuspended in 2.00 ml THF and homogenized and centrifuged again. The supernatant was collected and combined with the first extracted supernatant. The extraction procedure was repeated twice more until the supernatant was colorless. The sediment was discarded and the combined 4 supernatants were placed in a water bath at 40 °C and reduced to 0.50 ml using nitrogen gas (Model N-EVAP 111, Organomation Inc., Berlin, Mass.). 2.50 mL of MeOH and 2.00 mL of THF were added to the 0.50 mL sample, vortexed, and filtered through a 0.2 μm polytetrafluoroethylene (PTFE) filter (Model Econofilter PTFE 25/20, Agilent Technologies, Palo Alto, Calif.) using a 5 mL syringe (Becton, Dickinson and Company, Franklin Lakes, N.J.) prior to high performance liquid chromatography (HPLC) analysis.

A HPLC unit with photodiode array detector (Agilent 1100, Agilent Technologies, Palo Alto, Calif.) was used for pigment separation. All samples were analyzed for carotenoid compounds using a Vydac RP C_{18} 5.0 μm 250 x 4.6 mm

column (Model 201TP54, Phenomenex, Torrance, Calif.) fitted with a 4 x 3.0 mm, 7.0 μm guard column compartment. The column was maintained at 16 °C using a thermostatic column compartment. Eluents were A: 74.05% acetronitrile, 20% methanol, 5% hexane, 0.05% BHT, and 0.013% triethyamine (TEA)(v/v) and B: 49.987% acetonitrile, 25% THF, 25% hexane and 0.013% TEA (v/v). The flow rate was 0.70 mL min^{-1} and the gradient was 100% eluent A for 30 min; 50% A and 50% B for 2 min; 100% B for 2 min; and 50% A and 50% B for 2 min. The eluent was returned to 100% A for 10 min prior to the next injection. Eluted carotenoids and chlorophyll compounds from a 20.0 μL injection were detected at 452, 652, and 665 nm, with data collected, recorded and integrated using 1100 HPLC ChemStation Software (Agilent Technologies, Palo Alto, Calif.). Peak assignment was performed by comparing retention times and line spectra obtained from the photodiode array detection with authentic standards (lutein from Carotenature, Lupsingen, Switzerland; β-carotene, Chl *a*, Chl *b* from Sigma Chemical Co., St. Louis, Mo). Recovery rates of ethyl-β-apo-8'-carotenoate during extraction were above 90%.

Statistical Analysis

Data sets were analyzed by a split plot, GLM procedure using Systat (Systat Software Inc., Richmond, Calif.). ANOVA determined significance of the main effects of treatments and cultivars, and for their interactions. The relationship between experimental dependent variables and nitrogen treatments were determined by regression analysis using SAS (SAS Institute, Cary, N.C.).

Results and Discussion

Tissue Biomass Accumulation

Shoot tissue fresh mass (FM) was influenced by N treatment level ($p \leq 0.01$) and spinach cultivar ($p \leq 0.001$). A small but significant amount of the model's variation was accounted for by the interaction of N treatment level and spinach cultivar ($p = 0.03$). Average FM increased from 45.6 to 273.2 g for 'Springer' and 127.0 to 438.6 g for 'Melody' as N treatment level increased from 13 to 105 mg L^{-1} (Table 1.1[a]). Increases in FM were found for 'Springer' (FM=25.5 + 2.4(T), $p \leq 0.001$) and 'Melody' (FM = 150 + 3.3(T), $p \leq 0.001$; Table 1.1). Average shoot tissue dry mass (DM) differed among N treatment level ($p = 0.01$) and between spinach cultivar ($p \leq 0.001$). A minor amount of variation for DM occurred due to the interaction of N treatment level and spinach cultivar ($p = 0.06$). As N treatment levels increased, there was an increase in DM for 'Springer' (DM= 6.7 + 0.1(T), $p \leq 0.001$) and 'Melody' (DM = 13.9 + 0.1(T), $p \leq 0.001$; Table 1.1). The largest FM and DM were observed at the highest N treatment level (105 mg L^{-1}) for both varieties.

Macro- and Micronutrient Accumulation

The N treatment level influenced the accumulation of N ($p \leq 0.001$), P ($p = 0.005$), Ca ($p = 0.01$), Mg ($p = 0.01$), and sulfur (S) ($p = 0.05$) in spinach leaf tissues. Choice of spinach cultivar influenced tissue N ($p = 0.04$) and S ($p \leq 0.001$). Tissue S

[a] All tables and figures are located in the appendix at the end of this part.

($p = 0.005$) was the only element to be influenced by the interaction between N treatment level and spinach cultivar. Elemental nutrient levels were within reported ranges for mature, greenhouse-grown spinach (Table 1.2 -3)(Mills and Jones, 1996). Increases in tissue N were observed for 'Springer' (Leaf N = 28.5 + 0.3(T), $p \leq 0.001$) and 'Melody' (Leaf N = 28.6 + 0.4(T), $p \leq 0.001$; Table 1.2). Decreases in tissue P were found for both 'Springer' (Leaf P = 23.3 - 0.1(T), $p \leq 0.001$) and 'Melody' (Leaf P = 23.0 - 0.1(T), $p \leq 0.001$; Table 1.2). Increases in tissue Ca were found for both 'Springer' (Leaf Ca = 5.9 + 0.01(T), $p = 0.002$) and 'Melody' (Leaf Ca = 5.4 + 0.01(T), $p \leq 0.001$; Table 1.2). Increases in tissue Mg were found for 'Springer' (Leaf Mg = 4.3 + 0.01(T), $p \leq 0.001$) and 'Melody' (Leaf Mg = 3.8 + 0.01(T), $p \leq 0.001$; Table 1.2). Changes in tissue S were found for 'Springer' (Leaf S = 2.4 + 0.01(T) - $0.0001(T)^2$, $p = 0.018$) and 'Melody' (Leaf S = 2.1 + 0.01(T) - $.0001(T)^2$, $p \leq 0.001$; Table 1.2). As NO_3-N is absorbed, the synthesis of organic anions increases, resulting in an increase in accumulated inorganic cations, such as Ca and Mg (Marschner, 1997). Data from this study demonstrates increases in both Ca and Mg in response to increases in N treatment level. Negative correlations between tissue %N and tissue %P have also been reported previously (Mills and Jones, 1996).

The accumulation of some micronutrient elements in spinach tissues was influenced by increasing N treatment level. Choice of spinach cultivar influenced tissue B ($p \leq 0.001$) and Mn ($p \leq 0.001$). A small portion of variation in the model for tissue Mn ($p = 0.005$) and Mo ($p = 0.018$) could be attributed to the interaction

between N treatment level and choice of spinach cultivar. Tissue Cu (Leaf Cu= 11.89 - 0.06(T), $p = 0.002$) and Mo (Leaf Mo= 2.25 - 0.01(T), $p = 0.03$) in 'Springer' decreased in response to increases in N treatment level (Table 1.3). Tissue Zn (Leaf Zn= 229.9 - 1.40(T), $p = 0.04$) in 'Melody' decreased in response to increases in N treatment concentration (Table 1.3).

Copper and Molybdenum both decreased in 'Springer' as N treatment level increased. Mo ions are critical components of the enzyme nitrate reductase, which converts NO_3-N to nitrite, which is then further converted to NH_4^+ before incorporation into organic constituents (Marschner, 1997; Taiz and Zeiger, 1998). The nutrient solution utilized in this experiment had a NO_3^-:NH_4^+ ratio of 3:1. The increase in Mo concentration at the lower N rates may signify a need by the plant to convert NO_3^- to NH_4^+. Higher levels of N usually result in increased Cu uptake (Marschner, 1997). There have been reported increases in Cu accumulation in parsley (*Petroselinum crispum* Nym.) as N treatment levels increased from 6 to 105 mg L^{-1} (Chenard et al., 2005). In this experiment, Cu accumulation in spinach was inversely proportional to N concentrations, the cause of which is still unknown. Zinc is required for plant growth in a number of enzymes, including RNA polymerase. It has also been reported that Zn was required for increased N levels in soybean (*Glycine max* L.; Johnson and Simons, 1979). However, Zn decreased as N level increased in this experiment. Differences in the behavior of Cu and Zn to previous reports could be caused by the physiology of spinach, or possible interactions among the elemental nutrients in the nutrient solutions.

Carotenoid and Chlorophyll Pigment Accumulation

Maximum tissue lutein accumulation expressed on a fresh mass basis for 'Springer' (109.6 μg g^{-1}) and 'Melody' (75.5 μg g^{-1}) occurred at the N treatment level of 52 mg L^{-1} (Table 1.4). Leaf tissue lutein concentrations responded to spinach cultivar ($p \leq 0.001$), but not to changes in N treatment level. Leaf tissue lutein responded quadratically to increases in N treatment level for 'Springer' (Lutein = $59.6 + 1.6(T) - 0.01(T)^2$, $p = 0.039$; Table 1.4). Previously, lutein in parsley increased from 40 to 86 μg g^{-1} as N treatment concentrations increased from 6 to 105 mg L^{-1}, respectively (Chenard et al., 2005). Maximum β-carotene accumulation for 'Springer' (85.4 μg g^{-1}) and for 'Melody' (57.0 μg g^{-1}) both occurred at the N treatment level of 52 mg L^{-1} (Table 1.4). β-carotene in the leaf tissues of the spinach responded to spinach cultivars ($p \leq 0.001$), but not to changes in N treatment level. Leaf tissue β-carotene increased, then decreased in response to increases in N treatment level for 'Springer' (β-carotene = $41.4 + 1.4(T) - 0.01(T)^2$, $p = 0.031$), but no significant trends were identified for 'Melody' (Table 1.4). Increases in β-carotene from 39 to 79 μg g^{-1} were previously identified for parsley as the N concentration in nutrient solutions increased from 6 to 105 mg L^{-1} (Chenard et al., 2005).

Spinach leaf tissues accumulated much higher chlorophyll concentrations when compared to carotenoid pigments (Table 1.4). Similar to the carotenoid pigments, maximum chlorophyll accumulation occurred at the N treatment level of 52 mg L^{-1} for both spinach cultivars. Choice of spinach cultivar influenced Chl *a* ($p \leq$

0.001) and Chl *b* ($p \leq 0.001$). A small amount of variation for Chl *a* ($p = 0.02$) and Chl *b* ($p = 0.03$) could be attributed to the interaction between N treatment level and choice of cultivar. The chlorophyll pigments had a quadratic response to increasing N treatment level for 'Springer' (Chl *a* = $723.4 + 30.9(T) - 0.2(T)^2$, $p = 0.013$; Chl *b* = $184.7 + 8.3(T) - 0.06(T)^2$, $p = 0.006$), but no response was recorded for 'Melody' (Table 1.4).

Nitrogen has been correlated to chlorophyll in a number of studies, with lower N levels resulting in less chlorophyll production. Under limited N, both Chl *a* and Chl *b* pigments are reduced, resulting in leaf tissue chlorosis (Marschner, 1997). Grunwald et al. (1977) reported field tobacco (*Nicotiana tabacuin* L.) grown with limited N had a reduction in chlorophyll of 51% when N rates were reduced from 336 to 112 kg ha^{-1}. In a field study with winter wheat (*Triticum aestivum* L.), Follett et al. (1992) reported a 19% increase in chlorophyll with an increase in N leaf concentration from 28 to 38 g kg^{-1}. Although not significant, the lowest levels of Chl *a* and Chl *b* accumulation in the current study occurred at the lowest N treatment concentration (Table 1.4).

Genetic differences for carotenoid accumulation were identified between the two spinach cultivars in the current study. Genetic variation for carotenoid accumulation can be found for broccoli (*Brassica oleracea* L. var. *italica* Plenck), red pepper (*Capsicum frutescens*, L.), and kale. Kurilich et al. (1999) reported that 79% of β-carotene variation in broccoli heads was due to differences among fifty accessions. Other studies have shown significant cultivar differences for lutein and β-carotene accumulation in red pepper (Almela et al., 1991; Daood et al., 1996).

Kopsell et al. (2004) reported lutein and β-carotene concentrations differed among twenty three *B. oleracea* cultigens. These studies demonstrate a strong genetic influence on carotenoid accumulations in *B. oleracea* subspecies, as well as other vegetable crops.

The carotenoid content of vegetable crops is normally reported on a fresh mass basis to equate to typical consumption patterns (Gil et al., 1999; Holden et al., 1999). Interestingly, different trends resulted when the spinach carotenoid pigments were calculated on a dry mass (DM) basis (Table 1.5). Spinach tissue expressed as % DM was influenced by N treatment level ($p = 0.008$), choice of cultivar ($p \leq 0.001$), and for the interaction between N treatment level and cultivar ($p \leq 0.001$). Average % DM decreased in response to increasing N treatment level for both 'Springer' (% DM = 13.1 - 0.1(T), $p < 0.001$) and 'Melody' (% DM = 8.9 - 0.03(T), $p = 0.01$; Table 1.5). When tissue lutein is expressed on a dry mass (mg g^{-1}) basis, it is influenced by N treatment level ($p = 0.02$) and by choice of spinach cultivar ($p = 0.002$). Increases in lutein expressed on a dry mass basis were computed for 'Springer' (Lutein DM = 0.64 + 0.01(T), $p < 0.001$) and 'Melody' (Lutein DM = 0.65 + 0.03(T), $p < 0.001$). Increases in β-carotene expressed on a dry mass basis were also found for 'Springer' (β-carotene DM = 0.47 + 0.01(T), $p < 0.001$) and 'Melody' (β-carotene DM= 0.48 + 0.02(T), $p = 0.01$; Table 1.5).

Nitrogen can affect the production of plant secondary compounds. Hochmuth et al. (1999) reported a quadratic response in carrot (*Daucus carota* L.) tissue carotenoids N rates increased, with maximum carotenoid production occurring at 160

kg ha^{-1}. Grunwald et al. (1977) reported total carotenoid levels increased in tobacco on a dry mass basis with increasing N rates. As N increased from 112 to 336 kg ha^{-1}, total carotenoid levels in the tobacco leaves, expressed on a dry mass basis, increased 14%, (from 0.29 to 0.33 mg g^{-1}, respectively). In the current study, spinach tissue lutein, as a function of dry mass, increased 49% in 'Springer' and 80% in 'Melody' as the N level was increased by a factor of 8 times. 'Springer' plants produced the most lutein and β-carotene at the 105 mg L^{-1} N treatment level, while 'Melody' plants had a maximum at the N treatment of 52 mg L^{-1} (Table 1.5).

Aside from the typical expression of concentration, carotenoid pigments can also be expressed as total content on a per plant basis. Total plant lutein content (TL; mg $plant^{-1}$) responded to N treatment levels ($p = 0.02$) and to choice of spinach cultivar ($p \leq 0.001$). Total plant β-carotene content (TBC; mg $plant^{-1}$) was influenced by N treatment levels ($p = 0.02$) and choice of spinach cultivar ($p \leq 0.001$). Increases in TL in response to increasing N treatment levels were calculated for 'Springer' (TL = 2.90 + 0.23(T), $p < 0.001$) and 'Melody' (TL= 11.2+ 0.22(T), $p = 0.002$; Table 1.5). As N treatment levels increased from 13 to 105 mg L^{-1}, TL increased >500% for 'Springer' and 270% for 'Melody'. Increases in TBC in response to increases in N treatment levels were computed for 'Springer' (TBC= 2.1 + 0.2(T), $p < 0.001$) and 'Melody' (TBC= 8.3 + 0.2(T), $p = 0.002$; Table 1.5). As N treatment levels increased from 13 to 105 mg L^{-1}, TBC increased >600% for 'Springer' and 300% for 'Melody'.

Dried spinach is currently used in commercial dietary capsules as a source of antioxidants. Pool-Zobel et al. (1997) and Müller et al. (1999) conducted

experiments to determine if carotenoids could protect against DNA damage and oxidative stress. The researchers used dried spinach powder (obtained from Völpel GmbH, Königmoos, Germany) mixed in either water or milk. The lutein concentration for the dried spinach was 11.3 mg $10g^{-1}$, while the β-carotene concentration was 3.1 mg $10g^{-1}$. The dried spinach increased serum lutein and β-carotene 2-fold over the two week feeding intervention, and significantly reduced the number of DNA strand breaks in the test subjects. This research suggests nutritional impacts from consuming dried spinach materials.

Conclusion

Most spinach is consumed fresh (Lucier and Plummer, 2003). However, changes in water content of the spinach due to growing environment, handling, storage, or food processing can cause a change in percent moisture found in the tissue. Such biomass changes will influence the expression of the nutritional value of the plant. The %DM of spinach from this experiment averaged between 5.7% and 12.7%, while the U.S. Dept. Agr. reports 8.4 %DM for the spinach samples used in the food composition database. When we calculated carotenoid concentrations on a fresh mass basis, there were no significant differences in lutein or β-carotene accumulations for the spinach cultivars over the N levels tested. However, when we calculated the spinach carotenoid concentration on a dry mass basis, there were significant increases in both lutein and β-carotene concentrations and total plant content of these carotenoid pigments. Identifying the effects of different N levels on

carotenoid accumulation in spinach expressed on a fresh mass basis can be applicable to production practices for fresh markets, whereas data expressed on a dry mass basis may be important for the carotenoid supplement market. Thus, reporting lutein and β-carotene on both a fresh and dry mass basis may be the most accurate way to express the carotenoid values of spinach.

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List of References

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Appendix

Table 1.1. Mean values[z] of leaf tissues of two spinach cultivars grown under increasing nitrogen levels in nutrient solution culture.

Nitrogen	Biomass (g plant^{-1})	
mg L^{-1}	Fresh mass	Dry mass
	Springer	
13	45.6	5.8
26	92.1	10.1
52	169.0	14.2
105	273.2	15.2
Contrasts		
Linear	***	***
Quadratic	***	***
	Melody	
13	127.0	12.2
26	255.1	19.2
52	408.1	25.2
105	438.6	26.9
Contrasts		
Linear	***	***
Quadratic	***	***

[z] Mean composition of sampled leaf tissue of 4 replications and 6 plants each.

*** Significance at $p \leq 0.001$.

Table 1.2. Mean values[z] of macronutrient accumulation on a dry mass basis in the leaf tissues of two spinach cultivars grown under increasing nitrogen concentrations in nutrient solution culture.

Nitrogen	Elemental composition (g kg^{-1})					
mg L^{-1}	N	P	K	Ca	Mg	S
			Springer			
13	30.3	22.3	82.5	6.5	4.8	2.7
26	36.8	19.6	81.9	5.8	5.1	3.1
52	50.0	14.4	81.4	7.1	7.2	3.3
105	60.5	9.2	75.0	7.9	8.5	2.8
Contrasts						
Linear	***	***	NS	**	***	NS
Quadratic	***	***	NS	**	***	*
			Melody			
13	30.8	24.1	78.2	6.6	4.4	2.9
26	36.8	19.4	74.9	5.3	4.8	3.0
52	53.0	17.4	88.0	7.0	7.3	3.9
105	63.3	10.1	83.9	8.9	8.9	3.2
Contrasts						
Linear	***	***	NS	***	***	NS
Quadratic	***	***	NS	***	***	**

[z] Mean composition of sampled leaf tissue of 4 replications and 6 plants each.

NS, *, **, *** Non-significant or significance at $p \leq 0.05$, 0.01, 0.001, respectively.

Table 1.3. Mean values[z] of micronutrient accumulation on a dry mass basis in the leaf tissues of two spinach cultivars grown under increasing nitrogen concentrations in nutrient solution culture.

Nitrogen mg L^{-1}	Elemental composition (mg kg^{-1})					
	B	Cu	Fe	Mn	Mo	Zn
			Springer			
13	46.0	12.3	219.1	275.1	2.14	354.0
26	44.9	9.5	158.1	179.4	2.03	133.1
52	42.4	8.3	184.3	194.4	2.09	121.7
105	40.2	6.4	189.8	198.5	1.57	168.1
Contrast						
Linear	NS	**	NS	NS	*	NS
Quadratic	NS	**	NS	NS	NS	NS
			Melody			
13	46.9	10.8	181.3	213.2	1.90	253.6
26	48.8	8.4	153.3	132.2	1.79	145.7
52	52.8	8.0	207.9	177.6	2.57	154.7
105	49.4	5.9	204.0	205.2	2.03	91.0
Contrasts						
Linear	NS	NS	NS	NS	NS	*
Quadratic	NS	NS	NS	NS	NS	*

[z] Mean composition of sampled leaf tissue of 4 replications and 6 plants each.

NS, *, ** Non-significant or significance at $p \leq 0.05$, 0.01, respectively.

Table 1.4. Mean values[z] for carotenoid and chlorophyll pigments expressed on a fresh mass basis in the leaf tissues of two spinach cultivars grown under increasing nitrogen concentrations in nutrient solution culture.

Nitrogen mg L^{-1}	Pigment Concentration (μg g^{-1})			
	Lutein	β-carotene	Chl *a*[y]	Chl *b*[y]
		Springer		
13	78.4	57.9	1086.1	279.2
26	94.4	71.1	1381.9	365.8
52	109.6	85.4	1708.0	444.4
105	89.6	69.6	1427.4	364.8
Contrasts				
Linear	NS	NS	NS	NS
Quadratic	*	*	*	**
		Melody		
13	65.0	47.0	860.9	226.7
26	74.1	56.3	953.7	276.1
52	75.5	57.0	1001.1	291.1
105	67.6	51.4	948.4	257.2
Contrasts				
Linear	NS	NS	NS	NS
Quadratic	NS	NS	NS	NS

[z] Mean composition of sampled leaf tissue of 4 replications and 6 plants each.

[y] Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*

[NS, *, **] Non-significant or significance at $p \leq 0.05$, 0.01, respectively.

Table 1.5. Plant pigment % dry matter (%DM) and comparison between the concentrations of lutein and β-carotene. Expressed on a dry mass basis and total plant content in the leaf tissues of two spinach cultivars grown under increasing nitrogen concentrations in nutrient solution culture.

Nitrogen		Concentration[z] (mg g^{-1})		Content[z] (mg $plant^{-1}$)	
mg L^{-1}	% DM	Lutein	β-carotene	Lutein	β-carotene
			Springer		
13	12.7	0.59	0.43	0.60	0.44
26	11.0	0.80	0.60	1.46	1.10
52	8.4	1.03	0.80	3.14	2.46
105	5.7	1.06	0.82	4.14	3.20
Contrasts					
Linear	***	***	***	***	***
Quadratic	***	***	***	***	***
			Melody		
13	9.7	0.59	0.43	1.34	0.97
26	7.5	0.76	0.57	3.10	2.35
52	6.2	0.90	0.68	5.10	3.86
105	6.3	0.88	0.67	5.04	3.81
Contrasts					
Linear	**	**	**	**	**
Quadratic	***	***	***	***	***

[z] Mean composition of sampled leaf tissue of 4 replications and 6 plants each.

, * significance at $p \leq 0.01$ or 0.001, respectively.

Part 2

Air Temperature Affects Biomass and Carotenoid Pigment Accumulation in Kale and Spinach Grown in a Controlled Environment

Air Temperature Affects Biomass and Carotenoid Pigment Accumulation in Kale and Spinach Grown in a Controlled Environment

This part is a lightly revised version of a paper by the same name published in the journal HortScience in 2005 by Mark G Lefsrud, Dean A Kopsell, David E. Kopsell and Joanne Curran-Celentano:

Lefsrud, M.G., D.A. Kopsell, D.E. Kopsell, and J. Curran-Celentano. 2005. Air temperature affects biomass and carotenoid pigment accumulation in kale and spinach grown in a controlled environment. HortScience 40(7): 2026-2030.

My use of "we" in this part refers to my co-authors and myself. My primary contributions to this paper include (1) selection of the topic and development of the problem into a work relevant to my study of temperature stress on kale and spinach, (2) determination of species, variety and temperature levels, (3) plant propagation and nutrient control, (4) sampling and analysis, (5) most of the gathering and interpretation of the literature, (6) compiling the information into a single paper, and (7) most of the writing and editing.

Abstract

Crop plants are adversely affected by a variety of environmental factors, with air temperature being one of the most influential. Plants have developed a number of methods in the adaptation to air temperature variations. However, there is limited research to determine what impact air temperature has on the production of secondary plant compounds, such as carotenoid pigments. Kale (*Brassica oleracea* L.) and spinach (*Spinacia oleracea* L.) have high concentrations of lutein and β-carotene carotenoids. The objectives of this study were to determine the effects of different growing air temperatures on plant biomass production and the accumulation of elemental nutrients, lutein, β-carotene, and chlorophyll pigments in the leaves of kale and spinach. Plants were grown in nutrient solutions in growth chambers at air temperatures of 15, 20, 25, 30 °C for 'Winterbor' kale and 10, 15, 20, 25 °C for 'Melody' spinach. Maximum tissue lutein and β-carotene concentration occurred at

30 °C for kale and 10 °C for spinach. Highest carotenoid accumulations were 16.1 and 11.2 mg 100 g^{-1} fresh mass for lutein and 13.0 and 10.9 mg 100 g^{-1} fresh mass for β-carotene for the kale and spinach, respectively. Lutein and β-carotene concentration increased linearly with increasing air temperatures for kale, but the same pigments showed a linear decrease in concentration for increasing air temperatures for spinach. Quantifying the effects of air temperature on carotenoid accumulation in kale and spinach, expressed on a fresh mass basis, is important for growers producing these crops for fresh markets.

Introduction

Various environmental factors can affect plant growth and development, such as light, water and air temperature. Significant air temperature variations can limit plant growth at both low and high temperature extremes. Environmental conditions between these two extremes provide an optimum air temperature range for plant growth that allows for maximum productivity (Abrami, 1972). Exposing plants to low air temperatures can damage the photosynthetic apparatus, inhibit the synthesis and/or degradation of proteins, damage the thylakoid membrane, and reduce the electron transfer capacity of the plant (Guy et al., 1985; Holaday et al., 1991; Taiz and Zeiger, 1998). An increase in growing air temperatures above optimum levels results in damage to the thylakoid membranes, along with reduction in the rates of photosynthesis and respiration (Arvidsson et al., 1997; Maevskaya et al., 2003; Rokka et al., 2000; Taiz and Zeiger, 1998). At high air temperatures, membrane disruption results from loses in tissue stability and membrane integrity.

Plants have adapted to potential air temperature changes in the growing environment and can usually adjust to conditions slightly above and below optimum air temperature ranges. Under cold air temperatures, plants increase the percentage of unsaturated fatty acid chains in membranes, specifically thylakoid membranes, and increase abscisic acid concentrations (Jun et al., 2001; Shewfelt, 1992; Taiz and Zeiger, 1998). Physiological adaptations to high air temperatures include increased leaf wax, leaf rolling, change in leaf orientation, change in leaf size and the production of heat shock proteins (Taiz and Zeiger, 1998).

Carotenoids are lipid soluble yellow, orange, and red plant pigments. In plants, carotenoids function as light harvesting antennae pigments, as important free radical scavengers, and have photoprotective roles (Demmig-Adam et al., 1996, Miki, 1991; Tracewell et al., 2001). Carotenoids cannot be synthesized in mammals, making plants the primary source of carotenoids in their diet. Two important carotenoids in human health maintenance are lutein and β-carotene. Dietary intake of foods rich in lutein and β-carotene has been associated with reduced risk of lung cancer and chronic eye diseases, such as cataracts and age-related macular degeneration (Ames et al., 1995; Landrum and Bone, 2001; Le Marchand et al., 1993; Semba and Dagnelie, 2003).

Kale (*Brassica oleracea* L.) ranks highest, and spinach (*Spinacia oleracea* L.) ranks second among vegetable crops for carotenoid content, including lutein and β-carotene (Holden et al., 1999; U.S. Dept. Agr., 2002). Kale is also an excellent source of Ca, Mg, and K (Mills and Jones, 1996). However, kale has low consumption rates in the United States, with per capita fresh intake of less than 0.33

kg·year^{-1} (Lucier and Plummer, 2003). Spinach has one of the highest rates of consumption among green-leafy vegetables in the United States, with per capita intakes of 0.73, 0.09, and 0.36 kg year^{-1} for fresh, canned, and frozen product, respectively (Lucier and Plummer, 2003). Spinach is high in Fe, which is bioavailable in human diets (Zhang et al., 1989), and is also high in Ca, Mg, K, and carotenoids (Holden et al., 1999; Mills and Jones, 1996; U.S. Dept. Agr., 2002).

Environmental air temperatures can have significant impacts on plant performance and metabolism. What remains unclear, however, is the effect of air temperature regimes on the production and accumulation of secondary plant compounds, such as carotenoids. High concentrations of lutein and β-carotene carotenoids are found in both kale and spinach. Carotenoids are membrane-bound compounds that serve important functions in photosynthesis. Both low and high temperature conditions can affect the integrity of thylakoid membranes, and thus affect the stability of carotenoid compounds. Therefore, the goal of this study was to investigate the influences of different air temperatures on plant biomass, elemental concentrations, and accumulation of lutein, β-carotene, and chlorophyll in kale and spinach.

Materials and Methods

Plant Culture

'Winterbor' kale and 'Melody' spinach (Johnny's Selected Seed, Winslow, Maine) were seeded into rockwool growing cubes (Grodan A/S, Dk-2640, Hedehusene, Denmark) and germinated in a greenhouse (22 °C day/ 14 °C night)

under natural lighting conditions (Durham, N.H., Lat. 43° 09' N). Kale was seeded on Aug. 8, Aug. 30 and Nov. 7, 2002 and spinach was seeded on June 12, 2002 and May 19 and June 19, 2003. Peter's 20N-6.9P-16.6K water-soluble fertilizer (Scotts, Marysville, Ohio) was applied at a rate of 200 mg L^{-1} every five days. After two weeks for the kale and three weeks for the spinach, the plants were transferred to 38 L plastic containers (Rubbermaid Inc., Wooster, Ohio). For each study, eight plants were placed in 2 cm round holes set at 10.6 x 9.5 cm spacing on each container lid. The plants were grown in 30 L of a nutrient solution (Hoagland and Arnon, 1950), with elemental concentrations of (mg L^{-1}): N (105.0); P (15.3), K (117.3), Ca (80.2), Mg (24.6), S (32.0), Fe (0.5), B (0.25), Mo (0.005), Cu (0.01), Mn (0.25), and Zn (0.025). The electrical conductivity (EC) of the starting nutrient solution was 0.7 $mS \cdot cm^{-1}$ and pH was measured at 5.6. Four containers were placed in a growth chamber (Model E15, Conviron, Winnipeg, Man.), with each chamber representing a distinct air temperature treatment.

Individual chambers provided plants with a set point air temperature treatment (T) of 15, 20, 25, or 30 °C for the kale, and 10, 15, 20, or 25 °C for the spinach. Each air temperature treatment was replicated three times in separate studies for each species. Photosynthetically active radiation (PAR) was measured (Model QSO-ELEC, Apogee Instruments; Logan, Utah) at six locations on top of each container (without plants present) at the four corner plant holes and between the two side middle plant holes and averaged for each of four containers. Irradiance inside each chamber was measured at the beginning and confirmed at the end of each replication

and averaged 500 ± 100 μmol m^{-2} s^{-1}. The photoperiod for all studies was set at 16hr light and 8hr dark. Both cool white fluorescent and incandescent bulbs were used during the experiment. Solutions were aerated with an air blower (Model 25E133W222, Spencer, Winsor, Conn.) connected to air stones. Nutrient solutions were replaced weekly throughout the experiment to refresh the solution to the initial nutrient concentrations.

The kale plants were cultured for three weeks, while the spinach plants were grown for four weeks. At harvest, shoot and root tissues were separated and weighed. A fully developed, non-shaded leaf from each of the eight plants was randomly selected and a 4 cm^2 piece of the leaf was removed. This treatment sample was stored at –20 °C prior to lyophilization. The remaining shoot material was dried at 60 °C for no less than 72 hr, at which time shoot dry mass was determined.

Elemental Determination

The dried kale and spinach shoot tissues were ground to pass a 0.5 mm screen using a sample mill grinder (Model 1093, Cyclotec-Tector, Höganäs, Sweden). A 0.300 g sample of the ground tissue homogenate was mixed with 10.0 mL of 70% concentrated nitric acid (HNO_3) and digested in a microwave accelerated reaction system (MARS5, CEM Corp., Matthews, N.C.). The digested solution was cooled to room temperature and deionized water was added to result in a final volume of 40.0 mL. Elemental analysis was determined by Inductively Coupled Argon Plasma –

Atomic Emission Spectrometry (ICP-AES model Vista AX, Varian, Inc., Palo Alto, Calif.).

Carotenoid and Chlorophyll Determination

The frozen kale and spinach samples were lyophilized for a minimum of 72 hr (Model 6 L FreeZone, LabConCo, Kansas City, Mo.). The freeze-dried tissues samples were ground with dry ice in a kitchen grinder (Handy Chopper Plus, HC 3000, Household Products Inc., Shelton, Conn.). Samples were extracted and separated according to the method of Kopsell et al. (2004), which was based on the method of Khachik et al. (1986). A 0.100 g sub-sample was placed into a Potter-Elvehjem tissue grinder tube (Kontes, Vineland, N.J.) and hydrated with 0.80 mL of deionized water. The sample was placed in a 40 °C water bath for 20 min. After hydration, 0.80 mL of the internal standard, ethyl-β-apo-8'-carotenoate (Sigma Chemical Co., St. Louis, Mo.) and 2.50 mL of tetrahydrofuran (THF) stabilized with 25 ppm 2,6-Di-*tert*-butyl-4-methoxyphenol (BHT) were added. The sample was homogenized in the tube with ~25 insertions with a Potter-Elvehjem tissue grinder pestle attached to a drill press (Model Craftsman 15 inch Drill Press, Sears, Roebuck and Co., Hoffman Estates, Ill.) at 540 rpm. The sample tube was kept immersed in ice during the grinding process. The tube was placed into a clinical centrifuge for 3 min at 500 g_n. The supernatant was removed with a Pasteur pipet, placed into a conical 15 ml test tube, capped and held on ice. The sediment was re-suspended in 2.00 ml THF and homogenized with ~25 insertions of the grinding pestle. The tube was centrifuged for 3 min at 500 g_n and the supernatant was collected and combined

with the first extracted supernatant. The extraction procedure was repeated twice more until the supernatant was colorless. The sediment was discarded and the combined four supernatants were placed in a 40 °C water bath and reduced to 0.50 ml using nitrogen gas (Model N-EVAP 111, Organomation Inc., Berlin, Mass.). Added to the 0.50 mL reduced sample was 2.50 mL of MeOH and 2.00 mL of THF, and the combined sample solution was vortexed. The sample was filtered through a 0.2 μm polytetrafluoroethylene (PTFE) filter (Model Econofilter PTFE 25/20, Agilent Technologies, Wilmington, Del.) using a 5 mL syringe (Becton, Dickinson and Company, Franklin Lakes, N.J.).

A HPLC unit with photodiode array detector (Agilent 1100, Agilent Technologies, Palo Alto, Calif.) was used for pigment separation. All samples were analyzed for carotenoid compounds using a Vydac RP C_{18} 5.0 μm 250 x 4.6 mm column (Model 201TP54; Phenomenex; Torrance, Calif.) fitted with a 4 x 3.0 mm, 7.0 μm guard column compartment. The column was maintained at 16 °C using a thermostatic column compartment. Eluents were A: 75% acetronitrile, 20% methanol, 5% hexane, 0.05% BHT, and 0.013% triethyamine (TEA)(v/v) and B: 50% acetonitrile, 25% THF, 25% hexane and 0.013% TEA (v/v). The flow rate was 0.70 $mL \cdot min^{-1}$ and the gradient was 100% eluent A for 30 min; 50% A and 50% B for 2 min; 100% B for 2 min; and 50% A and 50% B for 2 min. The eluent was returned to 100% A for 10 min prior to the next injection. Eluted compounds from a 20.0 μL injection were detected at 452 (carotenoids and internal standard), 652 (chlorophyll *a*), and 665 (chlorophyll *b*) nm and data were collected, recorded, and integrated

using 1100 HPLC ChemStation Software (Agilent Technologies). Peak assignment was performed by comparing retention times and line spectra obtained from the photodiode array detection of authentic standards (lutein from Carotenature, Lupsingen, Switzerland; β-carotene, chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) from Sigma Chemical Co., St. Louis, Mo.). Recovery rates of ethyl-β-apo-8'-carotenoate during extraction were above 90%.

Statistical Analysis

Data were analyzed according to a one-way ANOVA using SAS (SAS Institute, Cary, N.C.). The ANOVA determined the significance of the main effects of the air temperature treatments. The relationship between experimental dependent variables and temperature treatments were determined by regression analysis using SAS. Significance for trend analysis was determined by goodness-of-fit of the regression model (r^2).

Results and Discussion

Tissue Biomass Accumulation

Shoot tissue fresh mass (FM) was significantly influenced by changes in air temperature for both 'Winterbor' kale ($F = 9.2$, $p = 0.01$) and 'Melody' spinach ($F = 5.2$, $p = 0.03$). Average shoot FM for 'Winterbor' kale increased from 42.3 to 68.6 g plant^{-1} as air temperature treatments increased from 15 to 20 °C (Table 2.1[a]).

[a] All tables and figures are located in the appendix at the end of this part.

Average shoot FM increased then decreased quadratically (FM = -221.1 + 26.6(T) – 0.61(T)^2; r^2 = 0.77) for 'Winterbor' kale with increasing air temperatures. 'Melody' spinach increased linearly in shoot FM from 33.1 to 156.0 g plant^{-1} as the air temperature increased from 10 to 20 °C (Table 2.1). However, average shoot FM increased then decreased quadratically (FM = 48.8 – 1.2(T) – 333.7(T)^2; r^2 = 0.66) for 'Melody' spinach over the air temperature range investigated (Table 2.1). Average shoot tissue dry mass (DM) for both 'Winterbor' kale and 'Melody' spinach was not significantly affected by changes in air temperature (Table 2.1).

Kale can grow successfully under a wider range of air temperatures than spinach, with optimum growth occurring at 20 °C (Decoteau, 2000; Paul, 1991). Optimum air temperatures are lower for spinach, and occur between 16 and 20 °C (Decoteau, 2000). Spinach is also able to withstand very low air temperatures during germination (Roeggen, 1984). Previous research shows that air temperatures exceeding 35 °C will affect the efficiency of spinach metabolism and reduce yields (Foyer et al., 1997; Maevskaya et al. 2003; Rokka et al, 2000). As the air temperature increases beyond an optimum range, decreases in yield are often reported (Abrami, 1972). This decrease in yield can be due to a number of factors including increased dephosphorylation (Rokka et al. 2000), increases in hydrogen peroxide (Foyer et al., 1997), decreases in efficiency of photosystem II of photosynthesis (Karim et al., 2003), and reduction of nitrate reductase (Maevskaya et al., 2003). All of these factors contribute to a reduction in the overall growth of the plant. Results

from the current study confirm optimum air temperature conditions for biomass production in both kale and spinach.

