

PLANT ADAPTATION AND ENHANCEMENT OF PHYTOCHEMICALS IN LETTUCE IN  
RESPONSE TO ENVIRONMENTAL STRESSES

by

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B.S., Seoul National University, 1999  
M.S., Seoul National University, 2003

AN ABSTRACT OF A DISSERTATION

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Department of Horticulture, Forestry and Recreation Resources  
College of Agriculture

KANSAS STATE UNIVERSITY  
Manhattan, Kansas

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## Abstract

Studies were conducted to examine the role of antioxidants in adaptation of lettuce (*Lactuca sativa* L.) to unprotected environmental conditions and various environmental stresses. Antioxidants, in addition to being a plant defense mechanism, are phytochemicals that provide significant health-promoting and nutritive value in human diet. Various approaches involving mild environmental stresses and cultural and management practices have been used to enhance the quality of this commonly consumed leafy vegetable by improving its phytochemical and health-promoting attributes. Lettuce plants grown in protective environments adapt to unprotective environmental conditions by activating antioxidant genes such as phenylalanine ammonia-lyase (PAL),  $\gamma$ -tocopherol methyl transferase ( $\gamma$ -TMT), and L-galactose dehydrogenase (L-GalDH) involved in the biosynthesis of phenolic compounds,  $\alpha$ -tocopherol and ascorbic acid, respectively. Mild environmental stresses such as heat shock, chilling, water stress and high light also activate these genes leading to the accumulation of secondary metabolites and phytochemicals without any adverse effect on biomass accumulation. The phytochemicals included chlorogenic acid, chicoric acid, caffeic acid, quercetin-3-O-glucoside and luteolin-7-O-glucoside. However, under field conditions, application of drought stress did not produce consistent results with regard to the phytochemical composition of lettuce. Plants grown in open field have higher phenolic content and higher antioxidant capacity than those grown in high tunnel. However, these plants also had less biomass accumulation. Many factors such as plant age, variety, fertilization, transplanting shock affected the phytochemical composition of lettuce. The red leaf variety 'Red Sails' had higher antioxidant capacity than 'Baronet' and younger

plants had the highest phytochemical content. With regard to the management practices, there was no significant difference in phytochemical composition between organically and conventionally managed crops. Also, low fertility favored the accumulation of phytochemicals and increased the antioxidant capacity. Thus, the results show that mild environmental stresses along with appropriate cultural and management practices can enhance the quality of lettuce by improving their phytochemical composition.

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## **Dedication**

I dedicate this dissertation to my almighty God who gave me wisdom to research these studies, taught me patience to overcome difficulties, suggested to me a vision for God's World, and always led me into a right path.

## CHAPTER 1 - Introduction

Lettuce (*Lactuca sativa* L.) has been a common leafy vegetable in salads since ancient times (Harlan, 1986). It is still one of the most popular leafy vegetables consumed fresh or in salad mixes, and ranks among the top 5 vegetables in the U.S. in both its production and consumption (Lucier and Jerardo, 2006). Lettuce is rich in phytochemicals such as phenolic compounds, vitamin A, C, and E as well as minerals such as calcium and iron which are essential in preventing diseases and promote health and wellness in people (Caldwell, 2003; Llorach et al., 2004; Nicolle et al., 2004; Romani et al., 2002). In recent years, the potential importance of vegetables including lettuce in the daily diet is being increasingly recognized as many epidemiological studies suggest that daily intake of fruits and vegetables has health benefits in preventing chronic and degenerative diseases, including cardiovascular disease and several types of cancer (Arai et al., 2000; Birt et al., 2001; Hu, 2003; Jang et al., 1997).

Phytochemicals are defined as compounds found in plants that are not required for normal functioning of human body but nonetheless have a beneficial effect on health or have an active role in the amelioration of disease. Thousands of phytochemicals have been identified in fruits and vegetables although a great many are yet to be identified (Boyer and Liu, 2004). Phytochemicals include a vast array of chemicals some of which are secondary metabolites like phenolics, terpenoids, and alkaloids and vitamins (Blokhina et al., 2003; Grusak, 2002; Keleş and Öncel, 2002; Rice-Evans et al., 1997; Sofo et al., 2005). Many researchers have reported various biological functions for phytochemicals in plants. For example, secondary metabolites are suggested to have an adaptive function in plants against abiotic and biotic stresses (Herms and Mattson, 1992; Kliebenstein, 2004; Mithöfer et al., 2004).

## **Plant Defense against Environmental Stresses and Phytochemicals**

Plants respond to abiotic stresses including high or low temperatures, drought, salt, and UVB/high light, and acclimate to these conditions to varying degrees. The accumulation of osmolytes is a common phenomenon in plants exposed to abiotic stresses. Proline accumulated in plants in response to stressful conditions is known to play an important role in protecting plasma membrane against oxidative stress (Hare et al., 1999; Hasegawa et al., 2000; Kuznetsov and Shevyakova, 1997; Saradhi et al., 1995). Similarly, glycine betaine accumulation is known to protect plants against cold and drought stress (Rajashekar et al., 1999; Xing and Rajashekar, 2001). Late embryogenesis abundant (LEA) proteins and numerous similar proteins are also known to be involved in plant stress tolerance (Mowla et al., 2006; Roberts et al., 1993; Wise and Tunnacliffe, 2004).

In addition, environmental stresses trigger defense or adaptive mechanisms in plants resulting in the accumulation of a wide range of antioxidants. They include enzymatic antioxidants such as superoxide dismutase (SOD) and catalase (CAT), and non-enzymatic antioxidants such as ascorbic acid, tocopherols, carotenoids, and some phenolic compounds, which can quench the reactive oxygen species (ROS) (Mckersie and Leshem, 1994). Plants use these antioxidants to offset the oxidative damage resulting from these stresses (Monk et al., 1989). Typically, phytochemicals which are non-enzymatic antioxidants are also known to have health-promoting effects in humans. However, antioxidants have traditionally been viewed as a defense mechanism in plants against a host of biotic and abiotic stresses, but not so much in relation to their nutritive and health-promoting value in human diets.

Drought stress has been found to increase  $\beta$ -carotene and  $\alpha$ -tocopherol in wheat seedlings and lavender plants, respectively (Keleş and Öncel, 2002; Munné-Bosch et al. 2001). In addition, several essential phytochemicals in purple coneflower and St. John's wort were enhanced under

drought stress conditions (Gray et al., 2003a; Gray et al., 2003b; Zobayed et al., 2007). Chalcone synthase (CHS), an enzyme involved in biosynthesis of flavonoids, and PAL, the key enzyme in phenolic biosynthesis, were upregulated by drought stress in *Arabidopsis* (Bray, 2002).

Chilling stress has been shown to elevate total phenolic content and antioxidant capacity in petunia (Pennycooke et al., 2005). Similarly, soybean root exposed to 24 h of low temperature accumulated more phenolic compounds, including isoflavonoids (Janas et al., 2002). Flavanone-3-hydroxylase, which is a key enzyme in relation to the biosynthesis of flavonoids, was activated by cold stress in roots and leaves of *Arabidopsis* (Kreps et al., 2002).

Relationship between phytochemicals and high light or UVB radiation has been well established. UVB has been shown to induce a broad range of phytochemicals, which absorb the harmful radiation and protect plants against damaging ROS (Bieza and Lois, 2003; Burritt and Mackenzie, 2003; Gitz III et al., 2004). UVB radiation can also increase PAL activity which can facilitate phenolic accumulation and thus, induce stress tolerance in plants (Teklemariam and Blake, 2004).

Similarly, high temperature has been shown to induce phytochemical accumulation in several plant species as a defense strategy (Lefsrud et al., 2005; Sayre et al., 1953).

Finally, nutrient deficiency has also been implicated in phytochemical response in plants. Nitrogen deficiency in particular induced the accumulation of phytochemicals such as ascorbic acid, flavonoids, and flavonols in *Arabidopsis* and tomato (Bongue-Bartelsman and Phillips, 1995; Kandlbinder et al., 2004; Stewart et al., 2001). Other nutrients like phosphate and sulfur can also trigger similar phytochemical response in plants, if they become deficient (Kandlbinder et al., 2004; Stewart et al., 2001). In contrast, excessive nitrogen application decreased phenolic

compounds in olive fruits and apple leaves compared to those in control plants (Fernández-Escobar et al., 2006; Leser and Treutter, 2005).

### **Secondary Metabolism Models**

The plant defense mechanism against stresses is characterized by the activation of secondary metabolism (Herms and Mattson, 1992). A number of hypotheses have been advanced to explain the shift from primary metabolism to secondary metabolism in plants. Among these, two models, carbon-nitrogen balance (CNB) and protein competition model (PCM), address the broader aspects of metabolic balance and its relationship with environmental constraints. CNB hypothesizes that a shift in the balance between carbon and nitrogen allows for a transition from primary to secondary metabolism (Bryant et al., 1983). According to this hypothesis, the reduction of plant growth induced by nitrogen deficiency increases the carbon availability in plants. The carbon resources are used for making carbon-based secondary metabolites like phenolics and terpenes. PCM hypothesis is somewhat similar to CNB in that balance between the need for protein synthesis and the available phenylalanine pool in plants (Jones and Hartley, 1999) can induce a shift from primary and secondary metabolism in plants. Phenylalanine, one of the key aromatic amino acids, is typically used in protein synthesis in actively growing plants. If plant growth is stalled because of nutrient deficiency or environmental stresses, there is less carbon fixation and thus, less protein synthesized. This permits plants to use phenylalanine for synthesizing secondary metabolites, instead of protein (Fig. 1.1).

### **Phytochemicals and Human Health**

Phytochemicals, largely due to their antioxidant properties, appear to function in a similar fashion in humans as they do in plants. Humans are routinely exposed to oxidative damage during the course of normal metabolic processes (Boyer and Liu, 2004). Oxidative damage may

cause many degenerative diseases such as cancer, cardiovascular disease, Alzheimer disease, and cataracts (Ames et al., 1993). Many epidemiological studies have shown that daily intake of fruits and vegetables rich in phytochemicals can provide health benefits and prevent many of these diseases (Arai et al., 2000; Berger, 2005; Jang et al., 1997). Thus, there is an increasing awareness of the importance of phytochemicals in human diet.

### **Phytochemical Biosynthesis in Plants**

Most secondary metabolites with antioxidant properties including the diverse group of phenolic compounds are synthesized through the shikimic acid pathway (Fig. 1.1). Although malonic acid pathway is also involved in the biosynthesis of some of these compounds (flavonoids), its role is rather minor in higher plants (Taiz and Zeiger, 1998). The first key step in the shikimic acid pathway is the condensation of erythrose-4-phosphate from the pentose phosphate pathway and phosphoenol pyruvate from glycolysis producing 3-deoxy-D-arabino-heptulosonate 7-phosphate and inorganic phosphate (Herrmann, 1999). Shikimic acid pathway leads to the formation of three aromatic amino acids; tryptophan, tyrosine, and phenylalanine. Most classes of phenolic compounds are derived from phenylalanine although hydrolyzable tannin is directly produced through gallic acid by the shikimic acid pathway. The production of phenolic compounds is catalyzed by phenylalanine ammonia-lyase (PAL). PAL is a key gateway enzyme in secondary metabolic pathway leading to the synthesis of phenolic compounds. Control of PAL activity seems to be a key factor in regulating this pathway (Jones, 1984). The PAL gene that encodes for this enzyme is activated in response to a wide range of stresses including wounding, infection, and environmental stresses (Diallinas and Kanellis, 1994; Liu et al., 2006; Reymond et al., 2000).

To evaluate the role of PAL in plants, several studies have been conducted using 2-aminoindan-2-phosphonic acid (AIP). AIP is a key inhibitor of PAL activity and is considered as a stronger inhibitor than 2-aminoxyacetate (AOA) and 2-aminoxy-3-phenylpropanoic acid (AOPP) (Appert et al., 2003). As AIP inhibits PAL activity, it prevents browning in cut lettuce caused by the accumulation of phenolic compounds during cold storage (Hisaninato et al., 2001; Peiser et al., 1998). In addition, AIP-treated duckweed was more sensitive to UVB than the control because duckweed exposed to AIP had lower potential quantum yield during photosynthesis (Gitz III et al., 2004). Keski-Saari (2005) found that inhibition of PAL by AIP treatment significantly decreased the content of total phenolic compounds. Thus, these results suggest that not only PAL is the rate-limiting enzyme of secondary metabolites but also plays an important role in plant stress tolerance.

In addition to phenolic compounds, fruits and vegetables are generally rich in vitamins such as vitamin E and C which also have strong antioxidant properties. Vitamin E is a product of secondary metabolism and is synthesized from tyrosine, an important product of shikimic acid pathway (Fig. 1.2A). The key enzyme that regulates the synthesis of vitamin E ( $\alpha$ -tocopherol) is  $\gamma$ -tocopherol methyl transferase ( $\gamma$ -TMT) which methylates  $\gamma$ -tocopherol in the last step to form  $\alpha$ -tocopherol (Koch et al., 2003; Shintani and DellaPenna, 1998). Similarly, the key enzyme involved in the last critical step in the biosynthesis of vitamin C (ascorbic acid) is galactose dehydrogenase (L-GalDH) which oxidizes L-galactone-1,4-lactone to ascorbic acid (Gatzek et al., 2002) (Fig. 1.2B). These enzymes are rate limiting and the genes encoding for them play a critical role in the biosynthesis and accumulation of these antioxidants in plants.

Understanding the biosynthetic pathways of vitamins has allowed the researchers to embark on genetic manipulation of plants to improve their nutritive and health-promoting



qualities. However, the efforts to enhance phytochemicals in plants through genetic engineering, although successful in many cases, have been limited to only few crops and non-crop species like *Arabidopsis* (Hossain et al., 2004; Shintani and DellaPenna, 1998; Wheeler et al., 1998; Winkel-Shirley, 2002; Ye et al., 2000). Among these efforts, development of enhanced vitamin A rice, called Golden Rice, is the celebrated case of successful practical application of metabolic engineering to improve nutritive quality of food crops (Ye et al., 2000). Subsequently, after 5 years, a new improved variety called Golden Rice 2 was introduced which produces up to 23 times more  $\beta$ -carotene than the original variety of golden rice (Paine et al., 2005). However, because of health and environmental concerns and strong opposition from environmental and anti-globalization organizations neither variety has been popular in countries where rice is the staple food.

### **Enhancing Quality of Food and Future Perspectives**

In recent years, there has been an increasing interest in organically grown food because people believe that it has less pesticide residues and is more healthy (Zhao et al., 2006). However, most studies on this subject have led to conflicting results. It is not clear whether organically grown food contains more health-promoting phytochemicals as opposed to conventionally grown food. We also do not know if environmental conditions, cultural practice, and management approaches can impact the quality of food by their abilities to promote good health and well being. However, consideration of plant environmental stresses to improve the quality of plant-based foods may be a logical choice in view of the plants responses to a wide range of abiotic and biotic stresses.

In fact, new management strategies are emerging that use ecophysiological factors to elevate phytochemical concentrations in food crops. Some ecophysiological conditions that are

thought to have significant impact on enhancing the health-promoting phytochemicals in a number of vegetable crops include environmental conditions and cultural and management practices such as daily temperature, daily irradiation, fertilizer supply, irrigation, and production time (Schreiner, 2005). Thus, there is an increasing interest in using appropriate strategies and management practices to improve the quality of food crops by enhancing their nutritive and health-promoting properties.

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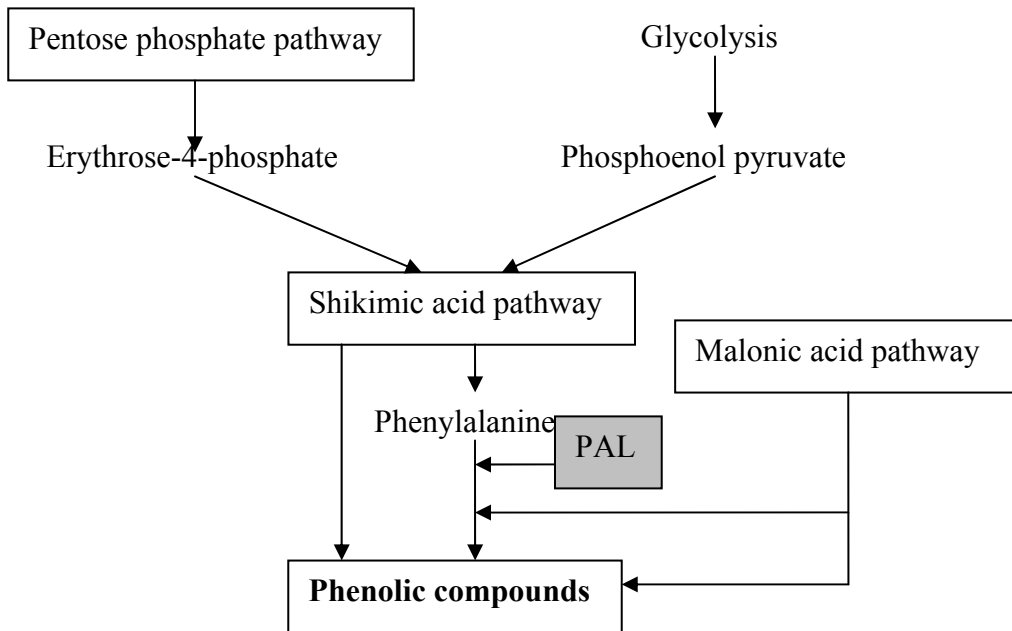


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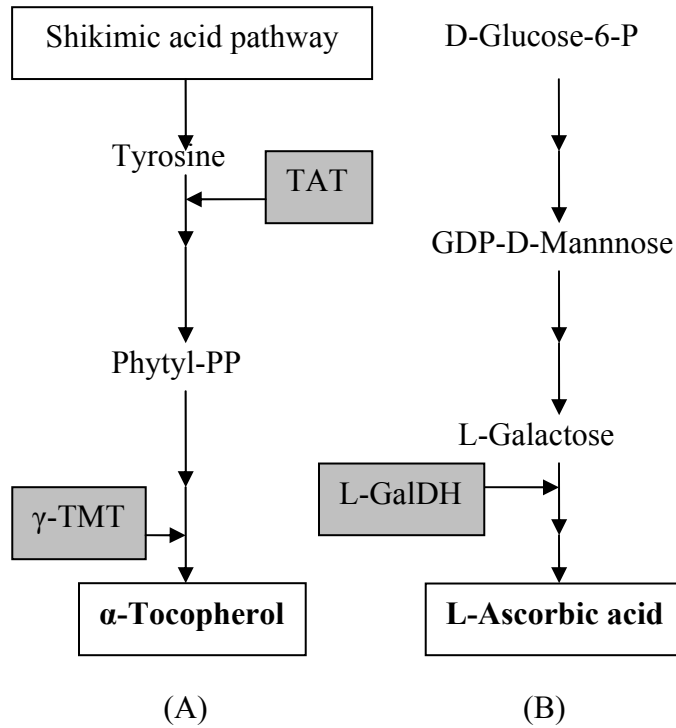
## Figures and Tables

**Figure 1.1. Simplified biosynthetic pathway of phenolic compounds.**



PAL, phenylalanine ammonia-lyase.

**Figure 1.2. Simplified biosynthetic pathways of vitamin E and vitamin C.**



TAT; tyrosine aminotransferase; Phytol-PP, phytol pyrophosphate;  $\gamma$ -TMT, gamma-tocopherol methyltransferase; D-Glucose-6-P, D-Glucose-6-phosphate, GDP-D-Mannose, guanosine diphosphate-D-mannose; L-GalDH, L-galactose dehydrogenase.

## **CHAPTER 2 - Secondary Metabolism and Antioxidants Are Involved in Environmental Adaptation and Stress Tolerance in Lettuce**

### **Abstract**

Lettuce (*Lactuca sativa*) plants grown in a protective environment, similar to *in vitro* conditions, were acclimated in a growth chamber and subjected to water stress to examine the activation of genes involved in secondary metabolism and biosynthesis of antioxidants. The expression of phenylalanine ammonia-lyase (PAL),  $\gamma$ -tocopherol methyl transferase ( $\gamma$ -TMT) and L-galactose dehydrogenase (L-GalDH) genes involved in the biosynthesis of phenolic compounds,  $\alpha$ -tocopherol and ascorbic acid, respectively, were determined during plant adaptation. These genes were activated in tender plants, grown under protective conditions, when exposed to normal growing conditions in a growth chamber. A large increase in transcript level for PAL, a key gene in phenylpropanoid pathway leading to the biosynthesis of a wide array of phenolics and flavonoids, was observed within one hour of exposure of tender plants to normal growing conditions. Plant growth, especially the roots, was retarded in tender plants when exposed to normal growing conditions. Furthermore, exposure of both protected and unprotected plants to water stress resulted in the activation of PAL. PAL inhibition by 2-aminoindan-2-phosphonic acid (AIP) rendered these plants more sensitive to chilling and heat shock treatments. These results suggest that activation of secondary metabolism as well as the antioxidative metabolism is an integral part of plant adaptation to normal growing conditions in lettuce plants.

### **Introduction**

Plants grown *in vitro* or in protective environments lack necessary adaptive mechanisms to thrive under harsher environmental conditions typically found in a growth chamber, greenhouse or field (Van Huylenbroeck and Debergh, 1996). These plants, therefore, need to acclimate before they can be successfully grown under *ex vitro* conditions which involve unregulated and often highly fluctuating environmental conditions (Pospíšilová et al., 1999; Van Huylenbroeck et al., 2000; Ziv, 1986). Tender plants grown *in vitro* without acclimation often fail to establish, and perform poorly in *ex vitro* conditions. Indeed, it is a common practice to acclimate *in vitro* propagated or protectively cultivated plants by gradual exposure to harsher environmental conditions to ensure their successful establishment and growth (Bañon et al., 2006; Dhawan and Bhojwani, 1987). Despite the recognition that these plants need to undergo an adaptive process to grow successfully under normal growing conditions, very little is known about the nature of such adaptation. Although plant responses and adaptations to various environmental stresses have been studied extensively (Guy, 1990; Levitt, 1980), they have not been examined in relation to environmental fluctuations typically found in normal conditions.

There is overwhelming evidence that many antioxidants play a key role in plant adaptation to both abiotic and biotic stresses (Burritt and MacKenzie, 2003; Dixon and Paiva, 1995; Vranová et al., 2002). Plants typically produce a diverse group of antioxidants as a protective mechanism against oxidative compounds which are produced in response to various stresses and known to have a damaging effect on membranes, organelles, and macromolecules (Mittler, 2002; Noctor and Foyer, 1998; Smirnoff, 1998). A significant part of antioxidants produced by plants in response to stress is secondary metabolites, including a vast array of simple and complex phenolic compounds derived primarily via the phenylpropanoid pathway (Dixon and Paiva, 1995). The two aromatic amino acids, phenylalanine and tyrosine, which are

the products of shikimate pathway, play an important role in this regard as the former is a precursor for a wide variety of phenolics while the latter a precursor for tocopherols. A well characterized and a key gateway enzyme in the phenylpropanoid pathway is phenylalanine ammonia-lyase (PAL; EC 4.3.1.5). It has been well documented that the gene encoding this protein is responsive to a number of abiotic and biotic stresses in many plant species (Diallinas and Kanellis, 1994; Liu et al., 2006; Reymond et al., 2000). In response to environmental stresses, an increase in PAL activity and an activation of a number of genes involved in phenylpropanoid pathway have been shown in many plant species (Keleş and Öncel, 2002; Leyva et al., 1995).

In addition, other important antioxidants such as tocopherols and ascorbic acid have been shown to be involved in plant adaptation to various stresses. Tocopherols are strong antioxidants which respond to various stresses and have been shown to protect plants against oxidative species, stabilize membranes (Havaux et al., 2003) and participate in intracellular signaling (Munné-Bosch, 2005). L-ascorbic acid is a commonly occurring major antioxidant in plants (Noctor and Foyer, 1998; Smirnoff et al., 2001) and is responsive to various stresses (Smirnoff and Wheeler, 2000). Hence it is thought to play an important role in plant adaptation (Conklin et al., 1996; Shalata and Neumann, 2001). Two enzymes,  $\gamma$ -tocopherol methyl transferase ( $\gamma$ -TMT; EC 2.1.1.95) and L-galactose dehydrogenase (L-GalDH; EC 1.1.1.117), play an important role in the biosynthesis of  $\alpha$ -tocopherol and ascorbic acid, respectively.  $\gamma$ -TMT participates in the last rate limiting step in the biosynthesis of  $\alpha$ -tocopherol by methylation of  $\gamma$ -tocopherol (Bergmüller et al., 2003; Shintani and DellaPenna, 1998). L-GalDH is a key regulating enzyme involved in the oxidation of galactose to L-galactono-1, 4-lactone leading to the formation of ascorbic acid (Gatzek et al., 2002; Wheeler et al., 1998). Although a number of these antioxidants have been

known to be involved in plant adaptation to various biotic and abiotic stresses, their role in plant function under non-stressful natural environmental conditions is not known.

The primary objective of this study is to determine the role of antioxidants, including some that are the products of secondary metabolism, in adaptation of lettuce to unprotective environmental conditions and to environmental stresses. We have examined plant responses and the activation of PAL,  $\gamma$ -TMT, and L-GalDH genes in response to unprotective environmental conditions and environmental stresses.

## **Materials and Methods**

### ***Plant material and growth conditions***

Lettuce plants (*Lactuca sativa* L., cv. Baronet) were grown under protective conditions similar to *in vitro* culture, (referred to here as tender) with relative humidity (RH) maintained around 100% under autotrophic conditions. The growing medium (Metro-Mix 350, Sun Gro, Canada) was maintained at field capacity using a capillary subirrigation system. The leaf water potential was maintained around -0.9 MPa. Seeds (2 seeds per pot) were planted on a sterile medium contained in plastic pots, 8 cm  $\times$  8 cm  $\times$  7 cm ( $L \times W \times H$ ), which were maintained in closed plastic troughs, 53 cm  $\times$  38 cm  $\times$  20 cm ( $L \times W \times H$ ), with clear glass tops with a provision for active air exchange and circulation so that the seedlings can perform as autotrophs. Seedlings were grown at 22/18 °C (day/night) with a photosynthetic photon flux density (PPFD) of 200  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  and a 12 h photoperiod for 26 d before transferring them to the unprotective environment.

The unprotective environment involved growing plants under conditions similar to those described above but in open growth chambers where RH was unregulated and typically



fluctuated between 60 and 70% and the light intensity was maintained at the same level as above. Seeds were planted on a sterile growing medium (Metro-Mix 350, Sun Gro, Canada) in a growth chamber at 22/18°C (day/night) with a PPFD of 200  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  and a 12 h photoperiod. The growing medium was maintained at field capacity with capillary subirrigation as described above. The leaf water potential of plants typically ranged from -0.95 to -1.1 MPa.

The seedlings grown under the protective environment were transferred to the unprotective environment and were also subjected to water stress to determine their growth response and activation of antioxidant genes. Water stress was imposed on plants by withholding watering for up to 7 d.

### ***Leaf water potential measurement***

Leaf water potential was measured using a Tru Psi water potential system (Model 3, Decagon Devices, Inc. Pullman, WA, USA). Leaf disks (1.2 cm in diameter) were cut with a cork borer and immediately transferred to sealed stainless steel chambers. They were equilibrated at 25°C for 30 min. The leaf water potential was measured with a cooling time of 15 s. Each treatment had three replications.

### ***Plant growth measurement***

Plant growth was evaluated in the protective environment (control) and during exposure to the unprotective environment. The plant growth parameters analyzed included dry and fresh weights of roots and shoots, total leaf area, total root length and volume and were measured using six plants per each treatment. Total leaf area was measured using a leaf area meter (LI-3100C, LI-COR, Lincoln, NE, USA). Total root length and volume were measured using a root scanning system (WinRHIZO, Regent Instruments Inc., Canada). Roots were washed thoroughly

in flowing tap water, blotted dry, excised, and stained with methyl blue solution ( $5 \text{ g} \cdot \text{L}^{-1}$ ) for 5 min. Stained roots were then rinsed with tap water and were carefully spread on a transparent plastic support making sure that they did not overlap before scanning.

### ***Photosynthetic rate***

Carbon dioxide ( $\text{CO}_2$ ) gas exchange of the whole-canopy was measured with a modified portable photosynthesis system (LI-6400, LI-COR, Lincoln, NE, USA). The instrument was equipped with a 0.64 L custom chamber to measure  $\text{CO}_2$  fixation from the whole canopy. Measurements were made sequentially under light and dark conditions. Three plants per treatment were used. The net  $\text{CO}_2$  assimilation rate for the canopy was determined as outlined by Bremer and Ham (2005).

### ***RNA gel blot analysis***

The specific probes for L-GalDH (497 bp, GenBank accession number AJ417563) with primers 5'-AACTTCTTCGACACCTCCCC-3' and 5'-TCATCCCAACCAACACCGAC-3', for  $\gamma$ -TMT (244 bp, GenBank accession number AF104220) with primers 5'-CATAGAAATCTATCTGCGGG-3' and 5'-CACTACGAAGCAGAGACACA-3', and for PAL (753 bp, GenBank accession number AF299330) with primers 5'-GCTTACAGTTTCTCAGGTGG-3' and 5'-TGATGCTTCAATTTGTGTGT-3' were amplified using the genomic DNA from lettuce plants with the primer pairs designed by a primer design program (Primer3, Whitehead Institute, Cambridge, MA, USA). Amplification of the probes was performed by PTC-100 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). The samples were denatured at  $94^\circ\text{C}$  for 5 min, subjected to 35 cycles, each consisting of 1 min at  $94^\circ\text{C}$ , 1 min at  $51^\circ\text{C}$  ( $\gamma$ -TMT and PAL) or  $54^\circ\text{C}$  (L-GalDH), and 1 min at  $72^\circ\text{C}$ , and then held at  $72^\circ\text{C}$  for 5

min. The fragment size of each probe was checked by electrophoresis on 1.2% (w/v) agarose gels in  $1 \times$  Tris-Acetate-EDTA buffer. After electrophoresis, the fragments stained with ethidium bromide were checked by UV transilluminator (Fisher Biotech, Fairlawn, NJ, USA). The probes were stored at  $-20^{\circ}\text{C}$  until use.