Carotenoid and Chlorophyll Pigment Accumulation

The accumulation of lutein in the leaf tissues was influenced by changes in air temperatures. Leaf tissue lutein concentrations responded significantly to changes in air temperatures for 'Winterbor' kale ($F = 10.0$, $p = 0.004$). Leaf tissue lutein increased linearly (Lutein = 8.75 + 0.24(T); $r^2 = 0.77$) as the air temperature increased from 15 to 30 °C for 'Winterbor' kale (Table 2.2). Increases in air temperatures from 10 to 25 °C resulted in a linear decrease in leaf tissue lutein (Lutein = 13.0 - 0.25(T); $r^2 = 0.41$) for 'Melody' spinach (Table 2.2). Maximum tissue lutein accumulation expressed on a fresh mass basis for 'Melody' spinach was 11.2 mg 100 g^{-1} and occurred at 10 °C (Table 2.2). Lutein accumulation for 'Winterbor' kale increased with increasing temperature treatments, reaching 16.1 mg 100 g^{-1} fresh mass at 30 °C (Table 2.2).

The accumulation of β-carotene in the leaf tissues was also influenced by changes in air temperatures. Leaf tissue β-carotene responded significantly to changes in air temperatures for both 'Winterbor' kale ($F = 14.4$, $p \leq 0.001$) and 'Melody' spinach ($F = 7.7$, $p = 0.01$). Increases in air temperatures for 15 to 30 °C resulted in linear increases in leaf tissue β-carotene (β-carotene = 5.0 + 0.26(T); $r^2 = 0.81$) for 'Winterbor' kale. Leaf tissue β-carotene decreased linearly (β-carotene = 12.8 - 0.27(T); $r^2 = 0.60$) as the air temperature increased from 10 to 20 °C for

'Melody' spinach (Table 2.2). β-carotene accumulation for 'Winterbor' kale increased with increasing temperature treatments, reaching 13.0 mg 100 g^{-1} fresh mass at 30 °C. Maximum β-carotene accumulation for 'Melody' spinach was 10.9 mg 100 g^{-1} under the 10 °C treatment (Table 2.2).

Temperature can affect the production of plant secondary compounds. Arvodsson et al. (1997) reported that conversion of violaxanthin to zeaxanthin was highly dependent on air temperature. In spinach, 50% of the violaxanthin was converted to zeaxanthin at 4 °C, but conversion increased to 70% at 25 °C and then to 80% at 37 °C. No further increase in conversion rate was noted at air temperatures >37 °C. Arvidsson et al. (1997) also noted that as the conversion rate increased for violaxanthin to zeaxanthin, the levels of lutein increased significantly while other carotenoids remained unaffected. Kale lutein concentrations in the current study increased as air temperature treatments increased, similar to the results of Arvodsson et al. (1997). However, lutein concentrations decreased in spinach as air temperatures increased, contradicting the results of Arvodsson et al. (1997). The β-carotene concentrations followed a similar trend as the lutein, but Arvodsson et al. (1997) reported no change in the content of this pigment. Increasing air temperatures from 12 to 32 °C also affected the production of glucosinolate compounds and myrosinase enzyme activity in rapid-cycling *B. oleracea* (Charron and Sams, 2004). Changes to plant secondary compounds should be noteworthy when growing *B. oleracea* under the different air temperature ranges provided in this study.

Kale and spinach leaf tissues accumulated high concentrations of chlorophyll (Chl) pigments (Table 2.2). Changes in air temperature did not significantly affect 'Winterbor' kale chlorophyll concentrations. However, Chl *a* ($F = 5.5$, $p = 0.02$) and Chl *b* ($F = 5.9$, $p = 0.02$) concentrations responded significantly to changes in air temperatures for 'Melody' spinach. Similar to the carotenoid pigments, maximum chlorophyll concentrations occurred at 30 °C for 'Winterbor' kale and 10 °C for 'Melody' spinach (Table 2.2). The accumulation of both Chl *a* (Chl $a = 233.2 - 4.65(T)$; $r^2 = 0.57$) and Chl *b* (Chl $b = 55.8 - 1.13(T)$; $r^2 = 0.56$) pigments decreased linearly as the air temperature increased from 10 to 25 °C for 'Melody' spinach (Table 2.2). Previously, positive correlations between chlorophyll and carotenoid pigments have been reported for several crop species (Terry and Abadía, 1986), including kale (Kopsell et al., 2004) and Swiss chard (*Beta vulgaris* L.; Ihl et al., 1994). Chlorophyll and carotenoid pigment concentrations followed a similar trend for the kale and spinach grown in the current study.

Percentage dry matter (%DM) accumulation for the spinach responded to increases in air temperatures ($F = 17.0$, $p \leq 0.001$). The spinach %DM increased, then decreased ($\%DM = 26.6 - 1.78(T) + 0.04(T)^2$; $r^2 = 0.85$) as the air temperatures increased from 10 to 25 °C (Table 2.3). Growing environment, handling, storage, or food processing can cause changes in tissue water content, resulting in changes in the concentration of carotenoids in the plant when expressed on a fresh mass basis (Ezell and Wilcox, 1959; Gil et al., 1999).

The carotenoid concentration of the kale and spinach calculated on a dry mass basis was not significantly affected by changes in air temperature (Table 2.3). Maximum lutein and β-carotene dry mass accumulation in kale and spinach occurred at the temperature treatments (25 and 20 °C, respectively) of maximum dry mass accumulation. Overall, dry mass carotenoid concentrations increased with increasing total dry matter production in both kale and spinach. Maximum lutein and β-carotene fresh mass accumulation for both kale and spinach occurred at the temperature treatments (30 and 10 °C, respectively) of minimum fresh mass accumulation. Overall, there tended to be a dilution of fresh mass carotenoid concentrations in both kale and spinach with increasing total fresh mass production.

Macro- and Micronutrient Accumulation

None of the essential macro- or micronutrients responded to changes in air temperatures for 'Winterbor' kale or 'Melody' spinach (Table 2.4 - 5). Marschner (1997) reported that mineral uptake is only slightly affected by air temperature, with changes in root zone temperature having a larger effect on ion uptake.

Previous research shows the potential for genetic differences in carotenoid accumulations within plant species (Howard et al., 2000; Kopsell et al., 2004; Kurilich et al., 2003; Nicolle et al., 2004). The current study demonstrates that air temperatures can also influences the production of carotenoid pigments in the leaves of kale and spinach. Carotenoid concentrations in the leaves of kale increased as the air temperatures increased from 15 to 30 °C, while the carotenoid concentrations

decreased in spinach as the air temperature increased from 10 to 25 °C. Changes in the growing air temperatures of these cool-season crops resulted in changes in fresh biomass production, and the accumulation of lutein, β-carotene, and Chl *b* pigments, when calculated on a fresh mass basis. In many parts of the country, cool-season crops such as kale and spinach can be planted in both spring and fall. Air temperatures in field conditions can be modified by growing kale and spinach at different times of the year. Therefore, the influence of air temperatures on kale and spinach carotenoid concentrations should be considered when selecting appropriate planting dates.

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List of References

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Appendix

Table 2.1. Mean value[z] of biomass production in the leaf tissues of 'Winterbor' kale (*Brassica oleracea* L.) and 'Melody' spinach (*Spinacia oleracea* L.) grown under increasing air temperatures in nutrient solution culture.

	Biomass (g plant^{-1})	
°C	FM[x]	DM[x]
	Winterbor kale	
15	42.3 ± 3.9	4.9 ± 1.6
20	68.6 ± 3.0	6.5 ± 1.0
25	65.0 ± 9.9	8.5 ± 2.6
30	30.2 ± 4.9	4.4 ± 1.4
Contrasts[y]		
L	ns	ns
Q	**	ns
	Melody spinach	
10	33.1 ± 15.5	3.1 ± 1.6
15	119.6 ± 25.5	6.6 ± 1.8
20	156.0 ± 28.9	6.8 ± 1.6
25	120.0 ± 19.4	4.4 ± 1.0
Contrasts[y]		
L	*	ns
Q	**	ns

[z] Mean leaf tissue mass of 3 replications, 8 plants ± standard error.

[y] Significance for linear (L) and quadratic (Q) orthogonal contrasts.

[x]FM = fresh mass, DM = dry mass.

[ns, *, **] Non-significant or significance at $p \leq 0.05$, 0.01 level, respectively.

Table 2.2. Mean value[z] of pigment accumulation expressed on a fresh mass basis in the leaf tissues of 'Winterbor' kale (*Brassica oleracea* L.) and 'Melody' spinach (*Spinacia oleracea* L.) grown under increasing air temperatures in nutrient solution culture.

	Pigment Concentration (mg $100g^{-1}$ FM)			
°C	Lutein	β-carotene	Chl *a*[x]	Chl *b*[x]
		Winterbor kale		
15	12.6 ± 0.6	9.3 ± 0.5	204.4 ± 15.1	50.5 ± 4.0
20	13.2 ± 0.1	9.8 ± 0.3	201.4 ± 13.8	49.5 ± 4.3
25	14.8 ± 0.5	11.8 ± 0.5	223.8 ± 31.4	56.1 ± 1.0
30	16.1 ± 0.5	13.0 ± 0.5	255.1 ± 3.5	61.1 ± 0.4
Contrasts[y]				
L	***	***	ns	*
Q	***	***	ns	*
		Melody spinach		
10	11.2 ± 1.6	10.9 ± 1.0	199.0 ± 17.8	47.5 ± 5.6
15	8.2 ± 0.8	7.7 ± 0.3	147.6 ± 7.8	35.8 ± 1.4
20	7.7 ± 1.1	7.1 ± 0.8	134.9 ± 14.8	29.9 ± 1.3
25	7.3 ± 0.6	6.7 ± 0.5	125.7 ± 13.3	30.5 ± 3.2
Contrasts[y]				
L	*	**	**	**
Q	*	**	**	**

[z] Mean composition of sampled leaf tissue of 3 replications, 8 plants ± standard error.

[y] Significance for linear (L) and quadratic (Q) orthogonal contrasts.

[x] Chl a = chlorophyll a; Chl b = chlorophyll b

[ns, *, **, ***] Non-significant or significance at $p \leq 0.05$, 0.01, 0.001, respectively.

Table 2.3. Mean value[z] of percent dry mass, and pigment concentration as expressed on a dry mass basis in the leaf tissues of 'Winterbor' kale (*Brassica oleracea* L.) and 'Melody' spinach (*Spinacia oleracea* L.) grown under increasing air temperatures in nutrient solution culture.

		Concentration (mg g^{-1} DM)	
°C	%DM	Lutein	β-carotene
		Winterbor kale	
15	14.3 ± 0.7	0.89 ± 0.08	0.65 ± 0.06
20	14.6 ± 1.4	0.92 ± 0.08	0.68 ± 0.06
25	15.3 ± 1.6	0.99 ± 0.10	0.80 ± 0.10
30	16.0 ± 1.4	1.02 ± 0.06	0.83 ± 0.05
Contrasts[y]			
L	ns	ns	ns
Q	ns	ns	ns
		Melody spinach	
10	13.2 ± 0.1	0.59 ± 0.05	0.43 ± 0.05
15	9.1 ± 0.5	0.76 ± 0.04	0.57 ± 0.04
20	8.2 ± 0.8	0.90 ± 0.07	0.68 ± 0.04
25	8.4 ± 0.6	0.88 ± 0.08	0.67 ± 0.06
Contrasts[y]			
L	**	ns	ns
Q	***	ns	ns

[z] Mean composition of sampled leaf tissue of 3 replications, 8 plants ± standard error.

[y] Significance for linear (L) and quadratic (Q) orthogonal contrasts.

[ns, **, ***] Non-significant or significance at $p \leq 0.01$, 0.001 level, respectively.

Table 2.4. Mean value[z] of macronutrient accumulation in the leaf tissues of 'Winterbor' kale (*Brassica oleracea* L.) and 'Melody' spinach (*Spinacia oleracea* L.) grown under increasing air temperatures in nutrient solution culture.

	Macronutrients (% DM)				
°C	P	K	Ca	Mg	S
			Winterbor kale		
15	1.07 ± 0.12	3.53 ± 0.37	3.81 ± 0.63	0.72 ± 0.14	0.67 ± 0.07
20	1.23 ± 0.05	4.92 ± 0.41	4.74 ± 0.23	0.89 ± 0.06	0.85 ± 0.09
25	1.10 ± 0.13	3.55 ± 0.57	3.15± 0.57	1.27 ± 0.20	0.71 ± 0.14
30	1.07 ± 0.05	3.83 ± 0.29	2.95 ± 0.28	0.70 ± 0.04	0.61 ± 0.06
Contrast[y]					
L	ns	ns	ns	ns	ns
			Melody spinach		
10	1.74 ± 0.36	10.86 ± 0.43	1.37 ± 0.22	1.66 ± 0.15	0.33 ± 0.08
15	2.58 ± 0.43	11.7 ± 0.59	1.18 ± 0.09	1.79 ± 0.12	0.33 ± 0.07
20	2.44 ± 0.44	11.3 ± 1.16	1.23 ± 0.11	1.68 ± 0.09	0.33 ± 0.07
25	1.90 ± 0.19	11.7 ± 0.65	0.98 ± 0.04	1.65 ± 0.08	0.31 ± 0.05
Contrast[y]					
L	ns	ns	ns	ns	ns

[z] Mean composition of sampled leaf tissue of 3 replications, 8 plants ± standard error.

[y] Significance for linear (L) and quadratic (Q) orthogonal contrasts.

[ns] Non-significant.

Table 2.5. Mean value[z] of micronutrient accumulation in the leaf tissues of 'Winterbor' kale (*Brassica oleracea* L.) and 'Melody' spinach (*Spinacia oleracea* L.) grown under increasing air temperatures in nutrient solution culture.

	Micronutrients (mg kg^{-1} DM)					
°C	B	Cu	Fe	Mn	Mo	Zn
			Winterbor kale			
15	47.4±8.4	3.0±1.1	76.0±19.9	150.2±28.4	0.77±0.16	73.3±51.6
20	55.7±11.2	20.6±17.7	59.5±32.6	168.1±9.1	0.77±0.13	52.0±24.1
25	46.0±0.5	16.3±9.8	32.4±9.7	127.0±20.5	0.81±0.11	42.8±7.9
30	55.2±7.2	17.2±14.8	45.7±20.0	108.8±11.7	0.59±0.15	110.3±73.8
Contrast[y]						
L	ns	ns	ns	ns	ns	ns
			Melody spinach			
10	37±12.4	9.6±6.5	159.4±19.9	296.6±64.6	0.71±0.21	149.3±24.1
15	41±7.3	8.2±2.9	172.9±20.2	309.5±21.8	0.92±0.08	78.5±19.2
20	46±7.5	6.8±1.2	138.1±21.8	297.2±40.0	0.68±0.09	131.2±42.5
25	47±8.5	7.0±0.6	115.7±32.8	342.0±68.9	0.87±0.08	135.1±32.4
Contrast[y]						
L	ns	ns	ns	ns	ns	ns

[z] Mean composition of sampled leaf tissue of 3 replications, 8 plants ± standard error.

[y] Significance for linear (L) and quadratic (Q) orthogonal contrasts.

[ns] Non-significant.

Part 3

Irradiance Levels Affect Growth Parameters and Carotenoid Pigments in Kale and Spinach Grown in a Controlled Environment

Irradiance Levels Affect Growth Parameters and Carotenoid Pigments in Kale and Spinach Grown in a Controlled Environment

This part is a lightly revised version of a paper by the same name published in the journal Physiologia Planatarum in 2006 by Mark G Lefsrud, Dean A Kopsell, David E. Kopsell and Joanne Curran-Celentano:

Lefsrud, M.G., D.A. Kopsell, D.E. Kopsell, and J. Curran-Celentano. 2006. Irradiance levels affect growth parameters and carotenoid pigments in kale and spinach grown in a controlled environment. Physiologia Planatarum In Press.

My use of "we" in this part refers to my co-authors and myself. My primary contributions to this paper include (1) selection of the topic and development of the problem into a work relevant to my study of irradiance stress on kale and spinach, (2) determination of species, variety and irradiance levels, (3) plant propagation and nutrient control, (4) sampling and analysis, (5) most of the gathering and interpretation of the literature, (6) compiling the information into a single paper, and (7) most of the writing and editing.

Abstract

Carotenoids play critical roles in both light harvesting and energy dissipation for the protection of photosynthetic structures. However, limited research is available on the impact of irradiance on the production of secondary plant compounds, such as carotenoid pigments. Kale (*Brassica oleracea* L.) and spinach (*Spinacia oleracea* L.) are two leafy vegetables high in lutein and β-carotene carotenoids. The objectives of this study were to determine the effects of different irradiance levels on tissue biomass, elemental nutrient concentrations, and lutein, β-carotene and chlorophyll pigment accumulation in the leaves of kale and spinach. 'Winterbor' kale and 'Melody' spinach were grown in nutrient solution culture in growth chambers at average irradiance levels of 125, 200, 335, 460, and 620 μmol m^{-2} s^{-1}. Highest tissue lutein, β-carotene and chlorophylls occurred at 335 μmol m^{-2} s^{-1} for kale, and 200

μmol m^{-2} s^{-1} for spinach. The accumulations of lutein and β-carotene were significantly different among irradiance levels for kale, but were not significantly different for spinach. However, lutein and β-carotene accumulation was significant for spinach when computed on a dry mass basis. Identifying effects of irradiance on carotenoid accumulation in kale and spinach is important information for growers producing these crops for dry capsule supplements and fresh markets.

Introduction

Incoming solar radiation is required to provide energy for plant metabolic systems, however light can be one of the most common environmental stresses. As light strikes the surface of plant leaves, photons are absorbed by antenna pigments which funnel this energy to the photosynthetic reaction center. In the reaction center, chlorophylls (Chl), pheophytins, and quinones molecules convert light energy into chemical energy (Frank and Cogdell, 1996). Carotenoids are bound to pigment-protein complexes within the thylakoid membranes and are utilized as antenna pigments. In higher plants, carotenoid distribution between the two photosystems is unevenly distributed, with pigments of photosystem I having high levels of β-carotene, while lutein makes up the majority of carotenoid pigments in photosystem II (Demmig-Adams et al., 1996). At high light levels, excess energy must be removed from the photosynthetic system to prevent damage. Carotenoid molecules are in close proximity to the Chl molecules and quench the energetic triplet state of the chlorophyll molecule to prevent damage to the photosynthetic system (Frank and Cogdell, 1996; Miki, 1991; Taiz and Zeiger, 1998; Tracewell et al., 2001).

Irradiance can affect the production of mineral accumulation and plant secondary compounds, such as lutein and β-carotene (Havaux et al., 1998). Increases in photosynthesis will increase plant biomass and can result in a dilution effect on elemental concentrations (Mills and Jones, 1996). One important class of secondary plant metabolites is the carotenoids. Carotenoids are C_{40} isoprenoid polyene compounds that form yellow, orange, and red lipid soluble pigments in higher plants, algae and bacteria. In shade leaves, the content of lutein and β-carotene is less than in sun leaves (Demmig-Adam et al., 1996). Similarly, summer-grown kale has higher lutein and β-carotene concentrations than kale grown during winter months, when light levels are reduced (Azevedo and Rodriguez-Amaya, 2005).

Kale (*Brassica oleracea* L. var. *acephala* D.C.) ranks highest, and spinach (*Spinacia oleracea* L.) ranks second among vegetable crops for lutein and β-carotene concentrations (Holden et al., 1999; U.S. Dept. Agr., 2002). Kale is also an excellent source of Ca, Mg, and K (Mills and Jones, 1996). However, kale has low consumption rates, with per capita fresh intake at less than 0.33 kg $year^{-1}$ in the United States (Lucier and Plummer, 2003). Spinach has one of the highest rates of consumption among green-leafy vegetables in the United States, with per capita intakes of 0.73, 0.09, and 0.36 kg $year^{-1}$ for fresh, canned, and frozen markets, respectively (Lucier and Plummer, 2003). Spinach is also high in Fe, Ca, Mg, and K (Mills and Jones, 1996; U.S. Dept. Agr., 2002; Zhang et al., 1989).

Lutein ((*3R,3'R,6'R)*-β,ε-carotene-3,3'diol) and β-carotene (β,β-carotene) carotenoids possess important human health properties, but cannot be synthesized in mammals. Plants are a primary dietary source of carotenoids, and foods rich in lutein

and β-carotene has been associated with reduced risk of lung cancer and chronic eye diseases, such as cataracts and age-related macular degeneration (Ames et al., 1995; Landrum and Bone, 2001; Le Marchand et al., 1993; Semba and Dagnelie, 2003).

The amount of light is critical for plant growth and development and can be modified by growing crops at different times of the year, or under various shade environments. What remains unclear, however, is the effect of irradiance on the production of carotenoid pigments. Therefore, the goal of this study was to investigate the effects of different irradiance levels on plant biomass, elemental concentrations, and accumulation of lutein, β-carotene, and Chl pigments in kale and spinach.

Materials and Methods

Plant Culture

'Winterbor' kale and 'Melody' spinach (Johnny's Selected Seed, Winslow, Maine) were seeded into rockwool growing cubes (Grodan A/S, Dk-2640, Hedehusene, Denmark) and germinated in a greenhouse (22°C day/ 14°C night) under natural lighting conditions (Durham, N.H., Lat. 43° 09' N). The three replications of kale were seeded on Feb. 3, Sept. 3 and Oct. 27, 2003, and three replications of spinach were seeded on Sept. 25 and Dec. 20, 2002 and Sept. 19, 2003. Peter's 20N-6.9P-16.6K water-soluble fertilizer (Scotts, Marysville, Ohio) was applied at a rate of 200 mg L^{-1} every five days. After two weeks for the kale and three weeks for the spinach, the plants were transferred to 38 L plastic containers

(Rubbermaid Inc., Wooster, Ohio). Eight plants of one species were placed into 2 cm round holes set at 10.6 x 9.5 cm spacing on a container lid. Four containers were placed in a growth chamber (E15, Conviron, Winnipeg, Man.) with the air temperature set point for the experiment at 20 °C. The plants were grown in 30 L of nutrient solution (Hoagland and Arnon, 1950), with elemental concentrations of (mg L^{-1}): N (105), P (15.3), K (117.3), Ca (80.2), Mg (24.6), S (32.0), Fe (0.5), B (0.25), Mo (0.005), Cu (0.01), Mn (0.25), and Zn (0.025). The electrical conductivity (EC) of the starting nutrient solution was 0.7 mS cm^{-1} and pH was measured at 5.6. Solutions were aerated with an air blower (25E133W222, Spencer, Winsor, Conn.) connected to air stones. Deionized water was added daily to maintain 30 L in each container and the complete nutrient solution was replaced every week throughout the experiment to refresh the solution to the initial nutrient concentrations.

Plants were grown under different irradiance levels within the growth chambers using both fluorescent and incandescent bulbs. Photosynthetically active radiation (PAR) was measured (QSO-ELEC, Apogee Instruments, Logan, Utah) at six locations, without plants, on top of each container at the four corner plant holes and between the two side middle plant holes and averaged. Six containers were blocked together for an average irradiance treatment level (PAR) of 125 ± 30, 200 ± 50, 335 ± 60, 460 ± 40, and 620 ± 100 μmol m^{-2} s^{-1}. The irradiance treatment daily integral was 7.2, 11.5, 19.3, 26.5, and 35.7 mol m^{-2}. The daily photoperiod was16 hr, this correlates to the maximum possible field photoperiod in the region where the experiment was located. Irradiance levels were measured at the beginning and confirmed at the end of each replication.

The kale plants were grown for three weeks, while the spinach plants were grown for four weeks for each experimental replication. At harvest, shoot and root tissues were separated and shoot tissue was weighed. A fully developed, non-shaded leaf from each of the eight plants was randomly selected and a 4 cm^2 piece of the leaf was removed. This treatment sample was stored at –20 °C prior to lyophilization. The remaining shoot material was dried at 60 °C for 72 hr, at which time shoot dry mass was determined.

Elemental Determination

Dried plant tissues were ground in a sample mill grinder (1093, Cyclotec-Tector, Höganäs, Sweden) to pass a 0.5 mm screen. A 0.300 g tissue sample was mixed with 10.0 mL of 70% nitric acid (HNO_3) and digested in a microwave accelerated reaction system (MARS5, CEM Corp., Matthews, N.C.). The digested solution was cooled to room temperature and deionized water was added to result in a final volume of 40.0 mL. Elemental analysis was determined by Inductively Coupled Argon Plasma – Atomic Emission Spectrometry (Vista AX, Varian, Inc., Palo Alto, Calif.).

Carotenoid and Chlorophyll Determination

Frozen kale and spinach samples were lyophilized for 72 hr (6L FreeZone, LabConCo, Kansas City, Mo.). The dried tissue samples were ground with dry ice in a kitchen grinder (Handy Chopper Plus, HC 3000, Household Products Inc, Shelton,

Conn.). Samples were extracted and separated according to the method of Kopsell et al. (2004), which is based on the method of Khachik et al. (1986). A 0.100 g sub-sample was placed into a Potter-Elvehjem tissue grinder tube (Kontes, Vineland, N.J.) and hydrated with 0.800 mL of deionized water. The sample was placed in a 40 °C water bath for 20 min. After hydration, 0.800 mL of the internal standard, ethyl-β-apo-8'-carotenoate (Sigma Chemical Co., St. Louis, Mo.) and 2.50 mL of THF stabilized with 25 ppm BHT were added. The sample was homogenized in the tube with ~25 insertions with the tissue grinder pestle attached to a drill press (Craftsman 15 inch Drill Press, Sears, Roebuck and Co., Hoffman Estates, Ill.) set at 540 rpm. The sample tube was kept immersed in ice during the grinding process. The tube was placed into a clinical centrifuge for 3 min at 500 g_n. The supernatant was removed with a Pasteur pipet, placed into a conical 15 ml test tube, capped and held on ice. The sediment was re-suspended in 2.00 ml THF and homogenized with ~25 insertions of the grinding pestle. The tube was centrifuged for 3 min at 500 g_n and the supernatant was collected and combined with the first extracted supernatant. The extraction procedure was repeated twice more until the supernatant was colorless. The sediment was discarded and the combined four supernatants were placed in a 40 °C water bath and reduced to 0.50 ml using nitrogen gas (N-EVAP 111, Organomatic Inc., Berlin, Mass.). Added to the 0.50 mL reduced sample was 2.50 mL of MeOH and 2.00 mL of THF, and then the combined sample solution was vortexed. The sample was filtered through a 0.2 μm PTFE filter (Econofilter PTFE 25/20, Agilent

Technologies, Wilmington, Del.) using a 5 mL syringe (Becton, Dickinson and Company, Franklin Lakes, N.J.).

A HPLC unit with photodiode array detector (Agilent 1100, Agilent Technologies, Palo Alto, Calif.) was used for pigment separation. All samples were analyzed for carotenoid compounds using a Vydac RP C_{18} 5.0 μm 250 x 4.6 mm column (201TP54, Phenomenex, Torrance, Calif.) fitted with a 4 x 3.0 mm, 7.0 μm guard column compartment. The column was maintained at 16 °C using a thermostatic column compartment. Eluents were A: 75% acetronitrile, 20% methanol, 5% hexane, 0.05% BHT, and 0.013% triethyamine (TEA)(v/v) and B: 50% acetonitrile, 25% THF, 25% hexane and 0.013% TEA (v/v). The flow rate was 0.70 mL min^{-1} and the gradient was 100% eluent A for 30 min; 50% A and 50% B for 2 min; 100% B for 2 min; and 50% A and 50% B for 2 min. The eluent was returned to 100% A for 10 min to re-equilibrate the column prior to the next injection. Eluted compounds from a 20.0 μL injection were detected at 452 nm (carotenoids and internal standard), 652 nm for Chl *a*, and 665 nm for Chl *b*, with data collected and integrated using 1100 HPLC ChemStation Software (Agilent Technologies, Palo Alto, Calif.). Peak assignment was performed by comparing retention times and absorption spectra obtained from the photodiode array detection of authentic standards (lutein from Carotenature, Lupsingen, Switzerland; β-carotene, Chl *a*, and Chl *b* from Sigma Chemical Co., St. Louis, Mo.). Recovery rates of ethyl-β-apo-8'-carotenoate during extraction were above 90%.

Statistical Analysis

Data were analyzed according to a one-way ANOVA using SAS (Cary, N.C.). The ANOVA determined the significance of the main effects of the irradiance treatments. LSD was used to determination post hoc multiple comparison tests. The relationship between experimental dependent variables and irradiance treatments were determined by regression analysis using SAS.

Results

Tissue Biomass Accumulation

Shoot tissue FM was significantly influenced by irradiance treatment for both 'Winterbor' kale ($F = 26.5$, $p \leq 0.001$) and 'Melody' spinach ($F = 14.4$, $p \leq 0.001$). Average kale FM increased from 15.3 to 74.3 g per plant as irradiance treatment increased from 125 to 620 μmol m^{-2} s^{-1} (Table 3.1[a]). Kale FM responded linearly to increases in irradiance treatment levels (FM= 8.8 + 0.1(PAR), r^2=0.75). Spinach FM increased from 59.2 to 174.1 g per plant as the irradiance increased from 125 to 620 μmol m^{-2} s^{-1} (Table 3.1). A linear increase in FM was also found for spinach (FM = 25.5 + 0.3(PAR), r^2=0.55) as the irradiance levels increased.

Shoot tissue DM was significantly influenced by irradiance treatment for both 'Winterbor' kale ($F = 26.6$, $p \leq 0.001$) and 'Melody' spinach ($F = 9.9$, $p \leq 0.001$). Average kale DM increased from 1.2 to 6.9 g per plant as irradiance treatment increased from 125 to 620 μmol m^{-2} s^{-1} (Table 3.1). Kale DM responded linearly to

[a] All tables and figures are located in the appendix at the end of this part.

increasing irradiance treatment levels (DM= 0.12 + 0.01(PAR), r^2=0.80). Spinach increased in DM from 2.5 to 6.5 g plant^{-1} as the irradiance increased from 125 to 620 μmol m^{-2} s^{-1} (Table 3.1). A significant linear increase in DM was found for the spinach (DM = 2.67 + 0.01(PAR), r^2=0.46) as the irradiance levels increased.

Macro- and Micronutrient Accumulation

Changes in irradiance levels accounted for small but significant variation for elemental accumulation in kale. Those mineral elements affected were: P (F = 3.2, p = 0.030), K (F = 3.1, p = 0.035), Ca (F = 2.9, p = 0.041), Cu (F = 5.4, p = 0.003), and Mn (F = 3.5, p = 0.022; Table 3.2-3). Phosphorous in the leaf tissues of kale increased, then decreased (P = 0.79 + 0.001(PAR) - 0.000001(PAR)^2, r^2=0.30) in response to increased irradiance. Kale leaf tissue K decreased (K = 5.0 – 0.002(PAR), r^2=0.32) in response to increases in irradiance levels. Calcium accumulation in kale followed a linear decrease (Ca = 4.7 - 0.001(PAR), r^2=0.23) due to increasing irradiance level. Kale Cu accumulation also followed a linear decrease (Cu = 7.0 - 0.01(PAR), r^2=0.43) as irradiance levels increased. Manganese accumulation in kale decreased linearly (Mn = 1.7 - 0.001(PAR), r^2=0.34) with increasing irradiance levels. Spinach leaf tissue Ca (F = 2.9, p = 0.043) and Fe (F = 3.8, p = 0.014) accumulation were both significantly affected by irradiance level (Table 3.2 - 3). Spinach Ca followed a quadratic (Ca = 1.11 – 0.0005(PAR) + 0.000002(PAR)^2, r^2=0.21) trend due to increasing irradiance levels. Spinach Fe decreased, then increased (Fe = 440.8 – 1.71(PAR) + 0.003(PAR)^2, r^2=0.24) in response to increasing irradiance levels.

Carotenoid and Chlorophyll Pigment Accumulation

A small but significant amount of variation for kale leaf tissue lutein concentrations (F = 3.08, p = 0.03) resulted from changes in irradiance levels. Kale leaf tissue lutein accumulation ranged from 9.1 mg 100 g^{-1} at 125 μmol m^{-2} s^{-1}, to as high as 15.1 mg 100 g^{-1} at 335 μmol m^{-2} s^{-1} (Table 3.4). The trend in kale leaf tissue lutein accumulation was an increasing then decreasing quadratic trend (Lutein = 5.36 + 0.04(PAR) - 0.00005$(PAR)^2$, r^2= 0.21) in response to increasing irradiance levels. Similarly, β-carotene accumulation in the kale leaf tissues responded significantly to irradiance treatments (F = 4.77, p = 0.005). Kale leaf tissue β-carotene accumulation ranged from 5.7 mg 100 g^{-1} at 125 μmol m^{-2} s^{-1}, to as high as 11.1 mg 100 g^{-1} at 335 μmol m^{-2} s^{-1} (Table 3.4). Kale leaf tissue β-carotene increased, then decreased (β-carotene = 2.29 + 0.036(PAR) - 0.00004$(PAR)^2$, r^2 = 0.30) in response to irradiance treatments. Overall, there tended to be a dilution of fresh mass carotenoid concentrations in both kale and spinach with increasing total fresh mass production.

Chlorophyll pigments concentrations in the leaf tissues of kale and spinach were much higher when compared to carotenoid concentrations. Concentrations of Chl *a* (F = 4.1, p =0.011) and Chl *b* (F = 4.1, p = 0.011) pigments in kale leaf tissues differed among irradiance treatments. Similar to the carotenoid pigments, maximum Chl pigment accumulation in kale leaf tissues occurred at 335 μmol m^{-2} s^{-1}, with Chl *a* at 247.3 mg 100 g^{-1} and Chl *b* at 59.0 mg 100 g^{-1} (Table 3.4). Kale leaf tissue Chl *a* and Chl *b* accumulation both followed a quadratic trend (Chl *a*= 68.3 + 0.75(PAR) -

0.0008(PAR)^2, r^2= 0.32; Chl *b* = 16.6 + 0.19(PAR) - 0.0002(PAR)^2, r^2= 0.36) in response to increases in irradiance levels.

The largest accumulation of carotenoid and Chl pigments in spinach leaf tissues occurred at the irradiance level of 200 μmol m^{-2} s^{-1}, with lutein levels at 11.1 mg 100 g^{-1}, β-carotene levels at 9.2 mg 100 g^{-1}, Chl *a* at 179.2 mg 100 g^{-1}, and Chl *b* at 46.1 mg 100 g^{-1} (Table 3.4). However, spinach leaf tissue carotenoid and Chl FM concentrations were not statistically affected by irradiance treatment.

Leaf tissue % dry matter (%DM) was influenced by irradiance levels for both kale (F = 8.1, $p \leq 0.001$) and spinach (F = 4.3, $p = 0.009$). Average kale %DM increased from 9.7 to 15.1 % as irradiance levels increased from 125 to 620 μmol m^{-2} s^{-1} (Table 3.5). Spinach leaf tissue %DM increased from 6.8 to 7.7% as the irradiance increased from 125 to 620 μmol m^{-2} s^{-1} (Table 3.5). The trend of kale leaf tissue %DM followed a quadratic trend (%DM = 7.7 + 0.0001(PAR) – 0.000004(PAR)^2, r^2=0.54) in response to increasing irradiance levels. No trend was reported for spinach.

The concentration of spinach leaf tissue lutein (F= 4.6, p= 0.007) and β-carotene (F= 3.4, p= 0.023) measure on a DM basis responded to increases in irradiance levels (Table 3.5). Spinach lutein DM accumulation ranged from 0.9 mg g^{-1} at 620 μmol m^{-2} s^{-1}, to as high as 1.41 mg g^{-1} at 200 μmol m^{-2} s^{-1}. Spinach β-carotene DM accumulation ranged from 0.79 mg g^{-1} at 620 μmol m^{-2} s^{-1}, to as high as 1.17 mg g^{-1} at 200 μmol m^{-2} s^{-1}. The trend in lutein accumulation in the spinach as a function of DM was quadratic (Lutein DM= 1.13 +0.001(PAR) – 0.00001(PAR)^2, r^2

= 0.33) in response to increasing irradiance levels. The trend in spinach β-carotene DM accumulation was also quadratic (β-carotene DM= 0.90 + 0.001(PAR) – 0.00001(PAR)^2, r^2 = 0.23) in response to increasing irradiance levels.

Discussion

Irradiance level directly influences the photosynthetic rate of plants, resulting in increased production of carbohydrates and total biomass (Mills and Jones, 1996; Taiz and Zeiger, 1998). Results from the current study showed linear increases in both FM and DM as the light irradiance levels increased from 125 to 620 μmol m^{-2} s^{-1}. Research by Weng (1992) also showed a linear increase in FM for *B. oleracea* as the irradiance level was increased to 750 μmol m^{-2} s^{-1}. However, as light levels increase, the photosynthetic efficiency decreases when a light saturation point is reached. The level of the light saturation point can be affected by a number of factors including plant nutrition, species, variety, and specific genetic factors (Weng, 1992). The light saturation point for members of *B. oleracea* is estimated to be between 750 and 1000 μmol m^{-2} s^{-1}, but has been reported to be as high as 1600 μmol m^{-2} s^{-1} for one variety of rapeseed (Weng, 1992). The light saturation point can explain why both FM and DM for spinach and kale increased steadily and then leveled as irradiance treatments increased in the current study. From regression analysis of the data the potential light saturation point based on FM would be 650 and 775 μmol m^{-2} s^{-1} for the kale and spinach, respectively.

Irradiance levels can indirectly affect elemental concentrations in plants by first impacting enzymatic activity and photosynthetic rates. This increases the amount of FM produced by the plant and results in a dilution effect for elemental concentrations (Mills and Jones, 1996). Calcium concentrations can increase as a response to lower K concentration at low irradiance levels (Mills and Jones, 1996). A study using soybean (*Glycine max* L. Merr.) revealed that low light resulted in increases in P, K, Cu, Fe, and Mn, however the form of N can affect which minerals increase at low light levels (Mills and Jones, 1996). In our study, the kale concentrations of Ca, Cu, K and Mn all increased at low irradiance while the P concentrations decreased. The spinach mineral concentrations were not significantly different for most irradiance levels, but both Ca and Fe decreased at low light levels. The specific reason for the decrease of P in kale and decrease of Ca in spinach is not known.

Behera and Choudhury (2003) reported increases in wheat (*Triticum sativum* L.) lutein (858 to 1211 μg g^{-1}) and β-carotene (22 to 92 μg g^{-1}) concentrations as irradiance levels increased from 70 to 210 μmol m^{-2} s^{-1}. Chlorophyll pigments also responded to changes in irradiance levels, with Chl *a* increasing from 1694 to 2403 μg g^{-1} and Chl *b* increasing 480 to 537 μg g^{-1} for the studied light levels, respectively. When the plants were exposed to 1250 μmol m^{-2} s^{-1} of light, the pigment concentrations decreased. Behera and Choudhury (2003) results are very similar to our results, where the carotenoid and chlorophyll concentration increased linearly for kale from 125 to 300 μmol m^{-2} s^{-1} and for spinach from 125 to 200 μmol m^{-2} s^{-1}. At

irradiance levels above 300 μmol m^{-2} s^{-1} for the kale and 200 μmol m^{-2} s^{-1} for the spinach, the carotenoid and chlorophyll levels start to decrease and remain fairly constant after 400 μmol m^{-2} s^{-1} for both species. The decrease in carotenoid and chlorophyll concentrations could be due to a combined effect of photodegradation of the pigment molecules and dilution of the concentration as the plant grows. The moisture content of the plants changed significantly as the irradiance increased. The kale %DM increased from 9.7 to 15.0 % and spinach increased from 6.8 to 7.7 %. The larger change in %DM for kale followed an increasing linear trend while spinach had no change after removing the lowest irradiance level. This change in %DM may result in dilution of the carotenoids, which could explain the reason for the significant result irradiance had on kale but not spinach.

Results from the current study would suggest that irradiance level has no impact on the carotenoid concentrations in spinach expressed on a FM basis. Most spinach is consumed fresh (Lucier and Plummer, 2003) and changes in water content in the plant tissue using traditional agriculture based farming practices for growing, handling, storage, or food processing, can results in changes in the nutritional value of the plant (Ezell and Wilcox, 1959; Gil et al., 1999). Based on U.S. Dept. Agr. procedures for reporting food carotenoid content on a FM basis, there were no significant differences in lutein or β-carotene accumulations for the spinach over the irradiance levels tested. However, with the increased interest in dietary antioxidant supplements, such as dried spinach capsules, warrants investigations into DM pigment concentrations (Lefsrud et al., 2005). When spinach carotenoid data are

reported on a DM basis, there were significant decreases in both lutein and β-carotene concentrations. Thus, reporting lutein and β-carotene carotenoids on both a FM and DM basis may be a more accurate way to express the nutritional value of spinach.

Previous research shows that modification of the growing environment air temperature can influence carotenoid accumulation in both kale and spinach (Lefsrud et al., 2005). Environmental modification of irradiance levels of these cool-season crops resulted in changes in fresh biomass production, and the accumulation of lutein, β-carotene, and Chl *b* pigments. Carotenoid concentrations in the leaves of kale were maximized at 335 μmol m^{-2} s^{-1}, while spinach carotenoids concentrations were highest at 200 μmol m^{-2} s^{-1}. In many parts of the United States, cool-season crops such as kale and spinach are grown as both spring and fall crops. Average field irradiance levels can vary dependent on location, time of year, shading, and atmospheric conditions. Therefore, the influence of irradiance levels on kale and spinach carotenoid concentrations should be considered when selecting appropriate growing conditions for these cool-season crops. Changes in carotenoid concentrations would be expected to influence the nutritional value of kale and spinach.

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Appendix

Table 3.1. Mean fresh mass (FM) and dry mass (DM) biomass production in leaf tissues of 'Winterbor' kale (*Brassica oleracea* L.) and 'Melody' spinach (*Spinacia oleracea* L.) grown under increasing irradiance levels in nutrient solution culture. Mean composition of sampled leaf tissue of 6 replications, 8 plants each ± standard error. ANOVA results: *** significance at $p \leq 0.001$ level.

Irradiance	Biomass (g)	
(μmol m^{-2} s^{-1})	FM	DM
	Winterbor kale	
125	15.3 ± 1.2	1.2 ± 0.1
200	34.6 ± 6.0	2.5 ± 0.4
335	54.4 ± 4.1	4.3 ± 0.4
460	62.5 ± 2.9	5.4 ± 0.4
620	74.3 ± 6.4	6.9 ± 0.7
	***	***
	Melody spinach	
125	59.2 ± 10.1	2.5 ± 0.3
200	121.2 ± 13.4	4.9 ± 0.3
335	122.3 ± 13.3	5.5 ± 0.6
460	148.2 ± 6.7	5.7 ± 0.3
620	174.1 ± 11.5	6.5 ± 0.8
	***	***

Table 3.2. Mean macronutrient accumulation in the leaf tissues of 'Winterbor' kale (*Brassica oleracea* L.) and 'Melody' spinach (*Spinacia oleracea* L.) grown under increasing irradiance levels in nutrient solution culture. Mean composition of sampled leaf tissue of 6 replications, 8 plants each ± standard error. ANOVA results: ns, *, non-significant or significance at $p \leq 0.05$, level, respectively.

Irradiance (μmol $m^{-2}sec^{-1}$)	Macronutrients (% DM)				
	P	K	Ca	Mg	S
			Winterbor kale		
125	0.95 ± 0.03	4.85 ± 0.19	4.62 ± 0.18	0.73 ± 0.02	0.72 ± 0.03
200	0.97 ± 0.04	4.66 ± 0.10	4.28 ± 0.12	0.72 ± 0.02	0.68 ± 0.02
335	1.12 ± 0.04	4.51 ± 0.14	4.36 ± 0.10	0.75± 0.02	0.69 ± 0.01
460	1.09 ± 0.05	4.40 ± 0.23	4.33 ± 0.12	0.77± 0.03	0.70 ± 0.02
620	1.09 ± 0.05	4.02 ± 0.20	3.88 ± 0.21	0.72 ± 0.02	0.72 ± 0.03
	*	*	*	ns	ns
			Melody spinach		
125	1.83 ± 0.07	12.3 ± 0.4	1.02 ± 0.07	1.15 ± 0.08	0.27 ± 0.01
200	1.96 ± 0.13	10.2 ± 0.4	1.21 ± 0.16	1.36 ± 0.16	0.40 ± 0.06
335	1.74 ± 0.13	10.9 ± 0.8	0.98 ± 0.03	1.17 ± 0.06	0.29 ± 0.03
460	1.77 ± 0.13	10.2 ± 0.2	1.31 ± 0.08	1.23 ± 0.04	0.34 ± 0.03
620	1.86 ± 0.14	10.3 ± 0.9	1.42 ± 0.15	1.26 ± 0.05	0.33 ± 0.03
	ns	ns	*	ns	ns

Table 3.3. Mean micronutrient accumulation in the leaf tissues of 'Winterbor' kale (*Brassica oleracea* L.) and 'Melody' spinach (*Spinacia oleracea* L.) grown under increasing irradiance levels in nutrient solution culture. Mean composition of sampled leaf tissue of 6 replications, 8 plants each ± standard error. ANOVA results: ns, *, **, non-significant or significance at $p \leq 0.05$, 0.01 level, respectively.

Irradiance (μmol $m^{-2}sec^{-1}$)	Micronutrients (mg kg^{-1} DM)					
	B	Cu	Fe	Mn	Mo	Zn
			Winterbor kale			
125	35.8±1.7	5.9±0.6	84.4±11.6	161.7±4.8	0.7±0.1	65.5±21.7
200	37.3±2.4	6.1±0.9	80.1±26.5	150.9±9.6	0.6±0.1	87.5±18.1
335	37.2±2.3	4.1±0.4	56.1±10.2	150.3±4.9	0.6±0.1	56.5±19.2
460	40.6±1.9	3.3±0.5	62.1±6.8	130.6±9.6	0.5±0.1	38.0±3.0
620	38.9±2.5	2.7±0.7	57.3±11.8	120.3±13.	0.7±0.1	51.6±4.2
	ns	**	ns	*	ns	ns
			Melody spinach			
125	31.5±1.8	16.7±5.5	187.4±16.1	330.8±41.8	0.6±0.1	246.±29.1
200	43.9±6.1	25.5±6.8	349.7±88.5	317.9±35.7	0.5±0.2	81.2±25.7
335	31.2±2.3	9.4±3.6	153.7±29.9	288.8±18.1	0.6±0.1	95.3±16.4
460	36.9±3.0	17.7±6.1	205.9±43.1	337.9±24.6	0.6±0.1	131.±47.5
620	34.9±2.5	19.7±5.9	489.4±121.	361.6±47.1	0.6±0.1	179.±68.5
	ns	ns	*	ns	ns	ns

Table 3.4. Mean pigment accumulation as expressed on a fresh mass (FM) basis in the leaf tissues of 'Winterbor' kale (*Brassica oleracea* L.) and 'Melody' spinach (*Spinacia oleracea* L.) grown under increasing irradiance levels in nutrient solution culture. Mean composition of sampled leaf tissue of 6 replications, 8 plants each ± standard error. ANOVA results: ns, *, **, non-significant or significance at $p \leq 0.05$, 0.01 level, respectively. Post Hoc LSD test for multiple comparison: a, b.

Irradiance (μmol m^{-2} s^{-1})	Pigment Concentration (mg 100 g^{-1} FM)			
	Lutein	β-carotene	Chl *a*	Chl *b*
		Winterbor kale		
125	9.1 ± 1.4 a	5.7 ± 0.9 a	145.0 ± 23.1 a	35.1 ± 6.4 a
200	12.0 ± 1.6 ab	8.1 ± 1.1 ab	184.4 ± 26.0 ab	47.2 ± 6.7 ab
335	15.1 ± 1.4 ab	11.1 ± 1.2 ab	247.3 ± 10.0 ab	59.0 ± 2.2 b
460	12.0 ± 0.6 ab	8.6 ± 0.5 ab	211.4 ± 13.0 ab	55.1 ± 1.5 b
620	12.7 ± 0.5 b	9.3 ± 0.4 b	216.3 ± 18.2 b	55.2 ± 4.2 b
	*	**	*	*
		Melody spinach		
125	7.9 ± 0.7	6.4 ± 0.6	127.2 ± 10.8	34.1 ± 2.6
200	11.1 ± 1.5	9.2 ± 1.3	179.2 ± 24.7	46.1 ± 6.1
335	8.7 ± 0.6	7.2 ± 0.6	143.1 ± 11.5	35.7 ± 3.0
460	8.7 ± 1.2	7.4 ± 1.1	142.7 ± 19.8	32.5 ± 4.4
620	7.1 ± 0.9	6.2 ± 0.9	119.2 ± 17.1	29.1 ± 3.7
	ns	ns	ns	ns

Table 3.5. Mean % dry matter (DM) and pigment concentration as expressed on a dry mass basis in the leaf tissues of 'Winterbor' kale (*Brassica oleracea* L.) and 'Melody' spinach (*Spinacia oleracea* L.) grown under increasing irradiance levels in nutrient solution culture. Mean composition of sampled leaf tissue of 6 replications, 8 plants each ± standard error. ANOVA results: ns, *, **, ***, non-significant or significance at p≤ 0.05, 0.01, 0.001 level, respectively. Post Hoc LSD test for multiple comparison: a, b, and c.

Irradiance (μmol m^{-2} s^{-1})	% DM	Concentration (mg g^{-1} DM)	
		Lutein	β-carotene
		Winterbor kale	
125	9.7 ± 0.3 a	0.93 ± 0.13	0.56 ± 0.08
200	9.7 ± 1.0 a	1.20 ± 0.08	0.81 ± 0.06
335	12.7 ± 0.7 a	1.19 ± 0.11	0.88 ± 0.09
460	13.1 ± 0.7 ab	0.94 ± 0.09	0.67 ± 0.08
620	15.1 ± 1.1 b	0.86 ± 0.05	0.63 ± 0.05
	***	ns	ns
		Melody spinach	
125	6.8 ± 0.5 a	1.17 ± 0.09 a	0.94 ± 0.06 a
200	7.7 ± 0.6 ab	1.41 ± 0.1 ab	1.17 ± 0.09 ab
335	7.4 ± 0.6 ab	1.19 ± 0.04 abc	0.98 ± 0.04 ab
460	7.5 ± 0.6 b	1.14 ± 0.09 bc	0.96 ± 0.08 a
620	7.7 ± 0.4 b	0.91 ± 0.08 c	0.79 ± 0.08 ab
	**	**	*

Part 4

Biomass Production and Pigment Accumulation in Kale Grown Under Increasing Photoperiods

Biomass Production and Pigment Accumulation in Kale Grown Under Increasing Photoperiods

This part is a lightly revised version of a paper by the same name that has been submitted to be published in the journal HortScience in 2006 by Mark G Lefsrud, Dean A Kopsell, Robert Augé and A.J. Both:

Lefsrud, M.G., D.A. Kopsell, R.M. Augé and A.J. Both. 2006. Biomass production and pigment accumulation in kale grown under increasing photoperiods. HortScience In Press.

My use of "we" in this part refers to my co-authors and myself. My primary contributions to this paper include (1) selection of the topic and development of the problem into a work relevant to my study of photoperiod on kale, (2) determination of species, variety and photoperiod levels, (3) plant propagation and nutrient control, (4) sampling and analysis, (5) most of the gathering and interpretation of the literature, (6) compiling the information into a single paper, and (7) most of the writing and editing.

Abstract

Consumption of fruits and vegetable crops rich in lutein and β-carotene carotenoids is associated with reduced risk of cancers and aging eye diseases. Kale (*Brassica oleracea* L. var. *acephala* D.C.) ranks highest for lutein concentrations and is an excellent source of dietary carotenoids. Kale plants were grown under varied photoperiods to determine changes in the accumulation of fresh and dry biomass, chlorophyll *a* and *b*, and lutein and β-carotene carotenoids. The plants were cultured in a controlled environment using nutrient solutions under photoperiod treatments of 6, 12, 16 or 24 hr (continuous). Photoperiod treatments significantly affected kale fresh mass, dry mass, % dry matter, and accumulation of chlorophyll *a*, chlorophyll *b*, lutein, and β-carotene. Fresh and dry mass production increased linearly as photoperiod increased, reaching a maximum under the 24 hr photoperiod. Maximum accumulation of lutein, β-carotene, and chlorophyll *b* occurred under the 24 hr

photoperiod at 13.5, 10.4, and 58.6 mg 100 g^{-1} fresh mass, respectively. However, maximum chlorophyll *a* (235.1 mg 100 g^{-1} fresh mass) occurred under the 12 hr photoperiod. When β-carotene and lutein were measured on a dry mass basis the maximum accumulation was shifted to the 16 hr photoperiod. An increase in photoperiod resulted in increased pigment accumulation, but maximum concentrations of pigments were not correlated with maximum biomass production.

Introduction

The length of the photoperiod will influence a number of plant physiological factors including biomass production, bud formation, flowering, germination, leaf elongation, leaf emergence, and changes in secondary compounds (Degli Agosti et al., 1990; Densmore, 1997; Drozdova et al., 2004; Gottdenker et al., 2000; Junttila et al., 1997; Koontz and Prince, 1986; Masuda and Murage, 1998; Murage et al., 1997; Riihimaki and Savolainen, 2004; Taylor et al., 1994). Increases in photoperiod have consistently resulted in increased plant biomass production (Garner and Allard, 1931; Koontz and Prince, 1986; Masuda and Murage, 1998; Ohler and Mitchell, 1996). This increase in biomass result from actions related to increases in leaf area and chlorophyll content (Langton et al., 2003). Under continuous irradiance, some plants, including eggplant (*Solanum melongena* L.), potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* L.), and cowpea (*Vigna unguiculata* (L.) Walp.), have responded with limited biomass production and with interveinal chlorosis and necrosis (Bradley and Janes, 1985; Murage and Masuda, 1997; Murage et al., 1997; Ohler and Mitchell, 1996; Stutte et al., 1996).

For many plant species, the increase in chlorophyll concentration as a result of increasing photoperiod is well documented. Sironval (1958) showed that changing the photoperiod of hemp (*Cannabis sativa* L.), lupin (*Lupinus albus* L.), soybean (*Glycine max* (L.) Merr.), and strawberry (*Fragaria vesca* L.) from 8 to 16 hr resulted in increased leaf chlorophyll concentration. Chlorophyll concentration also increased as photoperiod increased in tomato (Hurd, 1973), geranium (*Pelargonium* X *hortorum* Bailey), impatiens (*Impatiens walleriana* Hooker), pansy (*Viola* X *wittrockiana* Kappert) and petunia (*Petunia* X *hybrida* (Hooker) Vilmorin) (Langton et al., 2003). Fukuda et al. (2000) reported additions of night supplemental lighting (to establish a 24 hr photoperiod) increased chlorophyll concentrations in lettuce (*Lactuca salvia* L.), pakchoi (*Brassica chinensis* L.) and tsukena (*Brassica rapa* L. subsp. *campestris* A.R. Clapham). The research into the effect of increased photoperiod on carotenoid accumulation is limited in higher plants. Arizmendi-Maldonado et al. (2003) reported adding supplemental lighting to extend the standard day to a 15 hr photoperiod did not affect the accumulation of β-carotene in bermudagrass (*Cynodon dactylon* Pers.) or African stargrass (*Cynodon nlemfuensis* Vandergst).

Carotenoids are yellow, orange, and red plant lipid-soluble pigments, produced by plants, algae and bacteria that cannot be synthesized by mammals. In plants, carotenoids are used as antenna pigments to funnel light energy to the photosynthetic reaction center. These carotenoids are in close proximity to the chlorophyll molecules and absorb energy to prevent damage to the photosynthetic system (Marschner, 1997; Miki, 1991; Taiz and Zeiger, 1998; Tracewell et al., 2001).

Lutein and β-carotene carotenoids possess important human health properties. Dietary intake of foods rich in lutein and β-carotene has been associated with reduced risk of lung cancer, cataracts, and age-related macular degeneration (Ames et al., 1995; Landrum and Bone, 2001; Le Marchand et al., 1993). Kale (*Brassica oleracea* L. var. *acephala* D.C.) is an excellent source of dietary carotenoids (Holden et al., 1999; Kurilich et al., 1999; U.S. Dept. Agr., 2002). The U.S. Dept. Agr. rates kale as the highest source of lutein and β-carotene of any vegetable (Holden et al., 1999; U.S. Dept. Agr., 2002). However, kale has low consumption rates, with a per capita fresh intake of less than 0.33 kg $year^{-1}$ in the United States (Lucier and Plummer, 2003).

Light is critical for plant growth and development, and the photoperiod can easily be controlled by growers in artificial growing environments. What remains unclear is the effect of photoperiod on the production of secondary plant pigments, such as carotenoids. Therefore, the goal of this study was to determine the influences of four different irradiance photoperiods on plant biomass and accumulation patterns of carotenoid pigments in the leaf tissues of kale.

Material and Methods

Plant Culture

'Winterbor' kale (Johnny's Selected Seed, Winslow, Maine) was sown into 3.8 cm rockwool growing cubes (Grodan A/S, Dk-2640, Hedehusene, Denmark) and germinated in a greenhouse (22°C day/ 14°C night) under natural lighting conditions (Durham, N.H., Lat. 43° 09' N). Peter's 20N-6.9P-16.6K water-soluble fertilizer

(Scotts Company, Marysville, Ohio) was applied at a rate of 200 mg L^{-1} every five days. After 2 weeks, the plants were transferred to 38 L plastic containers (Rubbermaid Inc., Wooster, Ohio). Eight plants were placed into 2 cm round holes cut at 10.6 x 9.5 cm spacing in each container lid. Four containers were placed into each of four growth chambers (E15, Conviron, Winnipeg, Man.). The growth chamber temperature was maintained at 20 ± 1 °C and the photosynthetically active radiation (PAR) was measured at 500 ± 100 $\mu mol \cdot m^{-2} s^{-1}$. PAR was measured (Model QSO-ELEC, Apogee Instruments; Logan, Utah) at six locations, without plants, on top of each tub at the four corner plant holes and between the two side middle plant holes and averaged. PAR levels were measured at the beginning and confirmed at the end of each replication. Cool white fluorescent (160W) and incandescent (60W) bulbs were used during the experiment. The chambers were randomly assigned experimental treatments, and the containers within each chamber were blocked together providing 32 plants for each sample. The four treatment levels for photoperiod were 6, 12, 16 or 24 hr. Treatments were replicated beginning on 20 October 2003, 17 November 2003, and 13 January 2004.

The plants were grown hydroponically in 30 L of nutrient solution (Hoagland and Arnon, 1950). Elemental concentrations of the nutrient solutions were (mg L^{-1}): N (105), P (15.3), K (117), Ca (80.2), Mg (24.6), S (32.0), Fe (0.5), B (0.25), Mo (0.005), Cu (0.01), Mn (0.25), and Zn (0.025). The electrical conductivity (EC) of the starting nutrient solution was 0.7 mS cm^{-1} and the pH was measured at 5.6. Solutions were aerated with an air blower (Model 25E133W222, Spencer, Winsor, Conn.) connected to air stones. Deionized water was added daily to maintain 30 L in

each container. Nutrient solutions were replaced every week throughout the experiment to refresh the solution to the initial nutrient concentrations.

The plants were grown for 3 weeks. At harvest, shoot and root tissues were separated and weighed. The fourth fully expanded leaf was selected and a 4 cm^2 piece of the leaf was removed from each of the 32 plants in the four containers and combined to form one sample. Samples were stored at –80°C prior to lyophilization. The remaining shoot material was dried at 60°C for no less than 72 hr, at which time shoot dry mass and % dry matter were determined.

Carotenoid and Chlorophyll Determination

Frozen kale samples were lyophilized at -20 °C for a minimum of 72 hr (Model 6L FreeZone, LabConCo, Kansas City, Mo.). The dried tissues samples were ground with dry ice in a kitchen grinder (Handy Chopper Plus, HC 3000, Household Products Inc., Shelton, Conn.). Pigments were extracted and separated according to Kopsell et al. (2004), a procedure which is based on the method of Khachik et al. (1986). A 0.100 g sub-sample was placed into a Potter-Elvehjem tissue grinder tube (Kontes, Vineland, N.J.) and hydrated with 0.80 mL of deionized water. The sample was placed in a 40 °C water bath for 20 min. After hydration, 0.80 mL of the internal standard, ethyl-β-apo-8'-carotenoate (Sigma Chemical Co., St. Louis, Mo.), and 2.50 mL of HPLC grade tetrahydrofuran (THF) were added to the sample. The sample was homogenized in the tube with 25 insertions with a Potter-Elvehjem tissue grinder pestle attached to a drill press at 540 rpm. The sample tube was kept immersed in ice

during extraction. The tube was placed into a clinical centrifuge for 3 min at 500 g_n. The supernatant was removed with a Pasteur pipette, placed into a conical 15 ml test tube, capped, and held on ice. The sample pellet was re-suspended in 2.00 ml THF and homogenized with 25 insertions of the grinding pestle. The tube was centrifuged for 3 min at 500 g_n and the supernatant was collected and combined with the first extracted supernatant. The extraction procedure was repeated twice more until the supernatant was colorless. The pellet was discarded and the combined 4 supernatants were placed in a 40°C water bath and reduced to 0.50 ml using nitrogen gas (Model N-EVAP 111, Organomatic Inc., Berlin, Mass.). 2.50 mL of MeOH and 2.00 mL of THF were added to the sample, which was then vortexed and filtered through a 0.2 μm polytetrafluoroethylene (PTFE) filter (Model Econofilter PTFE 25/20, Agilent Technologies, Wilmington, Del.) using a 5 mL syringe (Becton, Dickinson and Company, Franklin Lakes, N.J.) prior to high performance liquid chromatograph (HPLC) analysis.

A HPLC unit with photodiode array detector (Agilent 1100, Agilent Technologies, Palo Alto, Calif.) was used for pigment separation. All samples were analyzed for carotenoid compounds using a Vydac RP C_{18} 5.0 μm 250 x 4.6 mm column (Model 201TP54, Phenomenex, Torrance, Calif.) fitted with a 4 x 3.0 mm, 7.0 μm guard column compartment. The column was maintained at 16°C using a thermostatic column compartment. Eluents were A: 75% acetronitrile, 20% methanol, 5% hexane, 0.05% BHT, and 0.013% triethyamine (TEA) and B: 50% acetonitrile, 25% THF, 25% hexane and 0.013% TEA. The flow rate was 0.70 mL

min^{-1} and the gradient was 100% eluent A for 30 min; 50% A and 50% B for 2 min; 100% B for 2 min; and 50% A and 50% B for 2 min. The eluent was returned to 100% A for 10 min prior to the next injection. Eluted carotenoids and chlorophyll pigments from a 20.0 μL injection were detected at 453 (carotenoids and internal standard), 652 (chlorophyll *a*), and 665 (chlorophyll *b*) nm, with data collected, recorded and integrated using 1100 HPLC ChemStation Software (Agilent Technologies, Palo Alto, Calif.). Internal standard % recovery ranged from 70 to 96%. Peak assignment for individual pigments were performed by comparing retention times and line spectra obtained from photodiode array detection using external standards (lutein from Carotenature, Lupsingen, Switzerland; β-carotene, chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) from Sigma Chemical Co., St. Louis, Mo.). Concentrations of external standards were determined spectrophotometrically using the following $E_{1cm}^{1\%}$ values: lutein, 2550 in ETOH, λ_{max} = 445 nm; β-carotene, 2592 in hexane, λ_{max} = 452 nm; Chl *a*, 819 in ETOH, λ_{max} = 665 nm; and Chl *b*, 441 in ETOH, λ_{max} = 649 nm (Davies and Köst, 1988). Standard reference material® (Slurried Spinach 2385, National Institute of Science and Technology, Gaithersburg, Md.) was used for method validation.

Statistical Analysis

Main effects were analyzed by one-way ANOVA using SPSS (Chicago, Ill.). The experiment was a randomized split plot design, consisting of four chambers being assigned one of the four treatments. Each treatment was replicated twice for a

total of three runs. The relationship between experimental dependent variables and photoperiod treatments were determined by regression analysis. Orthogonal polynomials were used to study changes associated with increasing photoperiod treatments by partitioning the sum of squares into components that were associated with linear and quadratic terms (Nogueira, 2004).

Results

Kale shoot tissue fresh mass (FM) responded ($p \leq 0.001$) to increases in photoperiod treatments, and ranged from 7.7 to 92.8 g per plant for the 6 hr photoperiod to the 24 hr photoperiod, respectively (Table 4.1[a]). Kale shoot tissue dry mass (DM) responded ($p \leq 0.001$) to increases in photoperiod treatments, and ranged from 0.6 to 10.5 g per plant for the 6 hr photoperiod to the 24 hr photoperiod, respectively (Table 4.1). There were quadratic responses in both FM and DM to increases in photoperiod. Kale FM increased (FM = -37.9+ 8.2 PHOTO - 0.2 $PHOTO^2$; r^2=0.92, $p \leq 0.001$) as the photoperiod treatments increased from 6 to 24 hr, as did kale DM (DM = -2.1 + 0.4 PHOTO + 0.01 $PHOTO^2$; r^2=0.93, $p \leq 0.001$; Table 4.1). Both the largest FM and DM were observed at the longest photoperiod of 24 hr. Photoperiod treatment affected the % dry matter (%DM; $p \leq 0.001$) found in the kale shoot tissues, and ranged from 8.2 to 11.4% for the 6 hr photoperiod to the 24 hr photoperiod, respectively (Table 4.1). The %DM responded quadratically to increases in photoperiod treatments (%DM = 0.09 - 0.002 PHOTO + 0.001 $PHOTO^2$; $r^2 = 0.66$, $p \leq 0.001$; Table 4.1).

[a] All tables and figures are located in the appendix at the end of this part.

The effects of photoperiod treatments on the concentrations of lutein, β-carotene, and chlorophyll pigments were measured for the kale shoot tissues (Table 4.2). Lutein concentrations responded to changes in photoperiod ($p \leq 0.001$). Maximum lutein accumulation (13.5 mg 100 g^{-1} FM) occurred under the 24 hr photoperiod treatment, while the lowest lutein concentrations (8.8 mg 100 g^{-1} FM) occurred at the 6 hr photoperiod. Lutein concentrations increased in response to increasing photoperiod (Lutein = 3.6 + 1.0 PHOTO – 0.03 $PHOTO^2$; r^2= 0.51, $p \leq$ 0.001). β-carotene also responded to changes in photoperiod treatments ($p \leq 0.001$). Maximum β-carotene accumulation was 10.4 mg 100 g^{-1} FM for the 24 hr photoperiod treatment, while the lowest β-carotene accumulation (6.3 mg 100 g^{-1} FM) occurred during the 6 hr photoperiod treatment. β-carotene concentrations increased in response to increasing photoperiod (β-carotene = 3.0 + 0.6 PHOTO – 0.01 $PHOTO^2$; r^2= 0.54, $p \leq 0.001$).

The concentrations of Chl *a* ($p \leq 0.001$), Chl *b* ($p \leq 0.001$) and total chlorophyll (Total Chl; $p \leq 0.001$) pigments were influenced by changes in photoperiod treatments (Table 4.2). Maximum Chl *a* and Total Chl levels occurred at the 12 hr photoperiod treatment, while maximum Chl *b* accumulation occurred at the 24 hr photoperiod treatment. Chlorophyll pigments in the kale shoot tissues increased in response to increases in photoperiod. Quadratic regression models were fit to Chl *a* (Chl *a* = 118.0 + 12.9 PHOTO– 0.3 $PHOTO^2$; r^2= 0.33, $p \leq 0.001$), Chl *b* (Chl *b* = 21.4 + 4.1 PHOTO – 0.1 $PHOTO^2$; r^2= 0.58, $p \leq 0.001$), and Total Chl (Total Chl = 139.4 + 17.0 PHOTO – 0.4 $PHOTO^2$; r^2= 0.39, $p \leq 0.001$) pigments.

The carotenoid content of vegetable crops is normally reported on a FM basis to equate to typical consumption patterns (Holden et al. 1999); however, due to the popularity of dried materials in dietary supplements as sources of antioxidants, the accumulations of lutein and β-carotene were also calculated on a DM basis and on a per plant (TP) basis (Table 4.3). Photoperiod treatments affected lutein DM, β-carotene DM, lutein TP, and β-carotene TP, and were all significant at $p \leq 0.001$. The lutein dry mass initially increased, then decreased resulting in a quadratic effect (Lutein DM = 0.36 – 0.09 PHOTO – 0.003 PHOTO^2; $r^2 = 0.41$, $p \leq 0.001$), and β-carotene dry mass also increased then decreased (β-carotene DM = 0.27 + 0.06 PHOTO - 0.002 PHOTO^2; r^2=0.25, $p = 0.002$). The lutein total plant concentrations increased with increased photoperiod in a quadratic response (Lutein TP = -5.5 + 1.1 PHOTO– 0.01 PHOTO^2; $r^2 = 0.87$, $p \leq 0.001$), and β-carotene total plant also increased with increasing photoperiod (β-carotene TP = -3.5 + 0.69 PHOTO- 0.006 PHOTO^2; r^2=0.89, $p \leq 0.001$).

Chlorophyll *a* to lutein (Chl *a*:L), chlorophyll *b* to lutein (Chl *b*:L), chlorophyll *a* to β-carotene (Chl *a*:β), chlorophyll *b* to β-carotene (Chl *b*:β), total chlorophyll (Chl *a* and *b*) to lutein (Chl:L), total chlorophyll to β-carotene (Chl:β), and chlorophyll *a* to *b* (Chl *a*:*b*) ratios were calculated (Table 4.4). The Chl *a*:L (p = 0.005), Chl *a*:β ($p \leq 0.001$), Chl *b*:β ($p \leq 0.001$), Chl:L ($p = 0.013$), Chl:β ($p \leq 0.001$), Chl *a*:*b* ($p = 0.002$), and lutein:β-carotene (p =0.027) were all influenced by photoperiod, but Chl *b*:L ($p = 0.442$) was not influenced by treatment. Decreasing quadratic changes were calculated for Chl *a*:L (Chl *a*:L = 24.9 - 0.1 PHOTO + 0.02

PHOTO^2; r^2=0.25, $p \leq 0.001$), Chl *a*:β (Chl *a*:β = 31.8 - 0.5 PHOTO - 0.002 PHOTO^2; r^2=0.44, $p \leq 0.001$), Total Chl:L (Total Chl:L= 30.1 - 0.86 PHOTO + 0.02 PHOTO^2; r^2=0.22, $p = 0.004$), and Chl *a*:*b* (Chl *a*:*b* = 4.8 – 0.09 PHOTO+ 0.002 PHOTO^2; r^2= 0.28, $p \leq 0.001$). The maximum ratio of Chl *a* to lutein, β-carotene and Chl *b* occurred with the 6 hr photoperiod. Increasing then decreasing quadratic changes were measured for Chl *b*:β (Chl *b*:β = 66 + 0.04 PHOTO – 0.003 PHOTO^2; r^2=0.19, $p = 0.003$) and Total Chl:β (Total Chl:β = 38.5 - 0.41 PHOTO – 0.002 PHOTO^2; r^2=0.40, $p \leq 0.001$). The ratio of Total Chl to β-carotene and Chl *b* to β-carotene was largest during the 12 hr photoperiod.

Discussion

The 24 hr photoperiod (continuous irradiance) resulted in the largest FM and DM, lutein, β-carotene and Chl *b* accumulation, whereas increasing the photoperiod from 6 to 16 hr resulted in an increase in Total Chl of 27%. Hurd (1973) showed similar results with tomato, where changing the photoperiod from 8 to 16 hr increased the chlorophyll concentration of the leaves by 25 to 34%. Increasing photoperiod has also increased the chlorophyll concentration in several bedding plants, lettuce, pakchoi and tsukena (Fukuda et al., 2000; Langton et al., 2003).

In our study of kale, β-carotene increased 65% and lutein increased 64% from the 6 to 24 hr photoperiod treatment. However, β-carotene only increased 17% when the photoperiod was changed from 12 to 16 hr and no change was measured for lutein. This increase in the carotenoid accumulation is different from the results of Arizmendi-Maldonado et al. (2003) who found that increasing the photoperiod to 15

hr did not affect the accumulation of β-carotene in bermudagrass or stargrass. Nicklisch and Woitke (1999) found that half of the algae species tested had a significant change in lutein or β-carotene when the photoperiod was changed from 6 to 12 hr. However, only one algae species, a diatom (*Synedra acus* Kütz), showed increases in β-carotene as the photoperiod increased and no species has been reported that had an increase in lutein.

Interestingly, different trends resulted when the kale carotenoid pigments were calculated on a DM basis (Table 4.3). Kale tissue %DM was influenced by photoperiod, with the greatest %DM occurring during the 24 hr photoperiod. The lutein and β-carotene concentrations on a DM basis had peak accumulations during the 16 hr photoperiod. Measuring the carotenoid accumulation on a total plant basis resulted in maximum accumulation occurring at the 24 hr photoperiod.

Positive correlations between chlorophyll and carotenoid pigments exist in kale (Kopsell et al., 2004), Swiss chard (*Beta vulgaris* L.; Ihl et al., 1994), and lettuce (Mou, 2005). Measurements of chlorophyll concentrations, or green colorations, are more efficient and cost effective when compared to carotenoid pigment analysis. The high correlations between chlorophyll and carotenoid pigments suggest that selection for chlorophyll concentrations would be an effective way for breeders to select for higher carotenoid levels in leafy vegetable crops (Kopsell et al., 2004; Mou, 2005). Decreasing chlorophyll to carotenoid pigment ratios in the current study demonstrate that carotenoid pigments in kale increase relative to chlorophyll as the photoperiod increases. Decreasing quadratic trends show an initial drop in pigment ratios from the 6 hr to the 12 hr photoperiod. Stabilization in the pigment ratios from the 12 hr to

24 hr photoperiod would support previous suggestions for the indirect selection of carotenoid pigments in leafy vegetable crops using chlorophyll concentrations.

The largest FM, DM and fresh basis lutein, β-carotene, and Chl *b* accumulation occurred at the 24 hr photoperiod, with the maximum Chl *a* occurred during the 12 hr photoperiod. The largest accumulation of lutein DM and β-carotene DM occurred during the 16 hr photoperiod. Increased photoperiod resulted in increases in pigment accumulation but maximum concentrations of pigments were not required for maximum biomass production. Controlling the photoperiod allows plants to be grown for optimization of fresh mass, pigment accumulation or both.

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Appendix

Table 4.1. Mean fresh and dry biomass accumulation and % dry matter in leaf tissues of 'Winterbor' kale grown under increasing photoperiod.

Photoperiod (hr)	Plant Biomass[z] (g plant^{-1})		
	Fresh Mass	Dry Mass	% Dry matter
6	7.7 ± 0.4	0.6 ± 0.1	8.2 ± 0.1
12	41.3 ± 2.0	3.8 ± 0.2	9.1 ± 0.1
16	66.3 ± 3.1	5.7 ± 0.4	8.6 ± 0.1
24	92.8 ± 4.1	10.5 ± 0.4	11.4 ± 0.2
Contrasts			
Linear	***	***	***
Quadratic	***	***	***

[z] Mean composition of sampled leaf tissue of 3 replications and 32 plants per replication ± standard error.

*** Significance at $p \leq 0.001$.

Table 4.2. Mean pigments concentrations[z] expressed on a fresh mass basis in the leaf tissues of 'Winterbor' kale grown under increasing photoperiod.

Photoperiod (hr)	Pigment Concentration (mg·100 g^{-1} FM)				
	Lutein	β-carotene	Chl *a*[y]	Chl *b*[y]	Total Chl[y]
6	8.8 ± 0.4	6.3 ± 0.2	179.3 ± 5.0	41.2 ± 1.1	220.5 ± 5.9
12	12.8 ± 0.3	8.3 ± 0.3	235.1 ± 6.3	58.1 ± 1.4	293.3 ± 7.5
16	13.1 ± 0.8	9.7 ± 0.6	223.4 ± 11.7	56.7 ± 2.3	280.0 ± 13.7
24	13.5 ± 0.6	10.4 ± 0.4	228.7 ± 8.3	58.6 ± 1.6	287.2 ± 9.4
Contrasts					
Linear	***	***	**	***	***
Quadratic	***	***	***	***	***

[z] Mean composition of sampled leaf tissue of 3 replications and 32 plants ± standard error.

[y] Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*, Chl = chlorophyll.

, * Significance at $p \leq 0.01$ or 0.001, respectively.

Table 4.3. Mean pigment concentrations[z] as a function of dry mass and total plant pigment in the leaf tissues of 'Winterbor' kale grown under increasing photoperiod.