Total RNA was extracted from lettuce leaves using a modified CTAB extraction method as previously described (Chang et al., 1993). Total RNA was subjected to electrophoresis on 1% (w/v) agarose gels with 10% (v/v) formaldehyde and was subsequently transferred onto nylon membranes (Sigma, St. Louis, MO, USA) in a  $20 \times$  SSC solution. The probes were labeled with  $^{32}\text{P}$  using the random primer DNA labeling system (Sigma, St. Louis, MO, USA). During prehybridization, nylon membranes were incubated with 40 mL prehybridization buffer (1 mM EDTA, 0.5 M  $\text{NaH}_2\text{PO}_4$ , 7% SDS, pH 7.2) containing salmon sperm DNA ( $10 \text{ mg} \cdot \text{mL}^{-1}$ ) at  $50^{\circ}\text{C}$  for 6 h. The hybridization was performed as previously described (Sambrook and Russell, 2001). Probes were hybridized to RNA on nylon membranes at  $50^{\circ}\text{C}$  for 12 h in 40 mL hybridization buffer. The membranes were rinsed twice, first with wash I buffer (1 mM EDTA, 40 mM  $\text{NaH}_2\text{PO}_4$ , 5% SDS, pH 7.2) and then with wash II buffer (1 mM EDTA, 40 mM  $\text{NaH}_2\text{PO}_4$ , 1% SDS, pH 7.2) at  $50^{\circ}\text{C}$  for 1 h. The membranes were then exposed in cassettes at room temperature for 1 d and the films were scanned using a gel imaging system (Storm<sup>TM</sup>, GE Healthcare, Piscataway, NJ, USA).

### ***AIP treatment and adaptation to stresses***

To test if inhibition of PAL in lettuce plants can block their ability to adapt to stress conditions, plants were treated with 2-aminoindan-2-phosphonic acid (AIP), a strong PAL inhibitor, which is kindly provided by Dr. Jerzy Zoń, and subjected to chilling and heat shock

treatments. Lettuce seedlings were grown in growth chambers with a PPFD of  $250 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  and 12 h photoperiod up to 5 weeks before treating with AIP. Plant roots were fed with AIP by placing the pots in a trough containing aqueous solution of AIP ( $10 \mu\text{M}$ ) for 2 d prior to stress treatments. Plants were subjected to chilling by exposing them to  $4^\circ\text{C}$  for 24 h or to heat shock by exposing them to  $38^\circ\text{C}$  for up to 3 h in a growth chamber. During heat shock treatment, the growing medium was kept at field capacity using subirrigation system and plants were maintained at a high RH ( $\sim 85\%$ ) in the growth chamber to minimize water loss from the plants. Injury to plants was characterized by measuring chlorophyll fluorescence and electrolyte leakage of leaves. Chlorophyll fluorescence from the adaxial side of leaf was monitored using a portable chlorophyll fluorometer (PEA, Hansatech Instruments, Ltd., UK). Photochemical efficiency of leaves as determined by chlorophyll fluorescence ratios ( $F_v/F_m$ ) was monitored during and after the heat shock treatment and during the chilling treatment up to 24 h. Measurements were made during the light cycle on the leaves using the saturation pulse method after 30 min of dark adaptation. For electrolyte leakage, leaf samples were incubated in 15 mL of distilled water for 10 h to measure the initial electrolyte leakage using an YSI conductance meter (Model 32, YSI, Inc., Yellow Springs, OH, USA). The samples were subjected to  $80^\circ\text{C}$  for 2 h to release the total electrolytes and then held at room temperature for 10 h. The final conductivity on the leachate was measured to determine the percent electrolyte leakage from the leaf samples.

## **Results**

### ***Plant growth characteristics***

Lettuce seedlings had similar shoot biomass and leaf area in both the protective and unprotective environments during the first 4 weeks of their growth. However, morphologically,

there were striking differences in these two groups of plants. Seedlings grown in the protective environment had long, narrow leaves compared to those grown in the unprotective environment (Fig. 1). However, transferring these tender seedlings to and growing them in the unprotective environment for over 3 d diminished these morphological differences (data not shown). Also, when tender seedlings were transferred to the unprotective environment, the increase in their shoot dry matter accumulation over a 7-day period was less than that in seedlings that were continuously grown in the unprotective environment (Fig. 1A). After 7 d of exposure to the unprotective environment, the shoot dry matter accumulation in these seedlings was 22.8% lower than that in seedlings grown continuously in the unprotective environment. Similarly, leaf area expansion in tender seedlings did not keep pace with that in seedlings grown continuously in the unprotective environment (Fig. 1B).

Large differences in root growth characteristics were noted between seedlings growing in the protective and unprotective environments. Tender plants had very poor root system consisting of small and shallow roots (Fig. 2). At 4 weeks, these plants had a root mass which was about only 14%, and root length and volume which were less than 50% of those in seedlings growing in the unprotective environment. Furthermore, when these plants were transferred to the unprotective environment, very little root growth occurred during the first 3 d followed by a significant increase up to 7 d. However, consistently during this period, plants grown continuously under the unprotective environment showed better root growth. The results, thus, indicate that plants grown under the protective environment are generally sensitive to normal growing conditions found in a growth chamber.

### ***Photosynthesis and gene activation***

Although the photosynthetic efficiency appeared to be similar in these two groups of plants (data not shown), the total CO<sub>2</sub> fixed by the plants in the unprotective environment was higher because of their larger leaf area (Fig. 3A). The CO<sub>2</sub> assimilation in the unprotected plants was about two-fold higher than that in plants growing under the protective environment. However, tender plants tend to adapt after 6 d of exposure to the unprotective environment as suggested by their similar CO<sub>2</sub> assimilation rates.

Tender plants (at 0 h) had lower transcript levels of PAL,  $\gamma$ -TMT and L-GalDH than did the plants under the unprotective environment (Fig. 3B), suggesting that the biosynthetic pathways of these antioxidants are not active in plants growing under the protective environmental conditions. However, when these plants were transferred to the unprotective environment, PAL,  $\gamma$ -TMT and L-GalDH genes were activated within an hour, especially with a strong activation of PAL gene. Although the transcript levels for these genes during the 6-day acclimation seemed to vary, they remained markedly higher than in the plants growing under the protective environment.

### ***AIP treatment and plant adaptation***

As PAL is the key enzyme in the synthesis of a vast number of plant antioxidants, its role in plant adaptation was determined using AIP, a specific PAL inhibitor. AIP-treated plants grew slower than control, with 29.6 % less leaf area and 29.5 % less shoot biomass compared to untreated controls (Fig. 4) suggesting that PAL is essential for plant growth even under normal growing conditions. AIP-treated plants were subjected to 4 °C for 24 h or to 38 °C for 3 h to evaluate their response to these stresses. In response to cold shock the photochemical efficiency of photosystem II (chlorophyll fluorescence ratio, Fv/Fm) of AIP-treated plants decreased with

the time of exposure (Fig. 5A). In addition, cold shock induced a higher leakage of electrolytes in AIP-treated plants suggesting an impairment of plasma membrane integrity (Fig. 5B). Heat shock treatment of AIP-treated plants resulted in poor water status suggesting that high temperature interferes with the ability of these plants to maintain an optimal water status (Fig. 6A). However, no clear differences in photosynthetic fluorescence ratios were noted between the untreated and AIP-treated plants (Fig. 6B). Also, no consistent difference in electrolyte leakage was observed between heat-shocked AIP-treated and control plants (Fig. 6C). However taken together, these results indicate that PAL activity which leads to the synthesis of myriad phenylpropanoids including a number of antioxidants are essential for stress adaptation in lettuce plants.

### ***Plant growth response to water stress***

To examine the ability of tender plants to adapt to stressful condition, they were exposed to water stress over a 7-day period. This resulted in a decrease in leaf water potential to approximately -2 MPa (Fig. 7A). Tender plants did not perform as well as the plants that were grown under the unprotective environment as indicated by their slow shoot dry matter accumulation and leaf growth during this period (Figs. 7B and 7C). As expected, water stress affected shoot growth in both groups of plants, however the reduction in shoot biomass and leaf growth was much greater in tender plants than in the unprotective environment.

Imposing water stress stimulated root growth in both protected and unprotected plants (Fig. 8). However, root growth in tender plants fell short of that in unprotected plants, as reflected by relatively small increases in root mass, root length and root volume after 7 d of water stress. The results suggest that tender plants do not perform as well and also do not adapt to water stress conditions as well as those grown under unprotective environmental conditions.

### ***Photosynthesis and gene activation under water stress***

Based on CO<sub>2</sub> fixation in both groups of plants, it can be concluded that the plants adapt to water stress condition. However, the unprotected plants are better in CO<sub>2</sub> fixation than their protected counterparts during first 4 d of water stress (Fig. 9A). Furthermore, exposing both tender and unprotected plants to water stress resulted in the activation of PAL,  $\gamma$ -TMT and L-GalDH genes with clearly stronger response of PAL in the unprotected plants (Fig. 9B).

### **Discussion**

Lettuce seedlings grown in the protective environment did not grow as well as those in the unprotective environment (normal growth chamber conditions) as indicated by their poor development of roots. They also showed significant morphological differences with plants grown under the unprotective environment having typically smaller, longer and narrower leaves. Poor growth and altered morphological features in plants grown under *in vitro* conditions have been reported in other plant species (Smith et al., 1986; Wetzstein and Sommer, 1982). The poor root growth in tender plants can be attributed primarily to the high RH which is likely to limit water loss from the plants through transpiration. Both groups of plants were well-watered with no water stress. However, the plants in the unprotective environment would have a continuous stream of transpirational water loss while the plants under the protective environment have limited water loss because of relatively high ambient RH. It is not clear, however, as to whether this plays a role in limiting the root growth under the protective environment. When tender plants are exposed to normal conditions in a growth chamber, their growth, as indicated by shoot and root biomass and leaf area, is adversely affected compared to those continuously growing under growth chamber conditions. After 7 d of growth under the unprotective conditions, there was a large disparity in shoot biomass and particularly in root growth characteristics in these two



groups of plants suggesting that the adaptation of tender plants was not rapid enough. However, it is important to note that the photosynthetic efficiency in these groups of plants did not vary regardless of their growing conditions. Nevertheless the total photosynthetic activity and CO<sub>2</sub> fixed per plant did vary with plants growing under the unprotective environment, fixing substantially higher CO<sub>2</sub> than those in the protective environment because of the differences in plant leaf area. However, this difference was minimized with the adaptation of protected plants to the unprotective environment, although a large difference in root growth persisted even after this adaptation because of poor root growth in tender plants.

The key gateway gene of phenylpropanoid pathway, PAL, which is involved in the biosynthesis of a vast number of phytochemicals and antioxidants, has a very low expression level in tender plants. Similarly, the expression of other antioxidant genes studied which are known to have a key role in plant stress and adaptation, namely  $\gamma$ -TMT and L-GALDH, was also minimal in these plants. These genes were activated in lettuce when they were exposed to normal growing conditions in a growth chamber. These results suggest that activation of these genes occurs in response to benign normal growing conditions and may have a role in plants ability to cope with normal growing conditions. Van Huylbroeck et al. (1998) found that adaptation of *in vitro* plants results in increasing the activity of enzymatic antioxidants such as catalase (CAT; EC 1.11.1.6) and superoxide dismutase (SOD; EC 1.15.1.1). There is an overwhelming evidence that the activation of secondary metabolism, particularly the pathways that lead to accumulation of antioxidants including phenylpropanoids have been associated with plants stresses such as drought, UV, high light, and low temperatures (Chappell and Hahlbrock, 1984; Dixon and Paiva, 1995; Janas et al., 2002; Pennycooke et al., 2005; Sofu et al., 2005). Our results suggest that antioxidants play a role in plant function even under normal growing conditions. There are a

number of reports indicating that suppressing genes involved in the synthesis of these phytochemicals leads to many dysfunctions in plants and making them sensitive to stresses (Bergmüller et al., 2003; Conklin et al., 1996; Huang et al., 2005; Shirley et al., 1995). Inhibition of PAL is known to affect the accumulation of a number of downstream phenylpropanoid compounds including many phenolic compounds and others with antioxidant properties (Gitz et al., 2004; Keski-Saari, 2005). In our study, plant growth as indicated by biomass accumulation was much slower as a result of PAL inhibition. The plants became more sensitive especially to chilling than control plants. In response to chilling, AIP-treated plants showed a greater impairment in photochemical efficiency of photosystem II as indicated by leaf chlorophyll fluorescence ratio and a greater membrane dysfunction as indicated by loss of electrolytes from the leaf cells. This may be due to a decreased production of antioxidants by inhibition of PAL, which may in turn increase the levels of ROS such as superoxide and hydrogen peroxide in chloroplast (Asada, 1999). The adverse effects of chilling tended to get worse with longer period of chilling treatment in AIP-treated plants. This is consistent with the observations made by Gitz et al. (2004), who reported an increased sensitivity of AIP-treated duckweed plants to UV-B stress.

In response to water stress, tender plants did not do as well as unprotected plants. Unprotected plants were better able to adapt to water stress than the tender plants. Adaptation to the stress was associated with the activation of PAL,  $\gamma$ -TMT and L-GalDH. It is important to note that this response was somewhat similar to that when tender plants were exposed to the unprotective conditions. This suggests that plants respond as an adaptive mechanism by activating genes involved in biosynthesis of antioxidants whenever there is an environmental perturbation. This may explain why even under normal unprotective conditions activation of

secondary metabolism genes is important for successful plant growth. A number of studies have shown that genes involved in phenylpropanoid pathway, including PAL, and those involved in the biosynthesis of tocopherols in a number of plant species are activated in responses to abiotic stresses, including water stress (Christie et al., 1994; Collakova and DellaPenna, 2003; Leyva et al., 1995). Accumulation of antioxidants such as phenolics, tocopherols and ascorbic acid in response to a wide range of abiotic stresses and hence, their importance in plant adaptation has also been recognized (Bergmüller et al., 2003; Hernández et al., 2004; Keleş and Öncel, 2002; Li et al., 1993; Munné-Bosch and Alegre, 2000; Yaginuma et al., 2002). Furthermore, studies showing that mutants deficient in secondary metabolites or plants with blocked PAL activity are sensitive to environmental stresses clearly demonstrate the role of these antioxidants in plant adaptation (Gitz et al., 2004; Li et al., 1993). Our results, in addition to confirming these findings, suggested a possible role of secondary metabolism in the adaptation to environmental perturbations that occur under normal growing conditions in lettuce plants.

### **Acknowledgement**

We thank for Dr. Jerzy Zoń (Wrocław University of Technology, Wrocław, Poland) for kindly providing AIP.

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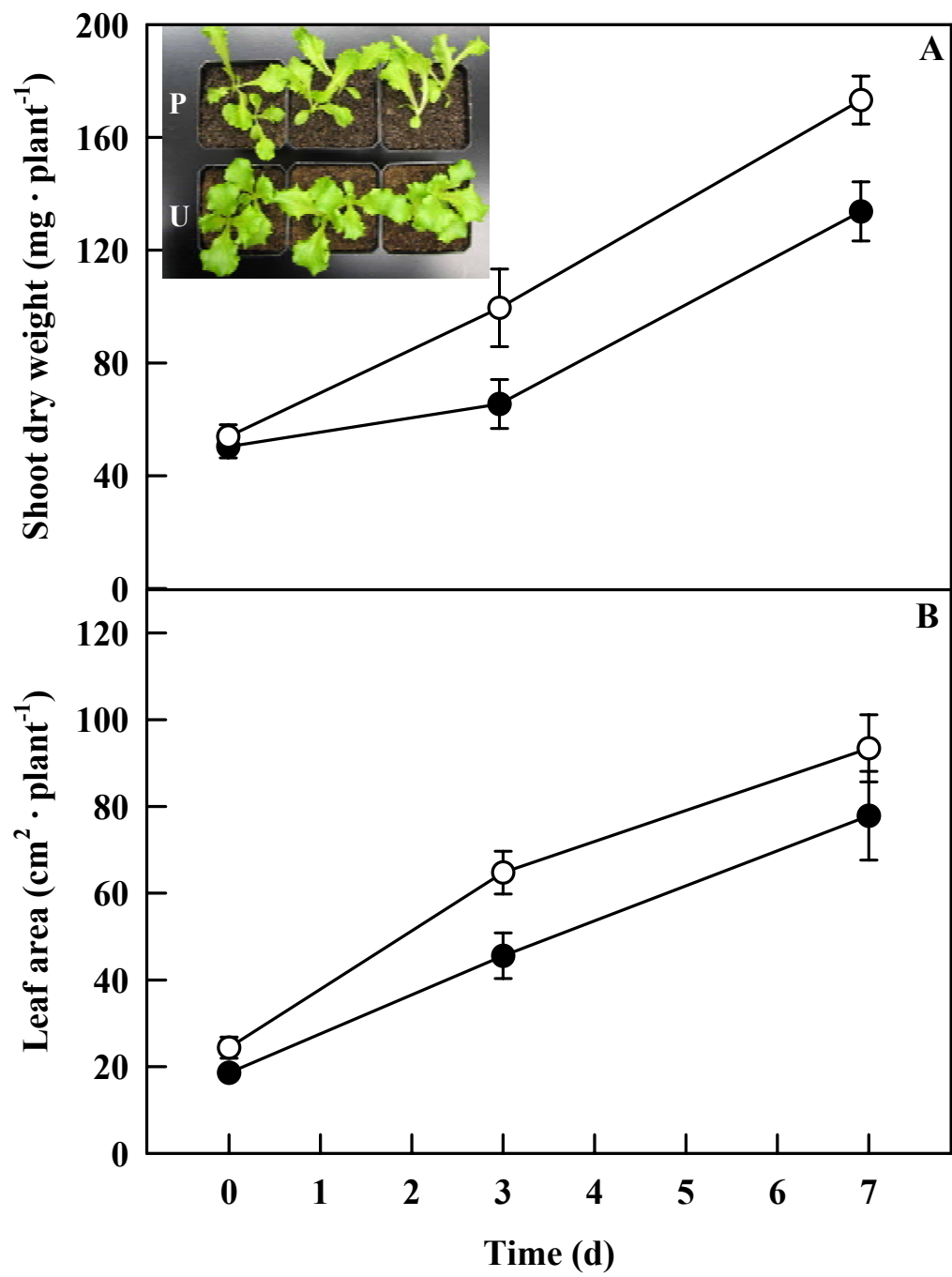


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## Figures and Tables

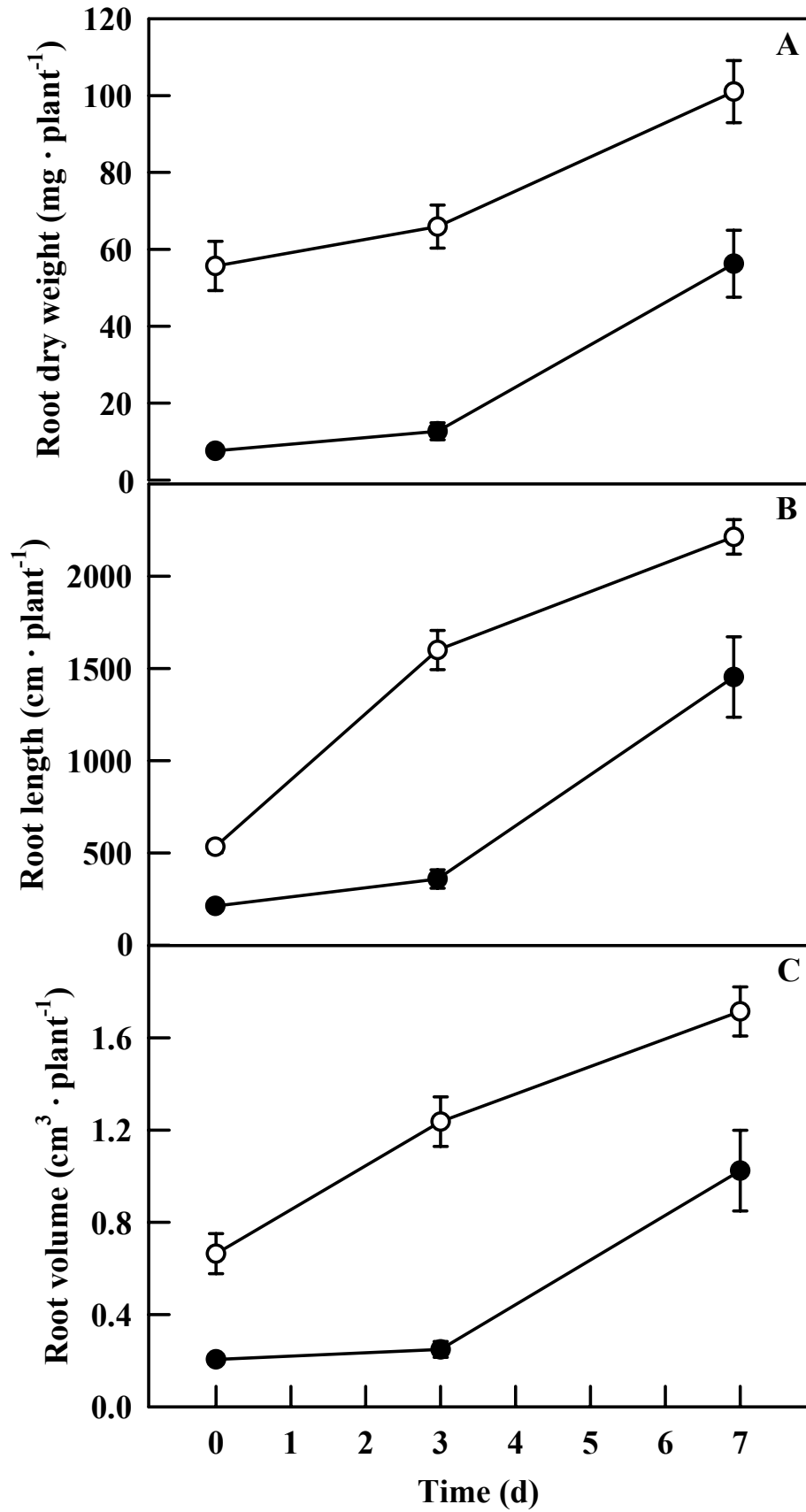
### **Figure 2.1. Plant growth characteristics of lettuce subjected to unprotective conditions.**

Plants grown in protective conditions (closed circle) were subjected to unprotective conditions for 7 d while control plants were continuously grown in unprotective conditions (open circle). Dry weight (A) and total leaf area (B) were measured. Plant morphological difference between plants grown under protective (P) and unprotective (U) conditions are shown (inset). The protective conditions consisted of 22/18°C (day/night), a PPFD of  $200 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ , a 12 h photoperiod and 100% RH. The unprotective conditions refer to the same environmental conditions as above with the exception of a lower RH of 60-70%. The data are means  $\pm$ S.E. (n = 6).



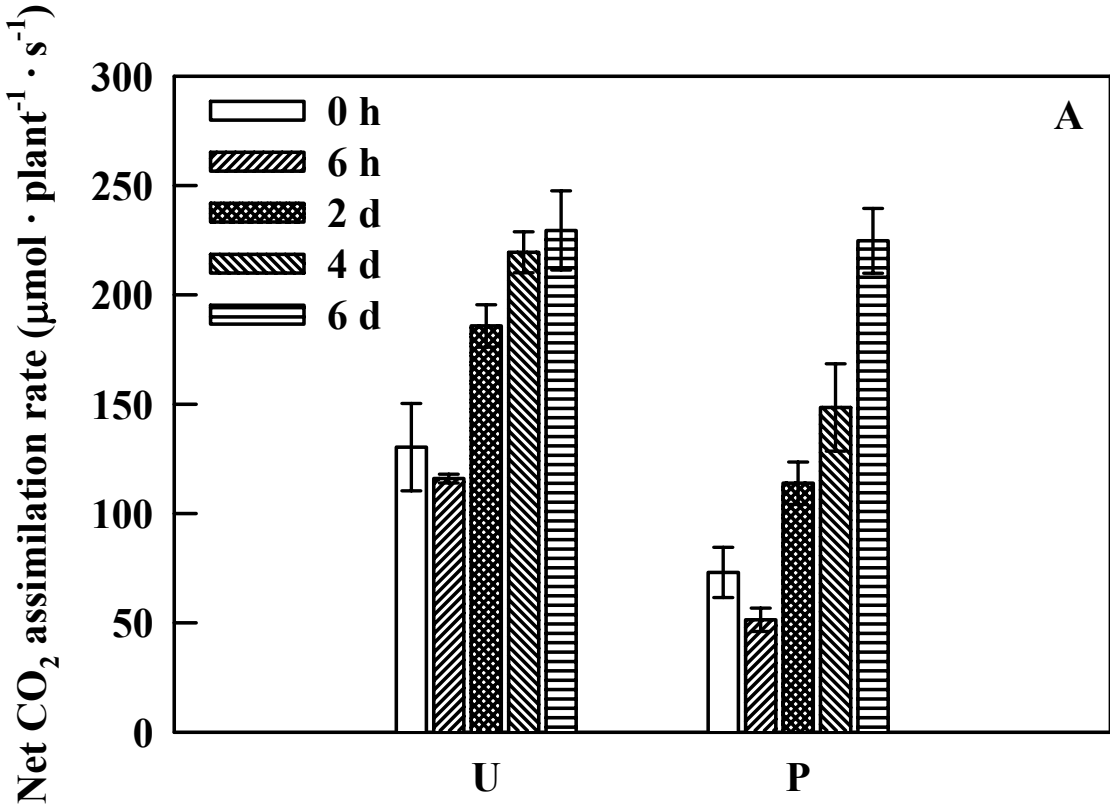
**Figure 2.2. Root growth characteristics of lettuce subjected to unprotective conditions.**

Root dry weight (A), total root length (B), and total root volume (C) were measured. Plants grown in protective conditions (closed circle) were subjected to unprotective conditions for 7 d while control plants were continuously grown in unprotective conditions (open circle). The data are means  $\pm$  S.E. (n = 6).

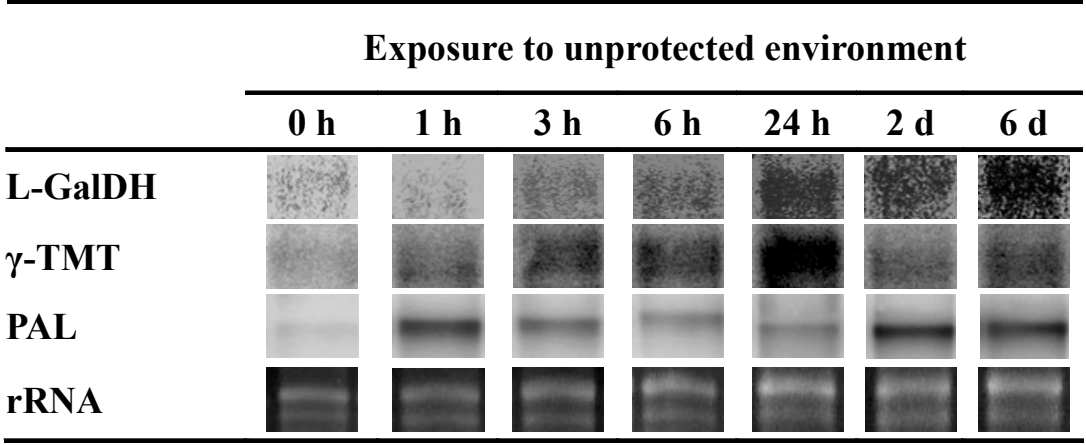


**Figure 2.3. Photosynthetic rate and gene expression in lettuce subjected to unprotective conditions.**

Plants grown in protective conditions (P) were subjected to unprotective conditions for 6 d while control plants were continuously grown in unprotective conditions (U). Leaf photosynthetic rates (A) were measured. The data are means  $\pm$  S.E. (n = 3). Gene expressions for L-GalDH,  $\gamma$ -TMT, and PAL (B) were determined in protected plants in response to unprotected conditions. rRNA was used as a loading standard.