Photoperiod (hr)	Pigment (mg g^{-1} DM)		Total Pigment (mg $plant^{-1}$)	
	Lutein	β-carotene	Lutein	β-carotene
6	0.77 ± 0.04	0.54 ± 0.02	0.7 ± 0.1	0.5 ± 0.1
12	0.94 ± 0.02	0.62 ± 0.03	5.3 ± 0.3	3.5 ± 0.2
16	0.96 ± 0.07	0.71 ± 0.06	8.7 ± 0.6	6.4 ± 0.4
24	0.67 ± 0.03	0.51 ± 0.02	12.5 ± 0.7	9.6 ± 0.5
Contrasts				
Linear	NS	NS	***	***
Quadratic	***	**	***	***

[z] Mean composition of sampled leaf tissue of 3 replications and 32 plants ± standard error.

NS, **, *** Non-significant or significance at $p \leq 0.01$, 0.001, respectively.

Table 4.4. Mean values[z] for pigment ratios in the leaf tissues of 'Winterbor' kale grown under increasing photoperiod.

	Pigment Ratio[y]							
Photoperiod (hr)	Chl *a*:L	Chl *b*:L	Chl *a*:β	Chl *b*:β	Total Chl:L	Total Chl:β	Chl *a*:*b*	L:β
6	20.9 ± 1.2	4.8 ± 0.3	28.7 ± 0.4	6.6 ± 0.1	25.6 ± 1.4	35.3 ± 0.5	4.35 ± 0.07	1.42 ± 0.07
12	18.4 ± 0.2	4.6 ± 0.1	28.4 ± 0.6	7.1 ± 0.3	22.9 ± 0.2	35.5 ± 0.9	4.05 ± 0.06	1.55 ± 0.04
16	17.2 ± 0.8	4.4 ± 0.2	23.5 ± 1.1	6.0 ± 0.3	21.6 ± 0.9	29.5 ± 1.4	3.92 ± 0.11	1.37 ± 0.04
24	17.1 ± 0.7	4.4 ± 0.2	22.3 ± 0.9	5.7 ± 0.2	21.6 ± 0.8	28.0 ± 1.1	3.91 ± 0.10	1.32 ± 0.06
Contrasts								
Linear	**	NS	***	**	**	***	***	NS
Quadratic	***	NS	***	**	**	***	***	NS

[z] Mean composition of sampled leaf tissue of 3 replications and 32 plants ± standard error.

[y] Chl *a* = Chlorophyll *a*; Chl *b* = Chlorophyll *b*; L = lutein; β = β-carotene.

NS, **, *** Non-significant or significance at $p \leq 0.01$, 0.001, respectively.

Part 5

Biomass Production and Pigment Accumulation in Kale Grown Under Different Radiation Cycles in a Controlled Environment

Biomass Production and Pigment Accumulation in Kale Grown Under Different Radiation Cycles in a Controlled Environment

This part is a lightly revised version of a paper by the same name that will be submitted to be published in the journal HortScience by Mark G Lefsrud and Dean A Kopsell:

Lefsrud, M.G. and D.A. Kopsell. Biomass production and pigment accumulation in kale grown under different radiation cycles in a controlled environment.

My use of "we" in this part refers to my co-authors and myself. My primary contributions to this paper include (1) selection of the topic and development of the problem into a work relevant to my study of radiation cycle on kale, (2) determination of species, variety and radiation cycle levels, (3) plant propagation and nutrient control, (4) sampling and analysis, (5) most of the gathering and interpretation of the literature, (6) compiling the information into a single paper, and (7) most of the writing and editing.

Abstract

Plant growing systems have consistently used the standard 24 hr Earth day as the radiation cycle for plant growth. However, the radiation cycle can be controlled using automated systems to regulate the exact amount of time plants are exposed to irradiance (and darkness). This experiment investigated the influence of different radiation cycle periods on plant growth and carotenoid accumulation in kale (*Brassica oleracea* L. var. *acephala* D.C.). Plants were grown using a controlled environment in nutrient solutions under radiation cycle treatments of 2, 12, 24 and 48 hr, with 50% irradiance and 50% darkness during each treatment period. Total integral irradiance was the same for each treatment. The radiation cycles significantly affected kale fresh mass, dry mass, % dry matter, and the accumulation of lutein, β-carotene, and chlorophyll *a* and *b*. Maximum fresh mass occurred under the 2 hr radiation cycle treatment, whereas maximum dry mass occurred under the 12

treatment. Maximum accumulation of lutein, β-carotene, and chlorophyll *a* occurred at the 12 hr radiation cycle at values of 14.5, 13.1 and 263.3 mg $100g^{-1}$ fresh mass, respectively. Increased levels of chlorophyll, lutein and β-carotene were not required to achieve maximum fresh mass production. Consumption of fruit and vegetable crops rich in lutein and β-carotene carotenoids is associated with reduced risk of cancers and aging eye diseases. Increases carotenoid concentrations in vegetable crops would therefore be expected to increase nutritional values.

Introduction

Plants on Earth have evolved to maximize efficiency of growth and development at a radiation cycle of 24 hr, with fluctuations occurring in the length of the photoperiods. Contrast this with the Moon, where the radiation cycle consists of 14 days of irradiance and 14 days of darkness for a total radiation cycle of 28 days. This paper defines radiation cycle as the hypothetical rotation of a planetary body relative to the sun. The growth of plants under controlled environments is not confined to the standard Earth day and past research has investigated the influence of irradiance on growth parameters. Radiation cycles are of importance since plants use irradiance cues as signals to determine such things as bud formation, flowering, leaf emergence and germination (Densmore, 1997; Gottdenker et al., 2000; Riihimaki and Savolainen, 2004). In general, increases in irradiance periods result in increases in plant biomass production. Moreover, many crop plants maximize biomass accumulations under continuous irradiance (Garner and Allard, 1931; Koontz and Prince, 1986).

There is conflicting literature on plant development responses to changes in radiation cycles. Changes in radiation cycles from the standard 24 hr can results in decreased fresh mass, dry mass, chlorophyll content, stem elongation and leaf area (Berry et al., 1986; Bonde, 1955; Bonde, 1956; Garner and Allard, 1931; Morrow et al., 1987; Takano et al., 1987). The amount of these decreases is usually dependent upon plant species, variety, and the radiation cycle chosen. Further, other research points to an increase in plant growth and pigment accumulation under changes in radiations cycles. Tomatoes (*Lycopersicon esculentum* Mill.) grown with a radiation cycle of 4 hr had greater biomass accumulation than plants grown with a 24 hr radiation cycle (Bonde, 1955). Tomatoes, lettuce (*Lactuca sativa* L.) and wheat (*Triticum aestivum* L.) biomass decreased when grown under a radiation cycle of 60:30 min (irradiance:dark conditions); however, an increase was reported for one variety of wheat (Morrow et al., 1987).

Carotenoids are C_{40} isoprenoid compounds that form lipid soluble yellow, orange, and red pigments in higher plants, bacteria, alga, and fungi (Sandmann, 2001; Zaripheh and Erdman, Jr., 2002). They are secondary plant compounds which are divided into two groups; the oxygenated xanthophylls such as lutein (3R,3'R,6'R β,ε-carotene-3,3'diol) and zeaxanthin (3,3'R-β,β-carotene-3,3'diol), and the hydrocarbon carotenes such as β-carotene (β, β-carotene) and lycopene (ψ, ψ-carotene) (Zaripheh and Erdman, Jr., 2002). In plants, carotenoids are used as antenna pigments to funnel light energy to the photosynthetic reaction center. Carotenoids are in close proximity to the chlorophyll molecules and absorb energy to prevent damage to the

photosynthetic system (Miki, 1991; Tracewell et al., 2001). Dietary intake of foods rich in lutein and β-carotene has been associated with reduced risk of lung cancer, cataracts, and age-related macular degeneration (Ames et al., 1995; Landrum and Bone, 2001; Le Marchand et al., 1993). The U.S. Dept. Agr. nutrient database ranks kale (*Brassica oleracea* L. var. *acephala* D.C.) as the highest vegetable source of lutein and β-carotene carotenoids, making it an excellent dietary source of these compounds (Holden et al., 1999; Kurilich et al., 1999; U.S. Dept. Agr., 2005).

Light is critical for plant growth and development, and irradiance cycles can be controlled by growers in artificial environments. What remains unclear is the effect of different radiation cycles on the production of plant secondary compounds, such as carotenoids. Therefore, the goal of this study was to investigate responses in biomass production and pigment accumulations in kale grown under different radiations cycles. In addition, it was our intent to determine which radiation cycle treatment resulted in maximum carotenoid concentrations.

Material and Methods

Plant Culture

'Winterbor' kale (Johnny's Selected Seed, Winslow, Maine) were seeded into rockwool growing cubes (Grodan A/S, Dk-2640, Hedehusene, Denmark) and germinated in a greenhouse (22 °C day/ 14 °C night) under natural lighting conditions (Durham, N.H., Lat. 43° 09' N) on 18 February, 23 March, and 8 May 2004. Peter's 20.0N-6.9P-16.6K water-soluble fertilizer (Scotts, Marysville, Ohio) was applied at

200 mg L^{-1} every five days. After 2 weeks, the plants were transferred to 38 L plastic containers (Rubbermaid Inc., Wooster, Ohio). Eight plants were placed into 2 cm round holes set at 10.6 x 9.5 cm spacing on each container lid. Four containers were placed into each of four growth chambers (E15, Conviron, Winnipeg, Man.), with chambers as experimental treatments and three replications over time. Growth chamber temperature set point was 20 ± 1 °C. The photosynthetically active radiation (PAR) was measured (QSO-ELEC, Apogee Instruments; Logan, Utah) at six locations, without plants, on top of each tub at the four corner plant holes and between the two side middle plant holes and averaged. Irradiance levels were measured at the beginning and confirmed at the end of each replication. Cool white fluorescent and incandescent bulbs delivered an average irradiance of 500 ± 100 μmol m^{-2} s^{-1}. Radiation cycles (CYCLE) treatments were 2, 12, 24 and 48 hr. For each treatment, the irradiance period was set at 50% of the cycle duration to produce irradianc:dark ratios of 1:1, 6:6, 12:12, and 24:24 hr for the radiation cycle of 2, 12, 24 and 48 hr, respectively. The bulbs reached 96% peak output within 5 min. This warm-up period resulted in the 2 hr radiation cycle having 0.4% less light than the other treatments.

The plants were grown in 30 L of a nutrient solution (Hoagland and Arnon, 1950). Elemental concentrations of the nutrient solutions were (mg L^{-1}): N (105), P (15.3), K (117), Ca (80.2), Mg (24.6), S (32.0), Fe (0.5), B (0.25), Mo (0.005), Cu (0.01), Mn (0.25), and Zn (0.025). The electrical conductivity (EC) of the starting nutrient solution was 0.7 mS cm^{-1} and pH was measured at 5.6. Nutrient solutions were replaced every week throughout the experiment to eliminate potential pH drift

and refresh solutions to initial nutrient concentrations. Solutions were aerated with an air blower (Model 25E133W222, Spencer, Winsor, Conn.) connected to air stones. Deionized water was added daily to maintain 30 L in each container. Nutrient solutions were replaced every week throughout the experiment to refresh the solution to the initial nutrient concentrations.

During each experimental treatment, plants were grown for 3 weeks and harvested on 25 March, 27 April, and 12 June 2004. At harvest, shoot and root tissues were separated and weighed. The fourth expanded leaf from the crown was selected and a 4 cm^2 piece was removed from the middle of each of eight plants in four container and then combined to form one sample. Samples were stored at –80 °C prior to lyophilization. Fresh mass was measured at harvest and the remaining shoot material was dried at 60 °C for no less than 72 hr, at which time shoot dry mass was calculated.

Carotenoid and Chlorophyll Determination

Frozen kale samples were lyophilized for a minimum of 72 hr (6L FreeZone, LabConCo, Kansas City, Mo.). Freeze-dried tissues were ground and homogenized prior to pigment extractions. Pigments were extracted and separated according to Kopsell et al. (2004), a procedure which is based on the method of Khachik et al. (1986). A 0.100 g sub-sample was placed into a Potter-Elvehjem tissue grinder tube (Kontes, Vineland, N.J.) and hydrated with 0.80 mL of deionized water. The sample was placed in a 40 °C water bath for 20 min. After hydration, 0.80 mL of an internal

standard, ethyl-β-apo-8'-carotenoate (Sigma Chemical Co., St. Louis, Mo.) and 2.50 mL of HPLC grade tetrahydrofuran (THF) were added to the sample. The sample was homogenized in the tube with ~25 insertions with a Potter-Elvehjem tissue grinder pestle attached to a drill press (Model Craftsman 15 inch Drill Press, Sears, Roebuck and Co., Hoffman Estates, Ill.) at 540 rpm. The sample tube was kept immersed in ice to dissipate excess heat from friction. The tube was placed into a centrifuge (Model 225, Fisher Scientific, Suwanee, Ga.) for 3 min at 500 g_n. The supernatant was removed with a Pasteur pipet, placed into a conical 15-mL test tube, capped and held on ice. The sediment was re-suspended in 2.00 mL THF and homogenized with ~25 insertions of the grinding pestle. The tube was centrifuged for 3 min at 500 g_n and the supernatant was collected and combined with the first extracted supernatant. The extraction procedure was repeated twice more until the supernatant was colorless. The sediment was discarded and the combined 4 supernatants were placed in a 40 °C water bath and reduced to 0.50 mL using nitrogen gas (N-EVAP 111, Organomatic Inc., Berlin, Mass.). Additions of 2.50 mL of MeOH and 2.00 mL of THF were made to the sample to achieve a final volume of 5.00 mL. Samples were vortexed and filtered through a 0.2 μm polytetrafluoroethylene (PTFE) filter (Model Econofilter PTFE 25/20, Agilent Technologies, Wilmington, Del.) using a 5-mL syringe (Becton, Dickinson and Company, Franklin Lakes, N.J.) prior to high performance liquid chromatograph (HPLC) analysis.

A HPLC unit with photodiode array detector (Agilent 1100, Agilent Technologies, Palo Alto, Calif.) was used for pigment separation. All samples were analyzed for carotenoid compounds using a Vydac RP C_{18} 5.0 μm 250 x 4.6 mm column (201TP54, Phenomenex, Torrance, Calif.) fitted with a 4 x 3.0 mm, 7.0 μm guard column compartment. The column was maintained at 16 °C using a thermostatic column compartment. Eluents were A: 75% acetronitrile, 20% methanol, 4.93% hexane, 0.05% BHT, and 0.013% triethyamine (TEA) and B: 50% acetonitrile, 25% THF, 24.98% hexane and 0.013% TEA. The flow rate was 0.70 $mL \cdot min^{-1}$ and the gradient was 100% eluent A for 30 min; 50% A and 50% B for 2 min; 100% B for 2 min; and 50% A and 50% B for 2 min. The eluent was returned to 100% A for 10 min prior to the next injection. Eluted carotenoid and chlorophyll compounds from a 20.0 μL injection were detected at 452 nm (carotenoid pigments and internal standard), 652 nm for chlorophyll *a* (Chl *a*), and 665 nm for chlorophyll *b* (Chl *b*), with data collected, recorded and integrated using 1100 HPLC ChemStation Software (Agilent Technologies, Palo Alto, Calif.). Peak assignment was performed by comparing retention times and line spectra obtained from the photodiode array detection with authentic standards (lutein from Carotenature, Lupsingen, Switzerland; β-carotene, Chl *a*, and Chl *b* from Sigma Chemical Co., St. Louis, Mo.). Concentrations of external standards were determined spectrophotometrically using the following $E_{1cm}^{1\%}$ values: lutein, 2550 in ETOH, λ_{max} = 445 nm; β-carotene, 2592 in hexane, λ_{max} = 452 nm; Chl *a*, 819 in ETOH, λ_{max} = 665 nm; and Chl *b*, 441 in ETOH, λ_{max} = 649 nm (Davies and Köst, 1988). Standard

reference material® (Slurried Spinach 2385, National Institute of Science and Technology, Gaithersburg, Md.) was used for method validation. Internal standard % recovery ranged from 70 to 96%.

Statistical Analysis

Main effects were analyzed by one-way ANOVA using SAS (Cary, NC). The relationship between experimental dependent variables and radiation treatments were determined by regression analysis. Orthogonal polynomials were used to study changes associated with increasing photoperiod treatments by partitioning the sum of squares into components that were associated with linear and quadratic terms (Nogueira, 2004).

Results and Discussion

Kale shoot tissue fresh mass (FM) responded ($p \leq 0.001$) to increases in radiation cycle treatments and ranged from 57.3 to 40.2 g plant^{-1} for the 2 hr and 24 hr radiation cycles, respectively (Table 5.1[a]). Kale shoot tissue dry mass (DM) responded ($p \leq 0.001$) to increases in radiation cycle treatments and ranged from 3.6 to 4.8 g plant^{-1} for the 48 hr and 12 hr radiation cycles, respectively (Table 5.1). Kale FM decreased, then increased in a quadratic response (FM = 492.0 – 8.8(CYCLE) + 0.1 (CYCLE)^2; r^2=0.39, $p \leq 0.001$) as the radiation cycle treatments increased from 2 to 48 hr, whereas kale DM first increased, then decreased (DM = 38.2 – 0.27(CYCLE) + 0.002(CYCLE)^2; r^2=0.27, $p \leq 0.001$) over increasing radiation cycle

[a] All tables and figures are located in the appendix at the end of this part.

treatments (Table 5.1). Radiation cycle treatments also influenced the % dry matter (%DM; $p \leq 0.001$) found in the kale shoot tissues. The %DM ranged from 7.9% to 9.4% for the 2 hr and 24 hr radiation cycle, respectively (Table 5.1). The %DM increased, then decreased in quadratic response to increases in radiation cycle treatments (%DM = 7.6 + 0.1(CYCLE) – 0.0001(CYCLE)^2; $r^2 = 0.54$, $p \leq 0.001$).

The 2 hr radiation cycle resulted in the largest FM production in kale, however maximum DM production occurred under the 12 hr radiation cycle. Garner and Allard (1931) also reported decreases in biomass in response to increases in radiation cycle for a number of other plant species. Higher biomass production was reported for beet (*Beta vulgaris* L.) grown under radiation cycles of 10 min, and soybean (*Glycine max* L. Merr.) grown under radiation cycles of 2 hr, both showed increases in fresh biomass when compared to a 24 hr radiation cycle treatments. Biomass decreases were also reported for tomato, lettuce, and wheat as radiation cycles increased from 60:30 min (irradiance:dark conditions) to 16:8 h (irradiance:dark conditions)(Morrow et al., 1987). However, one of the varieties of wheat had a minor increase in biomass with the 60/30 min radiation cycle, increasing slightly from 82.4 to 86.5 g plant^{-1}.

Kale leaf tissue lutein concentrations responded to increases in radiation cycles ($p \leq 0.001$). Maximum lutein accumulation (14.5 mg 100 g^{-1} FM) occurred under the 12 hr radiation cycle, whereas the lowest lutein concentration (11.2 mg·100 g^{-1} FM) occurred for the 2 hr radiation cycle. Lutein concentrations increased, then decreased in a quadratic response to increasing radiation cycle (Lutein = 11.5 + 0.19(CYCLE) – 0.0004 (CYCLE)^2; $r^2 = 0.27$, $p \leq 0.001$). Radiation cycle influenced

kale leaf tissue β-carotene concentrations ($p \leq 0.001$). Maximum β-carotene accumulation was 13.1 mg $100g^{-1}$ FM for the 12 hr radiation cycle, whereas the lowest β-carotene accumulation (8.3 mg 100 g^{-1} FM) occurred during the 24 hr radiation cycle. There was no reported trend in kale β-carotene concentrations when plotted against increasing radiation cycle (Table 5.2).

The concentrations of kale leaf tissue Chl *a* ($p \leq 0.001$), Chl *b* ($p \leq 0.001$) and total chlorophyll (TChl) ($p \leq 0.001$) pigments were influenced by increases in radiation cycle (Table 5.2). Maximum Chl *a* and Chl *b* levels occurred at the 12 hr and 24 hr radiation cycle, respectively. Maximum TChl accumulation occurred at the 24 hr radiation cycle. Chlorophyll pigment concentrations in the kale leaf tissues increased, then decreased in quadratic responses to increases in radiation cycle: Chl *a* (Chl $a = 179.9 + 4.9(\text{CYCLE}) - 0.09(\text{CYCLE})^2$; $r^2 = 0.42$, $p \leq 0.001$); Chl *b* (Chl $b = 38.3 + 0.17(\text{CYCLE}) - .004\ (\text{CYCLE})^2$; $r^2 = 0.69$, $p \leq 0.001$); and TChl (TChl $= 218.1 + 6.6(\text{CYCLE}) - 0.13(\text{CYCLE})^2$; $r^2 = 0.49$, $p \leq 0.001$). Bonde (1955) reported the highest accumulation of chlorophyll in tomato occurred at an 8 hr radiation cycle, with decreases in chlorophyll accumulation under 4, 12 and 19 hr radiation cycle. Previous research performed by our group has shown correlations between chlorophyll and carotenoid pigments in kale, due to both genetic and environmental effects (Kopsell et al., 2004). In the current study, the largest chlorophyll accumulation occurred for the 24 hr radiation cycle, whereas the highest accumulation of carotenoid pigments occurred under the 12 hr radiation cycle.

The carotenoid content of vegetable crops is normally reported on a FM basis to equate to typical consumption patterns (Holden et al., 1999); however, due to the popularity of dried plant materials in dietary capsules as antioxidant sources, concentrations of the kale leaf tissue pigments was also calculated on a DM basis (Table 5.3). Radiation cycle influenced lutein ($p \leq 0.001$) and β-carotene DM accumulations ($p \leq 0.001$). The lutein DM concentrations decreased linearly with increasing radiation cycle (Lutein DM = 1.1 – 0.002(CYCLE); $r^2 = 0.09$, $p = 0.036$). The β-carotene DM concentrations increased, then decreased in a quadratic response to increasing radiation cycle (β-carotene DM = 1.0 – 0.02(CYCLE) + $0.0003(\text{CYCLE})^2$; r^2=0.16, $p = 0.021$).

Kale leaf tissue biomass and lutein and β-carotene concentrations changed in response to increases in the radiation cycle. Lutein and β-carotene concentrations, expressed on both a FM and DM basis, peaked under the 12 hr radiation cycle treatment, whereas kale FM was highest under the 2 hr radiation cycle treatment. Heance, maximum FM production does not always occur concurrently with maximum accumulation of chlorophyll and carotenoid pigments. Radiation cycles can be manipulated in controlled environments to maximize plant biomass production and concentrations of plant secondary compounds, such as carotenoids. Enhancement of carotenoid pigment concentrations would be expected to increase the nutritional value of kale.

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Appendix

Table 5.1. Mean fresh and dry biomass accumulation (g plant^{-1}) in shoot tissues of 'Winterbor' kale grown under increasing radiation cycle treatments[z].

Time (hr)[y]	Plant Biomass		
	Fresh Mass	Dry Mass	% Dry mass
2	57.3 ± 2.8	4.5 ± 0.2	7.9 ± 0.2
12	55.3 ± 2.4	4.8 ± 0.2	8.8 ± 0.1
24	40.2 ± 2.0	3.8 ± 0.2	9.4 ± 0.1
48	43.0 ± 2.0	3.6 ± 0.2	8.5 ± 0.2
Contrasts			
Linear	***	***	NS
Quadratic	***	***	***

[z] Mean composition of sampled leaf tissue of 3 replications and 32 plants per replication ± standard error.

[y] Radiation cycle time composed of 50% irradiance and 50% darkness.

NS, *** Non-significant or significance at $p \leq 0.001$, respectively.

Table 5.2. Mean pigments concentrations expressed on a fresh mass basis (FM) in the leaf tissues of 'Winterbor' kale grown under increasing radiation cycle treatments[z].

Time (hr)[y]	Pigment Concentration (mg 100 g^{-1} FM)				
	Lutein	β-carotene	Chl *a*[x]	Chl *b*[x]	Total Chl[x]
2	11.2 ± 0.2	9.6 ± 0.2	184.5 ± 3.7	40.5 ± 0.7	224.9 ± 4.4
12	14.5 ± 0.6	13.1 ± 0.5	236.3 ± 12.0	56.9 ± 2.5	293.2 ± 14.4
24	12.8 ± 0.3	8.3 ± 0.3	235.1 ± 6.3	58.1 ± 1.4	293.3 ± 7.5
48	11.6 ± 0.2	10.3 ± 0.2	197.9 ± 3.8	41.6 ± 0.9	239.4 ± 4.6
Contrasts					
Linear	NS	NS	NS	NS	NS
Quadratic	***	NS	***	***	***

[z] Mean composition of sampled leaf tissue of 3 replications and 32 plants per replication ± standard error.

[y] Radiation cycle time composed of 50% irradiance and 50% darkness.

[x] Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*.

[NS, ***] Non-significant or significance at $p \leq 0.001$, respectively.

Table 5.3. Mean pigment concentrations as a function of dry mass (DM) in the leaf tissues of 'Winterbor' kale grown under increasing radiation cycle treatments[z].

Time (hr)[y]	Pigment ($mg \cdot g^{-1}$ DM)	
	Lutein	β-carotene
2	1.02 ± 0.02	0.87 ± 0.01
12	1.19 ± 0.03	1.07 ± 0.02
24	0.94 ± 0.02	0.62 ± 0.03
48	0.99 ± 0.02	0.88 ± 0.02
Contrasts		
Linear	*	NS
Quadratic	NS	*

[z] Mean composition of sampled leaf tissue of 3 replications and 32 plants ± standard error.

[y] Radiation cycle time composed of 50% irradiance and 50% darkness.

[NS, *] Non-significant or significance at $p \leq 0.05$, respectively.

Part 6

Effect of Wavelength on Carotenoid Accumulation in Kale

Effect of Wavelength on Carotenoid Accumulation in Kale

This part is a lightly revised version of a paper by the same name that will be submitted to be published in the journal HortScience by Mark G Lefsrud, Dean A Kopsell, and Carl Sams:

Lefsrud, M.G., D.A. Kopsell, and C.E. Sams. Effect of wavelength on carotenoid accumulation in kale.

My use of "we" in this part refers to my co-authors and myself. My primary contributions to this paper include (1) selection of the topic and development of the problem into a work relevant to my study of wavelength on kale, (2) determination of species, variety and LED wavelength levels, (3) plant propagation and nutrient control, (4) sampling and analysis, (5) most of the gathering and interpretation of the literature, (6) compiling the information into a single paper, and (7) most of the writing and editing.

Abstract

Kale plants (*Brassica oleracea* L. var. *acephala* D.C.) were grown with differing wavelength light emitting diode (LED) array's to determine changes in the accumulation of chlorophylls, carotenoids, and glucosinolates. The plants were cultured in a growth chamber in a hydroponic system under wavelength treatments of 730, 640, 525, 440 and 400 nm. The wavelength treatments significantly affected accumulation in kale leaves of chlorophyll *a*, chlorophyll *b*, and lutein, but not β-carotene nor the glucosinolates, when expressed on a fresh mass (FM) basis. The maximum accumulation of chlorophyll *a* and *b*, and lutein occurred at the wavelength of 640 nm measuring 85.7, 66.2 and 11.2 mg 100 g^{-1} FM, respectively. The maximum accumulation of β-carotene occurred at 440 nm measuring 4.0 mg 100 g^{-1} FM. However, when lutein was measured on a dry mass basis the maximum accumulation was shifted to 440 nm. Therefore, irradiance wavelength control can be used to change pigment accumulation in kale.

Introduction

Plant pigments have specific wavelength absorption patterns known as absorption spectra. However, absorption of light at certain wavelengths does not always directly correlate with biosynthesis of chlorophylls and carotenoids. The absorption of specific wavelengths of light required for biosynthesis of chorophyll is known as the action spectrum. Chlorophyll absorbs wavelengths of light strongly in the red and blue region, with less absorption occurring in the green wavelengths. In acetone, chlorophyll *a* (Chl *a*) exhibits peak absorption at 430 and 663 nm, while chlorophyll b (Chl *b*) peaks at 453 and 642 nm. The pigments lutein and β-carotene (in acetone) absorb strongly in the blue region of light with peaks occurring at 448 and 454 nm, respectively (Hopkins and Huner, 2004; Taiz and Zeiger, 1998). However, maximum light absorption can shift up to 38 nm, depending on the specific environment surrounding the chloroplasts (Heber and Shuvalov, 2005; Jouni and Wells, 1996). The maximum biosynthesis of pigments for the action spectrum of wheat (*Triticum aestivum* L.) occurs at 447 and 646 nm for chlorophylls and β-carotene, respectively (Ogawa et al., 1973), while corn's (*Zea mays* L.) action spectrum is largest at 445 and 650 nm (Koski et al., 1951). Wavelengths of light at 500 nm and levels greater than 700 nm result in very little biosynthesis of chlorophyll (Koski et al., 1951; Ogawa et al., 1973).

Research to determine environmental and genetic factors that affect the accumulation of plant pigments and glucosinolates has been completed by a number of researchers (Charron and Sams, 2004; Kopsell et al., 2003; Kushad et al., 1999;

Lefsrud et al., 2005; Lefsrud et al., 2006; Rosa, 1997; Rosa and Rodrigues, 1998). Environmental factors, such as temperature and irradiance levels, can have strong influences on the accumulation of pigments and glucosinolates (Charron and Sams, 2004; Lefsrud et al., 2005; Lefsrud et al., 2006). The use of filters, interference filters and/or bulbs to produce wavelengths of light in the desired range, resulted in reductions in the irradiance levels, with larger reductions occurring at the shorter wavelengths (Virgin, 1993). However, research into wavelength on plant growth has used very low irradiance levels, less than 13 μmol m^{-2} s^{-1} (Ogawa et al, 1973; Wang et al., 2001). Lefsrud et al. (2006) reported that changes in irradiance levels could result in significant changes in the measured carotenoid and chlorophyll levels in kale.

Changes in wavelength could result in changes in biomass production and morphology of the plant due to changes in the red/far red ratio (Brown et al., 1995; Heraut-Bron et al., 2001; Hoenecke et al., 1992; Taiz and Zeiger, 1998) as well as blue light (Hoenecke et al., 1992; Gauthier et al., 1997; Taiz and Zeiger, 1998). These traditional methods of wavelength control also showed changes in Chl *a*/*b* ratio (Walters and Horton, 1995), Chl biosynthesis and action spectrum (Anstis and Northcot, 1974; Björn, 1967; French, 1991; Koski et al., 1951; Ogawa et al., 1973; Virgin, 1993), β-carotene biosynthesis (Ogawa et al., 1973) and decreases in Chl *b* under UV-B light (Taiz and Zeiger, 1998; Quaderi and Reid, 2005). Work by Virgin (1993) showed that relative accumulation of Chl is wavelength dependent for bean (*Pinguicula vulgaris* L.), pea (*Pisum sativum* L.), potato (*Solanum tuberosum* L.) and wheat, with each species and individual tissues in the plant responding differently.

Secondary plant compounds can also be affected by wavelength. Wang et al. (2001) reported that the plant compound artemisinin found in wormwood (*Artemisia annua* L.) varied in concentration in the plant, depending on the wavelength of irradiance. Colored plastic mulches used to vary the irradiance spectrum showed that using blue mulches increased the glucosinolates concentration in the roots of turnip (*Brassica campestris* L.) (Antonious et al., 1996).

With the development of light emitting diodes (LED), specific wavelengths of light can now be applied at higher irradiance levels and allow further determination of the effects of wavelength on plant development. However, limited research has been preformed on plant growth under LED lights. Plants have been grown under red LED lights, resulting in plant elongation and reduced biomass that was corrected with the supplementation of blue LED light for both lettuce (*Lactuca sativa* L.; Hoenecke et al., 1992) and pepper (*Capsicum annuum* L.; Brown et al., 1995).

Kale (*Brassica oleracea* L. var. *acephala* D.C.) is an excellent source of glucosinolates (GS) (Stoewsand, 1995) and carotenoids (Holden et al., 1999; Kurilich et al., 1999; U.S. Dept. Agr., 2002). However, kale has low consumption rates, with per capita fresh intake in the U.S. averaging less than 0.33 kg $year^{-1}$ (Lucier and Plummer, 2003). GS are sulfur-containing compounds responsible for the distinct smell and flavor of *Brassica* vegetables, with 12 different GS compounds identified in the *Brassica* species. These GS can impart negative flavor characteristics in high concentrations (Kopsell et al., 2003). The U.S. Dept. Agr. rates kale as the highest source of lutein and β-carotene of any vegetable (Holden et al., 1999; U.S. Dept. Agr., 2002). Carotenoids are yellow, orange, and red plant lipid soluble pigments

produced by plants, algae and bacteria, which cannot be synthesized in mammals. In plants, carotenoids are used as antenna pigments to funnel light energy to the photosynthetic reaction center. These carotenoids are in close proximity to the chlorophyll molecules and quench the energetic triplet state of the chlorophyll molecule to prevent damage to the photosynthetic system (Marschner, 1997; Miki, 1991; Taiz and Zeiger, 1998; Tracewell et al., 2001). Lutein and β-carotene carotenoids possess important human health properties. Dietary intake of foods rich in lutein and β-carotene has been associated with reduced risk of lung cancer, cataracts, and age-related macular degeneration (Ames et al., 1995; Landrum and Bone, 2001; Le Marchand et al., 1993), while diets high in vegetables with GS reduce the risk of cancer (Stoewsand, 1995; van Poppel et al., 1999).

Light is critical for plant growth and development, and wavelengths can easily be controlled by growers in artificial growing environments. Therefore, the goal of this study was to investigate the influences of five different wavelengths of light on plant biomass and accumulation patterns of chlorophylls, carotenoids and GS compounds in the leaf tissues of kale.

Material and Methods

Plant Culture

'Winterbor' kale (Johnny's Selected Seed, Winslow, Maine) were seeded into rockwool growing cubes (Grodan A/S, Dk-2640, Hedehusene, Denmark) and germinated in a growth chamber (E30B, Percival, Boone, Iowa) at 20 ± 1 °C under cool white fluorescent (17W) and incandescent (40W) bulbs. The growth chamber

light intensity photosynthetic active radiation (PAR) was measured at 275 ± 10 μmol m^2 s^{-1} (Model QSO-ELEC, Apogee Instruments; Logan, Utah). Peter's 20N-6.9P-16.6K water-soluble fertilizer (Scotts, Marysville, Ohio) was applied every five days at a rate of 200 mg L^{-1}. After 2 weeks, the plants were transferred to 11 L plastic containers (Rubbermaid Inc., Wooster, Ohio). Six plants were placed into 2 cm round holes at 10.6 by 9.5 cm spacing in each container lid. One container was placed into a growth chamber. The growth chamber light intensity was measured at 275 ± 10 μmol m^2 s^{-1} and air temperature was held throughout the experiment at 20 ± 1 °C. The plants were grown in 10 L of half strength modified Hoagland nutrient solution (Hoagland and Arnon, 1950). Elemental concentrations of the nutrient solutions were (mg L^{-1}): N (105), P (15.3), K (117), Ca (80.2), Mg (24.6), S (32.0), Fe (0.5), B (0.25), Mo (0.005), Cu (0.01), Mn (0.25), and Zn (0.025). The EC of the starting nutrient solution was 7 S m^{-1} and pH was measured at 5.6. Solutions were aerated with an aquarium air pump (MK-1504, Wal-Mart, Bentonville, Ark.) connected to air stones. Deionized water was added daily to maintain 10 L in each container. After one week in the growth chamber with incandescent and fluorescent bulbs the tub and plants were moved to a growth chamber with an LED array. Air temperature was held at 20 ± 1 ºC and aeration was provided as described above.

The LED array capable of providing five wavelengths and independent control was acquired from ORBITEC (Madison, Wisc.). The wavelengths provided by the LEDs were 730, 640, 525, 440 and 400 nm. PAR for each wavelength was

measured at leaf canopy height at the beginning of each treatment (Table 6.1[a]). The LED array produced a small amount of background light (0.2 μmol m^{-2} s^{-1} PAR irradiance) of when the system was electrified but turned off. The experiment consisted of growing a single plant directly under the LED array with five guard plants surrounding it at the spacing determined by the holes in the container lid. Irradiance was only supplied by the LED array with the chamber light sealed. The plants were grown for seven days under the LED array with a 16 hr photoperiod. Final harvest occurred at 10 hours into the irradiance cycle. The experiment was replicated three separate times for all five wavelengths.

At harvest, shoot tissues, from the single plant directly underneath the LED array (6 by 6 cm) were removed and weighed. Leaves and parts of leaves that were more than 1 cm outside the boundaries of the array were removed from the sample and stored separately. Shoot tissues were washed with soap (Aquet, Bel-art Products, Pequannock, N.J.), rinsed, and blotted dry with paper towels. The sample was stored at –20 °C prior to lyophilization

Carotenoid and Chlorophyll Determination

Frozen kale samples were lyophilized for a minimum of 72 hr (6L FreeZone, LabConCo, Kansas City, Mo.). The dried tissues samples were ground with dry ice in a kitchen grinder (Handy Chopper Plus, HC 3000, Household Products Inc., Shelton, Conn.). Pigments were extracted and separated according to Kopsell et al. (2004). A 0.100 g sub-sample was placed into a Potter-Elvehjem tissue grinder tube

[a] All tables and figures are located in the appendix at the end of this part.

(Kontes, Vineland, N.J.) and hydrated with 0.80 mL of deionized water. The sample was placed in a 40°C water bath for 20 minutes. After hydration, 0.80 mL of the internal standard, ethyl-β-apo-8'-carotenoate (Sigma Chemical Co., St. Louis, Mo.) and 2.50 mL of tetrahydrofuran (THF) stabilized with 25 ppm 2,6-Di-*tert*-butyl-4-methoxyphenol (BHT) were added. The sample was homogenized in the tube with 25 insertions with a Potter-Elvehjem tissue grinder pestle attached to a drill press (Model Craftsman 15 inch Drill Press, Sears, Roebuck and Co., Hoffman Estates, Ill.) at 540 rpm. The sample tube was kept immersed in ice. The tube was placed into a clinical centrifuge for 3 minutes at 500 g_n. The supernatant was removed with a Pasteur pipet, placed into a conical 15 ml test tube, capped and held on ice. The sample pellet was resuspended in 2.00 ml THF and homogenized with 25 insertions of the grinding pestle. The tube was centrifuged for 3 minutes at 500 g_n and the supernatant was collected and combined with the first extracted supernatant. The extraction procedure was repeated twice more until the supernatant was colorless. The pellet was discarded and the combined four supernatants were placed in a 40 °C water bath and reduced to 0.50 ml using nitrogen gas (N-EVAP 111, Organomatic Inc., Berlin, Mass.). 2.50 mL of MeOH and 2.00 mL of THF were added to the sample, which was then vortexed and filtered through a 0.2 μm polytetrafluoroethylene (PTFE) filter (Econofilter PTFE 25/20, Agilent Technologies, Wilmington, Del.) using a 5 mL syringe (Becton, Dickinson and Company, Franklin Lakes, N.J.) prior to high performance liquid chromatograph (HPLC) analysis.

A HPLC unit with photodiode array detector (Agilent 1100, Agilent Technologies, Palo Alto, Calif.) was used for pigment separation. All samples were analyzed for carotenoid compounds using a Pronto SIL 5.0 μm 250 x 4.6 mm column (200-5-C_{30}, Bischoff Chromatography, Leonberg, Germany) fitted with a 4 x 3.0 mm, 7.0 μm guard column compartment. The column was maintained at 16 °C using a thermostatic column compartment. The eluent was 11% methyl-tert butyl ether (MTBE), 89% methanol, 0.1% triethyamine (TEA) (v/v). The flow rate was 1.00 mL min^{-1} for 55 minutes. Eluted compounds from a 20.0 μL injection were detected at 453 nm (carotenoids and internal standard) and 652 nm (chlorophylls), with data collected and integrated using 1100 HPLC ChemStation Software (Agilent Technologies, Palo Alto, Calif.). Peak assignment was performed by comparing retention times and line spectra obtained from the photodiode array detection with authentic standards (lutein from Carotenature, Lupsingen, Switzerland; β-carotene, Chl *a*, Chl *b* from Sigma Chemical Co., St. Louis, Mo.). Recovery rates of ethyl-β-apo-8'-carotenoate during extraction were above 90%.

Statistical Analysis

Main effects were analyzed by one-way ANOVA using SPSS (Chicago, Ill.). The relationship between experimental dependent variables and wavelength treatments were determined by regression analysis.

Results

The effects of wavelength treatments on the concentrations of lutein, β-carotene, and Chl pigments were measured for the kale shoot tissues (Table 6.2). Lutein concentrations responded to changes in wavelength ($p = 0.031$), but β-carotene was not significant ($p = 0.259$). Maximum lutein accumulation fresh mass (FM; 11.2 mg 100 g^{-1} FM) occurred under the highest PAR at 640 nm, while the lowest lutein concentrations (6.9 mg 100 g^{-1} FM) occurred under the lowest PAR at 730 nm wavelength. The concentrations of Chl *a* ($p = 0.007$), Chl *b* ($p \leq 0.001$) and Total Chl ($p = 0.002$) pigments were influenced by changes in wavelength PAR (Table 6.2). Maximum chlorophyll accumulation occurred under the highest PAR at 640 nm.

No data was collected on whole plant fresh or dry mass (DM), due to the sampling method. The carotenoid content of vegetable crops is normally reported on a FM basis to equate to typical consumption patterns (Holden et al., 1999); however, due to the popularity of dried materials in dietary supplements, as sources of antioxidants, the accumulation of the kale pigments were also calculated on a DM basis (Table 6.3). Wavelength treatment of % dry matter (%DM; $p = 0.679$) was not significant in the kale shoot tissues, which ranged from 7.9% to 9.1% (Table 6.3). The treatments had a significant effect on lutein DM ($p = 0.027$), but not on β-carotene DM ($p = 0.073$). However, no linear correlation was measured for the lutein DM but the β-carotene DM increased linearly (β DM = 0.63 + 0.0001 λ; $r^2 = 0.35$, $p = 0.023$) as the wavelength increased.

Total chlorophyll to lutein (Total Chl:L), total chlorophyll to β-carotene (Total Chl:β), and chlorophyll *a* to *b* (Chl *a*:*b*) ratios were calculated (Table 6.4). The Total Chl:L ($p = 0.028$), Total Chl:β ($p = 0.038$), and Chl *a*:*b* ($p = 0.007$) all had treatment effects. Decreasing then increasing quadratic changes were calculated for the Chl *a*:*b* ratio (Chl *a*:*b* = $9.28 - 0.03\ \lambda + 0.00002\ \lambda^2$; $r^2 = 0.58$, $p = 0.005$) as the wavelength increased.

The glucosinolate (GS) content was measured and recorded in Table 6.5. None of the GS compounds were statistically affected by wavelength, except for sinigrin, an aliphatic GS, that showed a significant trend. Sinigrin concentration increased linearly as the wavelength increased (Sinigrin = $-39.1 + 0.09\ \lambda$; $r^2 = 0.27$, $p = 0.050$).

Discussion

Specific wavelengths of light can influence growth and development of secondary compounds in plants. Numerous researchers have investigated the Chl action spectrum (Anstis and Northcot, 1974; Björn, 1967; French, 1991; Koski et al., 1951; Ogawa et al., 1973; Virgin, 1993) and Ogawa et al. (1973) measured the action spectrum for biosynthesis of β-carotene in wheat. Measurement of LEDs on secondary plant compounds is limited, with no studies found that used specific wavelength as the irradiance source. Under low levels of white light the Chl *a*/*b* ratio differed in *Arabidopsis* varieties, but were normalized under red light (Walters and Horton, 1995). In another study, the amount of glucosinolates in the roots of turnip

was controlled by using different colored mulches (Antonious et al., 1996). Plants grown using blue mulch had the highest level of glucosinolates, which decreased using green mulch.

In our study, peak accumulation of the chlorophylls, lutein and the glucosinolates, sinigrin, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin occurred under the 640 nm wavelength, which corresponded to the maximum measured irradiance. A second peak was also measured at 440 nm, corresponding to the second highest irradiance, for the chlorophylls, lutein, glucobrassicin, and neoglucobrassicin. The peak for β-carotene was reversed with the major peak at 440 nm and second peak at 640 nm. When lutein was measured on a dry mass basis the maximum accumulation was shifted from the 640 to 440 nm wavelength. Research by Wang et al. (2001) studying *Artemmisia annua* L. (wormwood), determined that wavelength also influenced the accumulation of the anti-malarial compound artemisinin. The wormwood plants were grown under white, red, green, blue and yellow light provided by spectrum shifted fluorescent bulbs. The researchers found plants grown under the red light produced the highest biomass after 30 days. Comparing the accumulation of artemisinin under the different wavelength showed the highest level under the red light with a drop of 40% under the white light, 57% for blue light, 62% for yellow light and an 86% drop for green light.

Irradiance can be a major factor in pigment accumulation within kale leaves (Lefsrud et al., 2006). Irradiance significantly affected lutein, β-carotene and the chlorophylls accumulation on a FM basis. However, maximum pigment

concentration did not occur at the maximum measured irradiance but at 335 μmol m^{-2} s^{-1}, compared to our maximum measured irradiance of 225 μmol m^{-2} s^{-1} for the 640 nm. Our research produced two peaks of maximum carotenoid pigment concentrations at 440 and 640 nm, which closely conforms to the action spectrum of wheat at 447 and 646 nm and corn at 445 and 650 nm (Koski et al., 1951; Ogawa et al., 1973). However, the chlorophylls only had one peak of maximum concentration occurring at 640 nm, which closely conforms to the maximum irradiance level from the LEDs (Table 6.1). Further research is required to determine if the results measured in this study were a result of wavelength or irradiance.

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Appendix

Table 6.1. Measured irradiance of each wavelength of the LED array at full intensity. LED array produced a small amount of background light even when wavelengths were turned off. Photosynthetic active radiation from experiment for five wavelengths and manufacture data and power usage for three wavelengths (PAR; Apogee Instruments; Logan, Utah; Li-Cor Bioscience, Lincoln, Nebr.).

	Treatment Data	Manufacturer Data	
Wavelength (nm)	Apogee Instruments PAR sensor (QSO-ELEC) ($\mu mol\ m^{-2}\ s^{-1}$)	Li-Cor Bioscience Quantum sensor (LI-190) ($\mu mol\ m^{-2}\ s^{-1}$)	Power usage (watt)
730	1.4		
640	226.5	225	10.1
525	5.7	47	9.8
440	10.5	154	9.9
400	2.9		
baseline	0.2		

Table 6.2. Mean pigments concentrations expressed on a fresh mass (FM) basis in the leaf tissues of 'Winterbor' kale grown under specific wavelength LEDs. Mean composition of sampled leaf tissue of three replications and one plant ± standard error. Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*, Chl = chlorophyll. [ns,] Non-significant

Wavelength (nm)	Pigment Concentration (mg 100 g^{-1} FM)				
	Lutein	β-carotene	Chl *a*	Chl *b*	Total Chl
730	6.9 ± 1.0	2.7 ± 0.4	31.1 ± 10.1	16.0 ± 6.0	47.1 ± 16.1
640	11.2 ± 0.4	3.7 ± 0.4	85.7 ± 7.2	66.2 ± 2.8	151.9 ± 10.0
525	7.8 ± 0.8	3.3 ± 0.4	51.2 ± 10.5	31.8 ± 6.5	83.0 ± 17.0
440	9.8 ± 0.7	4.0 ± 0.4	63.8 ± 4.3	33.9 ± 5.1	97.7 ± 9.3
400	8.1 ± 1.1	3.4 ± 0.3	57.9 ± 3.7	28.8 ± 3.9	86.7 ± 7.7
Contrasts					
Linear	ns	ns	ns	ns	ns
Quadratic	ns	ns	ns	ns	ns

Table 6.3. Mean pigment concentrations as a function of dry mass (DM) pigment in the leaf tissues of 'Winterbor' kale grown under specific wavelength LEDs. Mean composition of sampled leaf tissue of three replications and one plant ± standard error. $^{ns, *,}$ Non-significant or significance at $p \leq 0.05$, respectively.

Wavelength (nm)	%DM	Pigment (mg g^{-1} DM)	
		Lutein	β-carotene
730	8.9 ± 0.9	0.80 ± 0.16	0.31 ± 0.06
640	9.1 ± 0.3	1.23 ± 0.02	0.41 ± 0.05
525	8.7 ± 1.1	0.91 ± 0.09	0.38 ± 0.02
440	7.9 ± 0.6	1.25 ± 0.01	0.51 ± 0.04
400	7.9 ± 0.5	1.02 ± 0.10	0.43 ± 0.03
Contrast			
Linear	ns	ns	*
Quadratic	ns	ns	ns

Table 6.4. Mean values for pigment ratios in the leaf tissues of 'Winterbor' kale grown under specific wavelength LEDs. Mean composition of sampled leaf tissue of three replications and one plant ± standard error. Chl = chlorophyll, L= lutein, β = β-carotene. $^{ns, **}$Non-significant or significance at $p \leq 0.01$, respectively.

Wavelength (nm)	Pigment Ratio		
	Total Chl:L	Total Chl:β	Chl *a*:*b*
730	6.5 ± 1.2	16.7 ± 3.5	2.0 ± 0.1
640	13.6 ± 0.9	42.7 ± 7.1	1.3 ± 0.1
525	10.7 ± 2.0	26.0 ± 5.9	1.6 ± 0.1
440	10.0 ± 0.8	25.0 ± 3.9	1.9 ± 0.2
400	10.8 ± 0.8	25.6 ± 1.8	2.1 ± 0.2
Contrast			
Linear	ns	ns	ns
Quadratic	ns	ns	**

Table 6.5. Mean glucosinolate concentrations as a function of dry mass in the leaf tissues of 'Winterbor' kale grown under specific wavelength LEDs. Mean composition of sampled leaf tissue of three replications and one plant ± standard error. nd = non detected. [ns, *,] Non-significant or significance at $p \leq 0.05$, respectively.

	Glucosinolates (mg 100 g^{-1} DM)					
Wavelength (nm)	Glucoiberin	Sinigrin	Gluconapin	Gluco brassicin	4-meth oxyglucobrassicin	Neogluco brassicin
730	10.0 ± 2.7	21.7±21.7	10.9 ± 8.0	69.2± 57.9	8.5 ± 1.4	5.8 ± 3.5
640	12.6 ± 5.3	32.0±16.6	11.2 ± 1.1	303.2±191.7	12.1 ± 3.3	32.2 ± 21.8
525	23.1 ± 4.7	0.8 ± 0.8	11.9 ± 1.4	77.2 ± 13.7	11.9 ± 3.6	16.8 ± 8.8
440	10.1 ± 5.2	nd	7.9 ± 3.9	212.1± 85.6	10.2 ± 1.3	31.7 ± 13.4
400	18.6 ± 6.1	nd	16.0 ± 6.2	77.7 ± 29.5	9.1 ± 1.7	9.4 ± 3.0
Contrast						
Linear	ns	*	ns	ns	ns	ns
Quadratic	ns	ns	ns	ns	ns	ns

Part 7

Changes in Kale Carotenoid Pigment Concentrations during Leaf Ontogeny

Changes in Kale Carotenoid Pigment Concentrations during Leaf Ontogeny

This part is a lightly revised version of a paper by the same name has been submitted to be published in the journal Scientia Horticulturae in 2006 by Mark G Lefsrud, Dean A Kopsell, Adam Wenzel and Joseph Sheehan:

Lefsrud, M., D. Kopsell, A. Wenzel and J. Sheehan. Changes in kale carotenoid pigment concentrations during leaf ontogeny.

My use of "we" in this part refers to my co-authors and myself. My primary contributions to this paper include (1) selection of the topic and development of the problem into a work relevant to my study of leaf ontogeny in kale, (2) determination of species, variety and sampling method, (3) plant propagation and nutrient control, (4) sampling and analysis, (5) most of the gathering and interpretation of the literature, (6) compiling the information into a single paper, and (7) most of the writing and editing.

Abstract

There has been recent market interest in "baby" salad greens. However, little information exists on the nutritional differences between immature "baby" greens and produce traditionally sold at the fully mature stage. Kale (*Brassica oleracea* L. var. *acephala* D.C.) contains high levels of lutein and β-carotene, which possess important human health properties. Kale was grown in a controlled environment and pigments were measured in young (<1 week), immature (1-2 weeks), mature (2-3 weeks), fully developed (3-4 weeks) and senescing (>4 weeks) leaves using high-performance liquid chromatography (HPLC). Significant differences were observed for all pigments during leaf development. Pigment accumulation followed a quadratic trend, with maximums occurring between the 1st and 3rd week of leaf age. The highest concentrations of lutein measured 15.1 mg 100 g^{-1} fresh mass and occurred in 1-2 week old leaves. The remaining pigments reached maximum levels at 2-3 weeks (β-carotene at 11.6 mg 100 g^{-1}; chlorophyll *a* at 251.4 mg 100 g^{-1}; and chlorophyll *b* at

56.9 mg 100 g^{-1} fresh mass). Mature fully expanded kale leaves accumulated higher carotenoid concentrations than immature or "baby" leaves, with senescent leaves having the lowest carotenoid concentrations. Harvesting kale leaves at a mature stage of development resulted in maximum carotenoid values. Cultural management practices that increase carotenoid concentrations would be expected to improve nutritional quality for fresh markets.

Introduction

Young leaves emerge pale and immature with a small number of tiny chloroplasts. During leaf development and expansion, pigmentation increases to provide energy through photosynthesis. This development is associated with an up regulation in gene expression, an increase in the production of ribulose bisphosphate carboxylase (RUBISCO), along with an increase in chlorophyll content and electron flow (Taiz and Zeiger, 1998; Yoo et al., 2003). As the leaf continues to age, there is a decline in photosynthetic capacity and a down regulation in the photosynthetic associated gene expression (Barry et al., 1992; Lu et al., 2001; Yoo et al., 2003). This sequential degradation process, termed senescence, is associated with a decline in RUBISCO, a decline in chlorophyll content and electron flow, and mobilization and exportation of nitrogen and other elements resulting in eventual desiccation and abscission of the leaf. A characteristic of senescent tissue is that chlorophylls are destroyed more rapidly than carotenoids, which often results in the appearance of vibrant orange, red, and yellow colorations.

Consumption of leafy green vegetables has doubled in the last 2 years (Stein, 2004). This increase is attributed partially to an increased understanding of the health properties of fresh greens. One tenth of the supermarket sales in 2002 were fresh cut salads, creating a $200 million (U.S. dollars) a year industry (Lucier et al., 2004). A large portion of this salad market includes "baby", or immature leafy greens. Baby greens, including kale, are now mainstays on the foodservice market. Baby spinach sales increased 70% over a one-year period in 2002-2003 (Lucier et al., 2004). Because of the prevalence of baby greens in the salad market in the United States, it would be useful to identify nutritional differences between baby greens and mature produce. In a recent article, de Azevedo and Rodriguez-Amaya (2005) reported that Brazilian field-grown "immature" kale leaves (averaging 14 cm in length) had less lutein and β-carotene than "mature" leaves (averaging 29 cm in length), which indicates a reduction in nutritional potential.

Kale (*Brassica oleracea* L. var. *acephala* D.C.) is an excellent source of dietary carotenoids (Holden et al., 1999; Kurilich et al., 1999; U.S. Dept. Agr., 2002). According to the U.S. Dept. Agr., kale has the highest concentration of lutein and β-carotene of any vegetable (Holden et al., 1999; U.S. Dept. Agr., 2002). However, kale has low consumption rates, with per capita fresh intake at less than 0.33 kg/year (Lucier and Plummer, 2003). Dietary intake of foods rich in lutein and β-carotene is associated with reduced risk of lung cancer and chronic eye diseases, such as cataracts and age-related macular (Ames et al., 1995; Landrum and Bone, 2001; Le Marchand et al., 1993). Carotenoids are yellow, orange, and red plant lipid soluble pigments produced by plants, algae and bacteria, which cannot be synthesized in

mammals. In plants, carotenoids are used as antenna pigments to funnel light energy to the photosynthetic reaction center. These carotenoids are closely attached to the chlorophyll molecules to remove excess energy from the photosynthetic system to prevent damage (Marschner, 1997; Miki, 1991; Taiz and Zeiger, 1998; Tracewell et al., 2001).

The physiological age of leaves directly influences coloration and energy production within the plant, which is a result of changes in chlorophyll and carotenoid pigment concentrations. The increased coloration in vegetable and fruit tissues associated with maturity is often indicative of increases in carotenoid concentrations (Gross, 1991). Previous research also revealed carotenoid pigments increased between two different leaf sizes (indicative of maturity) within the same harvested bunch of kale (de Azevedo and Rodriguez-Amaya, 2005). What remain unclear are the potential changes in leaf carotenoid concentrations during leaf ontogeny. Therefore, the goal of this study was to investigate chlorophyll and carotenoid pigment accumulations within kale leaves of differing maturity stages.

Material and Methods

Plant Culture

Seeds of *Brassica oleracea* L. var. *acephala* D.C. cv. Winterbor (Johnny's Selected Seed, Winslow, Maine) were sown into rockwool growing cubes (Grodan A/S, Dk-2640, Hedehusene, Denmark) and germinated in a greenhouse (22 °C day/ 14 °C night) under natural lighting conditions (Durham, N.H., Lat. 43° 09' N). Peter's 20N-6.9P-16.6K water-soluble fertilizer (Scotts Company, Marysville, Ohio) was applied

every five days at a rate of 200 mg L^{-1}. After 2 weeks, the plants were transferred to 38 L plastic containers (Rubbermaid Inc., Wooster, Ohio). Eight plants were placed into 2 cm round holes set at 10.6 x 9.5 cm spacing on each container lid. Three containers were placed into a growth chamber (Model E15, Conviron, Winnipeg, Man.). Temperature was held throughout the experiment at 20 °C, with a light intensity of 500 ± 100 μmol m^{-2} s^{-1}. The experiment used both cool white fluorescent and incandescent bulbs with a photoperiod set at 16 hr light and 8 hr dark.

Kale plants were grown in 30 L of nutrient solution (Hoagland and Arnon, 1950). Elemental concentrations of the nutrient solutions were (mg L^{-1}): NO_3-N (79), NH_4-N (26.3), P (15.3), K (117), Ca (80.2), Mg (24.6), S (32.0), Fe (0.5), B (0.25), Mo (0.005), Cu (0.01), Mn (0.25), and Zn (0.025). The electrical conductivity (EC) of the starting nutrient solution was 0.7 mS cm^{-1} and pH was measured at 5.6. Solutions were aerated with an air blower (Model 25E133W222, Spencer, Winsor, Conn.) connected to air stones. Deionized water was added daily to maintain 30 L in each container. Nutrient solutions were replaced every week throughout the experiment to refresh the solution to the initial nutrient concentrations.

The experiment was replicated three separate times, with plants grown for 3 weeks during each replication. Harvests occurred on June 12, October 4, and November 2, 2004. At harvest, three individual plants were randomly selected from each of three containers, bulked together, and run separately for analysis for each replication. Shoot and root tissues were separated, washed with soap (Aquet, Bel-art Products, Pequannock, N.J.), rinsed, and blotted dry with paper towels before biomass was recorded.

After removal of petiole and major veins, leaves of each plant were separated into five different leaf stages (AGE), The young leaves that were less than a week old (<1 week) consisting of leaves less than 1 cm in length; immature leaves (1-2 weeks) still developing and expanding; mature leaves (2-3 weeks) almost fully expanded and forming the majority of visible leaves when viewed from above; fully developed leaves (3-4 weeks) which had expanded to peak size but were shaded by leaves in the upper canopy; and senescing leaves (>4 weeks) starting to change color and wilt. Each leaf group sample had between 2-3 leaves. Samples were stored at –20 °C prior to lyophilization.

Carotenoid and Chlorophyll Determination

Frozen kale samples were lyophilized for a minimum of 72 hr (Model 6L FreeZone, LabConCo, Kansas City, Mo.). Samples were extracted and separated according to the method of Kopsell et al. (2004) based on the method of Khachik et al. (1986). Briefly, a 0.100 g sub-sample of tissue was hydrated with 0.80 ml of deionized water in a 40 °C water bath for 20 min. After hydration, 0.80 ml of the internal standard, ethyl-β-apo-8'-carotenoate (Sigma Chemical Co. St. Louis, Mo.) and 2.50 ml of HPLC-grade tetrahydrofuran (THF) were added and the mixture was homogenized before being placed into a clinical centrifuge for 3 min at 500 g_n. The supernatant was removed with a Pasteur pipet, placed into a test tube, capped and held on ice. The sediment was re-suspended in 2.00 ml THF and homogenized a second time. The sample was then centrifuged for 3 min at 500 g_n and the

supernatant was collected and combined with the first extracted supernatant. The extraction procedure was repeated twice more, until the supernatant was colorless. The sediment was discarded and the combined supernatants were reduced to 1.50 mL under a stream of nitrogen gas (N-EVAP 111, Organomation Inc., Berlin, Mass.) in a water bath set at 40 °C, and brought up to a final volume of 5.0 ml with methanol (MeOH). A 2.0 ml aliquot was filtered through a 0.2 μm polytetrafluoroethylene (PTFE) filter (Model Econofilter PTFE 25/20, Agilent Technologies, Wilmington, Del.) using a 5 mL syringe (Becton, Dickinson and Company, Franklin Lakes, N.J.) prior to HPLC analysis.

A HPLC unit with photo diode array detection (Model 1100, Agilent Technologies, Palo Alto, Calif.) was used for pigment separation. All samples were analyzed for carotenoid compounds using a Vydac RP C_{18} 5.0 μm 250 x 4.6 mm column (Model 201TP54, Phenomenex, Torrance, Calif.) fitted with a 4 x 3.0 mm, 7.0 μm guard column compartment. The column was maintained at 16 °C using a thermostatic column compartment. Eluents were A: 75% acetronitrile, 20% methanol, 5% hexane, 0.05% BHT, and 0.013% triethyamine (TEA) and B: 50% acetonitrile, 25% THF, 25% hexane and 0.013% TEA. The flow rate was 0.70 ml min^{-1} and the gradient was 100% eluent A for 30 min; a change to 50% A and 50% B over the next 2 min; a change to 100% B over the next 2 min; followed by a change to 50% A and 50% B for the next 2 min. The eluent was instantly returned to 100% A for 10 min prior to the next injection. Eluted carotenoids and chlorophyll compounds from a 20.0 μL injection were detected at 452 (carotenoids and internal

standard), 652 (chlorophyll *b*), and 665 (chlorophyll *a*) nm (Figure 7.1[a]) (Davies and Köst, 1988), with data collected, recorded and integrated using 1100 HPLC ChemStation Software (Agilent Technologies, Palo Alto, Calif.). Pigment concentrations were measured at signal/noise (*S/N*') ratios of >1000 for limit of detection (LOD) and >300 for limit of quantification (LOQ). The LOQ and LOD values have a greater impact on analyses of the entire carotenoid profile in samples, included trace and minor carotenoids, than on the quantification limited to major carotenoids, such as β-carotene and lutein (Rodriquez-Amaya, 2001). Internal standard % recovery ranged from 70 to 96%, with a mean for all samples at 81%. Peak assignment for individual pigments was performed by comparing retention times and line spectra obtained from photodiode array detection using external standards (lutein from Carotenature, Lupsingen, Switzerland; β-carotene, chlorophyll *a*, chlorophyll *b* from Sigma Chemical Co., St. Louis, Mo.). External standard concentrations were determined spectrophotometrically using the following $E^{1\%}_{1cm}$ values: lutein, 2550 in ETOH, λ_{max} = 445 nm; β-carotene, 2592 in hexane, λ_{max} = 452 nm; Chlorophyll *a*, 819 in ETOH, λ_{max} = 665 nm; and Chlorophyll *b*, 441 in ETOH, λ_{max} = 649 nm (Davies and Köst, 1988). Standard reference material® (Slurried Spinach 2385, National Institute of Science and Technology, Gaithersburg, Md.) was used for method validation.

[a] All tables and figures are located in the appendix at the end of this part.

Statistical Analysis

Main effects were analyzed by one-way ANOVA using SPSS (Chicago, Ill.). The relationship between experimental dependent variables and radiation treatments were determined by regression analyses.

Results

Kale leaf tissue fresh mass (FM; $p \leq 0.001$) and leaf tissue dry mass (DM; $p = 0.020$) were influenced by leaf age (Table 7.1). Leaf tissue FM varied from 1.7 to 6.2 g for the young (<1 week) leaves and fully developed (3-4 week) leaves, respectively. Kale FM responded quadradically ($FM = -2.2 + 4.3(AGE) - 0.6(AGE)^2$; $r^2=0.46$, $p \leq 0.001$; Table 7.1). Likewise, kale DM responded quadratically ($DM = -0.3 + 0.7(AGE) - 0.1(AGE)^2$; $r^2=0.24$, $p = 0.004$) over leaf age (Table 7.1). The largest FM was observed with the 3-4 week fully developed leaves, while the largest DM accumulation occurred with the 2-3 week mature leaves. Leaf age also influenced % dry matter (%DM; $p \leq 0.001$; Table 7.1). The %DM responded quadratically ($\%DM = 15.3 + 0.7(AGE) - 0.4(AGE)^2$; $r^2=0.39$, $p \leq 0.001$) to changes in kale leaf age.

Kale leaf tissues were measured for lutein, β-carotene, and chlorophyll pigment concentrations (Table 7.2). Lutein concentrations were affected by kale leaf age ($p \leq 0.001$). Maximum lutein accumulation (15.1 mg 100 g^{-1}) occurred with the 1-2 week old leaf, while the lowest lutein shoot tissue concentrations (6.9 mg 100 g^{-1}) occurred with the leaves older than 4 weeks. There was a quadratic relationship between leaf lutein concentrations and increasing leaf age ($Lutein = 5.9 + 7.0(AGE) - 1.4(AGE)^2$; $r^2= 0.58$, $p \leq 0.001$). β-carotene in the leaf tissues also responded to leaf

developmental ages ($p \leq 0.001$). Maximum β-carotene accumulation was 11.6 mg 100 g^{-1} with 2-3 week old leaves. The lowest β-carotene accumulation (6.1 mg 100 g^{-1}) occurred with leaves older than 4 weeks. There was a quadratic relationship between leaf β-carotene concentrations and increasing leaf age (β-carotene = 2.9 + 5.9(AGE) – 1.1$(AGE)^2$; r^2= 0.39, $p \leq 0.001$).

The concentrations of both chlorophyll *a* (Chl *a*; $p \leq 0.001$), chlorophyll *b* (Chl *b*; $p \leq 0.001$) and total chlorophyll (TChl; $p \leq 0.001$) pigments were influenced by leaf age (Table 7.2). Maximum Chl *a*, Chl b, and TChl levels occurred with the 2-3 week old leaves. Chlorophyll pigments in the kale leaf tissues increased, then decreased in response to leaf age. Quadratic models were fit to Chl *a* (Chl *a* = 69.2 + 136.5(AGE) – 25.5$(AGE)^2$; r^2= 0.45, $p \leq 0.001$), Chl *b* (Chl *b* = 21.07 + 26.53(AGE) – 5.00$(AGE)^2$; r^2= 0.46, $p \leq 0.001$), and TChl (TChl = 90.3 + 163.0(AGE) – 30.5$(AGE)^2$; r^2= 0.46, $p \leq 0.001$) pigments.

Vegetable tissues can be dried and encapsulated for use as lutein and β-carotene dietary supplements (Müller et al., 1999). Therefore, lutein and β-carotene expressed on a DM basis were calculated (Table 7.3). Leaf age had an influence on lutein DM ($p \leq 0.001$) and β-carotene DM ($p \leq 0.001$). Both lutein DM (Lutein DM = 0.4 + 0.5(AGE) – 0.08$(AGE)^2$; r^2=0.27, $p \leq 0.001$) and β-carotene DM (β-carotene DM = 0.2 + 0.4(AGE) – 0.05$(AGE)^2$; r^2=0.56, $p \leq 0.001$) responded quadratically to increases in kale leaf age.

The ratios of chlorophyll *a* to chlorophyll *b* (Chl *a*:*b*), chlorophyll *a* to lutein (Chl *a*:L), chlorophyll *a* to β-carotene (Chl *a*:β), chlorophyll *b* to lutein (Chl *b*:L),

chlorophyll *b* to β-carotene (Chl *b*:β), total chlorophyll to lutein (TChl:L), and total chlorophyll to β-carotene (TChl:β) and were also calculated in the kale leaf tissues (Table 7.4). The ratio of Chl *b*:β responded to changes in kale leaf tissues age (p = 0.028). Quadratic changes were calculated for the ratio of Chl *b*:β (Chl *b*:β = 6.0 - 0.4(AGE) + 0.05(AGE)^2; r^2=0.16, p = 0.032) as kale leaf tissues increased in age. The ratio of TChl:β responded to changes in kale leaf age (p = 0.03). Quadratic changes were calculated for TChl:β (Chl:β = 28.9 + 0.5(AGE) – 0.3(AGE)^2; r^2=0.20, p = 0.01) as kale leaf age increased.

Discussion

In this study, maximum pigment concentrations occurred with leaves that were between 1 and 3 weeks of age. The lutein concentrations reached maximum levels within 1-2 week old leaves, while β-carotene, chlorophyll *a* and *b* had maximum levels at 2-3 week old leaves. This quadratic response of pigment concentrations is similar to work performed by Yoo et al. (2003) on the response of pigments in clover (*Trifolium repens* L.) leaves. Carotenoid distribution between the two photosystems is unevenly distributed, with pigments of photosystem I being enriched with β-carotene, while lutein dominates photosystem II (Demmig-Adams et al., 1996; Thayer and Bjorkman, 1992). PS II develops earlier in leaf tissues than photosystem I (PS I), resulting in the maximization of lutein concentrations before other pigments (Taiz and Zeiger, 1998). All of the pigments decreased during leaf senescence, resulting in a quadratic pigment response over the life of the kale leaf.

When lutein and β-carotene were measured on a DM basis, maximum lutein concentrations still occurred with the 1-2 week old leaves, but β-carotene peak concentration shifted to the 3-4 week old leaves. Yoo et al. (2003) proposed that this shift in pigment concentrations is due to the light harvesting complex II degrading at a faster rate during senescence.

When the pigment ratios were calculated for the leaf tissues, only the ratio of TChl:β and Chl *b*:β showed changes in response to increasing kale leaf age. Result from the current study differ from Lu et al. (2001), where significant responses in the ratio of TChl:L and Chl *a*:*b* were reported in senescencing leaves of field grown wheat (*Triticum aestivum* L.). Lu et al. (2001) also reported no response in the ratio of TChl:β. However, Yoo et al. (2003) reported no response for Chl *a*:*b* ratios in clover leaves during ontogeny. The exact reasons for the differences in pigment ratios during leaf ontogeny are not known, but may be related to differences in genetics or environmental growing conditions.

Results from the current study show maximum carotenoid concentrations occur in mature and fully developed kale leaves. These results are in agreement with de Azevedo and Rodriguez-Amaya (2005) who showed that lutein and β-carotene reached higher concentrations in mature kale leaves grown in Brazil. The researchers in this study identified carotenoid accumulations between two maturity levels of kale leaves, whereas five different leaf ages were evaluated in the current study. Decreases in the carotenoid concentrations occurred as the kale leaves underwent senescence. Kale leaf tissue lutein concentrations reach a maximum earlier than the other pigments. However, immature or baby kale leaves have less carotenoid

concentrations than the mature leaves, and only slightly better concentrations than the senescent leaves. Harvesting kale leaves at a more mature stage of development will allow for maximum carotenoid nutritional value.

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List of References

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Appendix

Table 7.1. Mean values[z] for fresh and dry leaf biomass accumulation and % dry mass for leaves sampled from 'Winterbor' kale.

Age (weeks)	Leaf Biomass (g)		
	Fresh Mass	Dry Mass	% Dry Mass
<1	1.7 ± 0.1	0.27 ± 0.07	15.8 ± 1.0
1-2	3.8 ± 0.4	0.53 ± 0.10	14.0 ± 1.6
2-3	5.4 ± 0.7	0.81 ± 0.17	14.5 ± 1.3
3-4	6.2 ± 1.7	0.71 ± 0.14	10.8 ± 1.1
>4	4.7 ± 0.3	0.41 ± 0.10	8.3 ± 0.6
Contrasts			
Linear	***	NS	***
Quadratic	***	**	***

[z] Mean composition of sampled leaf tissue from three replications of three individual samples (three plants each) ± standard error.

NS, **, *** Non-significant or significance at $p \leq 0.01$, 0.001, respectively.

Table 7.2. Mean value[z] for pigments expressed on a fresh mass basis for leaves sampled from 'Winterbor' kale.

Age (weeks)	Pigment[y] Concentration (mg 100 g^{-1} FM)				
	Lutein	β-carotene	Chl *a*	Chl *b*	Total Chl
<1	11.0 ± 0.7	7.7 ± 0.7	175.4 ± 13.8	41.5 ± 2.8	216. ± 16.6
1-2	15.1 ± 1.4	10.3 ± 0.9	248.5 ± 22.2	55.6 ± 4.8	304.1 ± 27.0
2-3	14.7 ± 0.8	11.6 ± 2.6	251.4 ± 20.3	56.9 ± 3.6	308.3 ± 23.9
3-4	10.8 ± 0.6	8.9 ± 0.9	197.7 ± 20.5	44.1 ± 3.6	241.8 ± 24.1
>4	6.9 ± 0.5	6.1 ± 0.7	120.4 ± 15.7	30.1 ± 3.3	150.5 ± 19.0
Contrasts					
Linear	***	NS	*	*	*
Quadratic	***	***	***	***	***

[z] Mean composition of sampled leaf tissue from three replications of three individual samples (three plants each) ± standard error.

Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*, Chl = chlorophyll.

NS, *, *** Non-significant or significance at $p \leq 0.05$, 0.001, respectively.

Table 7.3. Mean value[z] for pigment accumulation as a function of dry mass (DM) in the leaves sampled from 'Winterbor' kale.

	Pigment (mg g^{-1} DM)	
Age (weeks)	Lutein	β-carotene
<1	0.71 ± 0.05	0.49 ± 0.05
1-2	1.12 ± 0.11	0.75 ± 0.03
2-3	1.05 ± 0.06	0.81 ± 0.02
3-4	1.03 ± 0.05	0.82 ± 0.09
>4	0.86 ± 0.07	0.73 ± 0.04
Contrasts		
Linear	NS	***
Quadratic	***	***

[z] Mean composition of sampled leaf tissue from three replications of three individual samples (three plants each) ± standard error.

NS, *** Non-significant or significance at $p \leq 0.001$, respectively.

Table 7.4. Mean value[z] for pigment ratios in the leaves sampled from 'Winterbor' kale.

Age	Pigment Ratios[y]						
(week)	Chl *a*:*b*	Chl *a*:L	Chl *a*:β	Chl *b*:L	Chl *b*:β	TChl:L	TChl:β
<1	4.21± 0.11	15.8 ± 0.5	23.3 ± 1.2	3.8± 0.1	5.6± 0.3	19.5 ± 0.5	28.9 ± 1.5
1-2	4.44± 0.08	16.6 ± 0.5	24.1 ± 0.9	3.7± 0.2	5.4± 0.2	20.3 ± 0.6	29.6 ± 1.1
2-3	4.41± 0.19	17.0 ± 0.7	21.9 ± 1.2	3.9± 0.1	5.0± 0.1	20.8 ± 0.7	26.9 ± 1.3
3-4	4.42± 0.13	18.0 ± 1.1	22.2 ± 0.6	4.1± 0.2	5.1± 0.2	22.0 ± 1.2	27.3 ± 0.7
>4	3.97± 0.20	17.3 ± 1.6	19.5 ± 1.1	4.3± 0.2	5.0± 0.2	21.6 ± 1.8	24.4 ± 1.2
Contrasts							
Linear	NS	NS	**	**	*	NS	**
Quadratic	*	NS	**	*	*	NS	**

[z] Mean composition of sampled leaf tissue from three replications of three individual samples (three plants each) ± standard error.

Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll; L = lutein; β = β-carotene.

[NS, *, **] Non-significant or significance at $p \leq 0.05$, 0.01, respectively.