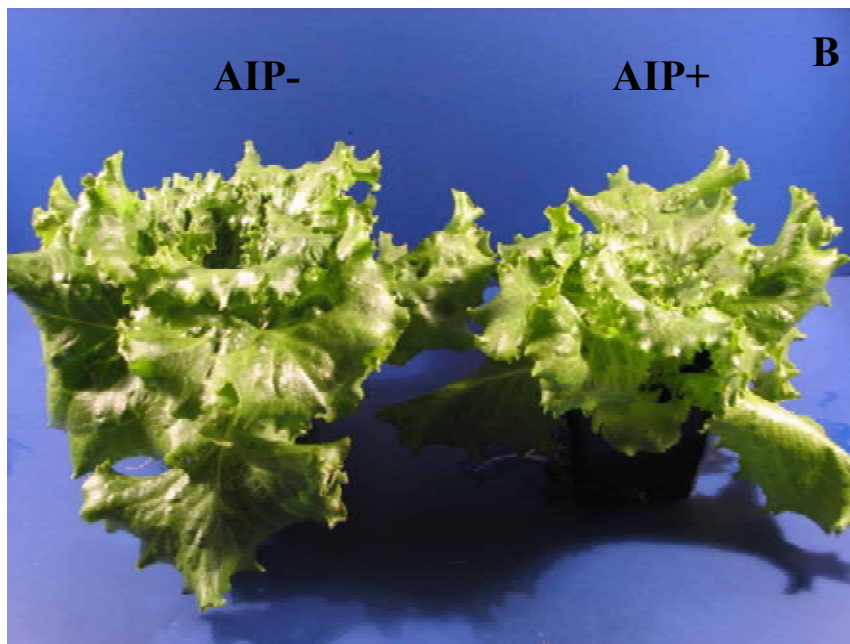
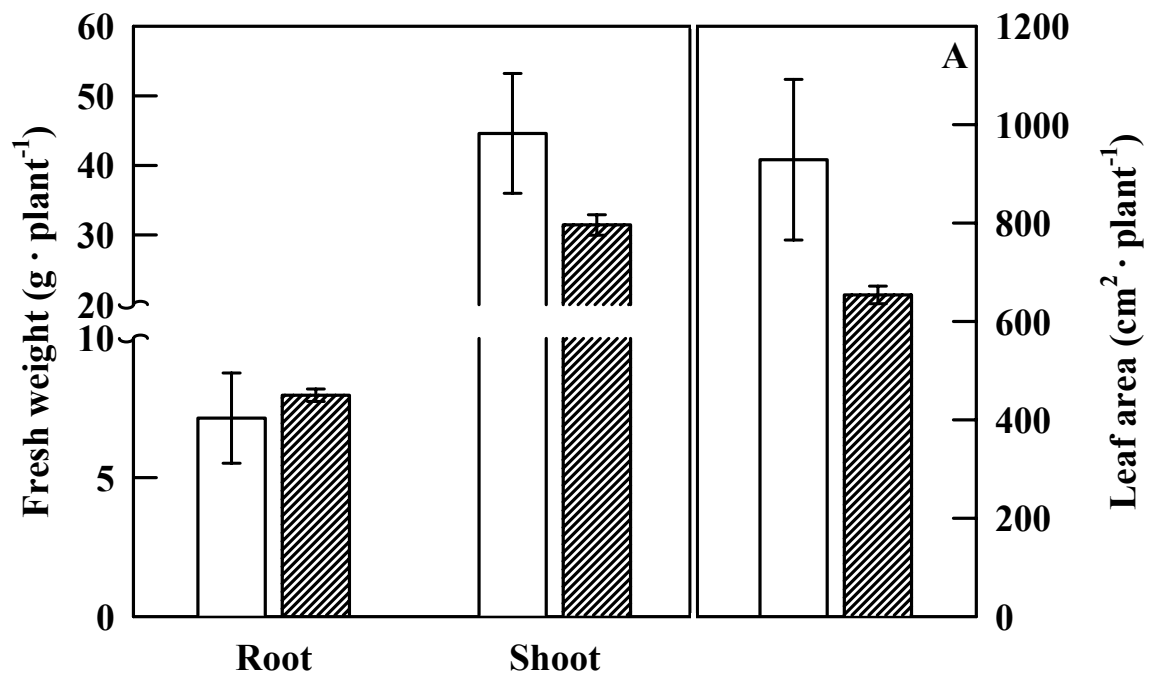
**B**



**Figure 2.4. Plant growth characteristics of AIP-treated lettuce.**

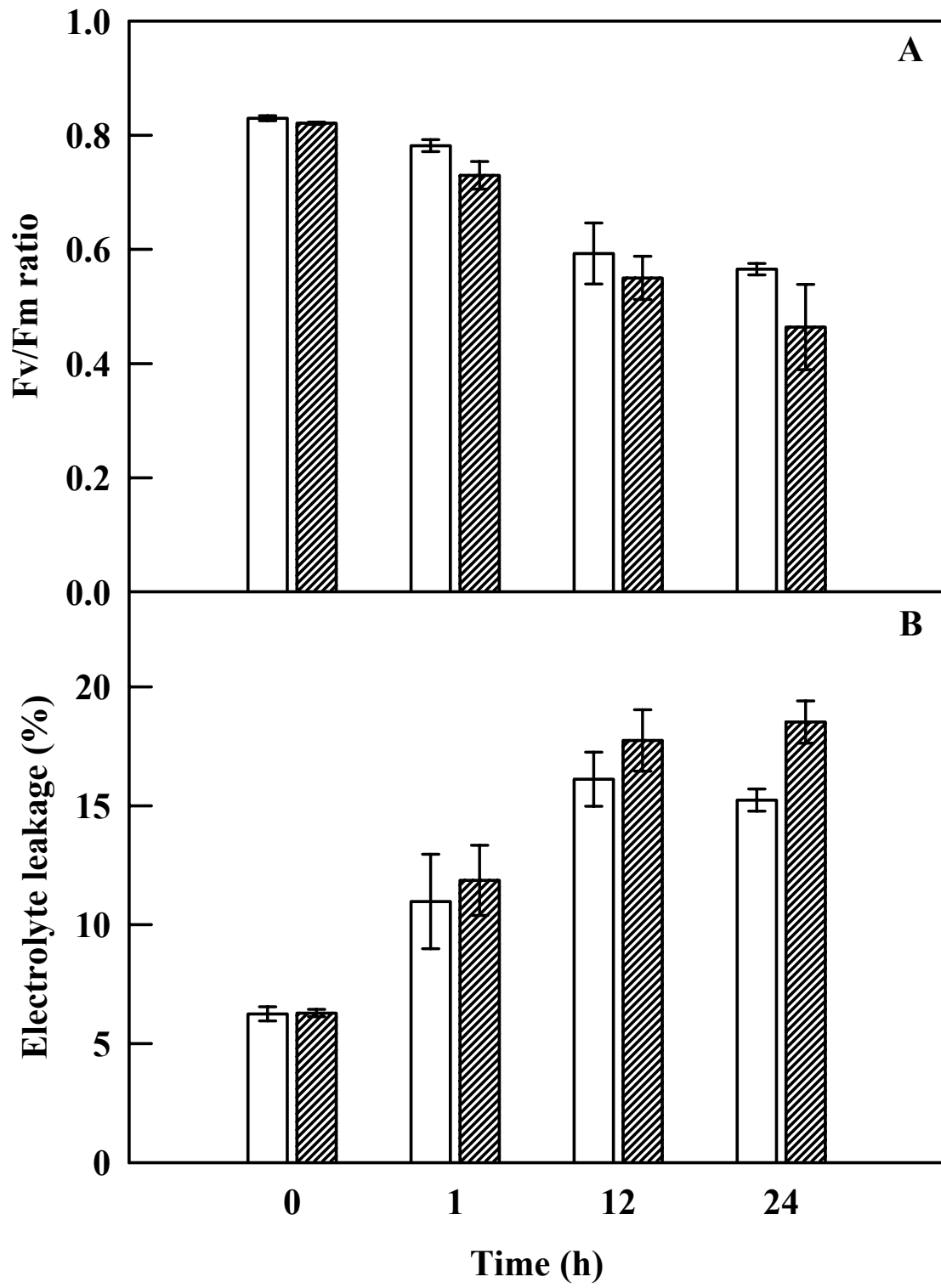
Shoot and root fresh weight and leaf area of untreated (open bars) and AIP-treated (shaded bars) plants (A) and the general growth characteristics of these groups of plants (B) are shown. The data are means  $\pm$ S.E. (n = 6).





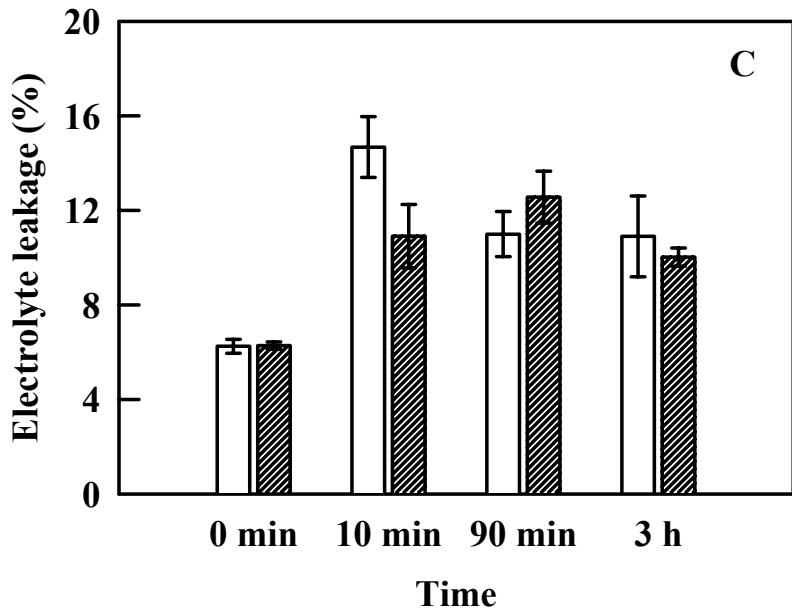
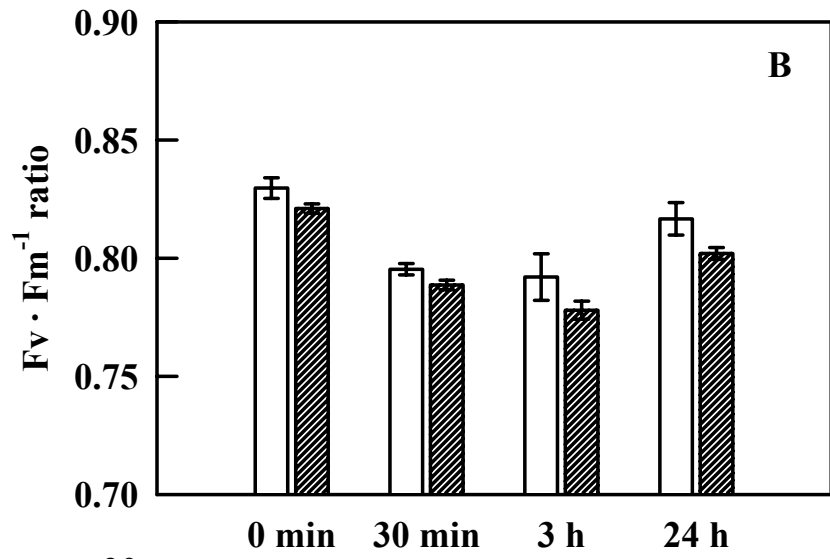
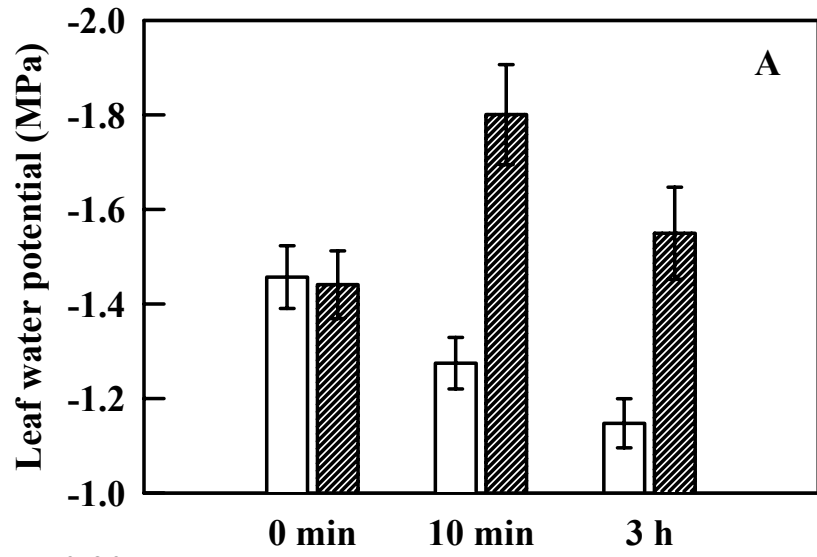
**Figure 2.5. Chlorophyll fluorescence and electrolyte leakage of lettuce in response to cold shock.**

Untreated (open bars) and AIP-treated lettuce (shaded bars) plants were exposed to low temperature (4 °C) for 24 h and the leaf fluorescence (A) and electrolyte leakage (B) were measured. The data are means  $\pm$ S.E. (n = 3).



**Figure 2.6. Leaf water potential, chlorophyll fluorescence, and electrolyte leakage of lettuce in response to heat shock.**

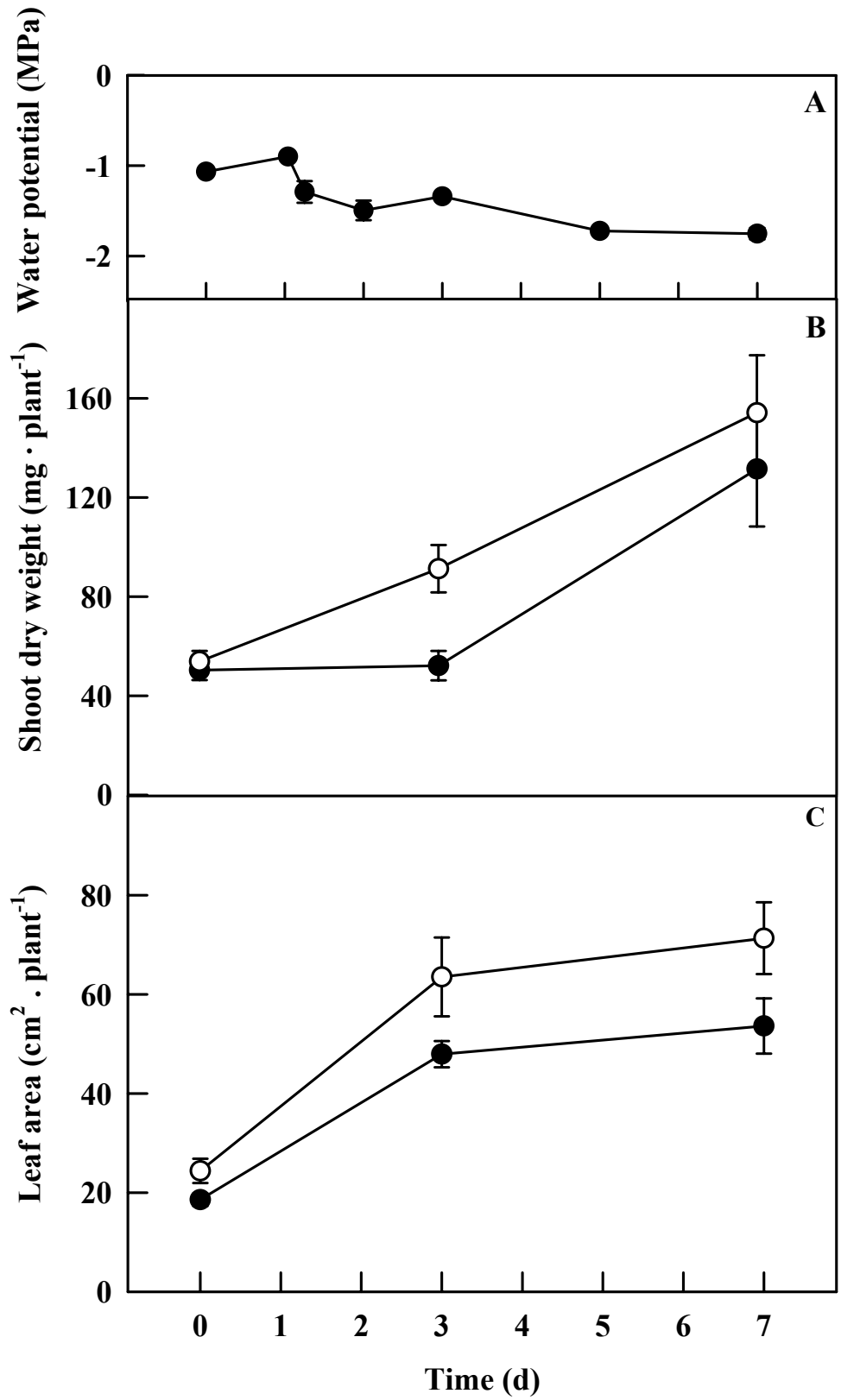
Untreated (open bars) and AIP-treated (shaded bars) plants were exposed to high temperature (38°C) for 3 h and the leaf water potential (A), fluorescence (B), and electrolyte leakage (C) were measured. The data are means  $\pm$ S.E. (n = 3).



**Figure 2.7. Leaf water potential and shoot growth characteristics of lettuce in response to water stress.**

Water stress was induced by withholding watering for 7 d and the leaf water potentials (A) were measured. Plants grown under unprotective (open circle) and protective (closed circle) conditions were subjected to water stress and their shoot dry weight (B) and leaf area (C) were measured.

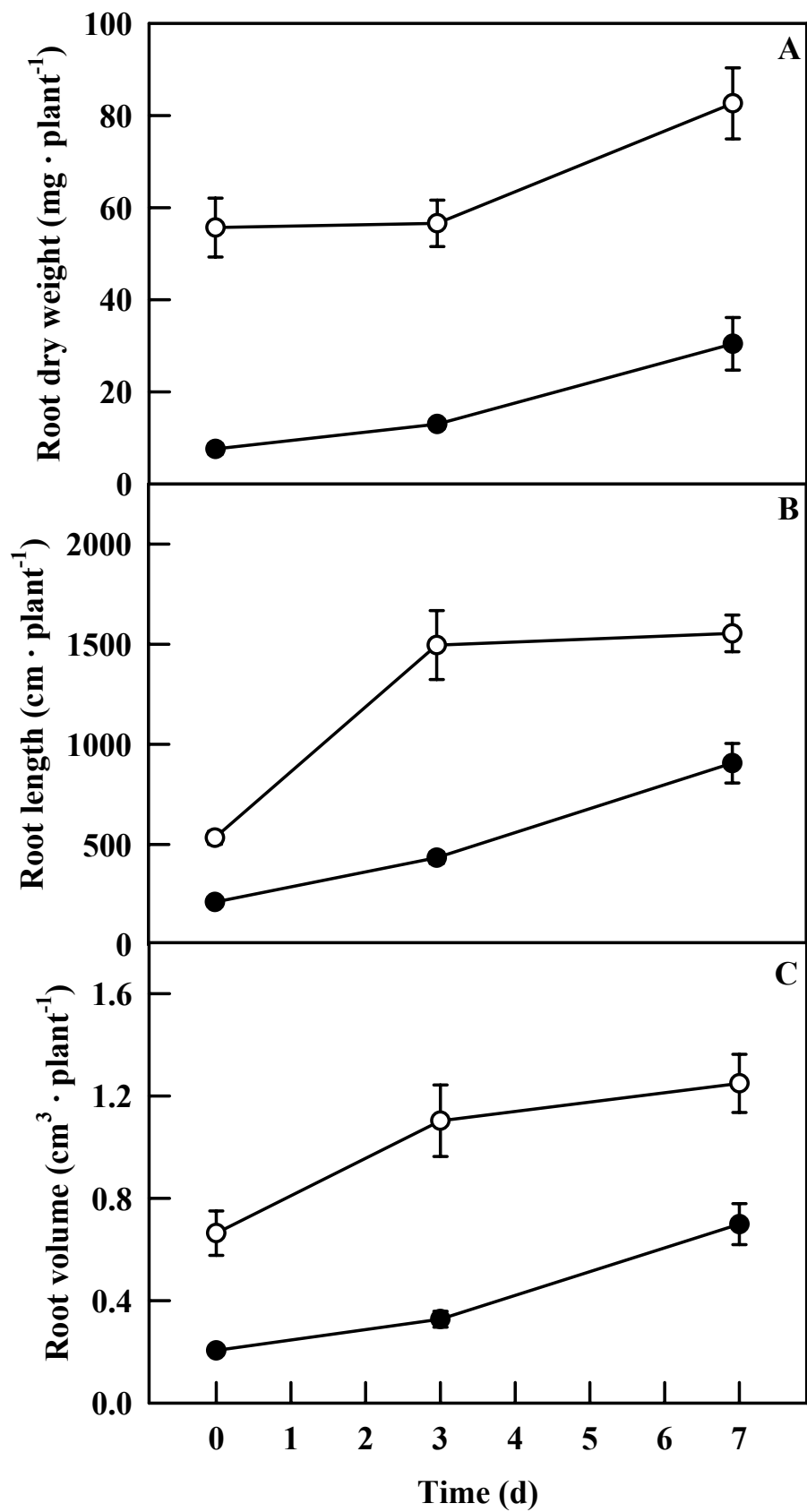
The data are means  $\pm$ S.E. (n = 6).



**Figure 2.8. Root growth characteristics of lettuce in response to water stress.**

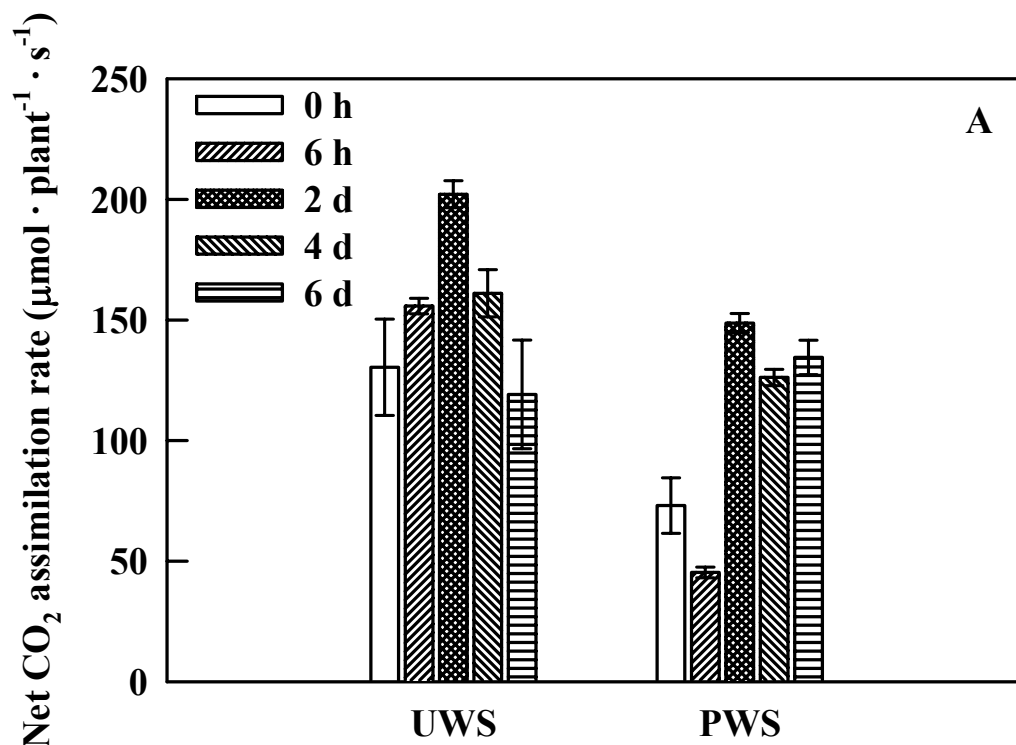
Plants grown under unprotective (open circle) and protective (closed circle) conditions were subjected to water stress for 7 d and their root dry weight (A), root length (B), and root volume (C) were measured. The data are means  $\pm$ S.E. (n = 6).



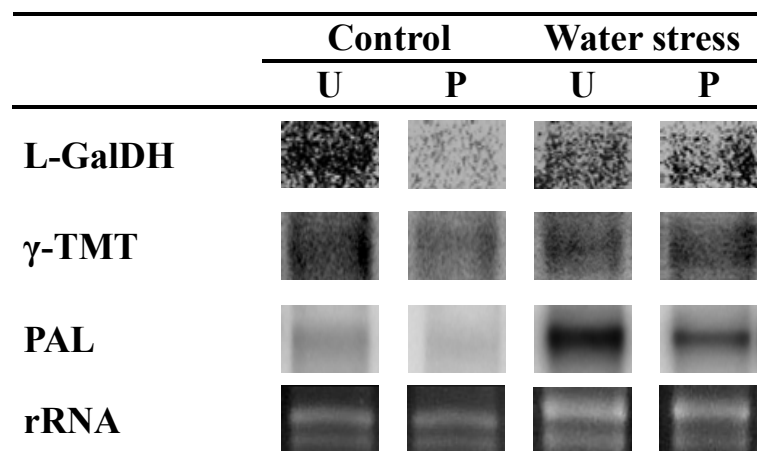


**Figure 2.9. Photosynthetic rate and gene expression in response to water stress.**

Leaf photosynthetic rates (A) of unprotected (UWS) and protected (PWS) plants subjected to water stress for 6 d were measured. The data are means  $\pm$ S.E. (n = 3). Gene expression for L-GalDH,  $\gamma$ -TMT, and PAL (B) were monitored for unstressed (control) and water stressed plants grown under unprotective (U) and protective (P) conditions. rRNA was used as a loading standard.



**B**



## CHAPTER 3 - Environmental Stresses Induce Health-promoting Phytochemicals in Lettuce

### Abstract

Lettuce (*Lactuca sativa* L., cv. Baronet) plants were subjected to various environmental stresses to determine their effects on the accumulation of phytochemicals with antioxidant properties and the activation of key genes involved in the biosynthesis of antioxidants. Five-week old plants grown in growth chambers were exposed to mild stresses such as heat (40°C for 10 min) or cold (4°C for 1 d) shocks, or high light intensity (800  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for 1 d). Plants were then harvested following a 3-d recovery period. The activation of genes for enzymes, L-galactose dehydrogenase (L-GalDH),  $\gamma$ -tocopherol methyltransferase ( $\gamma$ -TMT), and phenylalanine ammonia-lyase (PAL), involved in the biosynthesis of vitamin C, vitamin E, and phenolics, respectively, was characterized in response to stress treatments. A marked activation of these genes was observed in response to each of the stresses. The response of PAL was rapid and the transcript levels of PAL remained high during and after stress periods lasting until harvest, suggesting a sustained elevated level of secondary metabolism. This was further supported by a 2- to 3-fold increase in the total phenolic content and a significant increase in the antioxidant capacity of leaves. Moreover, all environmental stresses including high light, which was the strongest elicitor for phenolic accumulation, had a pronounced effect on the accumulation of chlorogenic acid, caffeic acid, chicoric acid, quercetin-3-O-glucoside, and luteolin-7-O-glucoside. Leaf growth, and root and shoot biomass accumulation were analyzed, and the results indicate that the mild environmental stresses had a negligible impact on the overall growth of lettuce. Thus, these results demonstrate that brief exposure of lettuce plants to

mild environmental stresses can have a positive effect on their health-promoting phytochemicals with antioxidant properties while having little or no adverse impact on their growth and yield.

## **Introduction**

Although a vast number of phytochemicals have been identified in fruits and vegetables, many still remain unidentified in plants (Boyer and Liu, 2004). Typically, phytochemicals include secondary metabolites like phenolics, terpenoids, and alkaloids, and numerous other chemicals (Grusak, 2002). Many of these are known to have various biological functions including protecting plants against herbivores and pathogen attacks (Herms and Mattson, 1992; Kliebenstein, 2004; Mithöfer et al., 2004). In addition, secondary metabolites especially phenolics play an important role in protecting plants against a wide range of environmental stresses including ultraviolet-B (UVB) radiation and low temperatures (Dixon and Paiva, 1995; Gitz III et al., 2004; Janas et al., 2002). Phenolics such as lignin being an important component of cell walls are also a major structural component of plants (Douglas, 1996).

However, the most significant role that phytochemicals play is to protect plants against stresses by providing them an adaptive mechanism. A number of phytochemicals including secondary metabolites and vitamins have antioxidant properties, which help in fending off the injury caused by stress-induced reactive oxygen species (ROS). ROS are known to damage a number of cell components including membranes in both plants and animals (Monk et al., 1989). Thus, the accumulation of antioxidants has a distinct adaptive advantage in plants. Typical phytochemicals with antioxidant properties are vitamins such as  $\alpha$ -tocopherol, ascorbic acid, and carotenoids and phenolic compounds (Blokina et al., 2003; Keleş and Öncel, 2002; Rice-Evans et al., 1997; Sofu et al., 2005).

Furthermore, phytochemicals appear to function in a similar fashion in humans as they do in plants. Humans are routinely exposed to highly oxidative environment resulting from the normal metabolic processes, which can often lead to harmful oxidative stress (Boyer and Liu, 2004). Oxidative damage may cause many degenerative diseases in humans such as cancer, cardiovascular disease, Alzheimer disease, and cataracts (Ames et al., 1993). Numerous epidemiological studies have reported that daily intake of fruits and vegetables rich in phytochemicals, has health benefits in the prevention of chronic diseases, including cardiovascular disease and several types of cancer (Arai et al., 2000; Jang et al., 1997). Many phenolics such as hydroxycinnamic acids (chlorogenic acid and caffeic acid) and flavonoids (chicoric acid, quercetin-3-O-glucoside, and luteolin-7-O-glucoside), present in most vegetables, have been extensively studied with regard to their antioxidant properties (Graefe and Veit, 1999; Olthof et al., 2001). Thus, there is an increasing awareness of the importance of fruits and vegetables in human diet. This is illustrated by the well known 5-A-Day Program, instituted by the National Research Council, which recommends eating at least five servings of fruits and vegetables a day to maintain good health and wellness. Thus, an increased awareness of the importance of phytochemicals in health and wellness has provided the impetus to improve the quality of fruits and vegetables, especially with regard to their nutritional benefits.

The primary objective of this study was to examine the role of various environmental stresses in producing health-promoting phytochemicals in lettuce, a commonly consumed leafy vegetable. In addition, the study also examined if this stress-induced phytochemical response is regulated by activation of genes involved in the biosynthesis of antioxidants, namely, phenolic compounds, vitamin C and vitamin E in lettuce.