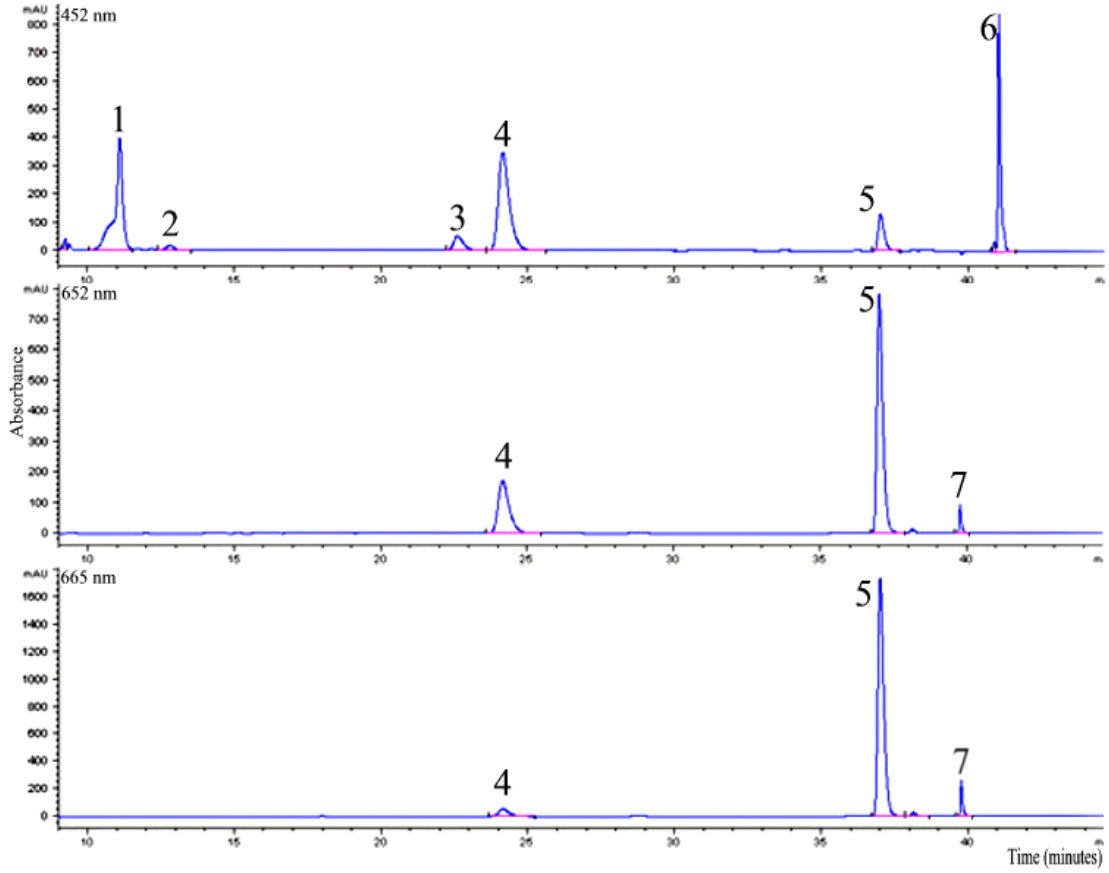

Figure 7.1. Representative HPLC chromatogram of kale extracts. Peak identification: 1. lutein, 2. zeaxanthin, 3. ethyl-β-apo-8'-carotenoate, 4. chlorophyll *b* (calibrated and measured at 652 nm), 5. chlorophyll *a* (calibrated and measured at 665 nm), 6. β-carotene, 7. unknown. HPLC conditions are described in the text.

Part 8

Kale Carotenoids are Unaffected while Biomass Production, Elemental Concentrations, and Selenium Accumulation Respond to Changes in Selenium Fertility

Kale Carotenoids are Unaffected while Biomass Production, Elemental Concentrations, and Selenium Accumulation Respond to Changes in Selenium Fertility

This part is a lightly revised version of a paper by the same name published in: Journal of Agriculture and Food Chemistry in 2006 by Mark G Lefsrud, Dean A Kopsell, David E. Kopsell and William M. Randle:

Lefsrud, M.G., D.A. Kopsell, D.E. Kopsell W.M. Randle. 2006. Kale carotenoids are unaffected while biomass production, elemental concentrations, and selenium accumulation respond to changes in selenium fertility. J. Agr. Food Chem. Web Published.

My use of "we" in this part refers to my co-authors and myself. My primary contributions to this paper include (1) selection of the topic and development of the problem into a work relevant to my study of selenium in kale, (2) determination of species, variety and selenium levels (3) plant propagation and nutrient control, (4) sampling and analysis, (5) most of the gathering and interpretation of the literature, (6) compiling the information into a single paper, and (7) most of the writing and editing.

Abstract

Selenium (Se) is a micronutrient in mammalian nutrition and is accumulated in kale (*Brassica oleracea* L.), which has high levels of lutein and β-carotene. Selenium, lutein and β-carotene have important human health benefits and possess strong antioxidant properties. The objectives of this study were to determine the influence of different Se (as sodium selenate (Na_2SeO_4) and sodium selenite (Na_2SeO_3)) fertility levels on (1) biomass accumulation, (2) the accumulation patterns of carotenoid pigments, and (3) elemental accumulation in the leaves of kale. 'Winterbor' kale was greenhouse-grown using nutrient solution culture with Se treatment concentrations of 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg L^{-1} Se as Na_2SeO_4 and 0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1} Se as Na_2SeO_3. Increases in either selenate (SeO_4^{-2}) or selenite (SeO_3^{-2}) resulted in decreases in kale leaf tissue

biomass. Neither of the Se treatments had an affect on the accumulation of lutein or β-carotene in leaf tissues. Increasing SeO_4^{-2} significantly increased the accumulation of kale leaf Se; however, leaf tissue Se did not significantly change over the SeO_3^{-2} treatments. Increases in SeO_4^{-2} affected the leaf tissue concentrations of P, K, Ca, Mg, S, B, Cu, Mn, and Mo, whereas SeO_3^{-2} only affected B and S. Growing kale in the presence of SeO_4^{-2} would result in the accumulation of high levels of tissue Se without affecting carotenoid concentrations.

Introduction

Selenium (Se) is an essential trace element in mammalian nutrition, but is not yet classified as an essential plant micronutrient (Mayland et al., 1989). Selenium is absorbed by plants in three forms: selenite (SeO_3^{-2}), selenate (SeO_4^{-2}), or as organic Se complexes, such as selenocysteine and selenomethionine (Mikkelsen et al., 1989; White et al., 2004). Plants preferentially accumulate SeO_4^{-2}, as compared to SeO_3^{-2}, under hydroponic or soil conditions (Marschner, 1997). Selenate competes with sulfate (SO_4^{-2}) for uptake by the roots, with increased levels of SO_4^{-2} resulting in decreases in SeO_4^{-2} uptake (Marschner, 1997). Selenate enters the root through normal sulfur (S) transporters in the plasma membrane (White et al., 2004). After long-distance transport to the leaves, the conversion of SeO_4^{-2} into organic Se compounds is believed to occur in the chloroplasts, where it enters into normal S metabolic pathways (Brown and Shift, 1982; White et al., 2004). Selenate is converted to SeO_3^{-2} by ATP sulfurylase before incorporation into various selenoether amino acids.

In most plant species, Se-amino acids replace corresponding S-amino acids in metabolic pathways (Anderson and Scarf, 1983). Phytotoxicity occurs mainly from Se interferences with normal S metabolism (Mikkelsen et al., 1989), resulting in chlorosis and decreases in protein and dry matter synthesis (Mengel and Kirkby, 1987). Plants are classified by their ability to accumulate Se, with non-concentrator species (< 25 mg kg^{-1} dry mass), secondary absorbers (25-100 mg kg^{-1} dry mass) and primary indicators (100-1000 mg kg^{-1} dry mass). Members of the *Brassica* family readily accumulate Se and have been characterized as primary indicators (Bañuelos and Meek, 1990; Mayland et al., 1989; Rosenfeld and Beath, 1964). It is this characteristic which makes *Brassica* vegetables candidate crops to deliver Se to human diets.

The distribution of Se in soils is highly variable, with toxic and deficient plant levels being reported worldwide. In areas of the world with low soil Se levels, fertilization is practiced to avoid potential mammalian deficiencies (Gissel-Nielsen et al., 1984). Consumption of Se-fertilized vegetables is a good way to insure the necessary level of Se in the human diet (Ip and Lisk, 1994). With the importance of *Brassica* plants as vegetable crops, increasing tissue Se concentration through Se applications would improve the overall Se contribution to human diets (Kopsell and Randle, 1999). Health benefits associated with increased Se intake include immune system enhancement (Manteroatienza et al., 1991), prevention of cardiovascular diseases (Korpela, 1993) and prevention of several forms of cancer (Ip and Lisk, 1994; Whanger, 1992).

Carotenoids are C_{40} isoprenoid polyene secondary compounds that form yellow, orange, and red lipid soluble pigments in higher plants, algae, and bacteria (Figure 8.1[a]). In plants, carotenoids are used as antenna pigments to funnel light energy to the photosynthetic reaction center. These carotenoids are closely associated with the chlorophyll molecules to prevent excess energy from entering the photosynthetic system (Marschner, 1997; Miki, 1991; Taiz and Zeiger, 1998; Tracewell et al., 2001). Lutein ((*3R,3'R,6'R)*-β,ε-carotene-3,3'diol) and β-carotene (β,β-carotene) possess important human health properties. Mammalian systems are unable to synthesize these compounds, so plants are one of the primary sources of dietary lutein and β-carotene. Moreover, intake of foods rich in lutein and β-carotene has been associated with reduced risk of lung cancer, cataracts, and age-related macular degeneration (Ames et al., 1995; Landrum and Bone, 2001; Le Marchand et al., 1993). The U.S. Dept. Agr. ranks kale (*Brassica oleracea* L. var. *acephala* D.C.) as the highest source of lutein and β-carotene among the vegetable crops, making it an excellent source of dietary carotenoids (Holden et al., 1999; Kurilich et al., 1999; U.S. Dept. Agr., 2002). However, kale has low consumption rates, with per capita fresh intake at less than 0.33 kg $year^{-1}$ (Lucier and Plummer, 2003).

The dietary carotenoid lutein is selectively deposited in the human retina and is responsible for the yellow pigmentation referred to as macular pigment (Bone et al., 1997; Khachik et al., 1997). Macular pigment is postulated to participate in photoprotection of the eye (Mares-Perlman and Klein, 1999; Wooten et al., 1999),

[a] All tables and figures are located in the appendix at the end of this part.

and increases can be achieved through dietary modification (Hammond et al., 1997) and supplementation (Landrum et al., 1997). However, studies have indicated that consumption of a variety of vegetables, providing a mixture of carotenoids, was more strongly associated with reduced eye disease and cancer risk than individual carotenoid supplements (Johnson et al., 2002; Le Marchand et al., 1993). The ability of plants to accumulate and incorporate Se into bioactive compounds can also influence human health and nutrition. Most notable is the anticarcinogenic activity of organic Se forms against certain types of cancers (Clark et al., 1996; Reid et al., 2002; Whanger, 2002). One of the most effective anticarcinogenic organic Se compounds is methylselenocysteine, which occurs in members of the *Brassica* genus (Ip and Ganther, 1992).

Brassica vegetables have high nutritional and medicinal value imparted from essential dietary minerals and secondary compounds in their tissues. Kale has the highest concentrations of lutein and β-carotene, providing antioxidant activity when consumed in the diet. *Brassica* vegetables also have the ability to accumulate high concentrations of Se. Therefore, the objectives of this study were to determine the influence of different Se (as SeO_4^{-2} or SeO_3^{-2}) fertility levels on (1) biomass accumulation, (2) the accumulation patterns of carotenoid pigments, and (3) elemental accumulation in the leaves of 'Winterbor' kale, a member of the *Brassica* family. Our research hypothesis contends that it will be possible to increase the concentration of Se in the leaf tissues of kale, while maintaining high concentrations of nutritionally valuable lutein and β-carotene.

Material and Methods

Plant Culture

'Winterbor' kale seeds (Johnny's Selected Seed, Winslow, Maine) were sown into rockwool growing cubes (Grodan A/S, Dk-2640, Hedehusene, Denmark) for germination on February 24, 2004 and grown in a greenhouse (22 °C day/ 14 °C night) under natural lighting conditions (Durham, N.H., Lat. 43° 09' N). Peter's 20N-6.9P-16.6K water-soluble fertilizer (Scotts Company, Marysville, Ohio) was applied every five days at a rate of 200 mg L^{-1}. After 2.5 weeks, the plants were transferred to 10 L plastic containers (Rubbermaid Inc., Wooster, Ohio). Six plants were placed into 2 cm round holes set at 10.6 x 9.5 cm spacing on each container lid.

The plants were grown in 9 L of a modified nutrient solution (Hoagland and Arnon, 1950). Elemental concentrations of the nutrient solutions were (mg L^{-1}): N (105), P (15.3), K (117.3), Ca (80.2), Mg (24.6), S (32.0), Fe (0.5), B (0.25), Mo (0.005), Cu (0.01), Mn (0.25), and Zn (0.025). Plants were grown in separate studies under increasing Se treatment concentrations at 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg L^{-1} Se as Na_2SeO_4 and at 0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1} Se as Na_2SeO_3. The EC of the starting nutrient solution was 1.0 dS m^{-1} and pH was measured at 5.6. Solutions were aerated with an air blower (Model 25E133W222, Spencer; Winsor, Conn.) connected to air stones. Water was added daily to maintain initial solution volumes in each container. Nutrient solutions were replaced every week throughout the experiment to refresh the solution to the initial nutrient concentrations. The experimental design was a randomized complete block for each study, with four replications of eight SeO_4^{-2} treatments and seven SeO_3^{-2} treatments, respectively.

Plants were harvested on April 4, 2004. At harvest, shoot and root tissues were separated and weighed. Shoot tissues were washed with soap (Aquet, Bel-art Products, Pequannock, N.J.), rinsed, and blotted dry with paper towels. The third fully expanded leaf from each of the 6 plants was selected, and a 4 cm^2 piece of the leaf was removed. This treatment sample was stored at –20 °C prior to lyophilization. The remaining shoot material was dried at 60 °C for no less than 72 hr.

Elemental Determination

A sample mill grinder (Model 1093, Cyclotec-Tector, Höganäs, Sweden) with a 0.5 mm screen was used to grind dried shoot tissue. A 0.300 g tissue sample was mixed with 10.0 mL of 70% concentrated nitric acid (HNO_3) and digested in a microwave accelerated reaction system (MARS5, CEM Corp., Matthews, N.C.). The digested solution was cooled to room temperature and deionized water was added to result in a final volume of 40.0 mL. Elemental analysis was determined by ICP-AES (Inductively Coupled Argon Plasma – Atomic Emission Spectrometry; Model Vista AX, Varian Inc., Palo Alto, Calif.) (Kopsell et al., 2000).

A wet-acid digest was used for Se analysis (Kopsell and Randle, 1997). Ground tissues were placed into 125-ml flask with 10.0 ml of concentrated nitric acid (70% NHO_3) and placed on a hot plate (Thermolynem, Model 2200, Dubuque, Iowa) under reflux for 4 hr at 165 ºC. Flasks were allowed to cool to room temperature and brought to a final volume of 50.0 ml with deionized water. The solutions were filtered through Whatman #1 filter paper (Maidstone, England) before total Se was

measured by graphite furnace atomic absorption spectrophotometry (GFAA; Perkin-Elmer Corporation, Model 4100ZL, Norwalk, Conn.) (Kopsell and Randle, 1997). The detection limit for Se by GFAA was 4.0 μg L^{-1}.

Carotenoid Determination.

The frozen plant samples were lyophilized for a minimum of 72 hr (Model 6L FreeZone, LabConCo, Kansas City, Mo.). The dried tissues samples were ground with dry ice in a kitchen grinder (Handy Chopper Plus, HC 3000, Household Products Inc, Shelton, Conn.). Pigments were extracted and separated according to previously published methods (Khachik et al., 1986; Kopsell et al., 2004). A 0.100 g sub-sample was placed into a Potter-Elvehjem tissue grinder tube (Kontes, Vineland, N.J.) and hydrated with 0.80 mL of deionized water. The sample was placed in a 40 °C water bath for 20 min. After hydration, 0.80 mL of the internal standard, ethyl-β-apo-8'-carotenoate (Sigma Chemical Co., St. Louis, Mo.) and 2.5 mL of tetrahydrofuran (THF), stabilized with 25 mg L^{-1} 2,6-Di-*tert*-butyl-4-methoxyphenol (BHT), were added. The sample was homogenized in the tube with ~25 insertions with a Potter-Elvehjem tissue grinder pestle attached to a drill press (Model Craftsman 15 inch Drill Press, Sears, Roebuck and Co., Hoffman Estates, Ill.) at 540 rpm. The sample tube was kept immersed in ice. The tube was placed into a clinical centrifuge for 3 min at 500 g_n. The supernatant was removed with a Pasteur pipet, placed into a conical 15 ml test tube, capped, and held on ice. The sediment was resuspended in 2.00 ml THF and homogenized with ~25 insertions of the grinding pestle. The tube was centrifuged for 3 min at 500 g_n, and the supernatant was collected and combined

with the first extracted supernatant. The extraction procedure was repeated twice more until the supernatant was colorless. The sediment was discarded, and the combined 4 supernatants were placed in a 40 °C water bath and reduced to 0.50 ml under a stream of nitrogen (Model N-EVAP 111, Organomatic Inc., Berlin, Mass.). Added to the 0.50 mL sample was 2.50 mL of MeOH and 2.00 mL of THF, with the solution vortexed. The sample was filtered through a 0.2 μm polytetrafluoroethylene (PTFE) filter (Model Econofilter PTFE 25/20, Agilent Technologies, Wilmington, Del.) using a 5 mL syringe (Becton, Dickinson and Company, Franklin Lakes, N.J.).

A HPLC unit with photodiode array detector (Agilent 1100, Agilent Technologies, Palo Alto, Calif.) was used for pigment separation. All samples were analyzed for carotenoid compounds using a Vydac RP C_{18} 5.0 μm 250 x 4.6 mm column (Model 201TP54, Phenomenex, Torrance, Calif.) fitted with a 4 x 3.0 mm, 7.0 μm guard column compartment (Khachik et al., 1986; Kopsell et al., 2004). The column was maintained at 16 °C using a thermostatic column compartment. Eluents were A: 75% acetronitrile, 20% methanol, 5% hexane, 0.05% BHT, and 0.013% triethyamine (TEA)(v/v) and B: 50% acetonitrile, 25% THF, 25% hexane and 0.013% TEA (v/v). The flow rate was 0.70 ml min^{-1} and the gradient was 100% eluent A for 30 min; 50% A and 50% B for 2 min; 100% B for 2 min; and 50% A and 50% B for 2 min. The eluent was returned to 100% A for 10 min prior to the next injection. Eluted carotenoids and chlorophyll compounds from a 20.0 μL injection were detected at 452, 652, and 665 nm, with data collected and integrated using 1100 HPLC ChemStation Software (Agilent Technologies, Palo Alto, Calif.). Peak

assignment was performed by comparing retention times and line spectra obtained from photodiode array detection with authentic standards (lutein from Carotenature, Lupsingen, Switzerland; β-carotene, chlorophyll *a*, chlorophyll *b* from Sigma Chemical Co., St. Louis, Mo.). Recovery rates of ethyl-β-apo-8'-carotenoate during extractions were > 90%.

Statistical Analysis

Data were analyzed by the ANOVA procedure using SPSS (Chicago, Ill.). The relationship between experimental dependent variables and selenium treatments were determined by regression analysis. Orthogonal polynomials were used to study changes associated with increasing SeO_4^{-2} and SeO_3^{-2} treatment levels by partitioning the sum of squares into components associated with linear and quadratic terms (Steel and Torrie, 1980).

Results and Discussion

Tissue Biomass Accumulation

Kale leaf tissue fresh mass (FM) and leaf tissue dry mass (DM) responded to the increases in SeO_4^{-2} ($p \leq 0.001$ and $p \leq 0.001$, respectively), but not to increases in SeO_3^{-2} ($p = 0.523$ and $p = 0.633$, respectively). Leaf tissue FM responded quadratically (FM $SeO_4 = 53.1 - 0.70(trt) - 0.98(trt)^2$), and ranged from 53.7 g plant^{-1} under 0.0 mg L^{-1} as SeO_4^{-2} to 39.1 g plant^{-1} under 3.5 mg L^{-1} as SeO_4^{-2} (Table 8.1). Leaf tissue DM also responded quadratically (DM $SeO_4 = 4.0 - 0.02(trt) - 0.08(trt)^2$), and ranged from 3.9 g plant^{-1} under 0.0 mg L^{-1} as SeO_4^{-2} to 2.9 g plant^{-1} under 3.5 mg

L^{-1} as SeO_4^{-2} (Table 8.1). The largest FM and DM were observed with the 0.0 mg L^{-1} SeO_4^{-2} treatment, whereas the largest biomass accumulations for kale grown under SeO_3^{-2} occurred at the 1.0 mg L^{-1} treatment. Decreases in biomass in response to increasing SeO_4^{-2} concentrations in the current study follow previously reported trends. Kopsell and Randle (1999) reported decreases in shoot FM and DM in rapid-cycling *B. oleracea* in nutrient solution from 0.0 to 9.0 mg L^{-1} Na_2SeO_4 (0.0 to 3.8 mg L^{-1}). *Brassica juncea* L. land races showed decreases in DM yields of 12 to 23% as the concentration of Se in solution culture increased from 0 to 4 mg L^{-1}, added as Na_2SeO_4 (Bañuelos et al., 1997a). Similarly, Bañuelos et al. (1997b) showed a decrease in shoot DM in canola (*Brassica napus* L. cv. Westar) when comparing plants grown in a low Se soil (0.1 mg kg^{-1}) with a high Se soil (40 mg kg^{-1}). Yield considerations in *Brassica* may be noteworthy when supplying Se in concentrations provided in this study.

Carotenoid and Chlorophyll Pigment Accumulation

Kale leaf tissues were measured for lutein, β-carotene, chlorophyll *a*, and chlorophyll *b* concentrations (Table 8.2). None of the pigments responded to increases in SeO_4^{-2} or SeO_3^{-2} concentrations. Values recorded for lutein and β-carotene concentrations were within previously reported ranges for 'Winterbor' kale (Kopsell et al., 2004). For kale grown under increasing SeO_4^{-2} concentrations, maximum lutein accumulation was 10.2 mg 100 g^{-1} FM and occurred under the 0.0 mg L^{-1} treatment. For kale grown under increasing SeO_3^{-2}, maximum lutein accumulation was 10.6 mg 100 g^{-1} FM and occurred under the 2.0 mg L^{-1} treatment.

Maximum β-carotene accumulation for kale grown under increasing SeO_4^{-2} treatments was 8.9 mg 100 g^{-1} FM and occurred under 0.0 mg L^{-1}, whereas maximum β-carotene accumulation for kale grown under increasing SeO_3^{-2} treatments was 9.3 mg 100 g^{-1} FM and occurred under 1.5 mg L^{-1} (Table 8.2). To the best of our knowledge, this is the first attempt to measure the influence of Se fertility on the accumulation of lutein and β-carotene in the leaf tissues of kale. One previous report in the literature described the influences of Na_2SeO_4 and Na_2SeO_3 on total carotenoid accumulation in the aquatic plant *Lemna minor* L. (Severi, 2001). There were no changes in total carotenoid values in *L. minor* as plants were grown in concentrations of Na_2SeO_4 and Na_2SeO_3 up to 32 μ*M* (equivalent to the highest Se treatments in the current study). Decreases in total carotenoid pigments were found only when *L. minor* was grown in Na_2SeO_4 and Na_2SeO_3 concentrations of 128 μ*M*, values that would be phytotoxic for *Brassica* plants (Kopsell and Randle, 1999). Data from the current study demonstrates that kale leaf tissue lutein and β-carotene concentrations are not affected by increases in either SeO_4^{-2} or SeO_3^{-2} within the ranges provided in this study.

Neither of the Se treatments affected the concentrations of chlorophyll *a* or *b* in the kale leaf tissues (Table 8.2). Previous research on the influences of Se on chlorophyll pigments has reported mixed results. Decreases in chlorophyll pigments in response to increases in both SeO_4^{-2} and SeO_3^{-2} occurred in maize (*Zea maize* L.)(Jain and Gadre, 1998), mung bean (*Vigna mungo* L.)(Padmaja et al., 1989), and ryegrass (*Lolium multiflorum* L.)(Hartkainen et al., 2000), whereas increases in

chlorophyll pigments were reported for potato (*Solanum tuberosum* L.)(Seppanen et al., 2003) and ryegrass (Hartkainen et al., 2000). Data from the current study demonstrates that kale leaf tissue chlorophyll concentrations are not affected by increases in either SeO_4^{-2} or SeO_3^{-2} within the ranges provided in this study.

Vegetable tissues can be dried and encapsulated for use as lutein and β-carotene dietary supplements (Müller et al., 1999; Pool-Zobel et al., 1997). Therefore, concentrations of lutein and β-carotene expressed on a DM basis were calculated for kale tissues in the current study (Table 8.3). The % dry matter (%DM) of the leaf tissues of kale was not affect by increasing concentrations of SeO_4^{-2}; however, a slight increase in %DM occurred in kale grown under increasing SeO_3^{-2} (Table 8.3). The accumulation of lutein in kale leaf tissues expressed on a DM basis was not affected by increasing concentration of either SeO_4^{-2} or SeO_3^{-2}. The accumulation of β-carotene in kale leaf tissues was not affected by increases in SeO_4^{-2}; however kale tissue β-carotene expressed on a DM basis was influenced by increases in SeO_3^{-2} (Table 8.3). β-carotene expressed on a DM basis responded quadratically (β-carotene DM $SeO_4 = 0.97 - 0.05(trt) + 0.01(trt)^2$) to increases in SeO_3^{-2} concentrations.

At low concentrations (< 10 mg kg^{-1}), Se acts as an antioxidant, but higher levels can result in pro-oxidant activity (Hartikainen et al., 2000). Selenium performs a similar role as lutein and β-carotene, which scavenge singlet oxygen and excited chlorophyll molecules (Seppanen et al., 2003). The dual role of Se seems to depend on the Se concentration in the tissue. Selenate has a much greater influence on plant

metabolism than does SeO_3^{-2}. Although not significant, there were decreases in lutein and β-carotene concentrations, when expressed on a FM basis, in the kale leaf tissues grown under increasing SeO_4^{-2} concentrations. Therefore, it may be possible that under higher levels of Se than those used in the current study, reduced concentrations of lutein and β-carotene may result. However, Se applied in concentrations within the range of the current study would not be expected to significantly lower carotenoid concentrations in kale.

Macro- and Micronutrient Accumulation

Elemental nutrient levels in the kale leaves were within reported ranges for mature, greenhouse-grown plants (Mills and Jones, 1996)(Table 8.4 - 5). Phosphorus levels in the leaves were influenced by increases in SeO_4^{-2} treatments ($p \leq 0.001$). Phosphorus concentrations responded quadratically to increases in SeO_4^{-2} (P SeO_4 = 0.71 - 0.09(trt) + 0.02(trt)^2) treatment concentrations and ranged from 0.57% under 3.5 mg L^{-1} to 0.73% under 0.0 mg L^{-1}. Leaf tissue K was affected by increases in SeO_4^{-2} treatments (p=0.032). Potassium increased, then decreased in a quadratic response (K SeO_4 = 4.0 + 0.27(trt) – 0.04(trt)^2) as the SeO_4^{-2} concentrations increased in the nutrient solutions, and ranged from 4.02% under 0.5 mg L^{-1} to 4.53% under 3.5 mg L^{-1}. Leaf tissue Mg responded to increasing SeO_4^{-2} treatments (p=0.013). Tissue Ca concentrations were influenced by SeO_4^{-2} treatments (Ca SeO_4 = 4.2 + 0.09(trt)) and ranged from 4.13% under 0.0 mg L^{-1} to 4.43% under 3.5 mg L^{-1}. Tissue Mg concentrations responded quadratically to increases in SeO_4^{-2} (Mg SeO_4 = 0.66 + 0.05(trt) – 0.01(trt)^2) and ranged from 0.66% under 0.0 mg L^{-1} to 0.77% under 3.5 mg

L^{-1}. Leaf tissue B was affected by both SeO_4^{-2} ($p \leq 0.001$) and SeO_3^{-2} ($p \leq 0.001$) treatments. Boron responded quadratically to increases in both SeO_4^{-2} (B SeO_4 = 61.5 – 12.3(trt) + 2.1(trt)^2) and SeO_3^{-2} (B SeO_3 = 60.3 – 10.3(trt) + 0.74(trt)^2). Leaf tissue Cu was affected by SeO_4^{-2} (p=0.006) and responded quadratically to increases in SeO_4^{-2} (Cu SeO_4 = 3.9 – 0.28(trt) + 0.16(trt)^2) treatments. Leaf tissue Mo was affected by SeO_4^{-2} (p=0.018) and the trend was quadratic (Mo SeO_4 = 2.48 – 1.07(trt) + 0.23(trt)^2), decreasing then increasing with increasing treatment concentrations. Kopsell et al. (2000) reported nutrient accumulation in rapid-cycling *B. oleracea* plants in response to increases in SeO_4^{-2} treatment concentrations. Data from their study revealed increases in K, and decreases in P, Fe, and B in the leaf tissues as the SeO_4^{-2} treatment concentrations increased from 0.0 to 3.8 mg L^{-1}. There was no response previously reported for the interaction between SeO_4^{-2} treatments and tissue Ca accumulation (Bañuelos et al., 1997b). Decreases in leaf tissue B in *Brassica* land races has been reported under increases in SeO_3^{-2} treatment concentrations (Bañuelos et al., 1997b). Results from the current study utilizing kale, a member of the *Brassica* family, confirms the earlier results with the rapid-cycling and land races of *Brassica*.

Leaf tissue S responded to increases in either SeO_4^{-2} ($p \leq 0.001$) or SeO_3^{-2} ($p \leq 0.001$) treatments (Table 8.4). The trends were a quadratic increase in response to increases in SeO_4^{-2} (S SeO_4 = 1.4 + 1.3(trt) – 0.23(trt)^2) or a decrease in S in response to SeO_3^{-2} (S SeO_3 = 1.36 - 0.12(trt) – 0.01(trt)^2) treatments. Sulfur increased from 1.40% to 3.15% as the SeO_4^{-2} treatment concentrations increased from 0.0 to 3.5 mg L^{-1}, respectively. However, leaf tissue S decreased from 1.35% to 1.06% as the SeO_3^{-2} treatment concentrations increased from 0.0 to 3.0 mg L^{-1}. Previous reports have

identified increases in leaf tissue S under increasing SeO_4^{-2} treatment concentrations (Kopsell and Randle, 1997; Kopsell and Randle, 1999). It has been postulated that either SeO_4^{-2} or Se metabolites antagonize the repression of SO_4^{-2} transporters by SO_4^{-2} and other S metabolites, thereby increasing S uptake in the presence of elevated media Se (White et al., 2004). The reason for the decrease in S as the SeO_3^{-2} treatments increased is not known.

Kale leaf tissue Se concentrations were affected by increases in SeO_4^{-2} treatments ($p \leq 0.001$); however, no changes in leaf tissue Se occurred when kale was grown in the presence of increasing SeO_3^{-2}. No Se was detected in the kale tissues under the 0.0 mg L^{-1} treatment. Kale leaf tissue Se concentrations ranged from non-detectable to as high as 1056.7 µg g^{-1} as the SeO_4^{-2} treatment concentrations increased from 0.0 to 3.5 mg L^{-1} (Table 8.5). As SeO_4^{-2} increased in nutrient solutions, the response in kale leaf tissue Se accumulation was quadratic ($SeO_4 = 168.5 + 53.7(trt) - 14.1(trt)^2$; Table 8.5). Bañuelos et al. (1997a) and Kopsell et al. (2000) reported increases in *Brassica* leaf tissue Se with increasing SeO_4^{-2} treatment concentrations.

Tissue Se results from the current study are within the ranges of reported Se accumulation in the leaves of rapid-cycling *B. oleracea* grown under increasing levels of SeO_4^{-2} in nutrient solutions (Charron et al., 2001; Kopsell et al., 2003; Kopsell and Randle, 1999; Kopsell et al., 2000). The concentration of Se in the leaf tissue of kale grown under SeO_3^{-2} treatments remained fairly consistent, averaging 209 µg g^{-1} over all of the treatments (Table 8.5). Selenate can accumulate in plants in concentrations much greater than those present in the surrounding media. In contrast, SeO_3^{-2} does not accumulate to levels surpassing those of the external environment (Brown and

Shift, 1982). When broccoli (*Brassica oleracea* L. var. *botrytis*), Indian mustard (*Brassica juncea* L.), or rice (*Oryza sativa* L.) were grown under SeO_4^{-2}, SeO_3^{-2}, or selenomethionine treatments, plants accumulated the greatest amount of shoot Se under SeO_4^{-2}, followed by those given selenomethionine (Zayed et al., 1998). Broccoli, Swiss chard (*Beta vulgaris* L. var. *cicla* L.), collards (*Brassica oleracea* L. var. *acephala* D.C.), and cabbage (*Brassica oleracea* L. var. *capitata* L.) grown in soils treated with 4.5 mg kg^{-1} as either SeO_3^{-2} or SeO_4^{-2} ranged in tissue Se concentration from 0.013 to 1.382 g kg^{-1} DM, and absorbed 10 times the amount of Se if treated with SeO_4^{-2} than with SeO_3^{-2} (Bañuelos and Meek, 1990). Time-dependent kinetic studies showed that Indian mustard absorbed SeO_4^{-2} up to 2-fold faster than SeO_3^{-2} (de Souza et al., 1998). The current study demonstrates that kale can accumulate much higher levels Se from SeO_4^{-2} than SeO_3^{-2}.

Kale can accumulate high concentrations of Se, as well as lutein and β-carotene. Results from this study show that kale can accumulate high levels of Se (provided as SeO_4^{-2}) without any negative effects on carotenoid concentrations. Selenite fertilization did not have a detrimental effect on the accumulation of lutein and β-carotene; however, kale did not accumulate Se from SeO_3^{-2} to the extent of SeO_4^{-2}. With the important health benefits associated with increased consumption of plant-derived lutein, β-carotene and Se, current results demonstrate that kale may be a good candidate crop to deliver all three to human diets.

List of References

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Appendix

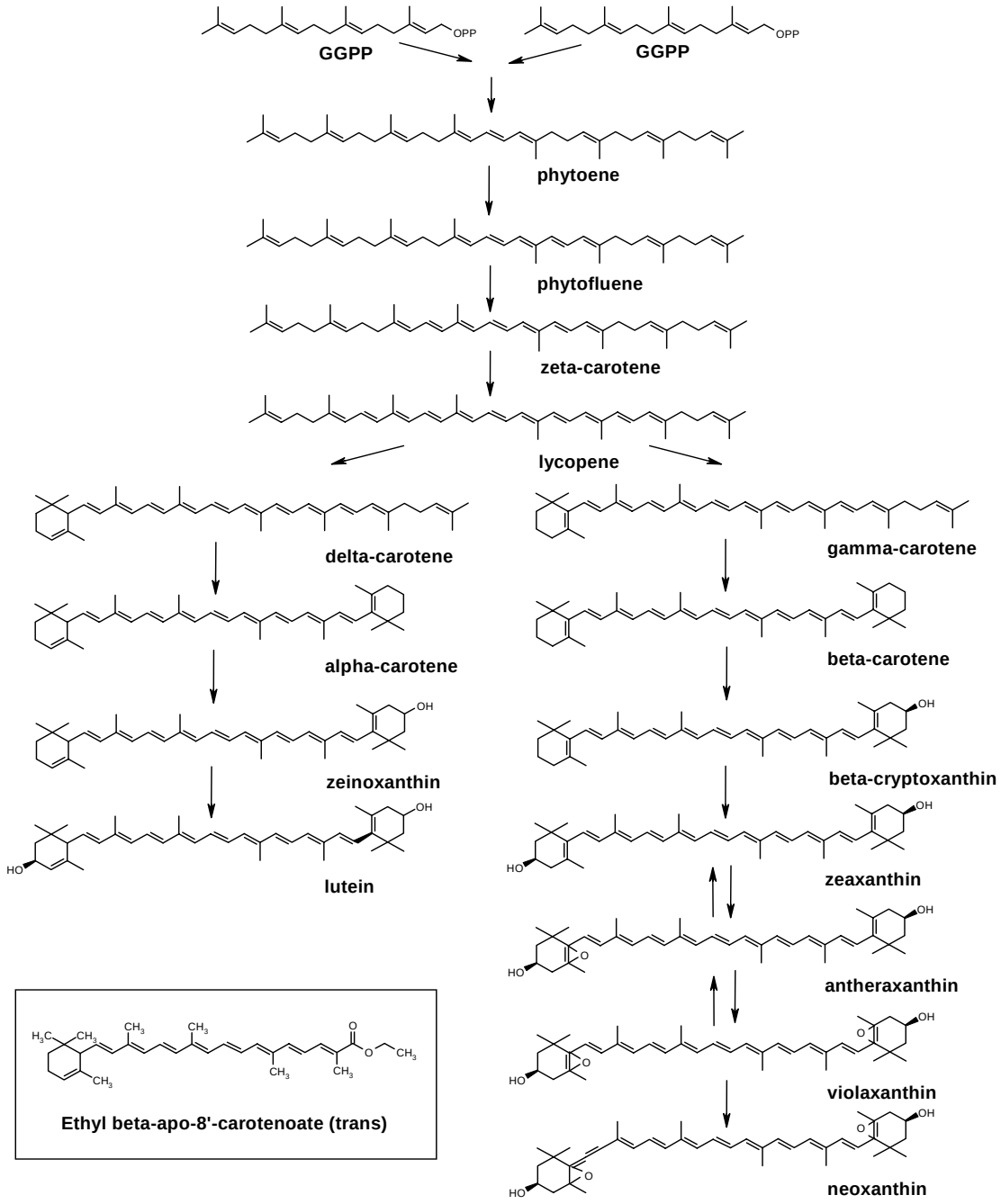

Figure 8.1. A simplified version of the carotenoid biosynthetic pathway in plants. GGPP, geranylgeranyl pyrophosphate. Ethyl β-apo-8'-carotenoate used as internal standard.

Table 8.1. Mean biomass[a] of leaf tissues of 'Winterbor' kale (*B. oleracea* L. *acephala* D.C.) grown under increasing selenium (selenate or selenite). Concentrations in nutrient solution culture.

	Plant Biomass (g)	
mg L^{-1} Se	Fresh Mass	Dry Mass
	Selenate	
0.0	53.7 ± 1.4	3.9 ± 0.2
0.5	53.1 ± 2.0	3.9 ± 0.2
1.0	47.2 ± 3.2	3.4 ± 0.2
1.5	52.3 ± 1.8	3.8 ± 0.1
2.0	48.5 ± 1.7	3.6 ± 0.1
2.5	46.2 ± 3.8	3.4 ± 0.3
3.0	40.3 ± 2.8	3.0 ± 0.2
3.5	39.1 ± 2.3	2.9 ± 0.2
Contrasts		
Linear	$p \leq 0.001$	$p \leq 0.001$
Quadratic	$p \leq 0.001$	$p \leq 0.001$
	Selenite	
0.0	50.7 ± 3.3	3.8 ± 0.3
0.5	49.7 ± 2.1	3.7 ± 0.2
1.0	51.3 ± 4.4	3.8 ± 0.3
1.5	49.3 ± 2.4	3.7 ± 0.1
2.0	44.5 ± 5.1	3.4 ± 0.3
2.5	45.3 ± 3.3	3.4 ± 0.2
3.0	43.7 ± 1.3	3.4 ± 0.1
Contrasts		
Linear	$p = 0.035$	ns[b]
Quadratic	ns[b]	ns[b]

[a] Mean composition of sampled leaf tissue of 4 replications, 6 plants each ± standard error. [b] Nonsignificant.

Table 8.2. Mean carotenoid and chlorophyll pigment concentrations[a] expressed on a fresh mass basis (milligrams per 100 g fresh mass) for leaf tissues of 'Winterbor' kale (*B. oleracea* L. *acephala* D.C.) grown under increasing selenium (selenate or selenite). Concentrations in nutrient solution culture.

	Pigment Concentration (mg 100 g^{-1} Fresh Mass)			
mg L^{-1} Se	Lutein	β-carotene	Chlorophyll *a*	Chlorophyll *b*
			Selenate	
0.0	10.2 ± 0.4	8.9 ± 0.5	177.0 ± 6.8	45.0 ± 2.2
0.5	9.5 ± 0.3	8.2 ± 0.2	166.1 ± 3.5	41.1 ± 2.0
1.0	9.5 ± 0.2	8.2 ± 0.2	144.1 ± 17.4	41.5 ± 2.3
1.5	9.6 ± 0.7	8.1 ± 0.6	161.0 ± 11.3	38.2 ± 3.9
2.0	9.8 ± 0.3	8.4 ± 0.3	168.7 ± 5.8	41.6 ± 1.7
2.5	9.7 ± 0.3	8.1 ± 0.1	169.0 ± 6.1	40.4 ± 1.5
3.0	9.9 ± 0.6	8.0 ± 0.3	169.9 ± 9.3	41.4 ± 3.0
3.5	8.7 ± 0.9	8.5 ± 0.2	170.0 ± 9.9	40.2 ± 1.6
contrasts				
linear	ns[b]	ns[b]	ns[b]	ns[b]
quadratic	ns[b]	ns[b]	ns[b]	ns[b]
			Selenite	
0.0	10.2 ± 0.5	8.9 ± 0.5	178.9 ± 5.5	45.0 ± 2.1
0.5	9.9 ± 0.5	8.7 ± 0.5	175.6 ± 8.0	43.7 ± 3.2
1.0	9.3 ± 0.7	8.6 ± 0.2	175.1 ± 7.3	43.2 ± 0.8
1.5	10.3 ± 0.5	9.3 ± 0.3	188.0 ± 7.4	46.0 ± 1.7
2.0	10.6 ± 0.4	8.9 ± 0.3	191.3 ± 11.0	44.9 ± 3.0
2.5	9.4 ± 0.6	8.5 ± 0.4	166.8 ± 6.1	40.5 ± 2.4
3.0	9.7 ± 0.6	8.9 ± 0.3	188.5 ± 7.1	44.1 ± 1.7
contrasts				
linear	ns[b]	ns[b]	ns[b]	ns[b]
quadratic	ns[b]	ns[b]	ns[b]	ns[b]

[a] Mean composition of sampled leaf tissue of 4 replications, 6 plants each ± standard error. [b] Nonsignificant

Table 8.3. Mean % dry matter and carotenoid pigment concentrations[a] expressed on a dry mass basis (mg per g dry mass) for leaf tissues of 'Winterbor' kale (*B. oleracea* L. *acephala* D.C.) Grown under increasing selenium (selenate or selenite). concentrations in nutrient solution culture.

		Pigment (mg g^{-1} Dry Mass)	
mg L^{-1} Se	% Dry Matter	Lutein	β-carotene
		Selenate	
0.0	7.3 ± 0.2	1.09 ± 0.03	0.94 ± 0.04
0.5	7.3 ± 0.2	1.09 ± 0.05	0.91 ± 0.04
1.0	7.3 ± 0.1	1.06 ± 0.07	0.90 ± 0.02
1.5	7.3 ± 0.1	1.07 ± 0.02	0.89 ± 0.02
2.0	7.4 ± 0.1	0.94 ± 0.05	0.90 ± 0.03
2.5	7.4 ± 0.2	1.02 ± 0.02	0.89 ± 0.02
3.0	7.3 ± 0.2	0.96 ± 0.07	0.83 ± 0.06
3.5	7.3 ± 0.1	1.10 ± 0.04	0.90 ± 0.03
contrasts			
linear	ns[b]	ns[b]	ns[b]
quadratic	ns[b]	ns[b]	ns[b]
		Selenite	
0.0	7.5 ± 0.1	1.09 ± 0.03	0.98 ± 0.01
0.5	7.4 ± 0.2	1.01 ± 0.05	0.91 ± 0.03
1.0	7.4 ± 0.1	1.07 ± 0.03	0.92 ± 0.02
1.5	7.5 ± 0.1	1.00 ± 0.06	0.90 ± 0.04
2.0	7.6 ± 0.1	1.00 ± 0.07	0.94 ± 0.01
2.5	7.5 ± 0.2	0.93 ± 0.05	0.80 ± 0.04
3.0	7.9 ± 0.2	1.02 ± 0.02	0.89 ± 0.02
contrasts			
linear	$p = 0.044$	ns[b]	$p = 0.010$
quadratic	$p = 0.044$	ns[b]	$p = 0.034$

[a] Mean composition of sampled leaf tissue of 4 replications, 6 plants each ± standard error. [b] Nonsignificant.

Table 8.4. Mean values of macronutrients[a] for leaf tissues of 'Winterbor' kale (*B. oleracea* L. *acephala* D.C.) grown under increasing selenium (selenate or selenite). Concentrations in nutrient solution culture.

mg L^{-1} Se	Macronutrients				
	%P	%K	%Ca	%Mg	%S
			Selenate		
0.0	0.73 ± 0.02	4.04 ± 0.16	4.13 ± 0.08	0.66 ± 0.01	1.40 ± 0.05
0.5	0.65 ± 0.01	4.02 ± 0.09	4.25 ± 0.08	0.69 ± 0.02	1.88 ± 0.06
1.0	0.63 ± 0.02	4.28 ± 0.03	4.42 ± 0.10	0.71 ± 0.01	2.56 ± 0.07
1.5	0.61 ± 0.01	4.35 ± 0.07	4.40 ± 0.17	0.74 ± 0.04	2.90 ± 0.13
2.0	0.60 ± 0.02	4.43 ± 0.11	4.41 ± 0.14	0.75 ± 0.02	3.00 ± 0.07
2.5	0.58 ± 0.01	4.39 ± 0.14	4.41 ± 0.12	0.74 ± 0.02	3.11 ± 0.10
3.0	0.58 ± 0.01	4.45 ± 0.15	4.40 ± 0.09	0.74 ± 0.02	3.12 ± 0.13
3.5	0.57 ± 0.01	4.53 ± 0.11	4.43 ± 0.07	0.77 ± 0.01	3.15 ± 0.08
contrasts					
linear	$p \leq 0.001$	$p \leq 0.001$	$p = 0.013$	$p \leq 0.001$	$p \leq 0.001$
quadratic	$p \leq 0.001$	$p \leq 0.001$	$p = 0.031$	$p \leq 0.001$	$p \leq 0.001$
			Selenite		
0.0	0.71 ± 0.02	4.10 ± 0.13	4.01 ± 0.06	0.65 ± 0.01	1.35 ± 0.03
0.5	0.68 ± 0.02	4.27 ± 0.02	4.02 ± 0.06	0.65 ± 0.01	1.28 ± 0.02
1.0	0.70 ± 0.01	4.01 ± 0.06	3.90 ± 0.04	0.65 ± 0.01	1.28 ± 0.06
1.5	0.69 ± 0.02	4.24 ± 0.09	3.83 ± 0.05	0.64 ± 0.01	1.19 ± 0.01
2.0	0.67 ± 0.01	4.25 ± 0.09	3.95 ± 0.09	0.65 ± 0.02	1.11 ± 0.04
2.5	0.69 ± 0.02	4.30 ± 0.08	4.01 ± 0.11	0.63 ± 0.02	1.06 ± 0.04
3.0	0.68 ± 0.01	4.26 ± 0.05	3.97 ± 0.04	0.64 ± 0.01	1.06 ± 0.03
contrasts					
linear	ns[b]	ns[b]	ns[b]	ns[b]	$p \leq 0.001$
quadratic	ns[b]	ns[b]	ns[b]	ns[b]	$p \leq 0.001$

[a] Mean composition of sampled leaf tissue of 4 replications, 6 plants each ± standard error. [b] Nonsignificant.

Table 8.5. Mean values of micronutrients[a] (μg per g) for leaf tissues of 'Winterbor' kale (*B. oleracea* L. *acephala* D.C.) grown under increasing selenium (selenate or selenite). Concentrations in nutrient solution culture.

	Micronutrients (μg g^{-1})						
mg L^{-1}	Se	B	Cu	Fe	Mn	Mo	Zn
				Selenate			
0.0	nd[c]	64.2± 5.5	3.6± 0.1	38.9± 3.8	147.1± 4.1	2.1± 0.6	24.5± 3.6
0.5	212.8 ± 22.5	52.7± 1.1	3.9± 0.2	42.3± 5.9	147.2± 3.9	2.7± 0.2	24.2± 3.0
1.0	280.0 ± 58.0	49.5 ± 0.8	4.1± 0.2	43.0± 4.5	142.5± 9.0	1.8± 0.2	21.5± 1.4
1.5	467.8 ± 27.8	48.2 ± 2.3	4.3± 0.5	44.1± 8.3	140.2± 6.0	0.8± 0.4	21.6± 1.0
2.0	603.3 ± 44.0	46.1 ± 1.6	3.8± 0.1	47.0± 3.7	134.8± 1.9	1.2± 0.3	23.1± 2.4
2.5	718.0 ± 50.1	45.5 ± 1.9	3.8± 0.1	31.4± 14.7	136.4± 2.4	1.4± 0.4	27.2± 3.6
3.0	988.3 ± 42.6	44.2 ± 1.2	4.3± 0.3	43.7± 4.5	134.3± 3.1	1.3± 0.1	24.9± 3.4
3.5	1056. ± 18.8	42.6 ± 1.5	5.3± 0.3	37.2± 4.6	138.4± 9.3	1.5± 0.2	22.0± 2.2
contrasts							
linear	$p \leq 0.001$	$p \leq 0.001$	p=0.004	ns[b]	p = 0.035	p=0.024	ns[b]
quadratic	$p \leq 0.001$	$p \leq 0.001$	p=0.004	ns[b]	ns[b]	p=0.011	ns[b]
				Selenite			
0.0	nd[c]	61.9 ± 5.9	3.5± 0.1	32.8± 6.3	139.8± 4.3	2.2± 0.4	23.8± 3.7
0.5	195.0 ± 4.7	51.5 ± 2.4	3.4± 0.4	38.0± 7.0	127.8± 3.6	2.6± 0.7	22.6± 3.7
1.0	209.0 ± 25.3	53.2 ± 2.9	3.6± 0.1	26.2± 6.7	139.9± 4.7	2.5± 0.7	20.4± 0.4
1.5	199.3 ± 22.0	46.7 ± 1.0	3.7± 0.3	37.4± 18.1	129.3± 6.1	2.9± 0.6	19.0± 0.7
2.0	235.0 ± 34.5	43.4 ± 2.6	3.5± 0.3	46.4± 3.4	127.9± 5.1	3.6± 0.1	21.2± 0.8
2.5	216.8 ± 12.7	38.1 ± 1.6	3.2± 0.1	38.3± 9.1	127.7± 4.1	3.1± 0.7	21.1± 1.2
3.0	198.0 ± 8.3	36.5 ± 1.7	3.1± 0.2	43.2± 3.2	127.2± 3.1	3.6± 0.1	22.0± 1.4
contrasts							
linear	ns[b]	$p \leq 0.001$	ns[b]	ns[b]	p = 0.048	p=0.029	ns[b]
quadratic	ns[b]	$p \leq 0.001$	ns[b]	ns[b]	ns[b]	ns[b]	ns[b]

[a] Mean composition of sampled leaf tissue of 4 replications, 6 plants each ± standard error. [b] Nonsignificant. [c] Nondetectable.

Part 9

Determination of % Dry Matter and Stability of Carotenoids in Kale and Spinach During Drying

Determination of % Dry Matter and Stability of Carotenoids in Kale and Spinach During Drying

This part is a lightly revised version of a paper by the same name that will be submitted to be published in the journal HortScience by Mark Lefsrud, Dean Kopsell, Carl Sams, Bob Augé, Jim Wills, and A.J. Both:

Lefsrud, M.G., D.A. Kopsell, C.E. Sams, R.M. Augé, J.B. Wills, and A.J. Both. Determination of % dry matter and stability of carotenoids in kale and spinach during drying.

My use of "we" in this part refers to my co-authors and myself. My primary contributions to this paper include (1) selection of the topic and development of the problem into a work relevant to my study of drying of kale and spinach, (2) determination of species, variety and drying methods, (3) plant propagation and nutrient control, (4) sampling and analysis, (5) most of the gathering and interpretation of the literature, (6) compiling the information into a single paper, and (7) most of the writing and editing.

Abstract

Drying of spinach (*Spinacia oleracea* L.) and kale (*Brassica oleracea* L. var. *acephala* D.C.) is required to determine the percentage of dry matter (%DM) and the pigment content of fresh leaves. 'Melody' spinach and 'Winterbor' kale were greenhouse grown in hydroponic nutrient solutions containing 13 or 105 mg L^{-1} N. Utilizing vacuum freeze dryers and convection ovens, plant tissues were dried for 120 hr at five different temperature treatments: (1) freeze drying at –25 ºC, (2) freeze drying at 0 ºC, (3) vacuum drying at +25 ºC, (4) oven drying at +50 ºC and (5) oven drying at +75 ºC. Spinach leaf tissue %DM was affected, but kale %DM was not affected by drying temperature. Spinach and kale leaf tissue %DM were both affected by N treatment. The high N spinach decreased from 7.3 to 6.4 %DM and low N spinach decreased from 12.7 to 9.6 %DM as the drying temperature increased. Kale ranged from 13.2 to 15.0 %DM for the high N treatment and from 21.4 to 22.5

%DM for the low N treatment. Lutein, β-carotene and chlorophyll levels for both spinach and kale leaf tissue were affected by drying temperature. Measured concentrations of all pigments decreased over 70% as the drying temperature increased. The largest pigment fresh and dry mass concentrations for spinach were measured at -25ºC freeze drying. Kale fresh and dry mass pigment concentrations were largest between –25 ºC freeze drying and +25 ºC vacuum drying. The spinach and kale samples dried between -25 and +25 ºC were not significantly different from each other in %DM or pigment concentration measured on a dry or fresh mass basis. Drying leaf tissue for accurate pigment analysis required temperatures below +25 ºC, using vacuum or freeze drying technology.

Introduction

Determination of carotenoid concentrations in plant tissue requires dried samples for analysis (Kopsell et al., 2004; Tai and Chen, 2000). Measurement of the water content within these biological samples can vary greatly depending on the plant growing environment (Lefsrud et al., 2005; Lefsrud et al., 2006). A number of methods are used for drying, including convection oven drying, freeze drying, vacuum drying, air drying, and microwave drying (Diaz-Maroto et al., 2004; Ingram et al., 2000; Karathanos, 1999; Litvin et al., 1998; Stegen et al., 1998).

To determine the effect of drying temperature on plant samples, Stegen et al. (1998) compared the water content of a number of plants using microwave, oven and vacuum drying (performed at 22ºC). Alfalfa (*Medicago sativa* L.), onion (*Allium cepa* L.), corn (*Zea mays* L.), and potato (*Solanum tuberosum* L.) had a final moisture

content that was not significantly different among the three drying methods, but bean (*Pinguicula vulgaris* L.), bean shell, and garlic (*Allium sativum* L.) were significantly different. The microwave oven and vacuum drying of the latter three plant samples were not significantly different, but oven drying resulted in a significant change in the final moisture content.

Moisture content within biological samples changes during drying and can result in the release of volatile organic compounds (VOC), destruction of pigments and changes in chemical composition. The loss of VOC through drying has been reported in a wide variety of plants (Diaz-Maroto et al., 2004; Diaz-Maroto et al., 2002a; Diaz-Maroto et al., 2002b; Ingram et al., 2000). At temperatures greater than 65 ºC, Karathanos (1999) reported that decomposition of solids in addition to evaporation of water could occur within the sample. While, Diaz-Maroto et al. (2002a) found that oven drying and air drying bay leaf (*Laurus nobilis* L.) did not affect the aroma of the fresh herb, but freeze drying resulted in a reduction. Diaz-Maroto et al. (2002b) reported that oven dried and freeze dried parsley (*Petroselinum crispum* L.) resulted in a decrease in VOC within the sample but air drying resulted in few losses. Oven dried and freeze dried basil (*Ocimum basilicum* L.) had a decrease in VOC, but air dried basil was not different compared to a fresh sample (Diaz-Maroto et al., 2004).

Carotenoids and other plant compounds can also be affected by drying temperature. A significant decrease in lutein and β-carotene was measured in daylily (*Hemerocallis disticha* Donn ex Sweet) when oven dried at 48 ºC, compared to –53 ºC freeze drying (Tai and Chen, 2000). Cinar (2004) reported that freeze drying

reduced the losses of carotenoids during storage in orange peel (*Citrus sinensis* (L.) Osbeck), potato and carrot (*Daucus carota* L.). Alomar et al. (1999) reported that oven dried silage resulted in a reduction in crude protein, and an increase in crude fiber, neutral detergent fiber, and acid detergent fiber when compared to freeze dried silage.

Carotenoids are lipid-soluble, yellow, orange, and red pigments produced by plants, algae and bacteria. In higher plants, carotenoids function in photoprotection as light harvesting antennae pigments and free radical scavengers (Miki, 1991; Taiz and Zeiger, 1998; Tracewell et al., 2001). Two important dietary carotenoids in human health maintenance are lutein and β-carotene. Increased intake of lutein and β-carotene has been associated with reduced risk of lung cancer and chronic eye diseases, such as cataracts and age-related macular degeneration (Ames et al., 1995; Landrum and Bone, 2001; Le Marchand et al., 1993; Semba and Dagnelie, 2003). Increasing the lutein and β-carotene concentrations in vegetable crops through cultural management techniques would be beneficial to the health status of consumers.

Kale (*Brassica oleracea* L. var. *acephala* D.C.) ranks highest, and spinach (*Spinacia oleracea* L.) ranks second among vegetable crops for lutein and β-carotene content (Holden et al., 1999; U.S. Dept. Agr., 2002). However, kale has low consumption rates, with per capita fresh intake of less than 0.33 kg $year^{-1}$ in the United States (Lucier and Plummer, 2003). Spinach has one of the highest consumption rates among green-leafy vegetables in the United States, with per capita

intakes of 0.73, 0.09, and 0.36 kg year^{-1} for fresh, canned, and frozen markets, respectively (Lucier and Plummer, 2003).

With increased interest in dried plant material for human health, the purpose of this study was to determine the effect that drying has on water content, measured % dry matter and stability of carotenoids and chlorophylls in kale and spinach.

Material and Methods

Plant Culture

'Melody' spinach and 'Winterbor' kale (Johnny's Selected Seed, Winslow, Maine) were seeded on February 22, 2005 into rockwool growing cubes (Grodan A/S, Dk-2640, Hedehusene, Denmark). The seeds were germinated and grown in a greenhouse (22 °C day/14 °C night) under natural lighting conditions (Knoxville, Tenn., Lat. 35° 57' N). Plants were grown according to the method described in Part 1, the N spinach study. Peter's 20N-6.9P-16.6K water-soluble fertilizer (Scotts, Marysville, Ohio) was applied every five days at a rate of 200 mg L^{-1}. After three weeks, the plants were transferred to 10 L plastic containers (Rubbermaid Inc., Wooster, Ohio). Six plants were transferred into 2 cm round holes arranged in a 10.6 by 9.5 cm spacing in each container lid. The plants were grown in 9 L of nutrient solution (Hoagland and Arnon, 1950). Plants were grown under two N treatment levels of 13 and 105 mg L^{-1}. The ratio of NO_3-N to NH_4-N was kept constant at 3:1 and solutions were changed every two weeks. Nitrogen treatments and variety were randomized and replicated in space in each of three separate containers.

Biomass Determination

Plants were harvested after four weeks in the hydroponic system on April 12, 2005. At harvest, shoot and root tissues were separated and weighed. Shoot tissues were washed with soap (Aquet, Bel-art Products, Pequannock, N.J.), rinsed, and blotted dry with paper towels. The third through sixth true leaves were removed from each plant, combined from each N treatment, randomly separated into approximately 5 g samples, weighed and assigned one of the drying treatments. These samples were stored at –80 °C prior to drying. The five drying treatments (T) were: (1) freeze drying at –25 ºC, (2) freeze drying at 0 ºC, (3) vacuum drying at +25 ºC, (4) oven drying at +50 ºC and (5) oven drying at +75 ºC. The freeze dryer was a LabConCo (Kansas City, Mo.) Freezezone 6 with the vacuum at 0.006 mbar, the condenser at –40 ºC and the stoppering tray dryer containing the samples set at the treatment temperature. The convection oven was a Fisher (Hampton, N.H.) isotemp oven 300 series, model 338F. Each drying treatment was replicated three times per species and N treatment. For each treatment, the shoot material was dried for 100 hr, weighed and dried for another 20 hr to confirm equilibrium moisture content. Samples were stored at –80 ºC prior to HPLC analysis.

Carotenoid and Chlorophyll Determination

The dried tissues samples were ground with dry ice in a kitchen grinder (Handy Chopper Plus, HC 3000, Household Products Inc., Shelton, Conn.). Pigments were extracted and separated according to Kopsell et al. (2004). A 0.100 g

sub-sample was placed into a Potter-Elvehjem tissue grinder tube (Kontes, Vineland, N.J.) and hydrated with 0.80 mL of deionized water. The sample was placed in a water bath at 40 °C for 20 min. After hydration, 0.80 mL of the internal standard, ethyl-β-apo-8'-carotenoate (Sigma Chemical Co., St. Louis, Mo.) and 2.5 mL of tetrahydrofuran (THF) stabilized with 25 mg L^{-1} 2,6-Di-*tert*-butyl-4-methoxyphenol (BHT) were added. The sample was homogenized in the tube with 25 insertions of the grinder pestle attached to a drill press (Model Craftsman 15 inch Drill Press, Sears Co., Hoffman Estates, Ill.) at 540 rpm while immersed in ice. The tube was placed into a clinical centrifuge for 3 min at 500 g_n. The supernatant was removed with a Pasteur pipet, placed into a conical 15 ml test tube, capped and held on ice. The sediment was resuspended in 2.00 ml THF and homogenized and centrifuged again. The supernatant was collected and combined with the first extracted supernatant. The extraction procedure was repeated twice more until the supernatant was colorless. The sediment was discarded and the combined 4 supernatants were placed in a water bath at 40 °C and reduced to 0.50 ml using nitrogen gas (Model N-EVAP 111, Organomation Inc., Berlin, Maine). 2.50 mL of MeOH and 2.00mL of THF were added to the 0.50 mL sample, vortexed, and filtered through a 0.2 μm polytetrafluoroethylene (PTFE) filter (Model Econofilter PTFE 25/20, Agilent Technologies, Palo Alto, Calif.) using a 5 mL syringe (Becton, Dickinson and Company, Franklin Lakes, N.J.) prior to high performance liquid chromatography (HPLC) analysis.

A HPLC unit with photodiode array detector (Agilent 1100, Agilent Technologies, Palo Alto, Calif.) was used for pigment separation. All samples were analyzed for carotenoid compounds using a Pronto SIL 5.0 μm 250 x 4.6 mm column (200-5-C_{30}, Bischoff Chromatography, Leonberg, Germany) fitted with a 4 x 3.0 mm, 7.0 μm guard column compartment. The column was maintained at 16 °C using a thermostatic column compartment. The eluent was 11% methyl-tert butyl ether (MTBE), 89% methanol, 0.1% triethyamine (TEA) (v/v). The flow rate was 1.00 mL min^{-1} for 55 minutes. Eluted compounds from a 20.0 μL injection were detected at 453 nm (carotenoids and internal standard), and 652 nm for chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*), with data collected and integrated using 1100 HPLC ChemStation Software (Agilent Technologies, Palo Alto, Calif.). Peak assignment was performed by comparing retention times and line spectra obtained from the photodiode array detection with authentic standards (lutein from Carotenature, Lupsingen, Switzerland; β-carotene, Chl *a*, and Chl *b* from Sigma Chemical Co., St. Louis, Mo.). Recovery rates of ethyl-β-apo-8'-carotenoate during extraction exceeded 90%.

Statistical Analysis

Data sets were analyzed by a split plot, GLM procedure using SPSS (Chicago, Ill.). ANOVA determined significance of the main effects of nitrogen treatments and drying temperature, and for their interactions. The relationship between experimental

dependent variables and N treatments were determined by regression analysis using SPSS (Steel and Torrie, 1980).

Results

Spinach

Biomass Accumulation

Percent dry matter (%DM) for spinach was influenced by N treatment level ($p \leq 0.01$) and drying temperature ($p = 0.011$). No significant variation was accounted for by the interaction between N treatment level and drying temperature ($p = 0.081$). Average %DM for the spinach increased from 7.0% for the high N treatment to 10.7% for the low N treatment (Table 9.1[a]; Figure 9.1). The average %DM for the high N spinach treatment varied among the drying temperature treatments and resulted in an increase from 6.4% for the +75ºC convection oven to 7.3% for the +25ºC vacuum drier. The average %DM for the low N spinach treatment increased from 9.6% for the +50 ºC convection oven to 12.7% for the –25 ºC freeze drier. Spinach leaf tissue %DM decreased linearly with increases in drying temperature for both the high N (%DM = 7.2 - 0.008 (T), $r^2 = 0.265$, $p = 0.05$) and low N treatments (%DM = 11.4 - 0.02(T), $r^2 = 0.487$, $p = 0.004$).

Carotenoid and Chlorophyll Pigment Accumulation

Maximum spinach tissue lutein accumulation expressed on a fresh mass (FM) basis was 9.8 mg $100g^{-1}$ for the low N treatment at the -25ºC drying temperature

[a] All tables and figures are located in the appendix at the end of this part.

(Table 9.1). Spinach leaf tissue lutein concentrations responded to drying temperature ($p \leq 0.001$), but not to N treatment ($p = 0.273$) nor interaction ($p = 0.203$). Spinach leaf tissue lutein decreased linearly with increases in drying temperature for both the high N (Lutein FM = 8.4 - 0.07(T), $r^2 = 0.837$, $p \leq 0.001$) and low N level (Lutein FM = 8.1 - 0.07(T), $r^2 = 0.760$, $p \leq 0.001$).

Maximum spinach tissue β-carotene accumulation expressed on a FM basis was 4.9 mg $100g^{-1}$ under the high N treatment at the –25 ºC drying temperature (Table 9.1). Spinach leaf tissue β-carotene concentrations responded to drying temperature ($p \leq 0.001$) and to N treatment ($p \leq 0.001$) but not to their interaction ($p = 0.056$). Spinach leaf tissue β-carotene decreased linearly with increases in drying temperature for both the high N (β-carotene FM = 4.3 - 0.04(T), $r^2 = 0.891$, $p \leq 0.001$) and low N level (β-carotene FM = 3.3 - 0.03(T), $r^2 = 0.661$, $p \leq 0.001$).

Spinach leaf tissue Chl *a* FM concentrations responded to drying temperature ($p \leq 0.001$), N treatment ($p \leq 0.010$), and to the interaction between N treatment and drying temperature ($p = 0.011$). Spinach leaf tissue Chl *b* concentrations responded to drying temperature ($p \leq 0.001$), N treatment ($p \leq 0.001$), and to the interaction between N treatment and drying temperature ($p = 0.012$). Spinach leaf tissue Chl *a* decreased linearly with increases in drying temperature for both the high N (Chl *a* FM = 79.3 – 1.0(T), $r^2 = 0.832$, $p \leq 0.001$) and low N treatments (Chl *a* FM = 57.7 - 0.8(T), $r^2 = 0.779$, $p \leq 0.001$) (Table 9.1). Spinach leaf tissue Chl *b* decreased linearly with increases in drying temperature for both the high N (Chl *b* FM = 28.7 - 0.4(T), r^2

= 0.822, $p \leq 0.001$) and low N treatments (Chl *b* FM = 21.6 - 0.3(T), $r^2 = 0.770$, $p \leq 0.001$) (Table 9.1).

Maximum spinach tissue lutein accumulation expressed on a dry mass (DM) basis was 1.25 mg g^{-1} for the high N treatment and the -25ºC drying temperature (Table 9.2). Spinach leaf tissue lutein concentrations responded to drying temperature ($p \leq 0.001$), N treatment ($p \leq 0.001$) and to the interaction between N treatment and drying temperature ($p = 0.008$). Spinach leaf tissue lutein decreased linearly with increases in drying temperature for both the high N (Lutein DM = 1.16 - 0.01(T), $r^2 = 0.836$, $p \leq 0.001$) and low N treatment (Lutein DM = 0.70 - 0.01(T), $r^2 = 0.781$, $p \leq 0.001$).

Maximum spinach tissue β-carotene accumulation expressed on a DM basis was 0.67 mg g^{-1} for the high N treatment and the –25 ºC drying temperature (Table 9.2). Spinach leaf tissue β-carotene concentrations responded to drying temperature ($p \leq 0.001$), N treatment ($p \leq 0.001$) and to the interaction between N treatment and drying temperature ($p \leq 0.001$). Spinach leaf tissue β-carotene decreased linearly with increases in drying temperature for both the high N (β-carotene DM = 0.60 - 0.01(T), $r^2 = 0.888$, $p \leq 0.001$) and low N treatments (β-carotene DM = 0.30 - 0.001(T), $r^2 = 0.738$, $p \leq 0.001$).

Spinach leaf tissue DM Chl *a* and *b* concentrations responded to drying temperature ($p \leq 0.001$), N treatment ($p \leq 0.001$), and to the interaction between N treatment and drying temperature ($p \leq 0.001$). Spinach leaf tissue Chl *a* decreased linearly with increases in drying temperature for both the high N (Chl *a* = 11.0 -

0.1(T), $r^2 = 0.834$, $p \leq 0.001$) and low N treatments (Chl *a* DM = 4.9 - 0.07(T), $r^2 = 0.795$, $p \leq 0.001$)(Table 9.2). Spinach leaf tissue Chl *b* decreased linearly with increases in drying temperature for both the high N (Chl *b* DM = 4.0 - 0.05(T), $r^2 = 0.822$, $p \leq 0.001$) and low N treatments (Chl *b* = 1.9 - 0.07(T), $r^2 = 0.790$, $p \leq 0.001$) (Table 9.2).

Kale

Biomass Accumulation

Percent dry matter for kale was influenced by N treatment level ($p \leq 0.01$). No significant influence was measured for the kale drying temperature ($p = 0.627$) or the interaction between N treatment and drying temperature ($p = 0.904$). Average %DM for the kale increased from 14.8% for the high N treatment to 21.8% for the low N treatment (Table 9.3). The average %DM for the high N kale treatment resulted in an increase from 13.2% for the +75 ºC convection oven to 15.9% for the +25 ºC vacuum drier. The average %DM for the low N kale treatment comparing the drying temperature increased from 21.4% for the –25 ºC freeze drier to 22.5% for the +25 ºC vacuum drier. No trend was measured for the kale %DM for either the high or low N treatment.

Carotenoid and Chlorophyll Pigment Accumulation

The largest lutein accumulation FM in kale was 13.1 mg $100g^{-1}$ for the high N treatment and the +25 ºC drying temperature (Table 9.3). Kale leaf tissue lutein

concentrations responded to N treatment ($p \leq 0.001$), drying temperature ($p \leq 0.001$), and to the interaction between N treatment and drying temperature ($p = 0.044$). Kale leaf tissue lutein decreased linearly with increases in drying temperature for both the high N (Lutein FM = 12.1 - 0.09(T), $r^2 = 0.603$, $p \leq 0.001$) and low N treatment (Lutein FM = 9.0 - 0.08(T), $r^2 = 0.683$, $p \leq 0.001$).

The largest kale β-carotene FM was 5.6 mg 100 g^{-1} with the high N treatment and the –25 ºC drying temperature (Table 9.3). Kale leaf tissue β-carotene concentrations responded to N treatment ($p \leq 0.001$), drying temperature ($p \leq 0.001$), but not to their interaction ($p = 0.300$). Kale leaf tissue β-carotene decreased linearly with increases in drying temperature for both the high N (β-carotene FM = 5.1 - 0.04(T), $r^2 = 0.713$, $p \leq 0.001$) and low N treatment (β-carotene FM = 3.1 + 0.03(T), $r^2 = 0.558$, $p \leq 0.001$).

Kale leaf tissue FM Chl *a* concentrations responded to N treatment ($p \leq 0.001$), drying temperature ($p \leq 0.001$), and to the interaction between N treatment and drying temperature ($p = 0.028$). Kale leaf tissue Chl *b* concentrations responded to N treatment ($p \leq 0.001$), drying temperature ($p \leq 0.001$), and to the interaction between N treatment and drying temperature ($p \leq 0.001$). Kale leaf tissue Chl *a* decreased linearly with increases in drying temperature for both the high N (Chl *a* FM = 109.9 – 1.2(T), $r^2 = 0.799$, $p \leq 0.001$) and low N treatments (Chl *a* FM = 68.2 - 0.9(T), $r^2 = 0.831$, $p \leq 0.001$) (Table 9.3). Kale leaf tissue Chl *b* decreased linearly with increases in drying temperature for both the high N (Chl *b* FM = 43.7 - 0.4(T), r^2

= 0.667, $p \leq 0.001$) and low N treatments (Chl *b* FM = 29.9 - 0.3(T), $r^2 = 0.844$, $p \leq 0.001$) (Table 9.3).

The kale maximum lutein DM was 0.84 mg g^{-1} with the high N treatment and the –25 ºC drying temperature (Table 9.4). Kale leaf tissue lutein concentrations responded to N treatment ($p \leq 0.001$), drying temperature ($p \leq 0.001$), and to the interaction between N treatment and drying temperature ($p = 0.003$). Kale leaf tissue lutein decreased linearly with increases in drying temperature for both the high N (Lutein DM = 0.81 - 0.01(T), $r^2 = 0.619$, $p \leq 0.001$) and low N treatments (Lutein DM = 0.42 - 0.01(T), $r^2 = 0.714$, $p \leq 0.001$).

The kale maximum β-carotene DM was 0.37 mg g^{-1} with the high N treatment and the –25 ºC drying temperature (Table 9.4). Kale leaf tissue β-carotene concentrations responded to N treatment ($p \leq 0.001$), drying temperature ($p \leq 0.001$), and to the interaction between N treatment and drying temperature ($p = 0.011$). Kale leaf tissue β-carotene decreased linearly with increases in drying temperature for both the high N (β-carotene DM = 0.34 - 0.002(T), $r^2 = 0.742$, $p \leq 0.001$) and low N treatments (β-carotene DM = 0.14 + 0.001(T), $r^2 = 0.607$, $p \leq 0.001$).

Kale leaf tissue DM Chl *a* and *b* concentrations responded to N treatment ($p \leq 0.001$), drying temperature ($p \leq 0.001$), and to the interaction between N treatment and drying temperature ($p \leq 0.001$). Kale leaf tissue Chl *a* decreased linearly with increases in drying temperature for both the high N (Chl *a* = 6.3 - 0.05(T), $r^2 = 0.668$, $p \leq 0.001$) and low N treatments (Chl *a* DM= 3.1 - 0.03(T), $r^2 = 0.868$, $p \leq 0.001$) (Table 9.4). Kale leaf tissue Chl *b* decreased linearly with increases in drying

temperature for both the high N (Chl *b* DM = 6.3 - 0.05(T), $r^2 = 0.668$, $p \leq 0.001$) and low N treatments (Chl *b* = 3.1 - 0.03(T), $r^2 = 0.868$, $p \leq 0.001$) (Table 9.4).

Discussion

Part 1 reported that %DM varied when growing spinach under varying N levels. In that study, spinach %DM varied from 9.6% at 13 mg L^{-1} N to 6.9% at 105 mg L^{-1} N when the leaf tissue of the plant was oven dried at 50 ºC. In the current study, the %DM of the spinach samples varied from 6.4 to 12.7% for the high and low N treatments, respectively. The %DM for the kale varied from 13.2 to 22.5%, for the high and low N treatments, respectively.

Karathanos (1999) and Stegen et al. (1998) reported decreases in the %DM when oven drying at temperatures greater than 65 ºC. Karathanos (1999) suggested that the decrease in %DM was the result of decomposition of solids, in addition to water evaporation. Other researchers also reported the loss of VOC as drying temperature increased (Diaz-Maroto et al., 2002a; Diaz-Maroto et al., 2002b; Diaz-Maroto et al., 2004). In the current study, the high N spinach samples decreased from 7.3 to 6.4 %DM and low N spinach samples decreased from 12.7 to 9.6 %DM as the drying temperature increased.

Nitrogen has been correlated to chlorophyll in a number of studies, with lower N levels resulting in less chlorophyll production. Under limited N, both Chl *a* and *b* pigments are reduced, resulting in potential leaf tissue chlorosis (Taiz and Zeiger, 1998). In the current study the spinach chlorophyll FM concentrations decreased 17 and 11% and the kale decreased 32 and 26% for the Chl *a* and *b*, respectively. Part 1

reported that for the 'Melody' spinach, no significant increase occurred in the chlorophyll concentration, but 'Springer' spinach FM increased around 23% when N rates were increased from 13 to 105 mg L^{-1}. Grunwald et al. (1977) reported field tobacco (*Nicotiana tabacuin* L.) grown with limited N had a reduction in chlorophyll of 51% when N rates were reduced from 336 to 112 kg ha^{-1}.

In the current study, the FM lutein and β-carotene decreased as the N treatments increased. When measured as a function of DM, lutein concentrations increased as the N level increased, similar to the results of Part 1. Hochmuth et al. (1999) reported a quadratic response in carrot (*Daucus carota* L.) tissue carotenoids as N rates increased, with maximum carotenoid production occurring at 160 kg ha^{-1}. Part 1 reported for 'Melody' spinach tissue lutein, as a function of FM, did not have a significant increase. However, when lutein was measured on a DM basis an increase of 80% in 'Melody' was measured as the N level was increased by a factor of eight times.