## **Materials and Methods**

### ***Plant material and growth conditions***

Seeds of lettuce (*Lactuca sativa* L., cv. Baronet) were sown in a plastic pots (8 cm × 8 cm × 7 cm;  $L \times W \times H$ ), containing growing medium (Metro-Mix 350, Sun Gro, Canada). The seedlings grown in ninety six pots, each containing one plant, were placed in 4 plastic trays (53 cm × 38 cm × 7 cm;  $L \times W \times H$ ). The growing medium was maintained at field capacity using a capillary subirrigation system. When seedlings were 3-week old, they were fertilized weekly with Peat-Lite Special (Scotts-Sierra Horticultural Products Co., Marysville, OH) with N-P-K of 20-10-20 at 250 ppm of nitrogen in irrigation water. Plants were grown at 22/18 °C (day/night), PPFD at 250  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ , and 12 h photoperiod in a growth chamber.

### ***Various environmental stresses***

Five week old lettuce seedlings were treated with various environmental stresses for a short period of time. For heat shock (HS) treatment, 24 seedlings were exposed to 40 °C for 10 min under high humidity (around 90%). To impose chilling stress (CH), another set of seedlings was placed at 4 °C in a growth chamber with a light intensity 250  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for 1 d. Seedlings were subjected to high light (HL) stress by exposing them to a light intensity of 800  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for 1 d. Following the treatments, seedlings were transferred to normal growing condition as indicated above and allowed to recover for 3 d.

### ***Total phenolic content***

To measure the total phenolic content of leaves, samples were collected 1 d prior to stress treatment (control) and at various times during the stress treatments and recovery. Total phenolic content of lettuce was analyzed using the modified Folin-Ciocalteu reagent method (Pennycooke et al., 2005). About 1 g fresh leaf tissue was macerated in 6 mL 80% (v/v) acetone with a mortar

and pestle. The samples were placed into 1.5 mL tightly covered micro-tubes and incubated in darkness at 4 °C overnight. Samples were centrifuged at 1000 rpm for 2 min. A mixture of 135 µL H<sub>2</sub>O, 750 µL 1/10 dilution Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA), and 600 µL 7.5% (w/v) Na<sub>2</sub>CO<sub>3</sub> was added to 50 µL of phenolic extract in 1.5 mL micro-tubes. After vortexing for 10 s, the mixture was incubated at 45 °C in a water bath for 15 min. Samples were allowed to cool to room temperature before reading the absorbance at 765 nm (U-1100 spectrophotometer, Hitachi Ltd. Japan). A blank was prepared from 50 µL 80% (v/v) acetone. A gallic acid standard curve was prepared from a freshly made 1 mg · mL<sup>-1</sup> gallic acid [(Acros Organics, Belgium) (in 80% (v/v) acetone)] stock solution. Each sample had three replications.

### ***Antioxidant capacity***

The antioxidant capacity of lettuce leaves was measured by the modified ABTS (Aminobenzotriazole) method (Awika et al., 2003; Miller and Rice-Evans, 1996; Pennycooke et al., 2005). Antioxidants were extracted with 5 mL extraction solution (acetone:water:acetic acid = 70:29.5:0.5) from 1 g of leaf samples. The extracted solution containing antioxidants was incubated in darkness at -20 °C overnight. Subsequently, the solution was centrifuged at 1000 rpm for 2 min. ABTS (2.5 mM) (Roche Diagnostics, Indianapolis, IN, USA) stock solution was prepared and about 0.4 g of MnO<sub>2</sub> (Acros Organics, Belgium) was added to stock solution to generate ABTS radical cation (ABTS\*), stirring the mixture occasionally for 30 min at room temperature. Excess MnO<sub>2</sub> was removed by first filtering the solution through a Buchner funnel and then with a 0.2 µm syringe-end filter (Millipore Corp., Bedford, MA, USA). The ABTS\* solution was incubated at 30 °C in a water bath and was diluted to an absorbance 0.7 (±0.02) at 730 nm using 5 mM PBS [phosphate buffer saline, pH 7.4, ionic strength (150 mM NaCl)]. A



100  $\mu$ L of the extract was added to 1 mL of ABTS\* solution. After vortexing for 10 s, the absorbance of the mixture was recorded at 730 nm by a spectrophotometer (U-1100, Hitachi Ltd. Japan) after 1 min of reaction period. Trolox [(6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxyl acid) (Acros Organics, Belgium)] standard curve was prepared using 0.5 mM stock solution. Each sample had three replications.

### ***High-performance liquid chromatography (HPLC) analysis for phenolics***

The extraction of phenolic compounds from lettuce leaves was carried out as described by Nicolle et al. (2004) with minor modifications. Frozen leaf sample (1 g) ground in a mortar with a pestle using liquid nitrogen was extracted with 50 mL of 70% methanol at 80 °C for 1 min. After stirring at room temperature for 1 h, the mixture was filtered with a filter paper (No. 1, Whatman plc., UK). The extract (25 mL) was evaporated to dryness by a rotary evaporator (Rotavapor R110, Brinkmann Instruments, Inc., Westbury, NY, USA) under reduced pressure at 50 °C and then resuspended in 5 mL of 70% methanol. The concentrated solution was filtered through a 0.45  $\mu$ m filter disc (Millex, Millipore Corp., Bedford, MA, USA) before HPLC analysis.

A 5  $\mu$ L aliquot of the sample extract was injected into a HPLC system equipped with an autosampler (SpectraSYSTEM AS1000, Thermo Separation Products, San Jose, CA, USA), a pump (HP 1050, Hewlett Packard, Palo Alto, CA, USA), an integrator (HP 3396, Hewlett Packard, Palo Alto, CA, USA), and an UV/VIS detector (Acutect 500, Thermo Separation Products, San Jose, CA, USA). Each phenolic compound was separated from the sample extract using a column (Discovery BIO Wide Bore C-18, 15 cm  $\times$  4.6 mm, 5  $\mu$ m, Supelco, Inc., Bellefonte, PA, USA) coupled to a guard column (Discovery BIO Wide Bore C-18, 2 cm  $\times$  4 mm, 5  $\mu$ m, Supelco, Inc., Bellefonte, PA, USA) at 60 °C. Compounds of the sample extract were

eluted with eluant A [ $\text{H}_2\text{O}:\text{CH}_3\text{COOH} = 338:1$  (v/v)] and eluant B [ $\text{H}_2\text{O}:\text{C}_4\text{H}_{10}\text{O}:\text{CH}_3\text{COOH} = 330:8:1$  (v/v/v)] at a flow of  $1.8 \text{ mL} \cdot \text{min}^{-1}$ . The gradient started at 20% B in A, which was held for 5 min, then increased to 100% B over 20 min. After equilibration for 2 min at 100% B, the composition of solution returned to the initial condition (20% B). Peaks from the sample extract were identified and quantified at 330 nm by comparing with standard compounds such as chlorogenic acid (Sigma-Aldrich, St. Louis, MO, USA), caffeic acid (Sigma-Aldrich, St. Louis, MO, USA), quercetin-3-O-glucoside (Sigma-Aldrich, St. Louis, MO, USA), chicoric acid (Indofine Chemical Co., Inc., Hillsborough, NJ, USA), and luteolin-7-O-glucoside (Indofine Chemical Co., Inc., Hillsborough, NJ, USA).

### ***RNA gel blot analysis***

The gene expression of key enzymes involved in biosynthesis of antioxidants including vitamin C, vitamin E, and phenolics was determined by RNA gel blot analysis. The genes for L-galactose dehydrogenase (L-GalDH),  $\gamma$ -tocopherol methyltransferase ( $\gamma$ -TMT), and phenylalanine ammonia-lyase (PAL) involved in the biosynthesis of vitamin C, vitamin E, and phenolic compounds, respectively (Bergmüller et al., 2003; Diallinas and Kanellis, 1994; Gatzek et al., 2002), were chosen in this study. The specific probes for L-GalDH (497 bp, accession number AJ417563) with primers 5'-AACTTCTTCGACACCTCCCC-3' and 5'-TCATCCCAACCAACACCGAC-3', and for  $\gamma$ -TMT (244 bp, accession number AF104220) with primers 5'-CATAGAAATCTATCTGCGGG-3' and 5'-CACTACGAAGCAGAGACACA-3', and for PAL (753 bp, accession number AF299330) with primers 5'-GCTTACAGTTTCTCAGGTGG-3' and 5'-TGATGCTTCAATTTGTGTGT-3' were amplified using genomic DNA from lettuce plants with the primer pairs designed by a primer design program (Primer3, Whitehead Institute, Cambridge, MA, USA). Amplification of probes was

performed by PTC-100 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA) after initial denaturation at 94 °C for 5 min by 35 cycles of 1 min at 94 °C, 1 min at 51 °C ( $\gamma$ -TMT and PAL) or 54 °C (L-GalDH), and 1 min at 72 °C, followed by 5 min at 72 °C. The fragment size of each probe was checked by electrophoresis on 1.2% (w/v) agarose gels in 1 × Tris-Acetate-EDTA buffer. After electrophoresis, the fragments stained with ethidium bromide were checked by a UV transilluminator (FisherBiotech, Fairlawn, NJ, USA). The probes were stored at -20 °C until use.

Total RNA was extracted from lettuce leaves using a modified cetyltrimethylammonium bromide (CTAB) extraction method as described by Chang et al. (1993). Total RNA (9 µg) was subjected to electrophoresis on 1% (w/v) agarose gels with 10% (v/v) formaldehyde. The RNA was transferred onto nylon membranes (Sigma-Aldrich, St. Louis, MO, USA) in a 20 × SSC solution (Sambrook and Russell, 2001). The probes were labeled with <sup>32</sup>P using random primer DNA labeling system (Sigma-Aldrich, St. Louis, MO, USA). During prehybridization, nylon membranes were incubated with 40 mL prehybridization buffer (1 mM EDTA, 0.5 M NaH<sub>2</sub>PO<sub>4</sub>, 7% SDS, pH 7.2) at 50 °C for 6 h with Salmon Sperm DNA (10 mg · mL<sup>-1</sup>). The hybridization was performed as described by Sambrook and Russell (2001). Probes were hybridized to RNA on nylon membranes at 50 °C for 12 h in 40 mL prehybridization buffer. The membranes were rinsed twice with wash I buffer (1 mM EDTA, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 5% SDS, pH 7.2) and then once with wash II buffer (1 mM EDTA, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 1% SDS, pH 7.2) at 50 °C for 1 h. The membranes were then exposed in cassettes at room temperature for 1 d and the films were scanned by a gel and blot imaging system (Storm<sup>TM</sup>, GE Healthcare, Piscataway, NJ, USA).

### ***Plant growth characteristics***

Plant growth was measured 3 d after recovering from the environmental stresses. Measurements on plant growth parameters such as dry and fresh weights of roots and shoots, total leaf area were conducted using four plants per each treatment. After measuring fresh weights of roots and shoots, the samples were dried at 70 °C in an oven for 3 d to measure dry biomass accumulation. Total leaf area was measured by a leaf area meter (LI-3100C, LI-COR, Lincoln, NE, USA).

### ***Statistical analysis***

Analysis of variance (ANOVA) was performed by the statistical analysis system (SAS) program. Duncan's multiple range test was used to compare means.

## **Results**

In response to all the stress treatments, total phenolic content of lettuce plants increased both during and after the treatments (Fig. 3.1). The increase in total phenolic content in lettuce was rather rapid in response to HS and CH. A significant increase in phenolic compounds occurred within 10 min of HS and within 1 h of CH. Even higher accumulation of phenolic compounds occurred following these stresses after 3 d of recovery. Of all the stress treatments, lettuce plants were particularly responsive to HL, resulting in a large accumulation of phenolic compounds. The increase in phenolic content of plants was approximately 3-fold after 1 d exposure to HL and the level of phenolic content remained high following the stress treatment as well.

The antioxidant capacity of lettuce plants increased in response to all the stresses, just as the total phenolic content. It increased readily in response to all the stresses and remained high consistently following the stress treatments, during the 3-d recovery period (Fig. 3.2). Although

the data for total phenolic content and antioxidant capacity presented here are based on fresh weight, a similar overall trend in plant response was observed using the dry weight basis as well (data not shown).

The key genes involved in the biosynthesis of antioxidants were activated in response to environmental stresses (Fig. 3.3). The gene for PAL, a gateway enzyme involved in the biosynthesis of numerous phenolics, was activated rapidly in response to all the stresses except HL. In fact, significant increase in PAL, L-GalDH, and  $\gamma$ -TMT activation occurred in response to 10-min HS and 1-h CH. However, high transcript levels were detected only after 1 d of HL. The transcript levels for all three antioxidant genes remained high following the stress treatments during the 3 d of recovery.

Figure 3.4 shows the accumulation of various phenolic compounds 3 d after HS, CH, and HL treatments during the recovery. The quantitative analyses of phenolic compounds in lettuce revealed that a number of major phenolic compounds increased significantly, a pattern consistent with the total phenolic content, in response to all the stresses. The amount of two major phenolic compounds in lettuce, chicoric acid and chlorogenic acid, increased significantly following all the stress treatments, with the greatest increase occurring in response to HL. The concentration of chlorogenic acid and chicoric acid of lettuce plants exposed to HL was 7.5- and 4.5-fold, respectively, higher than that of control plants. Interestingly, quercetin-3-O-glucoside and luteolin-7-O-glucoside were detected in plants exposed to HL and CH, but not in control plants.

There was very little adverse effect on lettuce plant growth as a result of environmental stresses (Table 3.1). There was no significant effect of HS, CH, and HL on shoot biomass accumulation. Interestingly, HL treatment significantly increased the root fresh weight and dry matter accumulation while it decreased the leaf area. Similarly, CH stress also adversely affected

the leaf area expansion in lettuce. However, the adverse effects of environmental stresses on plant growth appeared to be minimal.

## **Discussion**

Phenolic compounds confer unique taste, flavor, and health-promoting properties found in fruits and vegetables (Tomás-Barberán and Espín, 2001). Thus, increasing phenolic content in these plants can lead to their enhanced quality. Although several studies have examined the impact of crop production and management practice in maintaining or improving the quality of food crops (Schreiner, 2005; Zhao et al., 2006), very little attention has been directed toward examining the possible effects of mild environmental stresses on their phytochemical composition. However, there are numerous studies showing the effect of environmental stresses on antioxidant accumulation in relation to plant adaptation. For example, Pennycooke et al., (2005) reported that chilling stress led to elevated total phenolic content and antioxidant capacity in petunia. Similarly, high irradiation and cold stress can lead to elevated levels of flavonoids in plants (Krebs et al., 2002; Tattini et al., 2005).

Our results show that mild environmental stresses can readily induce key genes involved in the secondary metabolism in lettuce. PAL, a key gene in the phenylpropanoid pathway involved in the biosynthesis of numerous phenolic and flavonoid compounds with antioxidant properties, was activated by HS, CH, and HL. PAL transcript levels increased within 10 min of HS treatment and within 1 h of CH treatment. Jones and Hartley (1999) proposed a model called the protein competition model (PCM) to explain the shift of metabolic pathways in plants from primary to secondary. The shift from primary to secondary metabolism corresponds to a shift in synthesis from proteins to phenolics, which is determined by the key end product of shikimate pathway, namely, phenylalanine. According to this model, the demand for proteins or phenolics

could be determined by intrinsic factors such as plant growth and development, and by external factors such as environmental and growing conditions. Therefore, environmental constraints can depress plant growth, leading to a reduced demand for protein synthesis. This leads to the deamination of phenylalanine by PAL, a first step in the biosynthesis of phenolic compounds. Therefore, PAL plays a pivotal role in the synthesis of a myriad of phenolic compounds and their derivatives (Jones, 1984). Increased PAL activity and activation of PAL gene have been reported in a number of plant species in response to wounding, and abiotic and biotic stresses (Diallinas and Kanellis, 1994; Liu et al., 2006; Reymond et al., 2000). In addition, our results also show that other key genes (L-GalDH and  $\gamma$ -TMT) involved in the biosynthesis of antioxidants such as vitamin C and vitamin E are also activated by all the stresses.

The antioxidant capacity and the total phenolic content of lettuce plants were significantly increased by all the three stress treatments. A positive correlation between the total phenolic content and antioxidant capacity exists because phenolic compounds contribute to a major share of the antioxidant capacity (60%) in lettuce (Nicolle et al., 2004). A similar relationship between phenolic content and antioxidant capacity has also been observed in another study (Llorach et al., 2004). The increase in antioxidant capacity appears to be similar with regard to various stresses while the response of total phenolic content showed some degree of variability. The total phenolic content was typically the highest at the end of recovery period (3 d) regardless of the type of stress imposed. Of all the stress treatments, HL produced the highest total phenolic content during the recovery period, which was three times higher than in the control plants.

Similar to the response of total phenolic content of lettuce in relation to stress, many phenolic compounds (chlorogenic acid, caffeic acid, chicoric acid, quercetin-3-O-glucoside, and

luteolin-7-O-glucoside) accumulated in response to all the stress treatments. Again consistent with the results on total phenolic content, HL induced the highest accumulation of all the phenolic compounds examined in this study. The major phenolic compounds in lettuce, chicoric acid and chlorogenic acid (Nicolle et al., 2004; Romani et al., 2002), accumulated at significant levels in response to all the stresses. The amount of these phenolic compounds in HL-treated plants was 4- to 6-fold higher than that in the control plants. Quercetin-3-O-glucoside and luteolin-7-O-glucoside were not detected in the control plants and HS-treated plants. However, significant accumulation of these compounds occurred as a result of CH and HL treatments. These phenolic compounds were especially responsive to HL, accumulating at levels 3- to 7-fold higher than in CH-treated plants.

In summary, the results show that mild environmental stresses induced a number of key genes involved in secondary metabolism including PAL and those involved in the biosynthesis of vitamin C and vitamin E. Significant increase in several phenolic compounds including chicoric acid and chlorogenic acid occurred after brief exposure to several stresses. Lettuce plants were very responsive to HL and could accumulate many phenolic compounds at the highest levels compared to HS, CH, or control. Some phenolic compounds (quercetin-3-O-glucoside, and luteolin-7-O-glucoside) undetected in lettuce grown under normal conditions could be induced by CH and HL. Thus, the results indicate that environmental stresses can not only affect levels of phenolic compounds but also the composition of phenolic compounds in lettuce. As phenolic compounds constitute a major part of antioxidants in lettuce, environmental stresses could significantly enhance the antioxidant capacity of lettuce. As the stress treatments used in this study were mild and plants were exposed to these stresses for a brief period of time, their effects on plant growth were negligible. Thus, this study shows that mild environmental



stresses can be used successfully to enhance the health-promoting phytochemicals in lettuce with no adverse effect on crop yield.

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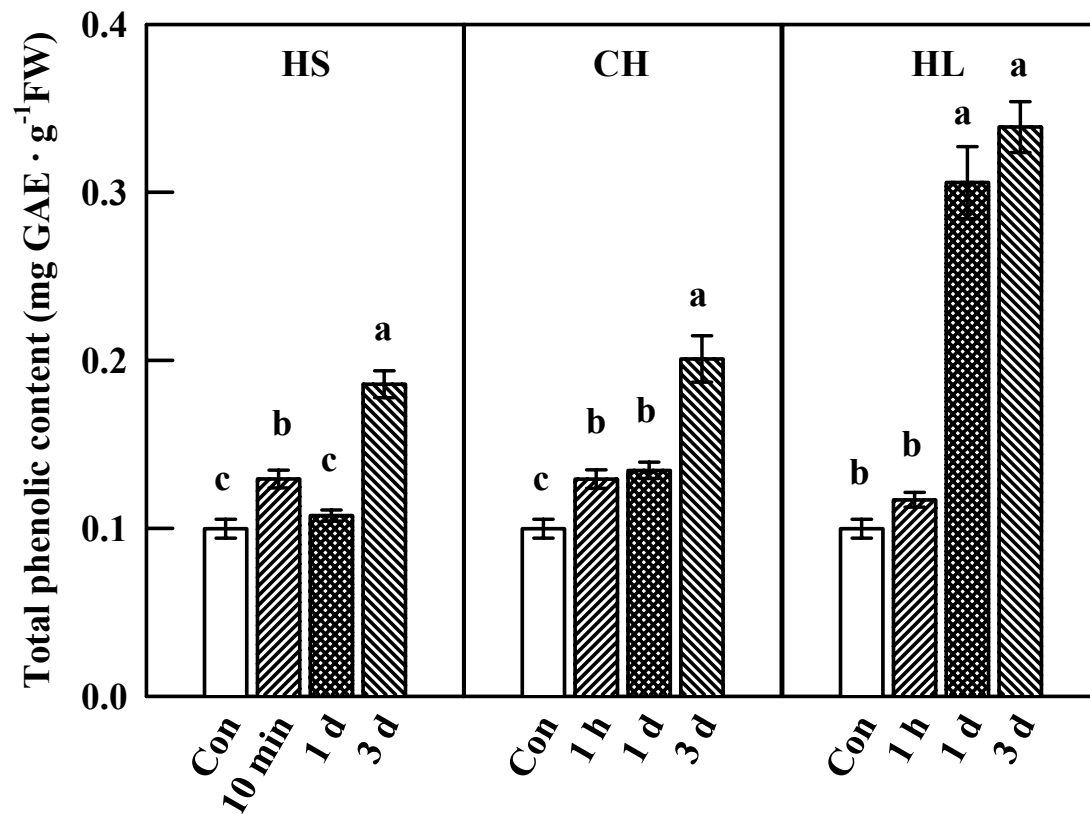
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## Figures and Tables

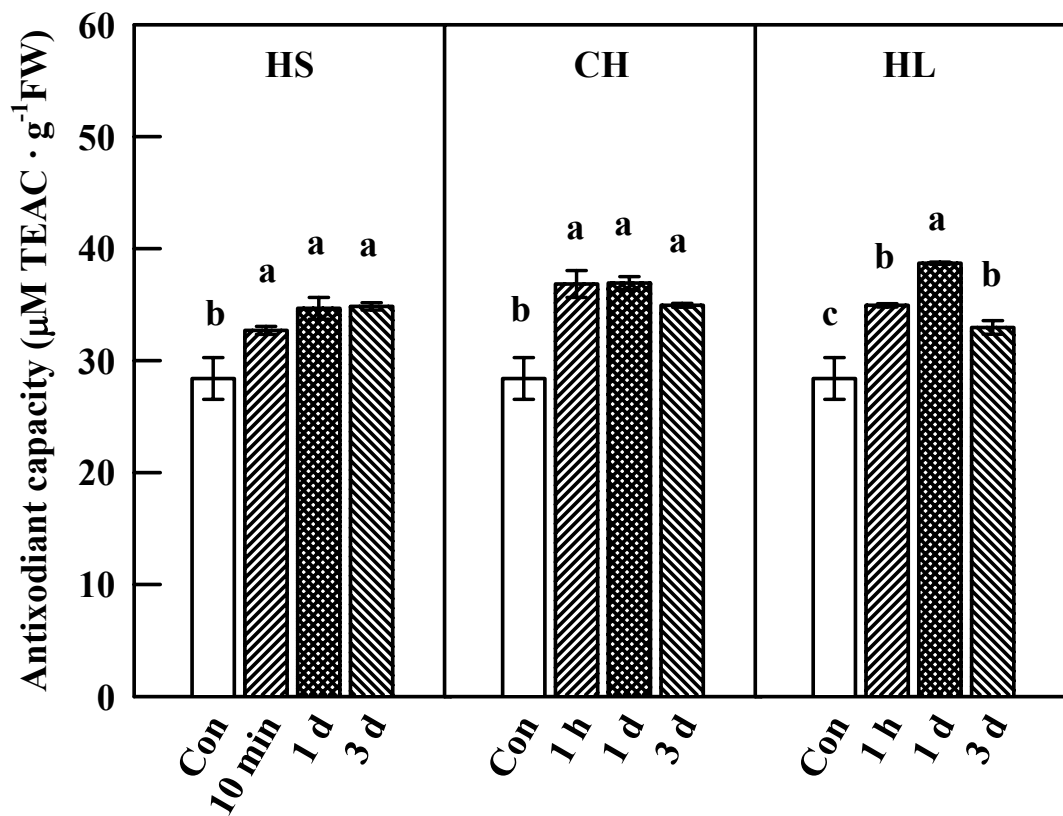
**Figure 3.1. Changes in total phenolic content of lettuce plants subjected to several environmental shocks.**

Five week old lettuce plants were exposed to heat shock (HS, 40°C for 10 min), chilling (CH, 4°C for 1 d), or high light (HL, 800  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for 1 d) condition. Control (Con) represents lettuce plants right before each stress treatment. The data are means  $\pm$  S.E. (n=3). Differences within a stress treatment indicated by different letters are significant at  $P=0.05$ .



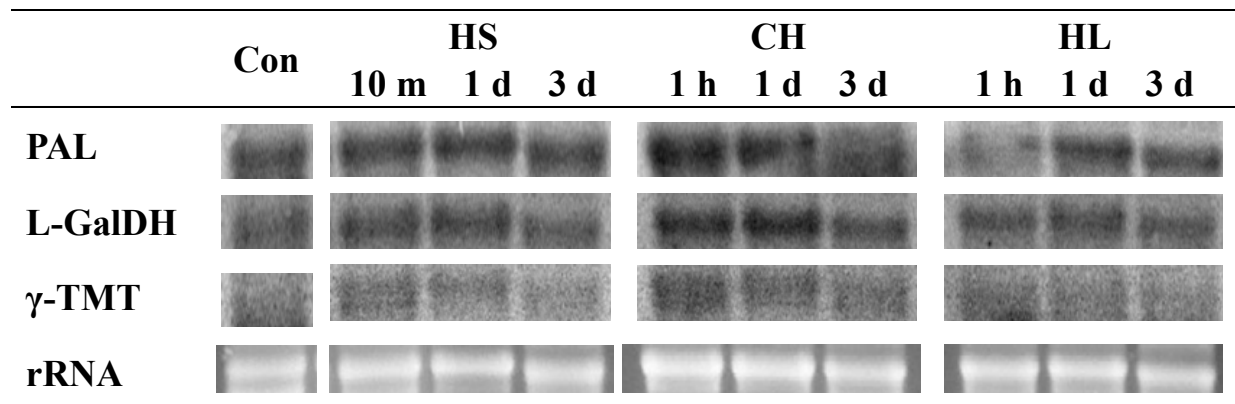
**Figure 3.2. Changes in antioxidant capacity of lettuce plants subjected to several environmental shocks.**

Five week old lettuce plants were exposed to heat shock (HS, 40°C for 10 min), chilling (CH, 4°C for 1 d), or high light (HL, 800  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for 1 d) condition. Control (Con) represents lettuce plants right before each stress treatment. The data are means  $\pm$  S.E. (n=3). Differences within a stress treatment indicated by different letters are significant at  $P=0.05$ .



**Figure 3.3. The gene expression of PAL, L-GalDH, and  $\gamma$ -TMT in lettuce subjected to several environmental shocks.**

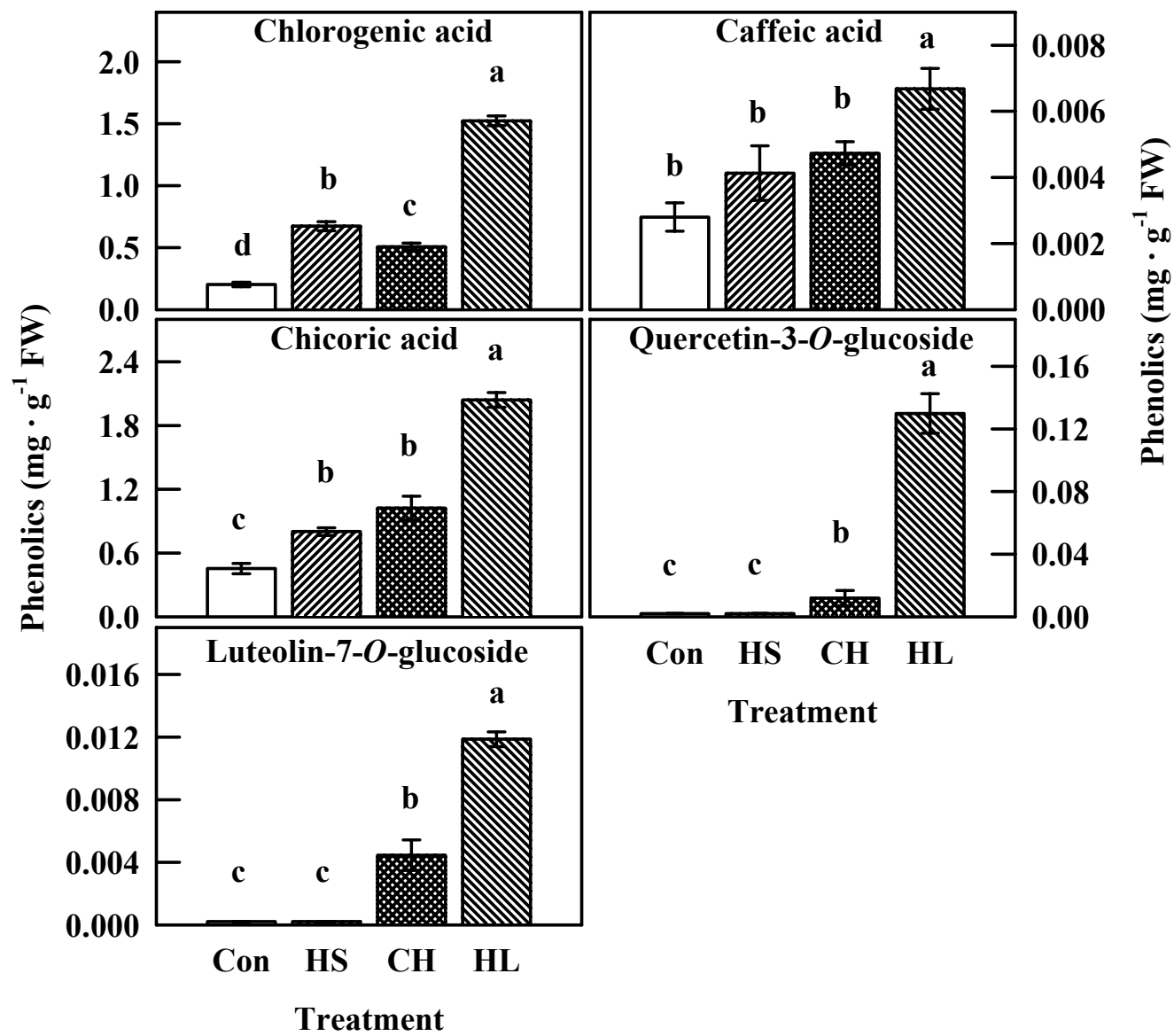
Five week old lettuce plants were exposed to heat shock (HS, 40°C for 10 min), chilling (CH, 4°C for 1 d), or high light (HL, 800  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for 1 d) condition. Control (Con) represents lettuce plants right before each stress treatment. rRNA was used as a loading standard.