Lutein, β-carotene and chlorophyll levels for both spinach and kale leaf tissue were affected by drying temperature. Concentrations of all four pigments decreased over 70% as the drying temperature increased. Tai and Chen (2000) reported that oven drying resulted in concentrations decreasing by 42% for lutein and 48% for β-carotene when compared to freeze drying. King et al. (2001) reported that chlorophyll concentration of freeze dried spinach remained higher than spinach that was dried at 1ºC. In the current study the largest spinach pigment FM and DM levels were measured at the –25 ºC freeze drier treatment. The largest kale pigment FM and DM levels were split between the –25 ºC freeze drier and +25 ºC vacuum drier

treatments. Comparing the data of the samples dried between -25 and +25 ºC resulted in no statistical difference between these drying temperatures. However, a linear decrease was observed from the data for drying temperature of +25, +50 and +75 ºC. The FM spinach β-carotene did not follow this trend, and decreased as drying temperature increased from -25 to +25 ºC. To limit possible degradation of pigments within kale and spinach tissue, samples should be dried at temperatures below +25 ºC. Hence, vacuum or freeze drying technology should be employed.

List of References

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Appendix

Table 9.1. Mean pigments concentrations[z] expressed on a fresh mass (FM) basis in the leaf tissues of 'Melody' spinach grown with two nitrogen levels and five drying temperatures.

Drying Temperature (ºC)	% DM	Pigment Concentration (mg 100g^{-1} FM)			
		Lutein	β-carotene	Chl *a*[y]	Chl *b*[y]
		Spinach High Nitrogen (105 mg L^{-1})			
+ 75	6.4 ± 0.1	2.2 ± 0.1	1.1 ± 0.1	0.8 ± 0.3	0.4 ± 0.1
+ 50	6.9 ± 0.2	5.1 ± 0.5	2.1 ± 0.2	9.5 ± 2.3	5.7 ± 1.3
+ 25	7.3 ± 0.1	8.0 ± 0.5	4.0 ± 0.3	79.2 ± 4.6	29.2 ± 1.6
0	7.2 ± 0.1	8.8 ± 0.6	4.5 ± 0.2	86.0 ± 5.6	31.8 ± 1.3
- 25	7.2 ± 0.2	9.0 ± 0.1	4.9 ± 0.1	91.6 ± 3.1	31.9 ± 0.8
Contrasts					
Linear	**	***	***	***	***
Quadratic	**	***	***	***	***
		Spinach Low Nitrogen (13 mg L^{-1})			
+ 75	10.5 ± 0.5	2.2 ± 0.4	1.3 ± 0.2	3.1 ± 1.7	1.5 ± 0.2
+ 50	9.6 ± 0.7	3.8 ± 0.2	1.2 ± 0.1	1.6 ± 0.6	2.2 ± 0.1
+ 25	11.4 ± 0.7	8.2 ± 0.6	3.2 ± 0.3	59.1 ± 6.2	22.3 ± 0.3
0	10.3 ± 0.1	7.0 ± 0.4	2.7 ± 0.2	51.5 ± 2.8	18.8 ± 0.3
- 25	12.7 ± 0.9	9.8 ± 1.4	4.2 ± 0.7	75.8 ± 9.9	28.3 ± 0.4
Contrasts					
Linear	*	***	***	***	***
Quadratic	ns	***	***	***	***

[z] Mean composition of sampled leaf tissue of three replications ± standard error.

[y] Chl *a* = *a*; Chl *b* = chlorophyll *b*.

[ns, *, **, ***] Non-significant or significance at $p \leq 0.05, 0.01, 0.001$, respectively.

Table 9.2. Mean pigments concentrations[z] expressed on a dry mass (DM) basis in the leaf tissues of 'Melody' spinach grown with two nitrogen levels and five drying temperatures.

Drying Temperature (ºC)	Pigment Concentration (mg g^{-1} DM)			
	Lutein	β-carotene	Chl *a*[y]	Chl *b*[y]
	Spinach High Nitrogen (105 mg L^{-1})			
+ 75	0.34 ± 0.01	0.16 ±0.01	0.1 ± 0.1	0.1 ± 0.1
+ 50	0.73 ± 0.04	0.31 ± 0.01	1.4 ± 0.3	0.8 ± 0.2
+ 25	1.10 ± 0.08	0.55 ± 0.05	10.9± 0.8	4.0 ± 0.3
0	1.22 ± 0.07	0.62 ± 0.02	11.9± 0.6	4.4 ± 0.1
- 25	1.25 ± 0.05	0.67 ± 0.03	12.7± 0.5	4.4 ± 0.2
Contrasts				
Linear	***	***	***	***
Quadratic	***	***	***	***
	Spinach Low Nitrogen (13 mg L^{-1})			
+ 75	0.20 ± 0.03	0.12 ± 0.01	0.3 ± 0.2	0.1 ± 0.1
+ 50	0.40 ± 0.04	0.12 ± 0.01	0.2 ± 0.1	0.2 ± 0.1
+ 25	0.72 ± 0.04	0.28 ± 0.02	5.1 ± 0.3	2.0 ± 0.1
0	0.68 ± 0.03	0.26 ± 0.02	5.0 ± 0.3	1.8 ± 0.1
- 25	0.77 ± 0.06	0.33 ± 0.03	5.9 ± 0.4	2.2 ± 0.2
Contrasts				
Linear	***	***	***	***
Quadratic	***	***	***	***

[z] Mean composition of sampled leaf tissue of three replications ± standard error.

[y] Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*.

*** significance at $p \leq 0.001$.

Table 9.3. Mean pigments concentrations[z] expressed on a fresh mass (FM) basis in the leaf tissues of 'Winterbor' kale grown with two nitrogen levels and five drying temperatures.

Drying Temperature (ºC)	% DM	Pigment Concentration (mg $100g^{-1}$ FM)			
		Lutein	β-carotene	Chl *a*[y]	Chl *b*[y]
		Kale High Nitrogen (105 mg L^{-1})			
+ 75	13.2 ± 0.5	2.1 ± 0.3	1.4 ± 0.1	6.5 ± 1.8	5.3 ± 1.2
+ 50	15.0 ± 1.4	10.0 ± 0.2	3.6 ± 0.2	42.1 ± 1.0	31.9 ± 1.4
+ 25	15.9 ± 0.4	13.1 ± 0.4	5.4 ± 0.2	112.5±12.4	46.9 ± 2.3
0	14.7 ± 1.8	11.6 ± 0.8	4.8 ± 0.4	118.4 ± 8.2	44.0 ± 2.4
- 25	15.0 ± 0.5	12.5 ± 0.7	5.6 ± 0.3	119.2 ± 4.6	44.8 ± 1.8
Contrasts					
Linear	ns	***	***	***	***
Quadratic	ns	***	***	***	***
		Kale Low Nitrogen (13 mg L^{-1})			
+ 75	21.7 ± 0.7	0.9 ± 0.2	0.5 ± 0.1	1.9 ± 0.9	2.6 ± 0.5
+ 50	22.0 ± 1.5	5.9 ± 0.3	1.9 ± 0.2	10.0 ± 2.4	11.1 ± 1.7
+ 25	22.5 ± 1.7	9.8 ± 0.7	3.6 ± 0.3	62.0 ± 12.7	28.4 ± 2.4
0	21.4 ± 1.0	9.3 ± 0.3	2.7 ± 0.8	75.0 ± 5.2	33.0 ± 2.0
- 25	21.4 ± 0.1	9.2 ± 0.3	3.4 ± 0.1	80.7 ± 4.3	32.9 ± 1.5
Contrasts					
Linear	ns	***	***	***	***
Quadratic	ns	***	***	***	***

[z] Mean composition of sampled leaf tissue of three replications ± standard error.

[y] Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*.

[ns, ***] Non-significant or significance at $p \leq 0.001$, respectively.

Table 9.4. Mean pigments concentrations[z] expressed on a dry mass (DM) basis in the leaf tissues of 'Winterbor' kale grown with two nitrogen levels and five drying temperatures.

Drying Temperature (ºC)	Pigment Concentration (mg g^{-1} DM)			
	Lutein	β-carotene	Chl *a*[y]	Chl *b*[y]
	Kale High Nitrogen (105 mg L^{-1})			
+ 75	0.15 ±0.02	0.10 ± 0.01	0.5 ± 0.1	0.4 ± 0.1
+ 50	0.68 ± 0.07	0.25 ± 0.03	2.2 ± 0.3	2.2 ± 0.2
+ 25	0.83 ± 0.04	0.34 ± 0.02	3.0 ± 0.9	3.0 ± 0.2
0	0.80 ± 0.06	0.33 ± 0.03	3.1 ± 0.5	3.1 ± 0.2
- 25	0.84 ± 0.03	0.37 ± 0.01	3.0 ± 0.4	3.0 ± 0.1
Contrasts				
Linear	***	***	***	***
Quadratic	***	***	***	***
	Kale Low Nitrogen (13 mg L^{-1})			
+ 75	0.04 ± 0.01	0.02 ± 0.01	0.1 ± 0.1	0.1 ±0.1
+ 50	0.28 ± 0.03	0.09 ± 0.01	0.5 ± 0.1	0.5 ± 0.1
+ 25	0.44 ± 0.01	0.15 ± 0.01	2.7 ± 0.4	1.3 ± 0.1
0	0.44 ± 0.03	0.12 ± 0.03	3.5 ± 0.1	1.6 ± 0.1
- 25	0.43 ± 0.01	0.16 ± 0.01	3.8 ± 0.2	1.5 ± 0.1
Contrasts				
Linear	***	***	***	***
Quadratic	***	***	***	***

[z] Mean composition of sampled leaf tissue of three replications ± standard error.

[y] Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*.

*** significance at $p \leq 0.001$.

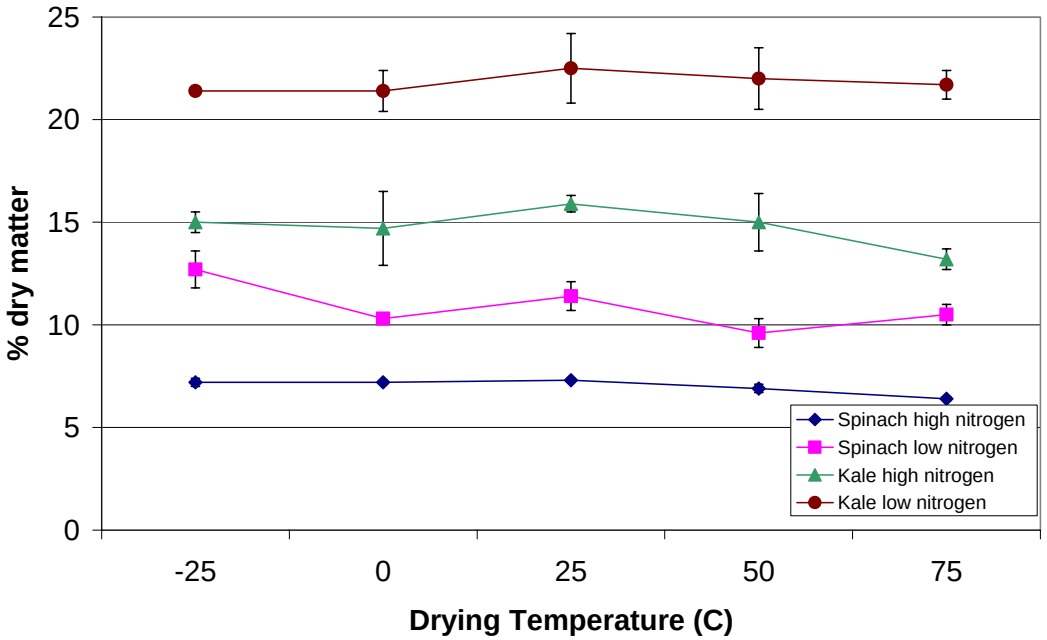

Figure 9.1. Drying effect on % dry matter of shoot tissue in kale and spinach grown under high (105 mg L^{-1}) and low (13 mg L^{-1}) nitrogen levels. Five drying temperatures: -25ºC freeze drying, 0ºC freeze drying, +25ºC vacuum drying, +50ºC convection oven, and +75ºC convection oven.

Part 10

Effect of % Dry Matter on the Measured Concentration of Carotenoids in Kale and Spinach Greens

Effect of % Dry Matter on the Measured Concentration of Carotenoids in Kale and Spinach Greens

This part is a lightly revised version of a paper by the same name that will be submitted to be published in the journal HortScience by Mark Lefsrud, Dean Kopsell, Jim Wills and A.J. Both:

Lefsrud, M.G., D.A. Kopsell, J.B. Wills, and A.J. Both. Effect of % dry matter on the measured concentration of carotenoids in kale and spinach greens.

My use of "we" in this part refers to my co-authors and myself. My primary contributions to this paper include (1) selection of the topic and development of the problem into a work relevant to my study of % dry matter and carotenoids in kale and spinach, (2) determination of species, variety and treatments, (3) analysis, (4) most of the gathering and interpretation of the literature, (5) compiling the information into a single paper, and (6) most of the writing and editing.

Abstract

Data was combined from spinach (*Spinacia oleracea* L.) and kale (*Brassica oleracea* L. var. *acephala* D.C.) growth chamber and greenhouse environmental manipulation experiments. The combined data % dry matter (%DM) ranged from 5 to 15% for spinach and from 9 to 23% for kale leaves. Comparing the %DM data to the pigment concentrations measured on both a fresh mass (FM) and dry mass (DM) basis resulted in a significant linear trend. Increases in %DM resulted in a linear increase in measured FM pigment concentration and a decrease in measured DM pigment concentration. Further evaluation of the data using principal components showed that the %DM could be used to explain approximately 40% of the variance in the pigment concentration. Results of this study would allow researchers to estimate pigment concentrations on a FM and DM basis based on the recorded %DM of the leaf tissue.

Introduction

Water is critical for plant growth and development, and through evapotranspiration, is responsible for transport, support and protection (Taiz and Zeiger, 1998). The amount of water within a plant can vary greatly depending on both genetic factors and environmental growing conditions (Davis et al., 2004; Kopsell et al., 2004; Lefsrud et al., 2005; Lefsrud et al., 2006). Under water stress, the water content within the plant decreases but the total dry matter remains constant, resulting in an increase in the measured % dry matter (%DM; Part 1). After harvest, the water content of the plant will change, and is dependent on harvesting and storage methods (Gill et al., 1999). Differences in water content have been suggested to cause variability in the concentration of secondary compounds within plants (Davis et al., 2004; Lefsrud et al., 2006). As the amount of water in the plant increases, the proportion of measured compound in the plant as a function of total plant mass decreases, a result of dilution (Lefsrud et al., 2006).

Research work into biological dilution effects has been limited. However, in chemistry it is well understood that a solution can be diluted by adding more solvent in relation to the solute. In biological systems this dilution has been suggested to occur, but has never been quantified. A number of researchers have listed dilution effects as the reason for reduced compound concentrations within biological samples.

Tomaselli et al. (1997) stated that in *Spirulina*, "the kinetic analysis suggested that the pigment decrease could be accounted for both dilution through growth and *in vivo* degradation." Tran et al. (1995) observed dilution of pigments in Orchidaceae hybrids containing regreening and wilting genomes. Lachaal et al. (2002) concluded

that the dilution effect may improve the salt tolerance of lentils (*Lens culinaris* Medik.) by increasing the time necessary for ion accumulation to reach toxic levels. It was hypothesized that new leaf growth was faster than the death caused by increased salt levels in the old leaves. Belkhodja et al. (1998) found that leaf chlorosis of peach trees (*Prunus persica* (L.) Batsch) due to Fe deficiency was the result of two separate processes, the dilution of chlorophyll due to plant growth and the subsequent inability to produce and/or stabilize new chlorophyll molecules. Genard et al. (2003) created a model that included dilution to determine the effect of sugar concentration within peaches. These researchers suggested that increases in water content of plants, relative to biomass, resulted in dilution of the compounds being measured.

Davis et al. (2004) compared 43 garden crops from U.S. Dept. Agr. food composition data collected in 1950 and 1999 to determine if crops had changed in nutritional value. The nutritional value of vegetables grown during the two periods was significantly different. However, a major factor that determined the nutritional quality of the crops was the moisture content of the plant tissues. For example, the moisture content of mustard greens (*Brassica juncea* (L.) Czern.) was measured at 7.8 %DM in 1950 and at 9.2 %DM in 1999. This 18% change in %DM was the major factor that explained the difference between the growing periods. No significant differences were measured when the nutritional values were calculated using a standardized %DM. Part 1 reported similar values in variability of %DM and carotenoid concentration within spinach leaves (*Spinacia oleracea* L.). 'Springer'

spinach carotenoid concentrations were not statistically significant when measured on a fresh mass basis but were significant when measured on a dry mass basis.

Kale (*Brassica oleracea* L. var. *acephala* D.C.) ranks highest, and spinach ranks second, among vegetable crops for carotenoid content, including lutein and β-carotene (Holden et al., 1999; Kurilich et al., 1999; U.S. Dept. Agr., 2002). However, kale has low consumption rates, with a per capita fresh intake of less than 0.33 kg $year^{-1}$ (Lucier and Plummer, 2003). Spinach has one of the highest rates of consumption among green-leafy vegetables in the United States, with per capita intakes of 0.73, 0.09, and 0.36 kg $year^{-1}$ for fresh, canned, and frozen product, respectively (Lucier and Plummer, 2003).

Carotenoids are yellow, orange, and red lipid soluble pigments produced by plants, algae and bacteria. In plants, carotenoids function as light harvesting antennae pigments, to funnel light energy to the photosynthetic reaction center (Demmig-Adam et al., 1996; Miki, 1991; Tracewell et al., 2001). These carotenoids also are important free radical scavengers, and have photoprotective roles (Demmig-Adam et al., 1996; Miki, 1991; Tracewell et al., 2001). These carotenoids are in close proximity to the chlorophyll molecules and quench the energetic triplet state of the chlorophyll (Chl) molecule to prevent damage to the photosynthetic system (Marschner, 1997; Miki, 1991; Taiz and Zeiger, 1998; Tracewell et al., 2001).

Lutein and β-carotene also possess important human health properties. However, these carotenoids cannot be synthesized in mammals, making plants the primary source of carotenoids in their diet. Dietary intake of foods rich in lutein and β-carotene has been associated with reduced risk of lung cancer, cataracts, and age-

related macular degeneration (Ames et al., 1995; Landrum and Bone, 2001; Le Marchand et al., 1993).

Most spinach is consumed fresh (Lucier and Plummer, 2003). Thus, changes in the water content due to growing environment, handling, storage, or food processing may influence the nutritional value of the plant (Gil et al., 1999). The goal of this research was to determine the influence of %DM on the nutritional value of kale and spinach.

Material and Methods

Data was collected from a number of previous studies that investigated the effect of environment and nutrition on %DM and the plant pigments Chl *a*, Chl *b*, lutein and β-carotene. Data collected for kale analysis consisted of hydroponic growth chamber experiments that measured the effect of air temperature (Lefsrud et al., 2005), irradiance (Lefsrud et al., 2006), photoperiod (Part 4), radiation cycle (Part 5) and leaf ontogeny (Part 7) on plant growth and pigment accumulation. Data collected for spinach analysis consisted of hydroponic growth chamber and greenhouse experiments that measured the effect of air temperature (Lefsrud et al., 2005), irradiance (Lefsrud et al., 2006), and nitrogen (Part 1) on plant growth and pigment accumulation. Procedures for plant production and data collection are presented in each of these papers. Plant samples were collected and analyzed with HPLC to determine pigment concentration. Freeze dried samples were used to determine %DM, according to results in Part 9.

Statistical Analysis

Data was analyzed comparing %DM and pigment concentration for one-way ANOVA analysis and principal component analysis (PCA) using SPSS (Chicago, Ill.). PCA with oblique rotation was used to determine variance of %DM and pigment concentrations (Cattell and Khanna, 1977; Cooper, 1983; Harman, 1976; Kaiser, 1958). The relationship between experimental dependent variables (lutein, β-carotene, chlorophyll *a* and chlorophyll *b* on both fresh and dry mass basis) and %DM were determined by regression analysis.

Results and Discussion

Spinach and kale data was compiled using the pigment concentrations measured on both a fresh (FM) and dry mass (DM) basis. When the pigment concentrations were compared to %DM, a linear correlation was observed (Figure 10.1-2[a]). The %DM in the data, ranged from 5 to 15% for the spinach and from 9 to 23% for the kale. A one-way ANOVA was performed with %DM as the factor and the pigments as the dependents. Lutein, β-carotene, Chl *a* and Chl *b* were significant ($p \leq 0.005$) for both spinach and kale when measured on both a FM and DM basis. Regression analysis of the data resulted in a significant linear trend for all of the pigments concentrations measured (Table 10.1). The FM pigment concentrations had a positive correlation with increasing %DM, whereas DM pigment concentration had a negative correlation with increasing %DM. The linear correlations were significant, but weak, with measured r^2 values ranging between 0.29 to a low of 0.09.

[a] All tables and figures are located in the appendix at the end of this part.

When PCA was applied to both the FM and DM data, the first two principal components explained 95% of the variance between the samples for spinach and 86% of the variance between the samples for kale. Principal component 1 explained 48% of the variance between samples for spinach and 43% of the variance between samples for kale. Principal component 1 has a strong correlation to the pigment concentration and principal component 2 had a strong correlation to %DM. The PCA was then obliquely rotated so that %DM directly correlated to principal component 2 (Table 10.2-3; Figure 10.3). Principal component 2 explained 98% of the variance in %DM for spinach and 99% of the variance in %DM for kale. Principal component 2 was then equated to %DM. Spinach %DM component (principal component 2) explained between 44 and 55% of the variance of the pigment concentration FM and between -29 and -44% of the variance of the pigment concentration DM. Kale %DM component (principal component 2) could explain between 35 and 47% of the variance of the pigment concentration FM and between -40 and -49% of the variance of the pigment concentration DM. Diaz-Maroto et al. (2002) reported very similar results in parsley (*Petroselinum crispum* L.) when dried using different methods. In their study, the first two principal components were able to explain approx. 88% of the variance of volatile plant compounds for parsley using different drying methods. These PCA results were very similar to our result with two components able to explain 86 and 95% of the variance of plant pigments for kale and spinach, respectively.

This variance explained by the %DM component was consistent with both the kale and spinach samples. Approximately 40% of the variance was explained in the

FM pigment concentrations and approximately -40% of the variance was explained in the DM pigment concentrations with the %DM component. This positive correlation suggests that as the %DM increases in the plant, the measured pigment concentration expressed on a FM basis increases. The negative correlation suggests that as the %DM increases, the measured pigment concentration expressed on a DM basis decreases. Principal component 1 has a large influence on determining the variance of the pigments with approximately 80% of the variance explained for both the FM and DM pigment concentrations.

Increases in %DM resulted in increases in measured pigment concentration expressed on a FM basis and decreases in measured pigment concentrations expressed on a DM basis. This result would allow researchers to estimate with a 40% confidence the pigment concentration expressed on a FM and DM basis after determining %DM.

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Appendix

Table 10.1. Linear regression equations expressing spinach and kale pigment concentrations based on dry matter content (%DM).

	Equation	r^2	Significance
	Spinach		
FM Lutein	3.7 + 0.5(%DM)	0.26	$p \leq 0.001$
FM β-carotene	2.6 + 0.5(%DM)	0.29	$p \leq 0.001$
FM Chl a	60.5 + 8.6(%DM)	0.22	$p \leq 0.001$
FM Chl *b*	18.1 + 3.7(%DM)	0.19	$p \leq 0.001$
DM Lutein	1.3 – 0.04(%DM)	0.13	$p \leq 0.001$
DM β-carotene	1.1 – 0.03(%DM)	0.18	$p \leq 0.001$
DM Chl a	21.3 – 0.6(%DM)	0.12	$p \leq 0.001$
DM Chl *b*	5.8 – 0.2(%DM)	0.09	$p = 0.002$
	Kale		
FM Lutein	7.4 + 0.4(%DM)	0.22	$p \leq 0.001$
FM β-carotene	6.0 + 0.3(%DM)	0.16	$p \leq 0.001$
FM Chl a	141.6 + 5.6(%DM)	0.14	$p \leq 0.001$
FM Chl *b*	30.7 + 1.6(%DM)	0.22	$p \leq 0.001$
DM Lutein	1.4 – 0.03(%DM)	0.22	$p \leq 0.001$
DM β-carotene	1.1 – 0.03(%DM)	0.15	$p \leq 0.001$
DM Chl a	24.0 – 0.6(%DM)	0.24	$p \leq 0.001$
DM Chl *b*	5.5 – 0.1(%DM)	0.12	$p \leq 0.001$

FM = fresh mass; DM = dry mass; Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*.

Table 10.2. Spinach principal component analysis data. Oblique rotation was performed to for principal component 1 (PC 1) and to equate PC 2 to %DM.

	Principal Components Oblique Rotation	
Spinach Factors	PC 1 (%)	PC2 (%DM (%))
%DM	0	98.6
FM Lutein	80.9	52.5
FM β-carotene	80.3	55.6
FM Chl a	86.1	48.7
FM Chl *b*	88.1	44.8
DM Lutein	88.5	-38.0
DM β-carotene	92.9	-29.6
DM Chl a	92.1	-35.7
DM Chl *b*	87.7	-44.0

FM = fresh mass; DM = dry mass; Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*.

Table 10.3. Kale principal component analysis data. Oblique rotation was performed for principal component 1 (PC 1) and to equate PC 2 to %DM.

	Principal Components Oblique Rotation	
Kale Factors	PC 1 (%)	PC2 (%DM (%))
%DM	0	99.1
FM Lutein	83.6	47.5
FM β-carotene	81.6	35.3
FM Chl a	83.6	38.7
FM Chl *b*	78.1	47.9
DM Lutein	83.3	-47.6
DM β-carotene	78.6	-40.1
DM Chl a	78.9	-49.6
DM Chl *b*	78.6	-47.2

FM = fresh mass; DM = dry mass; Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*.

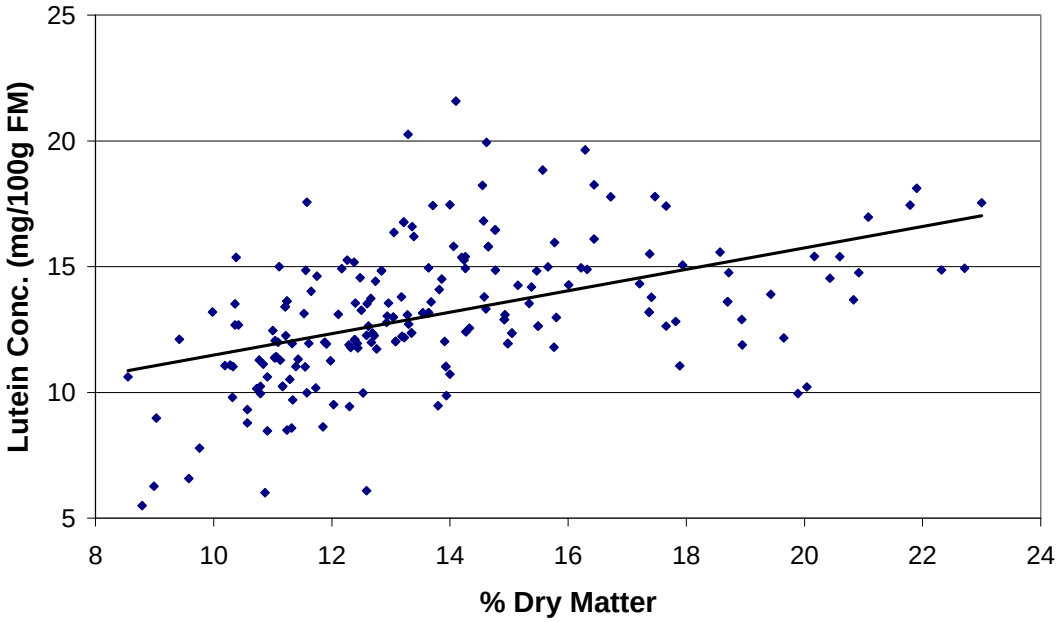

Figure 10.1. Combined data of kale lutein fresh mass (FM) concentration verse plant sample % dry matter.

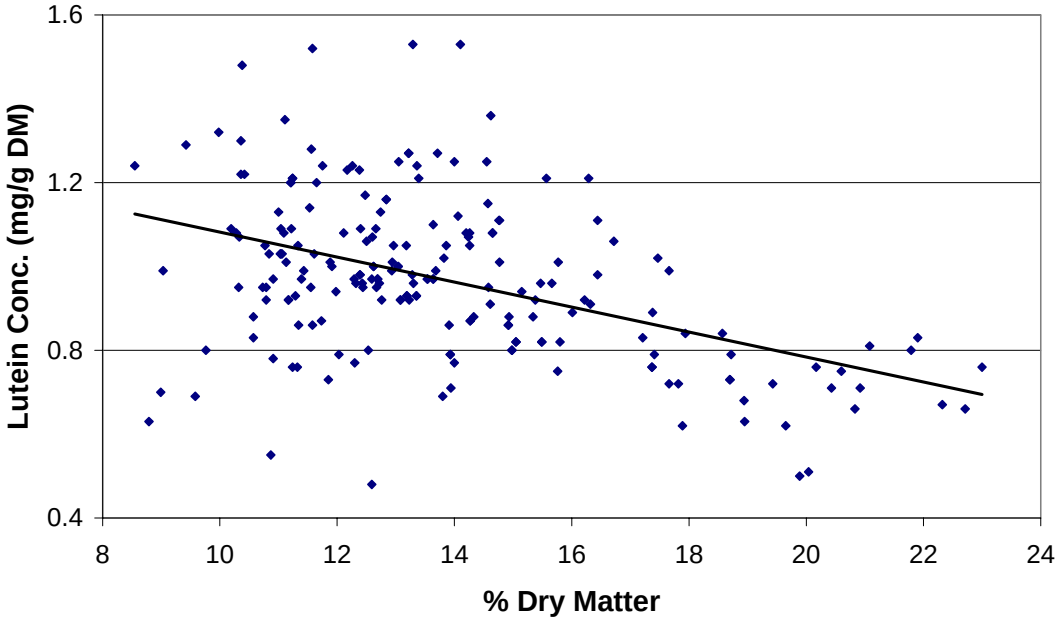

Figure 10.2. Combined data of kale lutein dry mass (DM) concentration verse plant sample % dry matter.

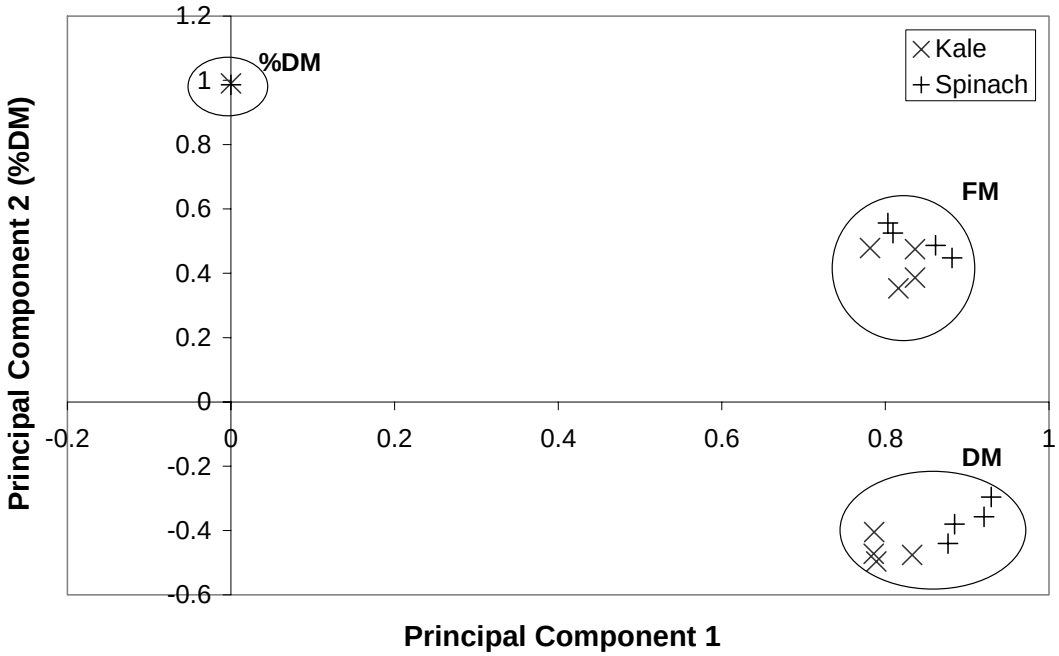

Figure 10.3. Principal components plot. Principal Component 1 and Principal Component 2 (% dry matter (%DM)). Combined data of spinach and kale, where regions are %DM, pigment concentration expressed on a fresh mass (FM) basis and pigment concentration expressed on a dry mass (DM) basis.

Conclusions

Conclusion

The purpose of the described studies was to determine how environmental stress affects pigment accumulation in kale and spinach. In the first study, two varieties of spinach were grown with varying N fertilization rates. Only the variety 'Springer' had a significant response to changes in N fertility, when measured according to the standard food composition fresh mass (FM) method. This method requires that plant samples be initially dried and then rehydrated to determine the carotenoid concentration of lutein and β-carotene. HPLC analysis determined the carotenoid concentration in the processed sample. To equate this HPLC measured value to a standard fresh sample, a mathematical calculation was performed using the original dried sample mass and the water content in the original harvested plant. Removing the water content from this calculation yields the carotenoid concentration on a dry mass (DM) basis. The same statistical procedures were followed on this DM data and both varieties of spinach were statistically affected by N treatment. This research proved that to provide a more thorough understanding of the nutritional value of the foods, both the FM and DM carotenoid concentrations should be reported.

A second observed result of the spinach N treatment study was that the largest pigment accumulation did not always correlate to maximum biomass. The maximum biomass production in both varieties occurred at the nitrogen treatment of 105 mg L^{-1} N. However, the largest pigment accumulation of 'Melody' occurred at 52 mg L^{-1} N, when expressed on both a FM and DM basis. 'Springer' had the largest pigment accumulation with the 52 mg L^{-1} N treatment on a FM basis and 105 mg L^{-1} N on a

DM basis. Further experiments tested the effect of irradiance, photoperiod, radiation cycle and temperature, confirmed the observation that increased carotenoids did not always correlate to increased biomass production.

During the air temperature experiment the largest pigment accumulation occurred at 30 ºC for kale on both FM and DM basis, while spinach had maximum accumulation at 10 ºC on a FM and 20 ºC on a DM basis. The maximum biomass production occurred at 20 ºC for both kale and spinach. In the irradiance experiment the maximum carotenoid accumulation occurred between 200 and 335 μmol m^{-2} s^{-1} for both kale and spinach, while maximum biomass occurred at the maximum treatment irradiance level of 620 μmol m^{-2} s^{-1}. When the photoperiod was varied for kale, the maximum biomass production and FM carotenoid accumulation occurred under continuous irradiance (24 hr) while the maximum DM accumulation occurred with a 16 hr photoperiod. Plants were grown under varying radiation cycles of 2, 12, 24 and 48 hr with 50% of the cycle illuminated. The maximum biomass production occurred with the short radiation cycle of 2 hr, but maximum pigment accumulation occurred under the 12 hr cycle.

Further experiments were performed to determine what other factors influence pigment accumulation in kale. Selenium fertility did not have any significant effect on lutein or β-carotene accumulation in kale, but selenium concentrations did increase in the plant. However, using different wavelength of LEDs did have an impact on pigment accumulation. Plants that were grown under red light (640 nm) accumulated the largest amount of pigment while blue light (440 nm) had increased levels relative to the other wavelength tested.

Two experiments were performed to determine if plant sampling method or drying procedures could cause variation in the measured carotenoid accumulation. When kale leaves were sampled according to age, a quadratic trend was observed with maximum lutein accumulation occurring between week 1-2 for both FM and DM leaves, while β-carotene peaked at week 2-3 FM and week 3-4 DM. The largest biomass was measured at week 3-4 leaves. My sampling procedure always selected leaves at the week 2-3 stage for all experiments. Drying method and temperature were tested to determine if freeze drying or oven drying at varying temperatures was optimum for conserving the pigment. Testing both spinach and kale showed that optimum pigment retention occurred at temperatures under +25ºC, while measurable loss in biomass and carotenoids occurred at higher temperatures. In my experiments, all drying occurred at temperatures below +25ºC.

The environmental manipulation data from the spinach and kale experiments was combined. The % dry matter (%DM) ranged from 5 to 15% for spinach and from 9 to 23% for kale. When plant pigment concentrations were analyzed in relation to %DM a linear trend was observed. Increases in %DM resulted in linear increases in measured FM pigment concentration and decreases in measured DM pigment concentration. Further analysis of the data using principal components showed that the %DM could be used to explain approximately 40% of the variance in the pigment concentration. The result of this research shows that water content strongly determines the measured carotenoid and chlorophyll concentrations in the plant. Reporting both FM and DM pigment concentrations along with %DM of the sample provides a more accurate method of determining the nutritional content of the plant.

Ultimately consumers are going to want to know what is in the foods they consume. Packaged foods are now required to list all of the ingredients and nutritional composition per serving. Similar labels may ultimately be required for fresh fruits and vegetables. Increased information provided to the consumer may lead to further improvements in the nutritional quality of fruits and vegetables. Through food selection, consumers will determine what specific nutritional qualities are important. As horticulturalists it will be our job to educate the public on critical health properties in foods and to determine critical methods to optimize their production and accumulation.

Vita

Mark Gregory Lefsrud was born in Edmonton, Alberta and raised on a mixed crop cereal farm 12 miles north of Viking, Alberta. He graduated from Viking School in 1992. He attended the University of Alberta, Edmonton in Mechanical Engineering and transferred to the University of Saskatchewan, Saskatoon where he was awarded a B.S. in Agricultural and Bioresource Engineering in 1997, with a focus on the construction of farm buildings and environmental engineering. His M.S was awarded in Agriculture Engineering from Rutgers University in 2001 and his thesis focused on the design of controlled environments and nutrient delivery systems. Mark then attended the University of New Hampshire, Durham where he worked on his doctorate to improve the human nutritional qualities of vegetable and fruits through genetic screening, environmental manipulation and fertilization. He then transferred to the University of Tennessee, Knoxville and is currently continuing his doctorate in plants, soils and insects, emphasis in horticulture.