**Figure 3.4. Concentration of phenolic compounds in lettuce plants at 3 d of recovery from several environmental shocks.**

Five week old lettuce plants were exposed to heat shock (HS, 40°C for 10 min), chilling (CH, 4°C for 1 d), or high light (HL, 800  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for 1 d) condition. Control (Con) represents lettuce plants right before each stress treatment. The data are means  $\pm$  S.E. (n=3). Differences within each phenolic compound indicated by different letters are significant at  $P=0.05$ .



**Table 3.1. Plant growth characteristics in lettuce subjected to several environmental shocks.**

Five week old lettuce plants were exposed to heat shock (40 °C for 10 min), chilling (4 °C for 1 d), or high light (800  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for 1 d) condition. Growth characteristics were measured 3 d after transferring to normal growing condition. The values are the means of three lettuce plants.

Treatment	Shoot			Root	
	Fresh weight (g)	Dry weight (g)	Leaf area (cm <sup>2</sup> )	Fresh weight (g)	Dry weight (g)
Control	10.19	0.79	239.35 a <sup>z</sup>	2.68 b	0.15 b
Heat shock	9.17	0.67	214.30 ab	2.88 b	0.15 b
Chilling	8.63	0.66	191.89 b	2.85 b	0.14 b
High light	8.68	0.87	182.84 b	3.27 a	0.17 a
Significance	NS	NS	*	*	*

<sup>z</sup> Mean separation within columns by Duncan's multiple range test.

<sup>NS</sup> Not significant.

\* Significant at  $P=0.05$ .

## **CHAPTER 4 - Enhancing Health-promoting Phytochemicals in Lettuce Using Regulated Water Deficit**

### **Abstract**

Lettuce (*Lactuca sativa*) plants, grown in a chamber, were used to evaluate the effect of plant age and drought stress on the health-promoting phytochemicals with antioxidant properties. Lettuce plants were treated with drought treatment once (OD) at 6 weeks (before harvest) for 2 d or multiple times (MD). For MD treatment, plants were subjected to water stress at 4 weeks for 4 d, at 5 weeks for 3 d, and at 6 weeks for 2 d. Lettuce seedlings immediately after germination had the highest total phenolic content and antioxidant capacity. OD and MD increased both the total phenolic content and antioxidant capacity in lettuce. Young seedlings were more responsive to drought stress treatments in accumulating the antioxidants than older plants under MD treatment. A marked activation of PAL and  $\gamma$ -TMT, involved in the biosynthesis of phenolic compounds and vitamin E, respectively, was observed in response to drought stress. Lettuce plants subjected to MD accumulated significant amounts of chicoric acid compared to control plants. Although the increase in the antioxidant activity in drought-treated plants at harvest was not as great as in young seedlings, it was significantly higher than the control. OD treatment of lettuce did not result in any adverse effect on plant growth. Thus, the results show that mild drought stress in lettuce prior to harvest can lead to significant increase in the quality of the crop in relation to their phytochemical content without loss of growth or yield.

### **Introduction**

In recent years, the potential importance of fruit and vegetables as a health-promoting food is growing in light of the increasing number of epidemiological studies suggesting that daily intake of fruits and vegetables has health benefits in preventing many chronic and degenerative diseases (Block et al., 1992; Buring and Hennekens, 1997; Arai et al., 2000; Berger, 2005). Plant-based foods such as fruits and vegetables provide these health benefits because they are rich in nutrients and phytochemicals, including antioxidants (DellaPenna, 1999). Lettuce (*Lactuca sativa*) contains various health-promoting phytochemicals with antioxidant properties such as vitamins and phenolic compounds (Caldwell, 2003; Llorach et al., 2004; Nicolle et al., 2004). Especially, the major phenolic compounds in lettuce such as hydroxycinnamic acids (chlorogenic acid and caffeic acid) and flavonoids (chicoric acid) are known to have positive effects on human health (Graefe and Veit, 1999; Olthof et al., 2001).

In response to drought, plants typically accumulate osmolytes such as proline, betaines, and fructans to maintain turgor and protect macromolecules in cells (Smirnoff, 1998). In addition, plants subjected to drought also accumulate a wide range of antioxidants, which consist of enzymatic antioxidants (peroxidases, superoxide dismutases, and catalases) and non-enzymatic antioxidants (carotenoids, ascorbate, tocopherols, glutathione, and phenolic compounds) to quench the reactive oxidative species (ROS) induced by stress (Sofa et al., 2005). Indeed, drought stress has been shown to induce  $\alpha$ -tocopherol,  $\beta$ -carotene, and flavonoids in a wide range of plant species (Munné-Bosch et al. 2001; Keleş and Öncel, 2002; Tattini et al., 2004; Zobayed et al., 2007). It is of interest to note that a number of phenolic compounds and vitamins such as vitamin A, C, and E having antioxidant properties that accumulate in plants in response to stress also have health-promoting benefits in humans (Nicolle et al., 2004).

Since lettuce is the most commonly consumed fresh leafy vegetable, our focus in this study was to improve its phytochemical content using mild drought stress at different growth stages. We examined if regulated drought stress can enhance the antioxidant capacity and phenolic content in lettuce. In addition, we determined if these changes are controlled by gene action by monitoring the activation of key genes involved in the biosynthesis of phenolic compounds, vitamin C and E in lettuce plants.

## **Materials and Methods**

### ***Plant material and growth conditions***

Lettuce (*Lactuca sativa* L. cv. Baronet) seeds (Johnny's Selected Seeds, Winslow, MA, USA) were germinated in plastic pots (8 cm × 8 cm × 7 cm ;  $L \times W \times H$ ), containing growing medium (Metro-Mix 350, Sun Gro, Canada). One hundred and ninety two plastic pots each containing 1 lettuce seed were placed in 8 plastic trays (53 cm × 38 cm × 7 cm;  $L \times W \times H$ ). The growing medium was maintained at field capacity using a capillary subirrigation system until drought stress treatments. Nutrient solution with N-P-K of 20-10-20 at 250 ppm of nitrogen was applied to seedlings once a week after 3 weeks of germination. Lettuce plants were grown at 22/18 °C (day/night), PPFD at 250  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ , and 12 h photoperiod in two growth chambers.

### ***Drought stress treatment***

Drought stress treatments were initiated when seedlings were 4-week old. Drought stress was induced in plants by withholding watering. To make sure that plants were treated with mild water stress, they were rewatered as the plants begin to lose turgor. Typically, withholding of water for 4 d resulted in a leaf water potential of -1.5 MPa. Lettuce plants were treated with drought treatment once (OD) at 6 weeks (before harvest) for 2 d or multiple times (MD). For MD

treatment, plants were subjected to water stress at 4 weeks for 4 d, at 5 weeks for 3 d, and at 6 weeks for 2 d.

### ***Leaf water potential***

Leaf water potential was measured by a thermocouple psychrometer (Tru Psi, Decagon Devices, Inc. Pullman, WA, USA). Leaf disks (1.2 cm diameter) were cut with a cork borer and immediately transferred to small sealed stainless steel chambers. They were equilibrated at 25 °C for 50 min before measurement. Water potential of lettuce leaves was measured with a cooling time of 15 s. Each treatment had three replications.

### ***Leaf fluorescence***

To evaluate the photosynthetic efficiency of lettuce, chlorophyll fluorescence was measured on the adaxial side of fully expanded leaf with a chlorophyll fluorometer (PEA, Hansatech Instruments Ltd., UK). The leaves selected for the measurement were from the middle of the canopy of 4-week old plants. Fv/Fm ratio, potential quantum yield, was measured 8 times during the day time using the saturation pulse method after 30 min of dark adaptation. Three lettuce plants per treatment were used.

### ***Total phenolic content***

Lettuce leaves were collected every 3 or 4 d after 1 week to 6 weeks of planting to monitor the changes of total phenolic content in relation to plant age. Samples treated with water stress were collected right before and at the end of the stress treatment after 4 weeks to 6 weeks of planting. Total phenolic content of lettuce leaves was analyzed by a modified Folin-Ciocalteu reagent method (Pennycooke et al., 2005). About 1 g fresh leaf tissue was ground in 6 mL 80% (v/v) acetone with a mortar and pestle. The sample (1 mL) was placed into a 1.5 mL tightly

covered micro-tube and incubated in darkness at 4 °C overnight. After sample centrifugation at 1000 rpm for 2 min, a mixture of 135 µL H<sub>2</sub>O, 750 µL 1/10 dilution Folin-Ciocalteu reagent and 600 µL 7.5% (w/v) Na<sub>2</sub>CO<sub>3</sub> was added with 50 µL of phenolic extract in a 1.5 mL micro-tube. The mixture vortexed for 10 s was incubated at 45 °C in a water bath for 15 min. Samples were allowed to cool at room temperature before measuring the absorbance at 765 nm by a spectrophotometer (U-1100, Hitachi Ltd. Japan). A blank was prepared from 50 µL 80% (v/v) acetone. A gallic acid (Sigma-Aldrich, St. Louis, MO, USA) standard curve was prepared from a freshly made 1 mg · mL<sup>-1</sup> gallic acid (in 80% (v/v) acetone) stock solution. Each treatment had three replications.

### ***Antioxidant capacity***

Leaf samples for antioxidant capacity were collected at the same time as for the determination of total phenolic content. Antioxidant capacity of lettuce leaves was monitored by a modified ABTS (Aminobenzotriazole) method (Awika et al., 2003; Miller and Rice-Evans, 1996; Pennycooke et al., 2005). Antioxidants were extracted from the lettuce leaves (about 1 g) using 5 mL extraction solution (acetone: water: acetic acid = 70:29.5:0.5, v/v). The extracted solution was incubated in darkness at -20 °C overnight. Supernatant containing antioxidants was obtained by centrifuging the solution at 1000 rpm for 2 min. Meanwhile, 2.5 mM of ABTS (Roche Diagnostics, Indianapolis, IN, USA) stock solution was prepared and about 0.4 g of MnO<sub>2</sub> (Acros Organics, Belgium) was added to stock solution to generate ABTS radical cation (ABTS\*) stirring the mixture occasionally for 30 min at room temperature. Excess MnO<sub>2</sub> was removed first by filtration using a Buchner funnel and then with a 0.2 µM syringe-end filter (Millipore Corp., Bedford, MA, USA). The ABTS\* solution was incubated at 30 °C in a water bath and was diluted to an absorbance of 0.7 (±0.02) at 730 nm using 5 mM PBS [phosphate



buffer saline, pH 7.4, ionic strength (150 mM NaCl)]. A 100  $\mu$ L of the extract was added to 1 mL of ABTS\* solution. After vortexing for 10 s, the optical density of the mixture was recorded at the absorbance at 730 nm (U-1100 spectrophotometer, Hitachi Ltd. Japan) 1 min after the start of the reaction. Trolox [(6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxyl acid) (Acros Organics, Belgium)] standard curve was prepared using 0.5 mM stock solution. Each treatment had three replications.

### ***RNA gel blot analysis***

RNA gel blot analysis was conducted to determine the activation of key genes involved in the biosynthesis of phytochemicals with antioxidant properties. The genes for L-galactose dehydrogenase (L-GalDH),  $\gamma$ -tocopherol methyltransferase ( $\gamma$ -TMT), and PAL involved in the biosynthesis of vitamin C, vitamin E, and phenolic compounds, respectively (Bergmüller et al., 2003; Diallinas and Kanellis, 1994; Gatzek et al., 2002), were selected in this study. The specific probes for L-GalDH (497 bp, accession number AJ417563) with primers 5'-AACTTCTTCGACACCTCCCC-3' and 5'-TCATCCCAACCAACACCGAC-3', for  $\gamma$ -TMT (244 bp, accession number AF104220) with primers 5'-CATAGAAATCTATCTGCGGG-3' and 5'-CACTACGAAGCAGAGACACA-3', and for PAL (753 bp, accession number AF299330) with primers 5'-GCTTACAGTTTCTCAGGTGG-3' and 5'-TGATGCTTCAATTTGTGTGT-3' were amplified using genomic DNA from lettuce plants with the primer pairs designed by a primer design program (Primer3, Whitehead Institute, Cambridge, MA, USA). Amplification of probes was performed by a PTC-100 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA) after initial denaturation at 94 °C for 5 min by 35 cycles of 1 min at 94 °C, 1 min at 51 °C ( $\gamma$ -TMT and PAL) or 54 °C (L-GalDH), and 1 min at 72 °C, followed by 5 min at 72 °C. The fragment size of each probe was checked by electrophoresis on 1.2% (w/v) agarose gels in 1  $\times$  Tris-Acetate-EDTA

buffer. After electrophoresis, the fragments stained with ethidium bromide were checked by a UV transilluminator (FisherBiotech, Fairlawn, NJ, USA). The probes were stored at -20 °C until use.

The samples were collected to check the transcript levels of above three interested genes at 6 weeks of planting. Total RNA was extracted from lettuce leaves using a modified cetyltrimethylammonium bromide (CTAB) extraction method as described by Chang et al. (1993). Total RNA (20 µg) was subjected to electrophoresis on 1% (w/v) agarose gels with 10% (v/v) formaldehyde. The RNA was transferred onto nylon membranes (Sigma-Aldrich, St. Louis, MO, USA) in a 20 × SSC solution (Sambrook and Russell, 2001). The probes were labeled with <sup>32</sup>P using random primer DNA labeling system (Sigma-Aldrich, St. Louis, MO, USA). The nylon membranes were incubated with 40 mL prehybridization buffer (1 mM EDTA, 0.5 M NaH<sub>2</sub>PO<sub>4</sub>, 7% SDS, pH 7.2) at 50 °C for 6 h with salmon sperm DNA (10 mg · mL<sup>-1</sup>). The hybridization was performed as described by Sambrook and Russell (2001). Probes were hybridized to RNA on nylon membranes at 50 °C for 12 h in 40 mL prehybridization buffer. The membranes were subsequently rinsed twice with wash I buffer (1 mM EDTA, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 5% SDS, pH 7.2) and then once with wash II buffer (1 mM EDTA, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 1% SDS, pH 7.2) at 50 °C for 1 h. The membranes were then exposed in cassettes at room temperature for 1 day and the film was scanned by a gel and blot imaging system (Storm<sup>TM</sup>, GE Healthcare, Piscataway, NJ, USA).

### ***High-performance liquid chromatography (HPLC) analyses***

#### ***Phenolic compounds***

The extraction of phenolic compounds from lettuce leaves was carried out as described by Nicolle et al. (2004) with minor modifications. Frozen leaf sample (1 g) ground in a mortar with a pestle using liquid nitrogen was extracted with 50 mL of 70% methanol at 80 °C for 1 min. After stirring at room temperature for 1 h, the mixture was filtered with a filter paper (No. 1, Whatman plc., UK). The extract (25 mL) was evaporated to dryness by a rotary evaporator (Rotavapor R110, Brinkmann Instruments, Inc., Westbury, NY, USA) under reduced pressure at 50 °C and then resuspended in 5 mL of 70% methanol. The concentrated solution was filtered through a 0.45 µm filter ascrodisc (Millex, Millipore Corp., Bedford, MA, USA) before HPLC analysis. A 5 µL aliquot of the sample extract was injected into a HPLC system equipped with an autosampler (SpectraSYSTEM AS1000, Thermo Separation Products, San Jose, CA, USA), a pump (HP 1050, Hewlett Packard, Palo Alto, CA, USA), an integrator (HP 3396, Hewlett Packard, Palo Alto, CA, USA), and an UV/VIS detector (Acutech 500, Thermo Separation Products, San Jose, CA, USA). Phenolic compounds were separated using a Discovery BIO Wide Bore C-18 column (15 cm × 4.6 mm, 5 µm, Supelco, Inc., Bellefonte, PA, USA) coupled to a guard column (Discovery BIO Wide Bore C-18, 2 cm × 4 mm, 5 µm, Supelco, Inc., Bellefonte, PA, USA) at 60 °C. Compounds of the sample extract were eluted with eluant A [H<sub>2</sub>O:CH<sub>3</sub>COOH = 338:1 (v/v)] and eluant B [H<sub>2</sub>O:C<sub>4</sub>H<sub>10</sub>O:CH<sub>3</sub>COOH = 330:8:1 (v/v/v)] at a flow of 1.8 mL · min<sup>-1</sup>. The gradient started at 20% B in A, which was held for 5 min, then increased to 100% B over 20 min. After equilibration for 2 min at 100% B, the composition of solution returned to the initial condition (20% B). Peaks from the sample extract were identified and quantified at 330 nm by comparing with standard compounds such as chlorogenic acid (Sigma-Aldrich, St. Louis, MO, USA), caffeic acid (Sigma-Aldrich, St. Louis, MO, USA), and chicoric acid (Indofine Chemical Co., Inc., Hillsborough, NJ, USA).

### ***Vitamin C***

Ascorbic acid was extracted from 1 g of lettuce leaves frozen in liquid nitrogen as described by Wimalasiri and Wills (1983) with some modifications. The sample was ground in a mortar with a pestle and then extracted with 10 mL of 3% metaphosphoric acid (HPO<sub>3</sub>) (Riedel-de Haën, Germany). Sample mixed with metaphosphoric acid solution was filtered with a filter paper (No. 1, Whatman plc., UK) and subsequently with a 0.45 µm filter ascrodisc (Millex, Millipore Corp., Bedford, MA, USA) under ice. The extract (5 µL) was immediately injected into a HPLC system equipped with a column (Discovery BIO Wide Bore C-18, 15 cm × 4.6 mm, 5 µm, Supelco, Inc., Bellefonte, PA, USA) and a guard column (Discovery BIO Wide Bore C-18, 2 cm × 4 mm, 5 µm, Supelco, Inc., Bellefonte, PA, USA) at 25 °C. The mobile phase was 10 mM ammonium dihydrogen phosphate [(NH<sub>4</sub>)H<sub>2</sub>PO<sub>4</sub>, pH 4] at a flow rate of 1 mL · min<sup>-1</sup>. Ascorbic acid was detected at 255 nm and was identified and quantified by standard ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA).

### ***Vitamin E***

The extraction of α-tocopherol was conducted following the method of Kim et al. (2007) with minor modifications. Homogenized leaf sample (1 g) was extracted with 3 mL hexane (Chromasolv plus, Sigma-Aldrich, St. Louis, MO, USA) containing 0.1% butylated hydroxyl toluene (BHT) (Sigma-Aldrich, St. Louis, MO, USA) and the organic phase was collected. The remaining pellet was extracted again with 3 mL hexane containing 0.1% BHT. The combined organic phase was adjusted to 5 mL and filtered through a 0.45 µm filter ascrodisc (Millex, Millipore Corp., Bedford, MA, USA) under the dark at 4 °C. The extract was evaporated to dryness on ice under nitrogen and then resuspended in 1 mL of methanol. The 20 µL extract was injected into a HPLC equipped with a column (Discovery BIO Wide Bore C-18, 15 cm × 4.6 mm,

5  $\mu\text{m}$ , Supelco, Inc., Bellefonte, PA, USA) and a guard column (Discovery BIO Wide Bore C-18, 2 cm  $\times$  4 mm, 5  $\mu\text{m}$ , Supelco, Inc., Bellefonte, PA, USA) using flow rate of 1.8 mL  $\cdot$  min<sup>-1</sup>. The mobile phase consisted of 97% methanol and 3% water. The wavelengths were set at 295 nm for excitation and 340 nm for emission to determine  $\alpha$ -tocopherol. The retention time of the standard was 5 min and samples were identified and quantified using standard  $\alpha$ -tocopherol (Sigma-Aldrich, St. Louis, MO, USA).

### ***Plant growth characteristics***

Plant growth characteristics were measured at the end of the experiment, 6 weeks after planting. Dry and fresh weights of roots and shoots, total leaf area, and total root length and volume were measured using four plants per each treatment. After measuring fresh weights of roots and shoots, the samples were dried at 70°C in an oven for 3 d to measure their dry weights. Total leaf area was measured by a leaf area meter (LI-3100C, LI-COR, Lincoln, NE, USA). Total root length and volume were measured using a root scanning system (WinRHIZO, Regent Instruments Inc. Canada). Roots were washed thoroughly in flowing tap water and stained with methyl blue solution (5g  $\cdot$  L<sup>-1</sup>) for 5 min. Stained roots were washed again with tap water and were carefully spread on a transparent plastic support before scanning.

### ***Statistical analysis***

Analysis of variance (ANOVA) was performed by the statistical analysis system (SAS) program. Duncan's multiple range test was used to compare means.

## **Results**

The highest amount of total phenolics in lettuce plants was in their early seedling stage, immediately after germination (Fig. 4.1). The total phenolic content subsequently declined sharply until the seedlings were approximately 2-week old. It declined by more than 65% during

this period (1 week) and only slightly thereafter to its lowest level at the time of harvest. The changes in antioxidant capacity of leaves with plant age were similar to those in the total phenolic content (Fig. 4.2)

When 4-week old plants were exposed to drought, slight decline in leaf water potential occurred after withholding water for 2 d. The leaf water potential declined sharply thereafter, reaching -1.5 MP on the 4<sup>th</sup> day of water withholding (Fig. 4.3). Plants were rewatered at this point as reflected by the recovery of leaf water potential. The photosynthetic efficiency appeared to be very sensitive to the early stages of water stress. Fv/Fm ratio began to decline after 1 d of water withholding.

When lettuce plants were subjected to MD, the total phenolic content of leaves increased sharply in response to the first drought stress treatment. This increase occurred over a 4-d period coinciding with the decreasing leaf water potential (Fig. 4.4). Although the second and third drought stress treatments increased the total phenolic content, the increases were not as large as the one in response to the first stress treatment. Similarly, leaf antioxidant capacity increased sharply in response to the first drought treatment (Fig. 4.5). However, it is worth noting that the results on antioxidant capacity are difficult to interpret because the data for the control plants were quite variable. The total phenolic content and antioxidant capacity of lettuce plants declined with plant age, and drought stress treatment just prior to harvest increased the total phenolic content over the control plants, although not to the same extent as the first drought stress treatment.

Both total phenolic content and antioxidant capacity of leaves increased significantly due to either OD or MD at the time of harvest (Figs. 4.6 and 4.7). The extent of increase in these was similar regardless of whether the plants were treated with drought one-time or multiple times. It

is interesting to note that both groups of plants were subjected with drought treatments when they were six-week old, although the plants with MD had received two prior stress treatments.

Drought stress activated PAL and  $\gamma$ -TMT in lettuce regardless of whether they were treated once or multiple times (Fig. 4.8). However, in the case of L-GalDH, no clear differences in transcript levels were detected as a result of drought stress treatments.

The amount of the most abundant phenolic acid in lettuce, chicoric acid, increased significantly in response to MD (Fig. 4.9). Although the level of other phenolic acids like chlorogenic acid and caffeic acid increased in numerical value in response to drought, the increases were not significant. Similarly, no significant differences were observed in ascorbic acid and  $\alpha$ -tocopherol contents between control and drought-treated plants (Fig. 4.10).

Plant growth was not affected by OD. There were no significant differences in biomass accumulation, leaf area, or root growth between drought-treated plants and control plants (Table 4.1). However, MD adversely affected the plant growth in lettuce. Most shoot growth parameters such as fresh weight and leaf area declined slightly except dry weight in response to MD. Interestingly, root growth negatively correlated with shoot growth. MD induced significantly a bigger formation of root as well as a higher accumulation of root biomass than control and OD in lettuce plants.

## **Discussion**

Young lettuce seedlings, immediately after germination, were rich in phenolic compounds and antioxidants. Both total phenolic compounds and antioxidant capacity declined rapidly and reached the lowest levels at the time of harvest. Typically, germinating seeds and young seedlings shift to secondary metabolism because of carbon-limitation in germinating seeds, and the accumulation of secondary metabolites, especially antioxidants, may aid young seedlings

against biotic and abiotic threats (Barton, 2007; Bryant et al., 1992; Clarke and Davison, 2004; Harper, 1977). As antioxidants declined with plant age to their lowest level at the time of harvest, the quality of lettuce in relation to its health-promoting value was also low compared to young seedlings. Health-promoting benefit of germinating seeds (sprouts) and young seedlings have long been known. For example, compared to mature plants, sprouts of radish, broccoli, and cauliflower contain 10 to 100 times of glucosinolates, which show a strong protective function against many types of cancers (Barillari et al., 2006; Fahey et al., 1997). In addition, sprouts of many species have been shown to protect cells against DNA-damage caused by oxidative stress and against bacterial infections (Gill et al., 2004; Ho et al., 2006).

When lettuce plants were exposed to mild water stress, there was a significant increase in their total phenolic content and antioxidant capacity. Numerous studies have shown that drought stress can induce a wide range of antioxidants in several diverse plant species (Bray, 2002; Keleş and Öncel, 2002; Munné-Bosch et al. 2001; Sofu et al., 2005). Our results showed that plant age may play a key role in the extent of this response in lettuce. Younger seedlings (4-week old) exposed to water stress increased their phenolic content and antioxidant capacity to the largest extent. In response to water stress, the increase in total phenolic content was approximately 4 times higher when plants were 4-week old compared to that in older plants. Thus, the results suggest that young lettuce plants are not only richer in phenolic compounds and higher in antioxidant capacity but also are more responsive to mild water stress than older plants. Furthermore, the increase in phenolic compounds occurred during water stress and rewatering plants appeared to decline the total phenolic content and antioxidant capacity. The results also suggested that exposure of plants to MD did not have a cumulative or elevated response compared to OD. Improvement of phytochemical content of lettuce plants treated with drought



once at 6 weeks before harvest or treated multiple times was identical. Thus, we can conclude that the response of phytochemicals in lettuce occurs only during water stress and the extent of response is largely dependent on the plant age. It should also be noted that although their response at the time of harvest (6 weeks) was much smaller than that in younger plants, the increase in phytochemicals was still quite significant.

In response to water stress, an increased activation of PAL was observed in lettuce. This response was consistent with the higher accumulation of phenolic compounds in lettuce. Expression of PAL in response to water stress is important since it is the gateway enzyme in the phenylpropanoid pathway that yields a wide range of phenolic compounds (Diallinas and Kanellis, 1994; Liu et al., 2006). Similarly, higher transcript level of  $\gamma$ -TMT was noted in response to water stress. However, no significant accumulation of  $\alpha$ -tocopherol was detected in the leaves. This clearly suggests that mild water stress can activate key genes involved in the biosynthesis of phytochemicals in lettuce. Thus, it is likely that phytochemical accumulation in response to drought stress in lettuce is as a result of genetic control rather than chemical or physiological modulation of biosynthetic pathways.

Analyses of individual phenolic compounds indicate that dominant phenolic compound in lettuce, chicoric acid, accumulates in response to water stress. However, no significant accumulations of chlorogenic acid, caffeic acid, and ascorbic acid occurred due to water stress.

As plant stresses including water stress are generally considered to produce adverse effects on growth and development, it is important to assess their impact on biomass accumulation and crop yield (Boyer, 1982). Exposure of lettuce plants to OD produced significant increase in phytochemicals without any adverse effect on plant growth. However,

plant exposure to MD produced a negative effect on biomass accumulation and other growth characteristics of plants.

Our results show that OD was as good as MD with regard to enhancing the phytochemical content in lettuce. Therefore, OD which did not have any adverse effect on plant growth or biomass accumulation would be of value in improving the health-promoting attributes in lettuce. Thus, these results show the potential use of regulated water stress as a crop management approach in improving the quality of lettuce crop by enhancing their nutritive and health-promoting value.

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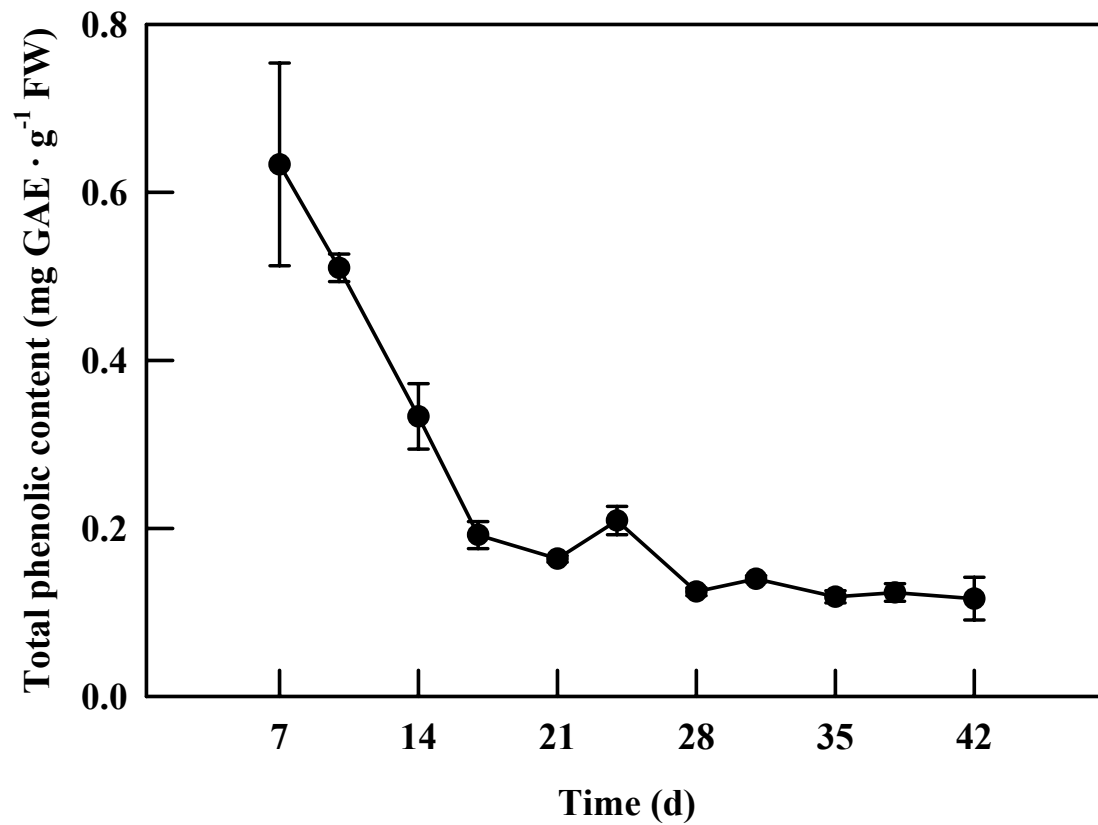
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## Figures and Tables

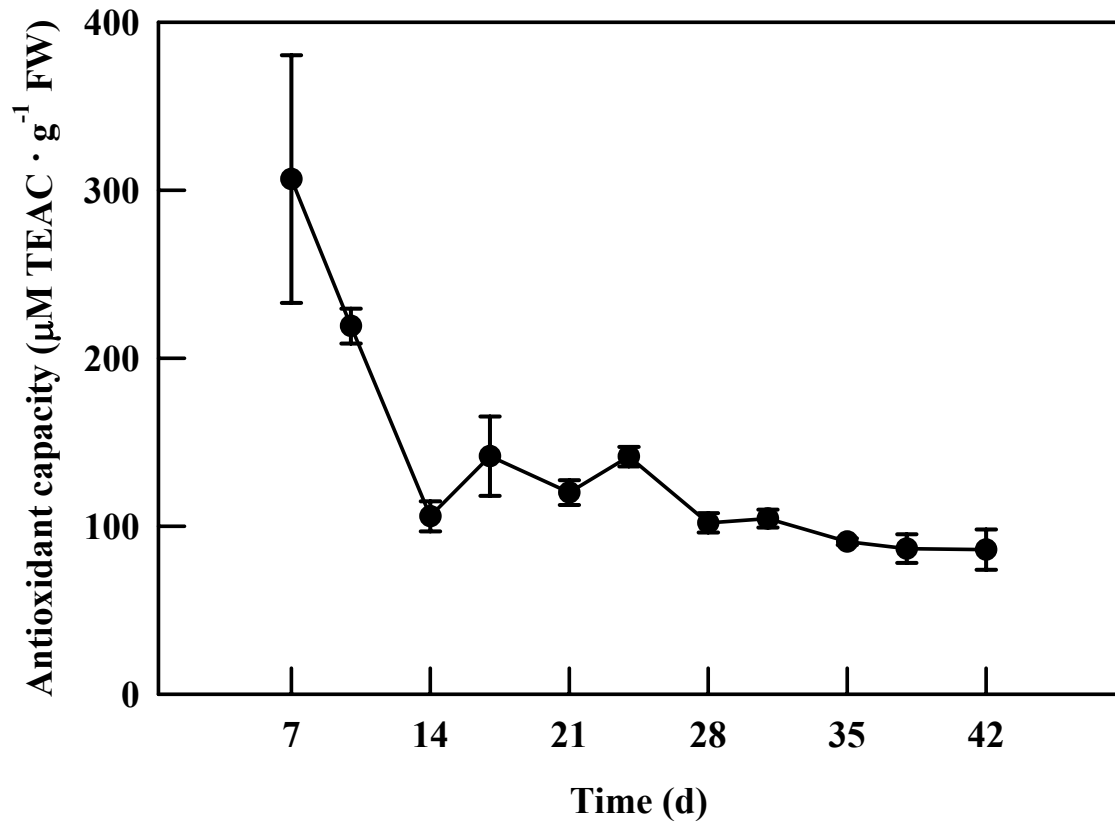
**Figure 4.1. Changes in total phenolic content in lettuce leaves with plant age.**

Lettuce plants were grown at 22/18 °C (day/night), PPFD at 250  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ , and 12 h photoperiod in a growth chamber. Total phenolic content was measured every 3 or 4 d after 1 week to 6 weeks of planting. The values are the means of three lettuce plants. Bars represent standard errors.



**Figure 4.2. Changes in antioxidant capacity in lettuce leaves with plant age.**

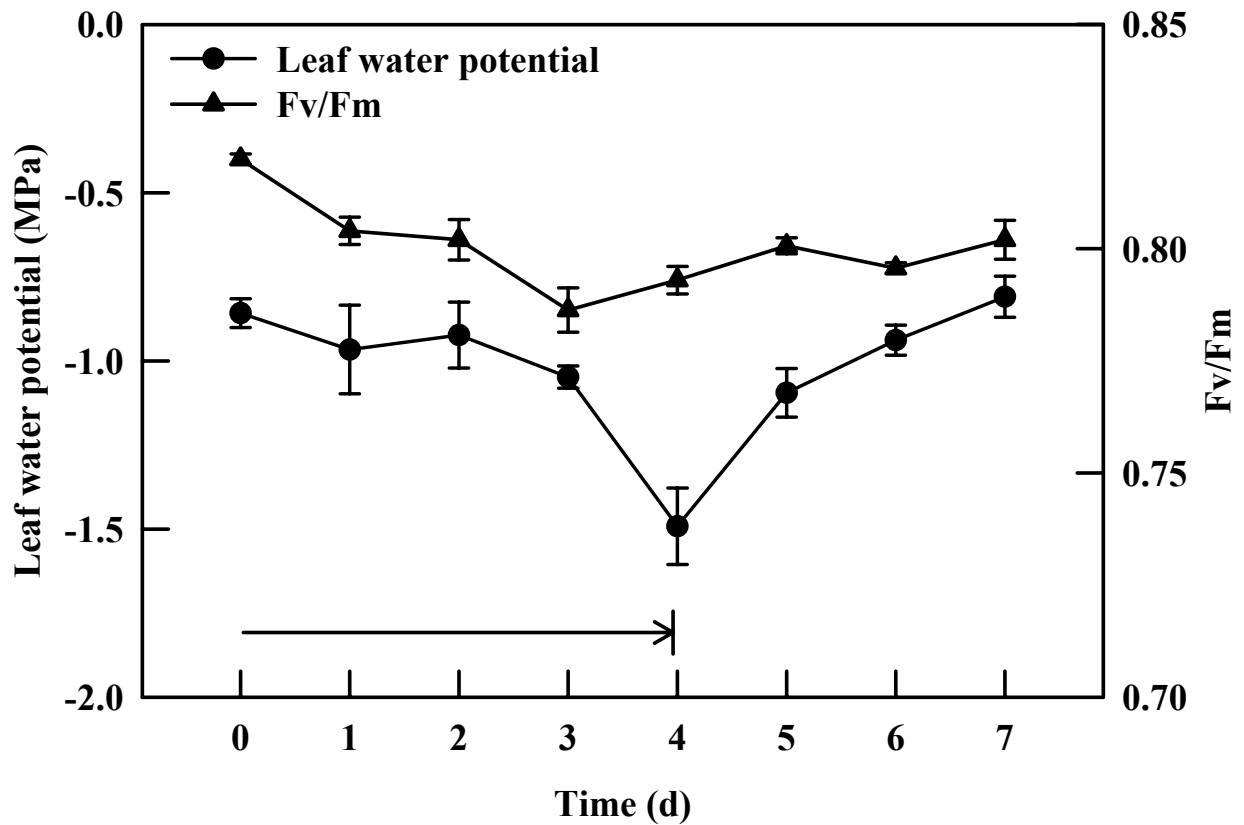
Lettuce plants were grown at 22/18 °C (day/night), PPFD at 250  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ , and 12 h photoperiod in a growth chamber. Antioxidant capacity was measured every 3 or 4 d after 1 week to 6 weeks of planting. The values are the means of three lettuce plants. Bars represent standard errors.





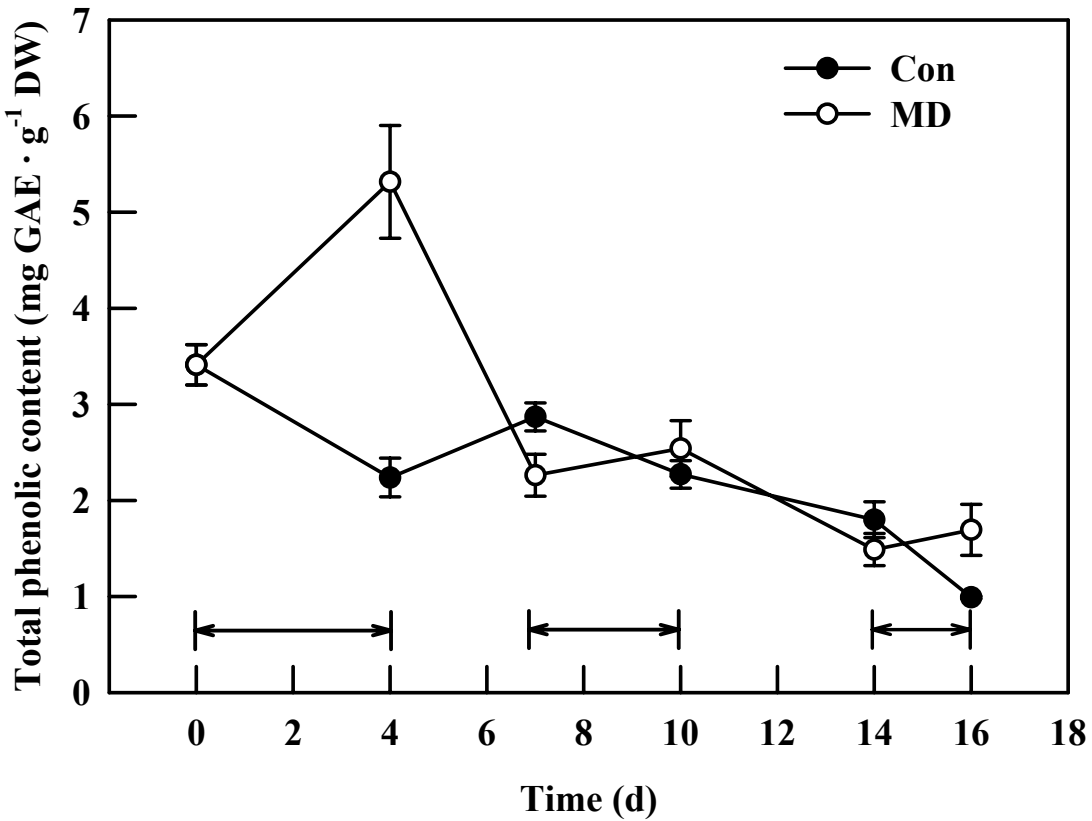
**Figure 4.3. Changes in leaf water potential and Fv/Fm ratio of lettuce plants subjected to 4 d of drought stress.**

Leaf water potential and Fv/Fm ratio were measured during the day. Drought stress period is indicated by the arrow. Plants were rewatered after this period. Three lettuce plants were used for each observation and bars represent standard errors.



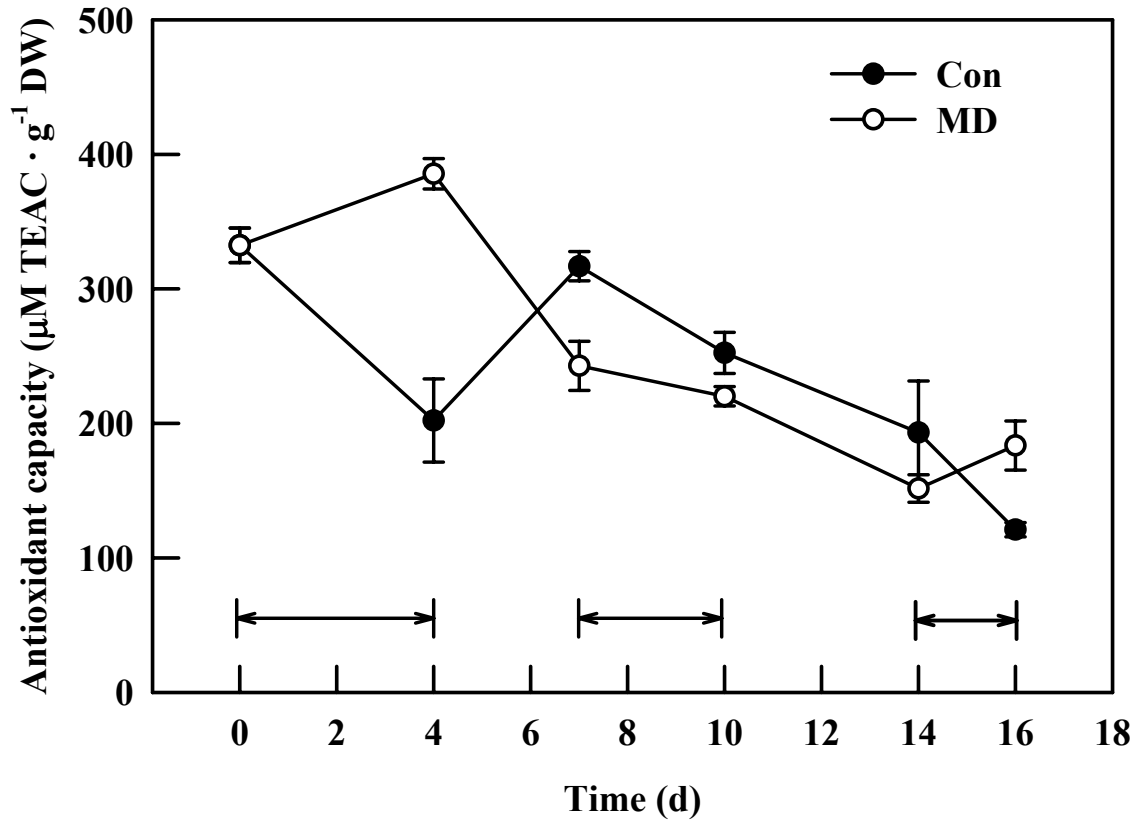
**Figure 4.4. Changes in total phenolic content of lettuce leaves subjected to multiple drought stress treatment.**

Lettuce plants were subjected to drought stress at 4 weeks for 4 d, at 5 weeks for 3 d, and at 6 weeks for 2 d after planting. Total phenolic content was measured right before and at the end of the stress treatment. Con and MD represent control and multiple drought stress, respectively. Three lettuce plants were used per treatment. Bars represent standard errors. Drought stress periods are indicated by arrows.



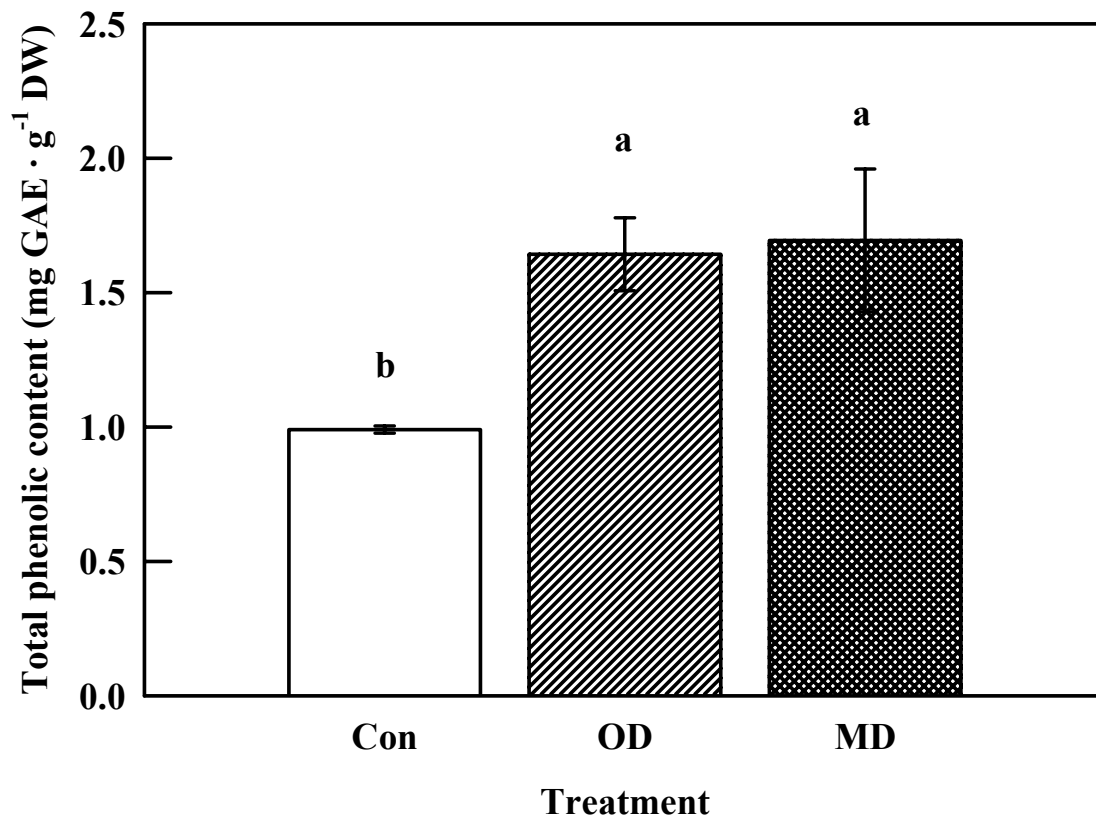
**Figure 4.5. Changes in antioxidant capacity of lettuce leaves subjected to multiple drought stress treatment.**

Lettuce plants were subjected to drought stress at 4 weeks for 4 d, at 5 weeks for 3 d, and at 6 weeks for 2 d after planting. Antioxidant capacity was measured right before and at the end of the stress treatment. Con and MD represent control and multiple drought stress, respectively. Three lettuce plants were used per treatment. Bars represent standard errors. Drought stress periods are indicated by arrows.



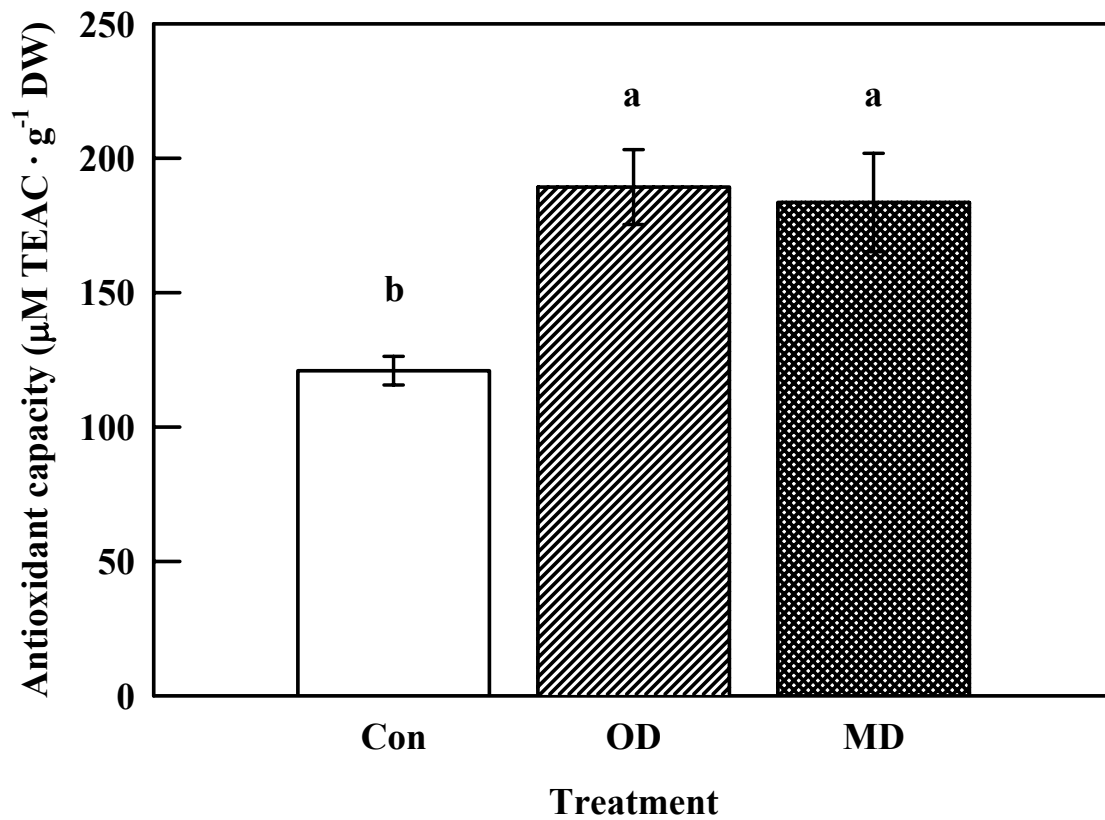
**Figure 4.6. Total phenolic content of lettuce leaves in plants subjected to one-time drought stress at the time of harvest or multiple drought stress treatment.**

Lettuce plants were harvested at 6 weeks after planting. Con, OD, and MD represent control, one-time drought stress (at 6 weeks for 2 d), and multiple drought stress treatment (at 4 weeks for 4 d, at 5 weeks for 3 d, and at 6 weeks for 2 d), respectively. Three lettuce plants were used per treatment. Bars represent standard errors. Significant differences ( $P=0.05$ ) between treatments are indicated by different letters.



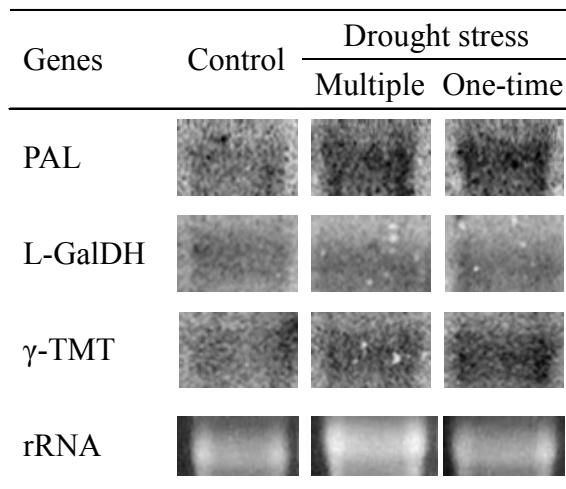
**Figure 4.7. Antioxidant capacity of lettuce leaves in plants subjected to one-time drought stress at the time of harvest or multiple drought stress treatment.**

Lettuce plants were harvested at 6 weeks after planting. Con, OD, and MD represent control, one-time drought stress (at 6 weeks for 2 d), and multiple drought stress treatment (at 4 weeks for 4 d, at 5 weeks for 3 d, and at 6 weeks for 2 d), respectively. Three lettuce plants were used per treatment. Bars represent standard errors. Significant differences ( $P=0.05$ ) between treatments are indicated by different letters.



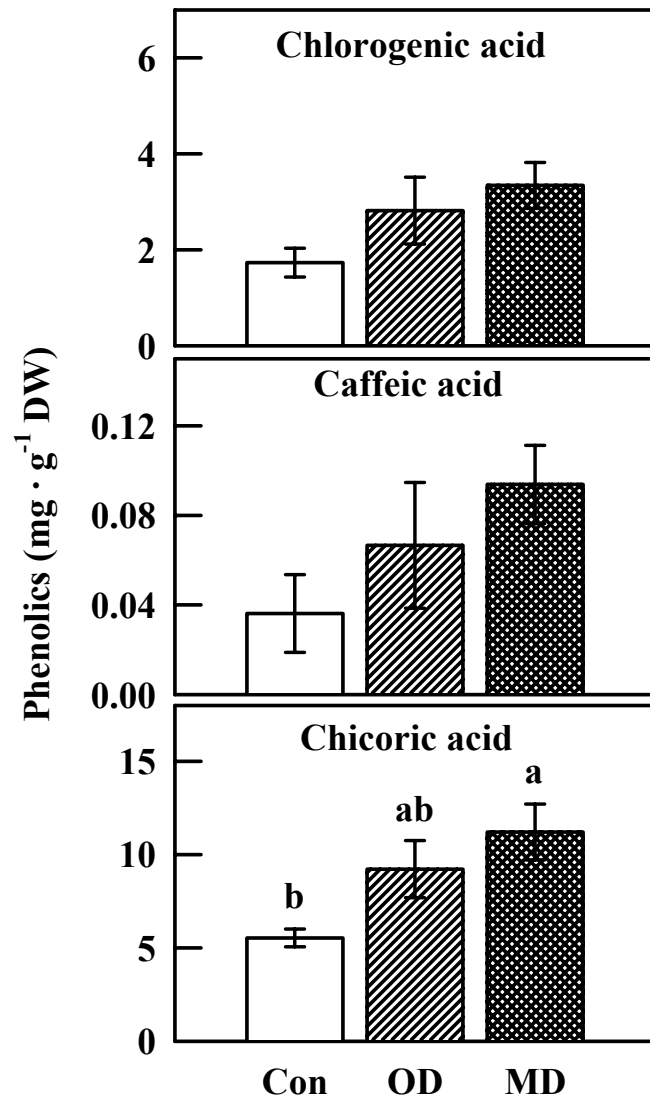
**Figure 4.8. The gene expression of PAL, L-GalDH, and  $\gamma$ -TMT in plants subjected to one-time drought stress at the time of harvest or multiple drought stress treatment.**

Lettuce plants were harvested at 6 weeks after planting. Lettuce plants were subjected to one-time drought stress (at 6 weeks for 2 d) or multiple drought stress treatment (at 4 weeks for 4 d, at 5 weeks for 3 d, and at 6 weeks for 2 d). Control represents transcript levels of lettuce plants normally grown in a growth chamber. rRNA was used as a loading standard.



**Figure 4.9. Concentration of phenolic compounds in lettuce plants under one-time drought stress at the time of harvest or multiple drought stress treatment.**

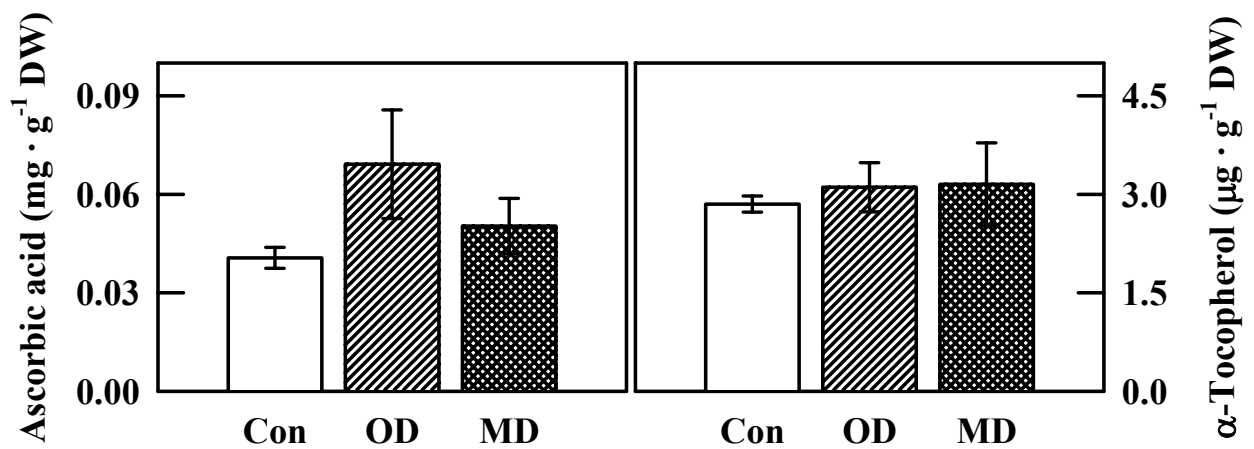
Lettuce plants were harvested at 6 weeks after planting. Con, OD, and MD represent control, one-time drought stress (at 6 weeks for 2 d), and multiple drought stress treatment (at 4 weeks for 4 d, at 5 weeks for 3 d, and at 6 weeks for 2 d), respectively. Three lettuce plants were used per treatment. Bars represent standard errors. Significant differences ( $P=0.05$ ) between treatments are indicated by different letters.





**Figure 4.10. Concentration of ascorbic acid and  $\alpha$ -tocopherol in lettuce plants under one-time drought stress at the time of harvest or multiple drought stress treatment.**

Lettuce plants were harvested at 6 weeks after planting. Con, OD, and MD represent control, one-time drought stress (at 6 weeks for 2 d), and multiple drought stress treatment (at 4 weeks for 4 d, at 5 weeks for 3 d, and at 6 weeks for 2 d), respectively. Three lettuce plants were used per treatment. Bars represent standard errors.



**Table 4.1. Plant growth characteristics of lettuce subjected to one-time drought stress at the time of harvest or multiple drought stress treatment.**

Lettuce plants were harvested at 6 weeks after planting. Lettuce plants were subjected to one-time drought stress (at 6 weeks for 2 d) or multiple drought stress treatment (at 4 weeks for 4 d, at 5 weeks for 3 d, and at 6 weeks for 2 d). Control represents lettuce plants normally grown in a growth chamber. The values are the means of four lettuce plants per treatment.

Treatment	Fresh weight (g)		Dry weight (g)		Total leaf area (cm <sup>2</sup> )	Total root length (cm)	Total root surface (cm <sup>2</sup> )	Total root volume (cm <sup>3</sup> )
	Shoot	Root	Shoot	Root				
Control	44.37 a <sup>z</sup>	5.19 b	2.15	0.20 b	882.5 a	4116.5 b	540.3 b	5.65 b
One-time	41.64 a	5.17 b	2.09	0.20 b	813.5 a	3921.0 b	538.5 b	5.89 b
Multiple	35.50 b	6.43 a	1.95	0.27 a	709.9 b	4730.1 a	655.8 a	7.25 a
Significance	*	*	NS	*	*	*	*	*

<sup>z</sup> Mean separation within columns by Duncan's multiple range test.

<sup>NS</sup> Not significant.

\* Significant at  $P=0.05$ .

## **CHAPTER 5 - Effects of Growing Conditions, Genotype, and Drought Stress on Health-promoting Phytochemicals in Lettuce**

### **Abstract**

The study examined the effect of mild drought stress on lettuce under contrasting growing conditions such as open field and high tunnel in two leaf lettuce varieties (*Lactuca sativa* L., cv. 'Baronet' and 'Red Sails'). Lettuce plants increased the total phenolic content and antioxidant capacity in response to transplanting to open field and high tunnel. Lettuce plants accumulated more phenolic compounds such as chlorogenic acid, chicoric acid, caffeic acid, quercetin-3-O-glucoside and luteolin-7-O-glucoside in open field than in high tunnel. Also, lettuce grown in open field did not accumulate as much biomass as those in high tunnel. Consistent with antioxidant accumulation, PAL,  $\gamma$ -TMT and L-GALDH, involved in the biosynthesis of phenolic compounds, vitamin E, and vitamin C, respectively, were activated in lettuce grown in open field. Drought stress treatments did not result in an increase in phytochemicals under field conditions. 'Red Sails' had higher amounts of phytochemicals than 'Baronet', especially grown in open field.

### **Introduction**

Lettuce has been a common leafy vegetable used in salads since ancient times (Harlan, 1986). However, only in recent years its value in human diet has been recognized as it contains a number of nutritive and health-promoting compounds such as phenolic compounds, vitamin A, C, and E, calcium, and iron that prevent many chronic diseases (Caldwell, 2003; Llorach et al., 2004; Nicolle et al., 2004; Romani et al., 2002). Many of these compounds found in lettuce have effective antioxidant properties (Caldwell, 2003, Wu et al., 2004), and of the various types of

lettuce commonly grown, leaf lettuce is most abundant in health-promoting phytochemicals (Liu et al., 2007).

A number of factors including plant genotype and growing conditions can have a large impact on the quality of vegetable crops, especially in relation to health-promoting phytochemicals (Schreiner, 2005). A variety of abiotic and biotic factors such as light, temperature, nutrient status, and pest infection can also directly affect the biosynthesis of phytochemicals in plants (Dixon and Paiva, 1995). Our earlier studies (chapter 2 and 3) and studies by others (Demmig-Adams and Adams, 2002) have found that harsher environmental conditions could lead to improved phytochemical content in lettuce. Thus, in addition to growing conditions, crop management and cultural practices are likely to influence the phytochemical composition in plants. In this study, lettuce plants were grown in open field and high tunnels to provide contrasting growing conditions.

High tunnels, which are unheated and passively ventilated production structures, have been widely used in Asia and Europe and are recently being used in the U.S. (Lamont et al., 2003; Spaw and Williams, 2004; Wells, 1996). High tunnels are known to produce higher yields and better quality of crops by extending their growth period and by providing protection from various adverse factors (Hodges and Brandle, 1996; Rader and Karlsson, 2006). However, there is limited information on crop quality in relation to health-promoting phytochemicals in crops grown in high tunnels relative to those grown in open fields.

In addition, mild drought stress in plants is known to result in accumulation of a wide range of antioxidants (Keleş and Öncel, 2002; Munné-Bosch et al. 2001; Sofó et al., 2005), which may actually lead to improved crop quality because of their higher health-promoting qualities. Most of the antioxidants like vitamin C and E and phenolic compounds induced by

environmental stresses have health-promoting qualities in humans (Berger, 2005; Nicolle et al., 2004). In previous growth chamber studies (chapter 3 and 4), we have found that mild drought stress improved the quality of lettuce crops in relation to phytochemicals without adversely affecting their biomass production or yield. Thus, in this study, we examined whether growing conditions involving open field and high tunnel, genotype, and mild water stress affect the quality of lettuce crop in relation to its phytochemical composition. Specifically, we determined if these factors can influence the activation of key genes involved in the biosynthesis of major phytochemicals and their composition in lettuce.

## **Materials and Methods**

### ***Experimental conditions and plant materials***

The study was conducted at K-State Horticulture Research and Extension Center, Olathe, Kansas State University from May 9 to June 4, 2007. One Haygrove high tunnel, 24.4 m × 7.3 m ( $L \times W$ ), covered with Luminance THB polythene (Visqueen Building Products, London, UK) and an adjacent open field, 24.4 m × 7.3 m ( $L \times W$ ), were used in this experiment. Seeds of two lettuce cultivars (Johnny's Selected Seeds, Winslow, ME, USA), 'Baronet', a green loose leaf type, and 'Red Sails', a red loose leaf type were germinated in plastic pots, 8 cm × 8 cm × 7 cm ( $L \times W \times H$ ), containing growing medium (Metro-Mix 350, Sun Gro, Canada). The seedlings were grown in a greenhouse in Manhattan, at Kansas State University for 3 weeks before transplanting to both high tunnel and open field. The fields containing Kennebec silt loam were fertilized with chicken manure compost 3N-4P-2K (Early bird compost, CMPP, Inc., High Point, MO, USA). Based on previous soil sample analyses at Kansas State University soils laboratory (Manhattan, KS), chicken manure compost was applied at a rate of 6.7 kg N · ha<sup>-1</sup> for plots in open field and high tunnel 1 week before transplanting. Drip irrigation system was used in this

study. A completely randomized block design (CRBD) was used for this experiment. Both high tunnel and open field were divided into four groups (blocks), and drought stress treatments were randomly assigned to the plots. Two lettuce cultivars were planted on either side of the drip tape. The distance between rows was 1.5 m and the seedlings were transplanted in a row at 0.3 m spacing. The layout of this study is shown in appendix A (Fig. A.1). Air temperature inside and outside was monitored by HOBO sensor (Onset Computer Corp., Pocasset, MA, USA). Figure 5.1 shows outside air temperature and the difference between day and night temperatures (DIF) in the research center, Olathe, during whole experiment period and continuous changes of air temperature in open field and high tunnel for 8 d until the time of harvest.

### ***Drought stress treatment***

Multiple drought stress treatments were applied to lettuce during crop growth. In each treatment, drought stress was induced by withholding irrigation for 4 d. The drought stress treatments were applied at 2-weeks (2WD), 4-weeks (4WD), and 2- and 4-weeks (2-4WD) after transplanting. Control plots were irrigated once in 2 or 3 days. Soil water content was periodically monitored by a time domain reflectometry (TDR) sensor (TRASE SYSTEM I, Soilmoisture Equipment Corp., Santa Barbara, CA, USA).

### ***Total phenolic content***

To measure total phenolic content of lettuce leaves, three samples per each treatment were collected from three different lettuce plants right before and at the end of each drought stress treatment. Fully expanded leaves from the middle of the canopy were collected. Leaf samples were frozen in liquid N<sub>2</sub> and stored at -20 °C until use. A modified Folin-Ciocalteu reagent method (Pennycooke et al., 2005) was used to analyze total phenolic content of lettuce leaves. About 1 g fresh leaf tissue was macerated with liquid N<sub>2</sub> and mixed with 3 mL 80% (v/v)

acetone in a mortar with pestle. All samples were placed into 1.5 mL tightly covered micro-tubes and incubated in darkness at 4 °C overnight. The sample was then centrifuged at 1000 rpm for 2 min. A mixture of 135 µL H<sub>2</sub>O, 750 µL 1/10 dilution Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) and 600 µL 7.5% (w/v) Na<sub>2</sub>CO<sub>3</sub> was added to 50 µL of phenolic extract in a 1.5 mL micro-tube. After vortexing for 10 s, the mixture was incubated at 45 °C in a water bath for 15 min. Samples were allowed to cool at room temperature before measuring the absorbance at 765 nm by a spectrophotometer (U-1100, Hitachi Ltd. Japan). A blank was prepared using 50 µL of 80% (v/v) acetone. A gallic acid standard curve was prepared from a freshly made 1 mg · mL<sup>-1</sup> gallic acid [(in 80% (v/v) acetone) (Acros Organics, Belgium)] stock solution.

### ***Antioxidant capacity***

Leaf samples for measuring antioxidant capacity were collected as described before. The antioxidant capacity of lettuce leaves was measured by a modified ABTS (Aminobenzotriazole) method (Awika et al., 2003; Miller and Rice-Evans, 1996; Pennycooke et al., 2005). Leaf sample (1 g) was extracted by 5 mL extraction solution (acetone:water:acetic acid = 70:29.5:0.5, v/v). The extracted solution was incubated in darkness at -20 °C overnight. Subsequently, the solution was centrifuged at 1000 rpm for 2 min. Meanwhile, 2.5 mM stock solution of ABTS (Roche Diagnostics, Indianapolis, IN, USA) was prepared and about 0.4 g of MnO<sub>2</sub> (Acros Organics, Belgium) was added to ABTS stock solution with occasional stirring to generate ABTS radical cations (ABTS\*). Excess MnO<sub>2</sub> was removed first by filtration using a Buchner funnel and then with a 0.2 µM syringe-end filter (Millipore Corp., Bedford, MA, USA). The ABTS\* solution was incubated at 30 °C in a water bath until needed. The ABTS\* solution was diluted to an absorbance of 0.7 (±0.02) at 730 nm using 5 mM PBS [phosphate buffer saline, pH 7.4, ionic strength (150 mM NaCl)]. Precisely, 100 µL of the extracted solution was add to 1 mL of

ABTS\* solution. After vortexing for 10 s, the optical density of the mixture was recorded at the absorbance at 730 nm (U-1100 spectrophotometer, Hitachi Ltd. Japan) at the end of 1 min reaction time. Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxyl acid) (Acros Organics, Belgium)] standard curve was prepared using 0.5 mM stock solution.

### ***High-performance liquid chromatography (HPLC) analyses***

#### ***Phenolic compounds***

The extraction of phenolic compounds from lettuce leaves was carried out as described by Nicolle et al. (2004) with minor modifications. Frozen leaf sample (1 g) ground in a mortar with a pestle using liquid N<sub>2</sub> was extracted with 50 mL of 70% methanol at 80 °C for 1 min. After stirring at room temperature for 1 h, the mixture was filtered with a filter paper (No. 1, Whatman plc., UK). The extract (25 mL) was evaporated to dryness by a rotary evaporator (Rotavapor R110, Brinkmann Instruments, Inc., Westbury, NY, USA) under reduced pressure at 50 °C and then resuspended in 5 mL of 70% methanol. The concentrated solution was filtered through a 0.45 µm filter ascrodisc (Millex, Millipore Corp., Bedford, MA, USA) before HPLC analysis. A 5 µL aliquot of the sample extract was injected into a HPLC system equipped with an autosampler (SpectraSYSTEM AS1000, Thermo Separation Products, San Jose, CA, USA), a pump (HP 1050, Hewlett Packard, Palo Alto, CA, USA), an integrator (HP 3396, Hewlett Packard, Palo Alto, CA, USA), and an UV/VIS detector (Acutect 500, Thermo Separation Products, San Jose, CA, USA). Each phenolic compound was separated from the sample extract using a column (Discovery BIO Wide Bore C-18, 15 cm × 4.6 mm, 5 µm, Supelco, Inc., Bellefonte, PA, USA) coupled to a guard column (Discovery BIO Wide Bore C-18, 2 cm × 4 mm, 5 µm, Supelco, Inc., Bellefonte, PA, USA) at 60 °C. Compounds of the sample extract were eluted with eluant A [H<sub>2</sub>O:CH<sub>3</sub>COOH = 338:1 (v/v)] and eluant B [H<sub>2</sub>O:C<sub>4</sub>H<sub>10</sub>O:CH<sub>3</sub>COOH =



330:8:1 (v/v/v)] at a flow of  $1.8 \text{ mL} \cdot \text{min}^{-1}$ . The gradient started from 20% B in A for 5 min, increasing 100% B in 20 min. After the equilibration for 2 min at 100% B, the composition of solution returned to the initial condition (20% B). Peaks from the sample extract were identified at 330 nm by comparing with standard compounds such as chlorogenic acid (Sigma-Aldrich, St. Louis, MO, USA), caffeic acid (Sigma-Aldrich, St. Louis, MO, USA), quercetin-3-*O*-glucoside (Sigma-Aldrich, St. Louis, MO, USA), chicoric acid (Indofine Chemical Co., Inc., Hillsborough, NJ, USA), and luteolin-7-*O*-glucoside (Indofine Chemical Co., Inc., Hillsborough, NJ, USA).

### ***Vitamin C***

Ascorbic acid was extracted from 1 g of lettuce leaves frozen under liquid N<sub>2</sub> as described by Wimalasiri and Wills (1983) with minor modifications. The sample ground in a mortar with a pestle was extracted with 10 mL of 3% metaphosphoric acid (HPO<sub>3</sub>) (Riedel-de Haën, Germany). A sample mixed with metaphosphoric acid solution was filtered with a filter paper (No. 1, Whatman plc., UK) and subsequently with a 0.45 µm filter ascrodisc (Millex, Millipore Corp., Bedford, MA, USA) under ice. The extract (5 µL) was immediately injected into a HPLC system equipped with a column (Discovery BIO Wide Bore C-18, 15 cm × 4.6 mm, 5 µm, Supelco, Inc., Bellefonte, PA, USA) and a guard column (Discovery BIO Wide Bore C-18, 2 cm × 4 mm, 5 µm, Supelco, Inc., Bellefonte, PA, USA) at 25 °C. The mobile phase was 10 mM ammonium dihydrogen phosphate [(NH<sub>4</sub>)H<sub>2</sub>PO<sub>4</sub>, pH 4] at a flow rate of  $1 \text{ mL} \cdot \text{min}^{-1}$ . Ascorbic acid was detected at 255 nm and was identified and quantified by standard ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA).

### ***RNA gel blot analysis***

RNA gel blot analysis was performed to determine the activation of three key genes, phenylalanine ammonia-lyase (PAL), γ-tocopherol methyl transferase (γ-TMT), and L-galactose

dehydrogenase (L-GalDH), involved in the biosynthesis of phenolic compounds, vitamin E, and vitamin C, respectively (Bergmüller et al., 2003; Diallinas and Kanellis, 1994; Gatzek et al., 2002). The three probes for L-GalDH (497 bp, accession number AJ417563) with primers 5'-AACTTCTTCGACACCTCCCC-3' and 5'-TCATCCCAACCAACACCGAC-3', for  $\gamma$ -TMT (244 bp, accession number AF104220) with primers 5'-CATAGAAATCTATCTGCGGG-3' and 5'-CACTACGAAGCAGAGACACA-3', and for PAL (753 bp, accession number AF299330) with primers 5'-GCTTACAGTTTCTCAGGTGG-3' and 5'-TGATGCTTCAATTTGTGTGT-3' were amplified using genomic DNA extracted from lettuce leaves with the primer pairs designed by a primer design program (Primer3, Whitehead Institute, Cambridge, MA, USA). Amplification of probes was performed by a PTC-100 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA) after initial denaturation at 94 °C for 5 min by 35 cycles of 1 min at 94 °C, 1 min at 51 °C ( $\gamma$ -TMT and PAL) or 54 °C (L-GalDH), and 1 min at 72 °C, followed by 5 min at 72 °C. The fragment size of each probe was checked by electrophoresis on 1.2% (w/v) agarose gels in 1 × Tris-Acetate-EDTA buffer. After electrophoresis, the fragments stained with ethidium bromide were detected by an UV transilluminator (FisherBiotech, Fairlawn, NJ, USA). The probes were stored at -20 °C until use.

The samples collected in the field were immediately stored under liquid N<sub>2</sub> and transported to deep refrigerator (-20 °C) in the laboratory. Total RNA was extracted from lettuce leaves using a modified CTAB extraction method as described by Chang et al. (1993). Total RNA (20 µg) was subjected to electrophoresis on 1% (w/v) agarose gels with 10% (v/v) formaldehyde. The RNA was transferred onto nylon membranes (Sigma-Aldrich, St. Louis, MO, USA) in a 20 × SSC solution (Sambrook and Russell, 2001). The probes were labeled with <sup>32</sup>P using random primer DNA labeling system (Sigma-Aldrich, St. Louis, MO, USA). During

prehybridization, nylon membranes were incubated with 40 mL prehybridization buffer (1 mM EDTA, 0.5 M NaH<sub>2</sub>PO<sub>4</sub>, 7% SDS, pH 7.2) at 50°C for 6 h with Salmon Sperm DNA (10 mg · mL<sup>-1</sup>). The hybridization was performed as described by Sambrook and Russell (2001). Probes were hybridized to RNA on nylon membranes at 50°C for 12 h in 40 mL prehybridization buffer. The membranes were rinsed twice with wash I buffer (1 mM EDTA, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 5% SDS, pH 7.2) and then once with wash II buffer (1 mM EDTA, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 1% SDS, pH 7.2) at 50°C for 1 h. The membranes were then exposed in cassettes at room temperature for 1 d and then the film was scanned by a gel and blot imaging system (Storm<sup>TM</sup>, GE Healthcare, Piscataway, NJ, USA).

### ***Plant growth characteristics***

Plant growth characteristics were measured at the time of harvest (4 weeks after transplanting). Fresh and dry weights of roots and shoots were determined using six plants per each treatment. After recording fresh weights, shoots and roots were dried at 70°C for 3 d in an oven to measure dry weights.

### ***Statistical analysis***

Analysis of variance (ANOVA) was performed by the statistical analysis system (SAS) program. Duncan's multiple range test was used to compare means.

## **Results**

### ***Total phenolic content***

The changes in total phenolic content of 'Baronet' and 'Red Sails' during the growing period in open field and high tunnels are shown in Figure 5.2. The total phenolic content was rather low in both varieties grown in greenhouse (preplant). However, sharp increase in total

phenolic content occurred following transplanting in both varieties. The increase in total phenolic content was higher when transplanted to open field compared to high tunnel in both varieties. However, 'Red Sails' showed a greater response than did 'Baronet.' Compared to pretransplant stage, the increase was more than 5- to 7-fold following transplanting (after 9 d) in 'Red Sails.' At the time of harvest (25 d after transplanting), this variety also had higher total phenolic content than did 'Baronet.' Both varieties grown in open field had significantly higher phenolic content during the early stages of their growth than did those grown in high tunnel. 'Baronet' grown in open field had higher phenolic content up to 22 d after transplanting compared to that in high tunnel. Similarly, 'Red Sails' in open field showed higher phenolic content up to 12 d after transplanting compared to that in high tunnel. However, at the time of harvest, no significant differences in phenolic content between plants in open field and high tunnel were observed regardless of variety.

### ***Antioxidant capacity***

The changes in the antioxidant capacity in 'Baronet' and 'Red Sails' were very similar to those in total phenolic content in response to transplanting to open field and high tunnel (Fig. 5.3). Thus, moving plants from greenhouse to field conditions resulted in a dramatic increase in total phenolic content and antioxidant capacity.

### ***Quantification of phenolic compounds and vitamin C***

Open field conditions significantly contributed to the accumulation of several phenolic compounds in both lettuce varieties at the time of harvest (Fig. 5.4). 'Baronet' grown in open field contained significantly higher amount of chlorogenic acid, chicoric acid, and luteolin-7-O-glucoside than the lettuce grown in high tunnel. Similarly, open field conditions induced significantly higher accumulation of chlorogenic acid, caffeic acid, and chicoric acid than did

high tunnel in ‘Red Sails.’ The accumulation of two major phenolic compounds, chlorogenic acid and chicoric acid, was approximately 4 times and 2 times respectively higher in ‘Red Sails’ in open field. Quercetin-3-O-glucoside and ascorbic acid did not show significant difference between plants grown in open field and high tunnel.

Between the two lettuce varieties, ‘Red Sails’ was a superior variety with regard to the accumulation of several phenolic compounds. Both under open field and high tunnel conditions, ‘Red Sails’ had higher levels of chlorogenic acid, but higher accumulations of caffeic acid, quercetin-3-O-glucoside, and ascorbic acid only under open field conditions than did ‘Baronet.’

### ***Gene expression***

Transcript levels for all three key genes, PAL, L-GalDH, and  $\gamma$ -TMT, involved in the biosynthesis of phenolic compounds, vitamin C, and vitamin E respectively, were higher under open field conditions than in high tunnels (Fig. 5.5). The activation of PAL under open field conditions was consistent with the results showing higher accumulation of phenolic compounds and consequent higher antioxidant capacity.

### ***Drought stress treatment***

#### ***Soil water content***

At the end of 4-d drought stress, soil water content under drought stress ranged about 16% to 21% regardless of growing conditions (Fig. 5.6). While soil water content ranged from 26% to 34% in well water plots. However, in open field because of rainfall, the water content data were not reliable and were not used in this study.

#### ***Total phenolic content and antioxidant capacity***

Drought stress treatments did not produce significant changes either in total phenolic content or antioxidant capacity in both varieties of lettuce (Fig. 5.7). Although the soil water

content was reduced by withholding irrigation, it was not clear whether this actually induced plant stress. Furthermore, in open field the results were compounded by rainfall. Nonetheless, both total phenolic content and antioxidants capacity were significantly higher in ‘Red Sails’ than in ‘Baronet’ both under control and stressed conditions. Data for open field were not presented because of compounding effects introduced by rainfall.

### ***Growth characteristics***

Growing conditions had a major influence on the biomass accumulation in both lettuce varieties (Table 5.1). In both ‘Baronet’ and ‘Red Sails’, fresh and dry weight accumulation in both shoots and roots were sharply higher in high tunnel than in open field for both control and drought stress treated plots. However, there was no significant difference in biomass (shoot and root) accumulation due to drought stress treatments. Also, there was no clear difference in this regard between the two varieties. However, the exception was root fresh and dry matter accumulations which were higher in ‘Red Sails’ than ‘Baronet’ both in open field and in high tunnel only under control (unstressed) conditions.

## **Discussion**

Our results showed that growing conditions have a striking effect on the quality of lettuce in relation to its leaf phenolic content and antioxidant capacity. Greenhouse-grown lettuce was rather poor in total phenolic content and antioxidant capacity. However, transplanting these plants to either open field or high tunnel resulted in a large increase in their total phenolic content and antioxidant capacity. These results are consistent with those reported by Romani et al. (2002) who observed the highest amount of flavonoids in lettuce following transplanting. When lettuce plants are transplanted from greenhouse to field, they are at least exposed to two types of stresses. The plants are going from somewhat protected environment to unprotected

field conditions. Secondly, they are exposed to a transplanting shock. Thus, both of these factors, especially the former as shown in our previous study (chapter 2), can result in the accumulation of antioxidants in lettuce.

Furthermore, lettuce plants accumulated higher amounts of antioxidants in open field than in high tunnel. Higher concentration of major phenolic compounds in lettuce such as chlorogenic acid and chicoric acid, as well as minor ones like caffeic acid, quercetin-3-O-glucoside, and luteolin-7-O-glucoside were observed under open field conditions. Although one would expect different microenvironments (soil and air) in open field and in high tunnel, surprisingly, the daily day and night temperature differences between open field and high tunnel were minimal during the 4-week growing period (Fig. 5.1). However, the obvious difference between these two growing conditions is the reduced light intensity in high tunnel. The polyethylene film covering the high tunnel transmits only 50-60% of the light, which may play a negative role in the production of secondary metabolites. Kleinhenz et al. (2003) found that shading did not increase anthocyanin contents of several lettuce varieties. On the other hand, high solar radiation is conducive to the accumulation of phytochemicals including flavonoids which may ward off oxidative stress induced by high light (Tattini et al., 2005). Furthermore, UV light as well as high light plays a key role in the accumulation of phytochemicals in plants. Most commercially available greenhouse films absorb short wavelength UV light because UV light could potentially inhibit plant growth and trigger the spread of fungi or insects (Raviv and Antignus, 2004, Schmitz-Hoerner and Weissenböck, 2003). Also, Chappell and Hahlbrock (1984) found that UV light could lead to the accumulation of pigments and phenolic compounds as a defensive mechanism in plants. They also found that UVB radiation activated genes such as PAL and chalcone synthase (CHS), which are involved in the phenylpropanoid pathway, and led

to the accumulation of phenolic compounds and other antioxidants (Caldwell and Britz, 2006; Luthria et al., 2006 ). We found that lettuce growing in open field with higher solar radiation and possibly higher UV had higher transcript levels for PAL, L-GalDH, and  $\gamma$ -TMT, all involved in the biosynthesis of antioxidants. The results also showed that lettuce plants accumulated key phenolic compounds.

From our previous study (chapter 4), we found that the amount of antioxidants declines with plant age and is usually very low at harvest time. Similar results were observed by Romani et al. (2002). In this study the total phenolic content decreased with plant age, but it showed an increase just before harvest both under open field and high tunnel conditions (Figs. 5.2 and 5.3). Several studies showed that fluctuations of temperature could lead to the accumulations of phenolic compounds, pigments (carotenoid and anthocyanin) in plants (Lefsrud et al., 2005; Nacif de Abreu and Mazzafera, 2005; Tomás-Barberán and Espín, 2001). The increase in the total phenolic content at the harvest time, observed in this study, may be due to the abrupt environmental perturbation prior to harvest, involving the largest fluctuation in day and night temperature 21 d after transplanting.

In our previous growth chamber study, we found that mild drought stress induced the accumulation of phytochemicals in lettuce. However, drought stress treatment did not appear to have a significant effect on the accumulation of phenolic compounds in lettuce under field condition. Under field conditions, it was not clear if the drought treatment (withholding irrigation for 4 d) actually caused water stress in lettuce plants. In addition, plants under field condition are typically subjected to a combination of multiple stresses (Jiang and Huang, 2001; Moffat, 2002) and it is possible that the effect of drought stress treatment would be masked by other stresses. If



plants were already pre-exposed to multiple stresses, they would be less sensitive to the drought treatment.

Comparison of phytochemical contents of the two lettuce varieties shows that 'Red Sails', which has loose red foliage, was generally higher in phenolic content and antioxidant capacity. 'Red Sails' had higher amounts of major phenolic compound, chlorogenic acid, than did 'Baronet.' In addition, 'Red Sails' was rich in several other phenolic compounds when grown in open field. The quality of both lettuce varieties was significantly improved by growing them in open field as opposed to in the high tunnel. However, lettuce plants in open field did not fare well in biomass accumulation as those in high tunnel. Although these plants were rich in phytochemicals, their growth and yield were not at the same level as those grown in high tunnel. In fact, the plants grown in open field had better health-promoting properties than did those grown in either greenhouse or high tunnel. It is worth noting, however, that lettuce grown in open field produces smaller heads compared to those produced in high tunnel, hence, it is possible to increase plant density under open field conditions and thus possibly make up for the reduced yield under open field conditions.

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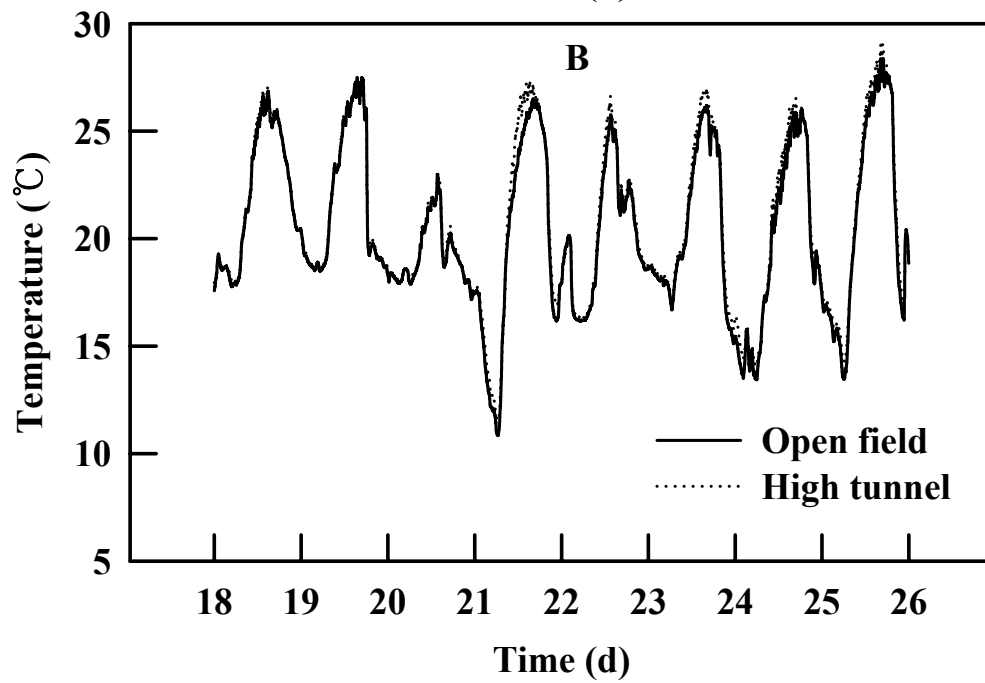
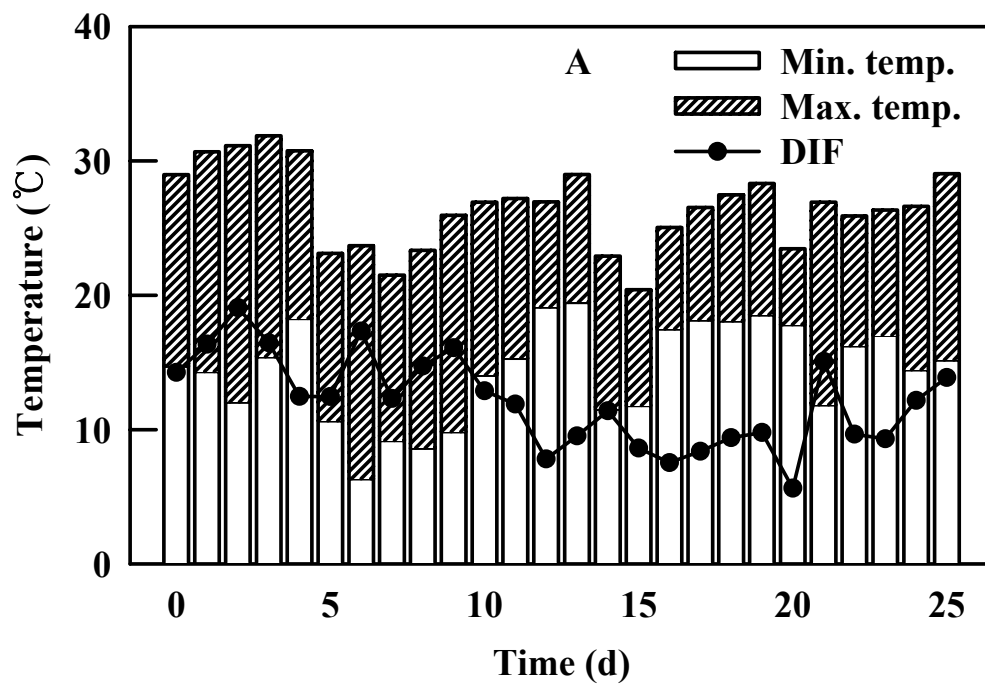
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## **Figures and Tables**

### **Figure 5.1. Field air temperature data.**

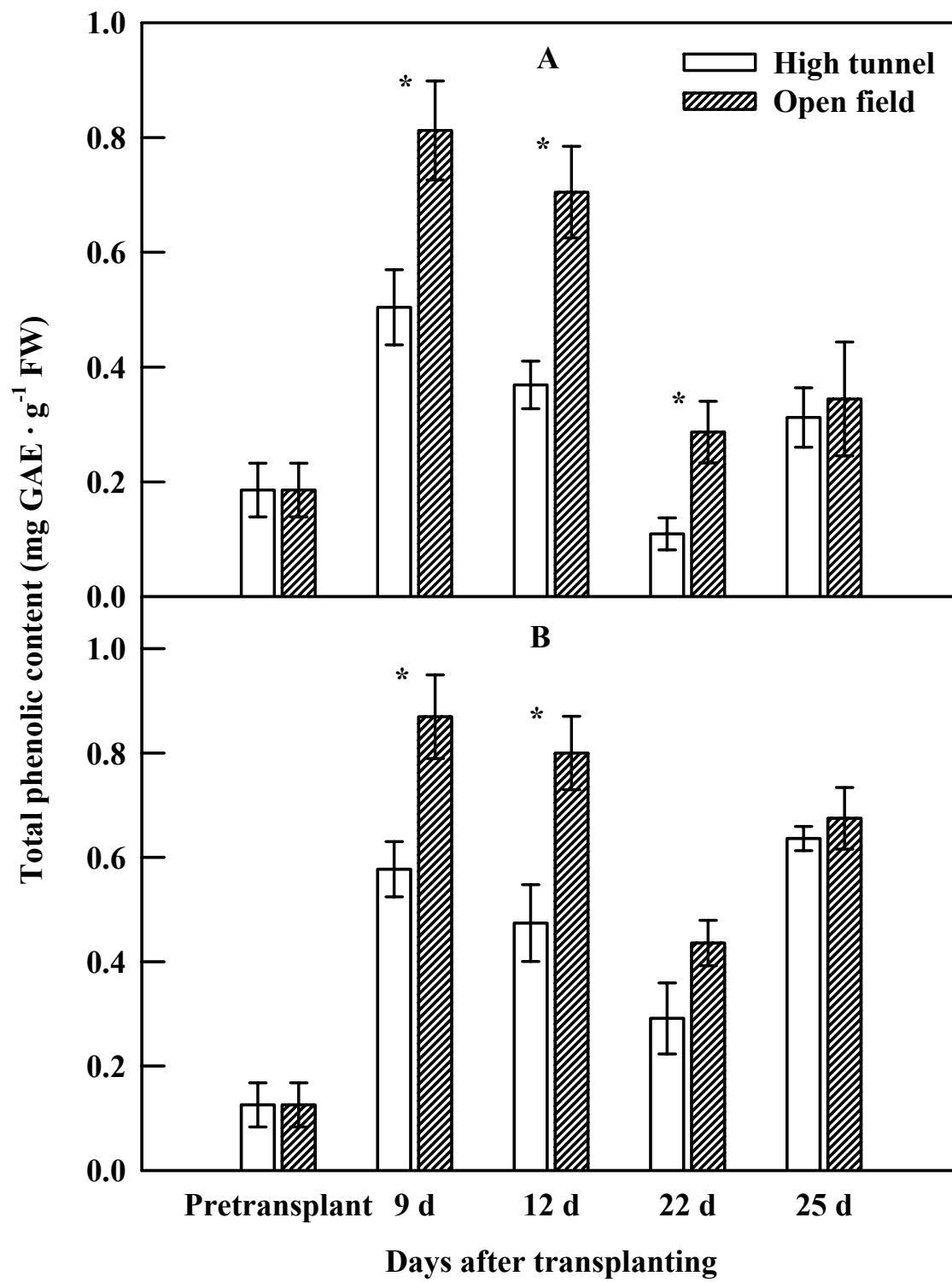
Outside air temperature and the difference between day and night temperatures (DIF) during the whole experimental period (A) and continuous changes of air temperature in open field and high tunnel for 8 d until the time of harvest (B) at K-State Horticulture Research and Extension Center, Olathe, KS are presented.



**Figure 5.2. Changes in total phenolic content of ‘Baronet’ (A) and ‘Red Sails’ (B) leaves in plants grown under high tunnel (open bars) and in open field (shade bars).**

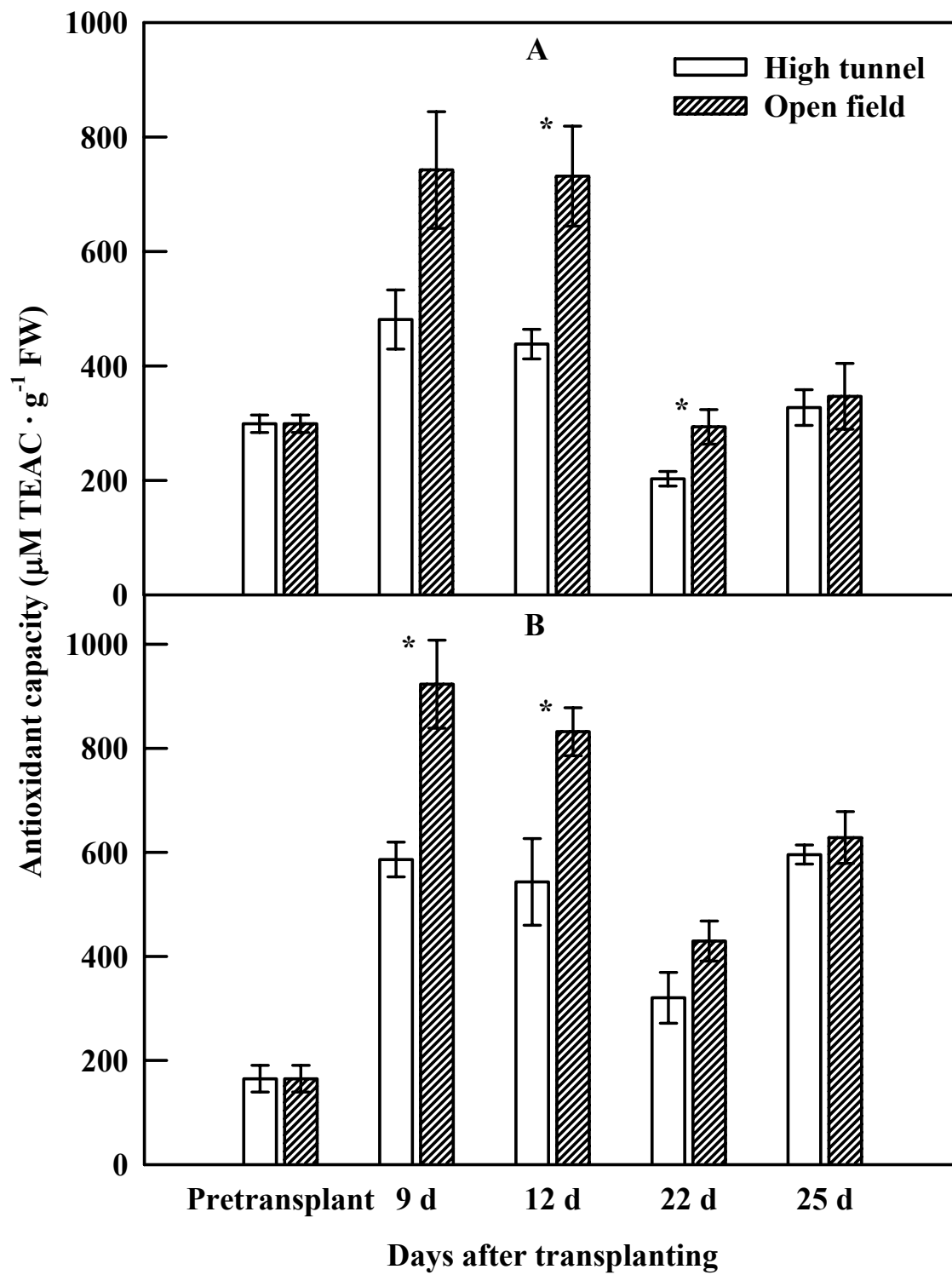
Two lettuce varieties were grown at K-State Horticulture Research and Extension Center, Olathe, KS after transplanting for 4 weeks. Total phenolic content was measured using three lettuce plants per treatment. The bars represent standard errors. Significant differences ( $P=0.05$ ) between high tunnel and open field treatments are shown with an asterisk (\*).





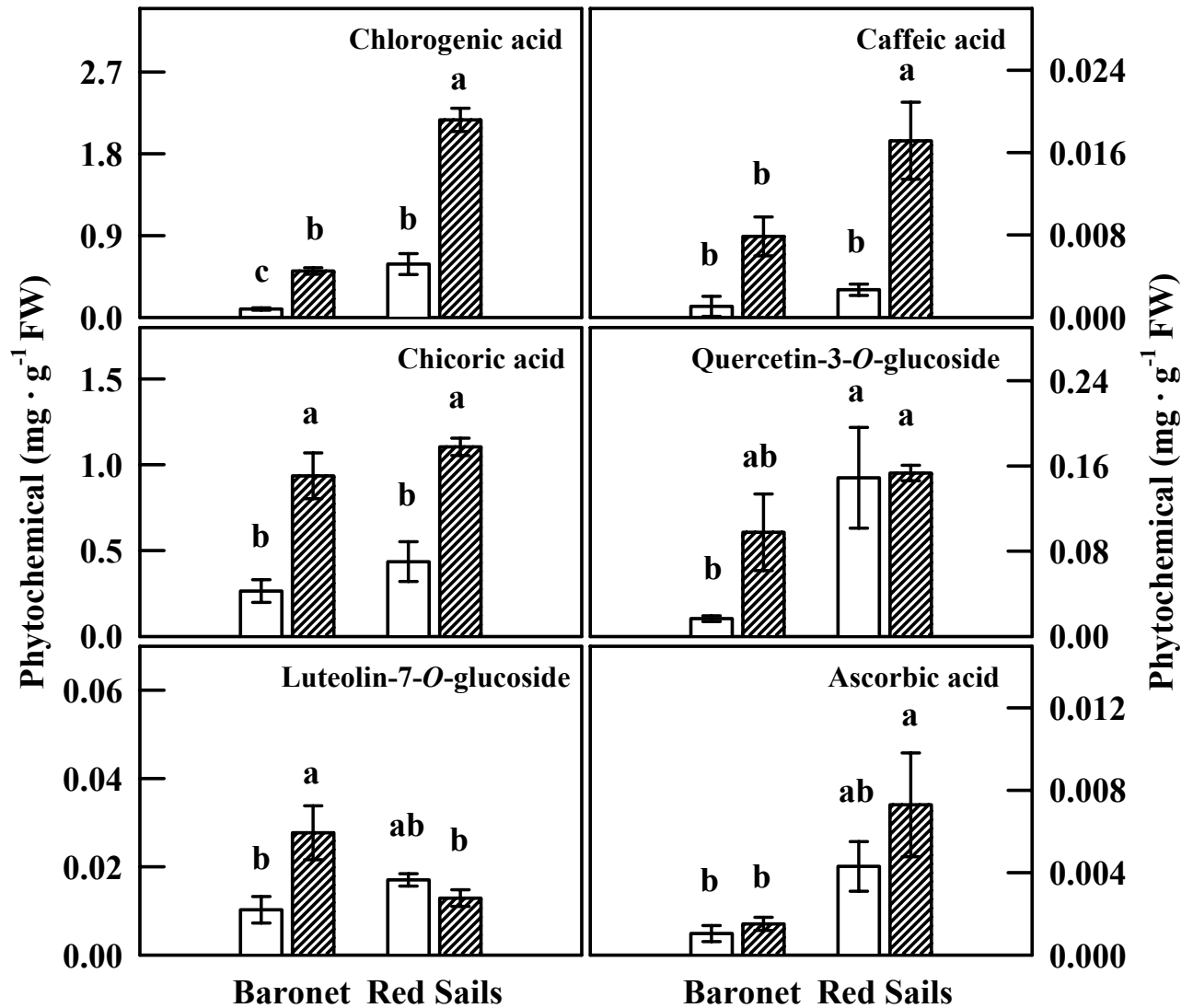
**Figure 5.3. Changes in antioxidant capacity of ‘Baronet’ (A) and ‘Red Sails’ (B) leaves in plants grown under high tunnel (open bars) and in open field (shade bars).**

Two lettuce varieties were grown at K-State Horticulture Research and Extension Center, Olathe, KS after transplanting for 4 weeks. Antioxidant capacity was measured using three lettuce plants per treatment. The bars represent standard errors. Significant differences ( $P=0.05$ ) between high tunnel and open field treatments are shown with an asterisk (\*).




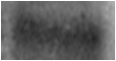






**Figure 5.4. Phenolic compounds and vitamin C of ‘Baronet’ and ‘Red Sails’ leaves in plants grown under high tunnel (open bars) and in open field (shade bars) at the time of the harvest.**

Lettuce plants were harvested 4 weeks after transplanting. Phenolic compounds and vitamin C were measured using three lettuce plants per treatment. The bars represent standard errors. Significant differences ( $P=0.05$ ) between the treatments are indicated by different letters.



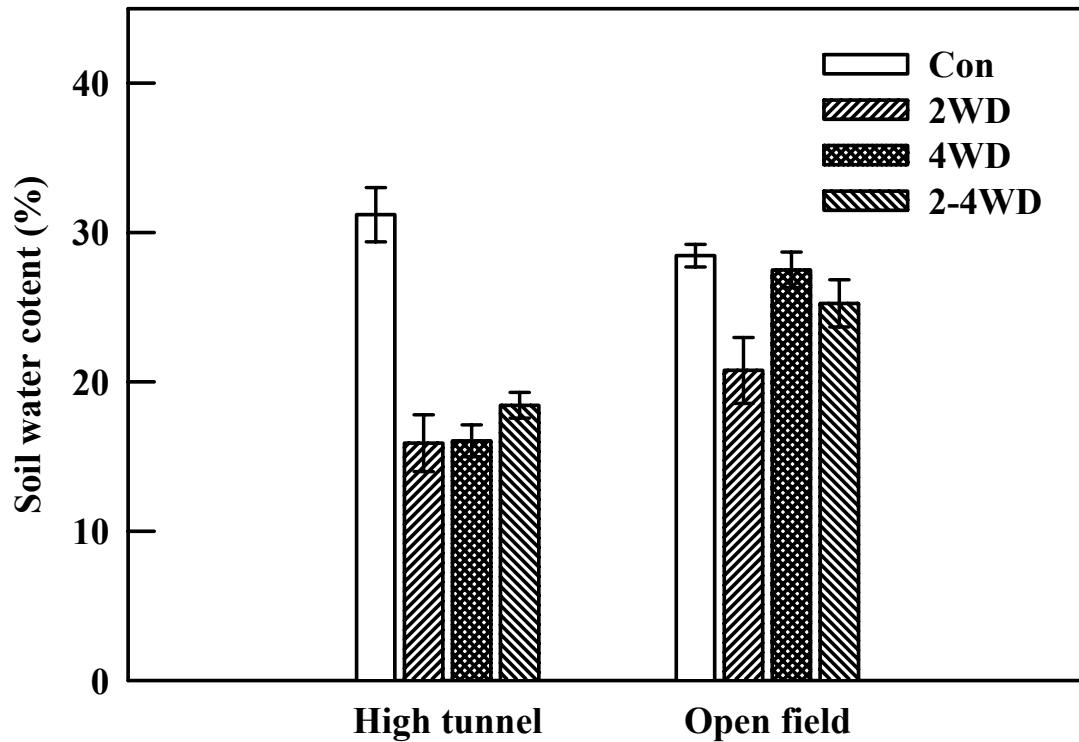
**Figure 5.5. The gene expression of PAL, L-GalDH, and  $\gamma$ -TMT in ‘Baronet’ plants grown under high tunnel and in open field at the time of harvest.**

Lettuce plants were harvested at 4 weeks after transplanting. rRNA was used as a loading standard.

Gene	High tunnel	Open field
PAL		
L-GalDH		
$\gamma$ -TMT		
rRNA		

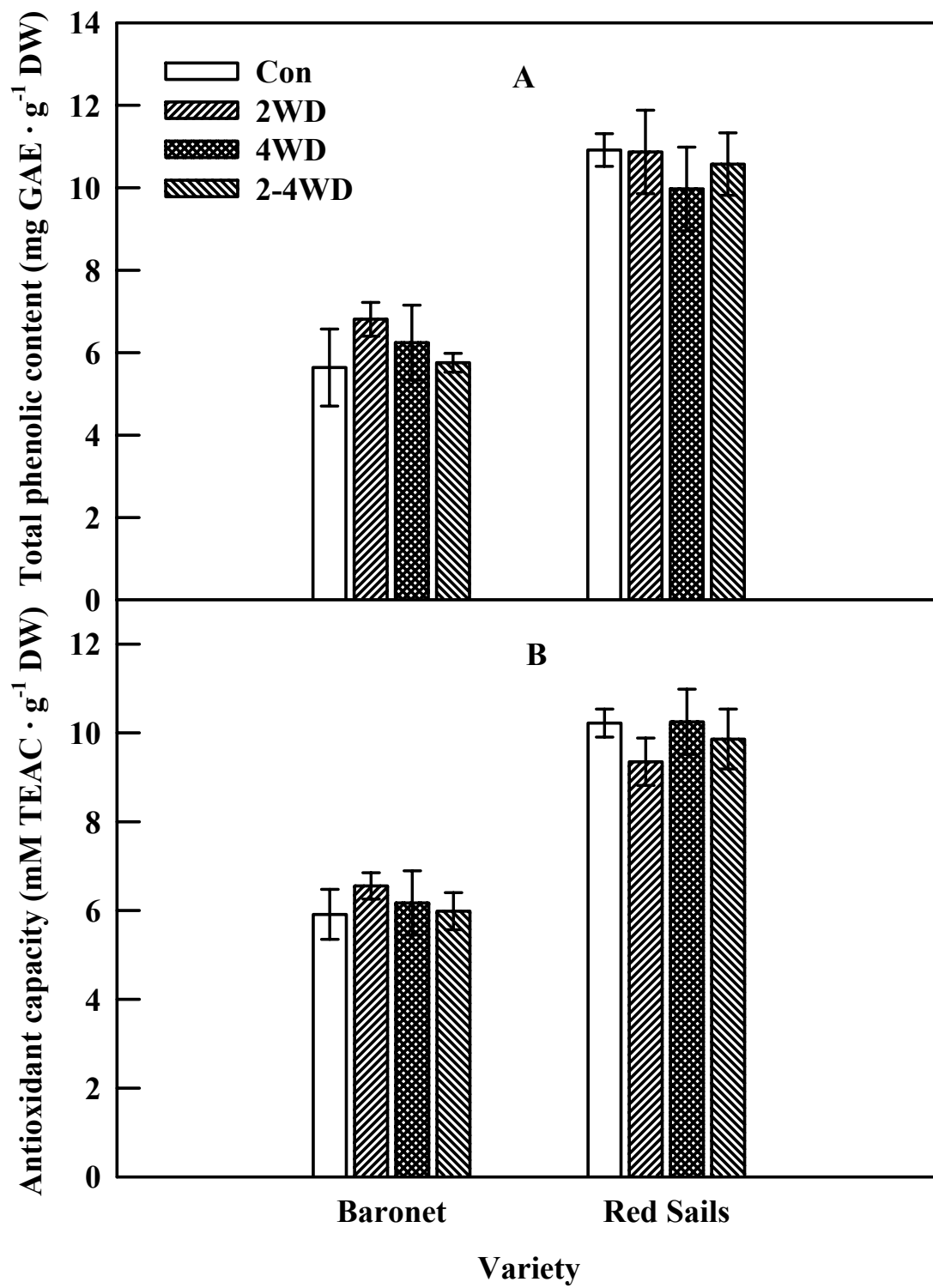
**Figure 5.6. Soil water content under drought stress treatments under high tunnel and in open field.**

Drought stress was induced by withholding irrigation for 4 d. Soil water content was measured at the last day of drought stress period. Four plots were used per treatment. The bars represent standard errors. Con- Control; 2WD - 4 days of drought stress at 2 weeks of transplanting; 4WD - 4 days of drought stress at 4 weeks of transplanting; 2-4WD – 4 days of drought stress at 2 and 4 weeks of transplanting.



**Figure 5.7. Total phenolic content (A) and antioxidant capacity (B) in ‘Baronet’ and ‘Red Sails’ plants subjected to the three different drought treatments under high tunnel at the time of harvest.**

Two lettuce varieties were grown at K-State Horticulture Research and Extension Center, Olathe, KS after transplanting for 4 weeks. Total phenolic content and antioxidant capacity were measured using three lettuce plants per treatment. The bars represent standard errors. Control; 2WD - 4 days of drought stress at 2 weeks of transplanting; 4WD - 4 days of drought stress at 4 weeks of transplanting; 2-4WD – 4 days of drought stress at 2 and 4 weeks of transplanting.





**Table 5.1. Growth characteristics of ‘Baronet’ and ‘Red Sail’ plants subjected to the three different drought stress treatments under high tunnel and in open field at the time of harvest.**

Two lettuce varieties were harvested 4 weeks after transplanting. The values are the means of six lettuce plants per treatment. 2WD - 4 days of drought stress at 2 weeks of transplanting; 4WD - 4 days of drought stress at 4 weeks of transplanting; 2-4WD – 4 days of drought stress at 2 and 4 weeks of transplanting.

Treatment	Growing Condition	Variety	Shoot		Root	
			FW (g)	DW (g)	FW (g)	DW (g)
Control	High tunnel	Baronet	258.4 ab <sup>z</sup>	14.3 cd	13.2 b	0.78 b
		Red sails	279.9 a	16.3 abc	16.5 a	1.06 a
	Open field	Baronet	85.8 cd	8.3 ef	3.3 e	0.30 d
		Red sails	118.9 c	11.2 de	7.0 c	0.49 c
2WD	High tunnel	Baronet	244.7 ab	13.9 cd	13.8 b	0.84 b
		Red sails	233.2 ab	14.4 c	13.6 b	0.88 b
	Open field	Baronet	62.8 d	6.6 f	3.4 e	0.39 cd
		Red sails	98.9 cd	9.2 ef	7.0 c	0.45 cd
4WD	High tunnel	Baronet	267.9 a	18.1 ab	12.0 b	0.85 b
		Red sails	211.3 b	13.5 cd	11.9 b	0.74 b
	Open field	Baronet	88.7 cd	8.4 ef	4.2 de	0.37 cd
		Red sails	129.3 c	11.1 de	7.7 c	0.42 c
2-4WD	High tunnel	Baronet	267.8 a	18.6 a	13.5 b	1.09 a
		Red sails	252.4 ab	15.4 bc	12.2 b	0.73 b
	Open field	Baronet	58.84 d	6.1 f	3.4 e	0.28 d
		Red sails	90.9 cd	8.0 ef	6.2 cd	0.35 cd
Significance			***	***	***	***

<sup>z</sup> Mean separation within columns by Duncan's multiple range test.

\*\*\* Significant at  $P=0.001$ .

## **CHAPTER 6 - Health-promoting Phytochemicals in Lettuce Grown under Organic and Conventional Fertilization**

### **Abstract**

Lettuce (*Lactuca sativa* L. cv. Baronet) plants were cultivated on organic and conventional plots with and without fertilizers under open field or high tunnel conditions. The concentration of total phenolics including several phenolic compounds, antioxidants, and vitamin C of lettuce leaves were measured in addition to monitoring the expression of PAL, L-GalDH, and  $\gamma$ -TMT, which are key genes involved in the biosynthesis of phenolic compounds, vitamin C, and vitamin E, respectively. Over all there were no significant differences in the total phenolic content, antioxidant capacity, and ascorbic acid of lettuce in both open field and high tunnel managed either organically or in conventional manner. However, in open field, organically managed lettuce had higher phenolic compounds such as chlorogenic acid, chicoric acid, and quercetin-3-O-glucoside than did the conventionally managed crop. The application of fertilizer had a negative effect on the accumulation of phytochemicals in lettuce compared to no fertilization although the response seemed to vary depending on the growing conditions. Biomass accumulation in lettuce organically managed with no fertilization was similar to those in organic and conventional plots with fertilization perhaps due to the residual nutrients in the organic plots.

### **Introduction**

Phytochemicals in fruits and vegetables play an important role in preventing degenerative diseases and chronic diseases (Arai et al., 2000; Jang et al., 1997; Joshipura et al., 1999; Liu, 2003). Among the diverse classes of phytochemicals found in fruits and vegetables, phenolic

compounds and vitamins are regarded to be important in maintaining and promoting human health (Liu, 2003).

As a commonly consumed vegetable in the U. S. (Lucier and Jerardo, 2005), lettuce (*Lactuca sativa* L.) contains a variety of health-promoting phytochemicals including several vitamins (vitamin A, C, and E) and a number of phenolic compounds such as hydroxycinnamic acids (chlorogenic acid and caffeic acid) and flavonoids (chicoric acid, quercetin-3-O-glucoside, and luteolin-7-O-glucoside) (Caldwell, 2003; Llorach et al., 2004; Nicolle et al., 2004; Romani et al., 2002). Compared to other popular vegetables like broccoli, spinach, carrot, tomato, and potato, the concentration of phytochemicals in lettuce based on weight basis is relatively low, however, since it is a commonly consumed vegetable, it is one of the major sources of phytochemicals in human diet (Ryder, 2001). Moreover, based on the total phenolic content, lettuce has higher scavenging activity especially for peroxy radical, which is one of the reactive oxygen species (ROS), than many other fruits and vegetables (Caldwell, 2003).

Consumers' perception that organic foods are healthier and safer than conventional foods has resulted in an increased demand for organic foods and thus, the consumption of organic foods has become one of the top consumer trends (Sloan, 2003). Many reasons for this perception are: less pesticide residues, protection of environment and farm worker, and potential quality improvement of organic products (Zhao et al., 2006). In addition, with increasing interest in good health and well-being, people tend to associate organic foods with better nutrition. Thus, the sale of organic products has increased annually since 1990 (Oberholtz et al, 2005).

A recent 10-year study of Mitchell et al. (2007) suggested that tomatoes organically grown accumulated significantly higher amount of flavonoids than those conventionally managed. However, the results of comparison between organic and conventional in other studies

showed that the difference is not always consistent (Bourn and Prescott, 2002; Brandt and Mølgaard, 2001) because the differences in phytochemical content may depend on the cultivar selection, growing condition, postharvest practice, and the method of sampling and analysis (Chassy et al., 2006, Magkos et al., 2003). Thus, it is not clear whether organic farming and organic produce can lead to healthy and safe foods due to lack of reliable research data (Zhao et al., 2006).

In this study, we examined if the quality of lettuce with regard to its phytochemical composition are affected by organic and conventional means of cultivation under different growing conditions, namely, in open field and high tunnel.

## **Materials and Methods**

### ***Experimental conditions and plant material***

This study was conducted at K-State Horticulture Research and Extension Center, Olathe, Kansas State University, starting May 9, 2007. Six high tunnels, 9.8 m × 6.1 m ( $L \times W$ ), covered with a single layer of 6-mil K-50 polyethylene (Klerk's Plastic Product Manufacturing, Inc., Richburg, SC, USA) and six adjacent open field plots (9.8 m × 6.1 m;  $L \times W$ ), were used for this experiment. A completely randomized block design (CRBD) was used for this experiment. All high tunnels and open field plots were divided randomly into two groups each; organic and conventional. The layout of this study is shown in appendix A (Fig. A.2).

Seeds of a green leaf lettuce, 'Baronet' (Johnny's Selected Seeds, Winslow, ME, USA), were germinated in plastic pots, 8 cm × 8 cm × 7 cm ( $L \times W \times H$ ), with a growing medium (Metro-Mix 350, Sun Gro, Canada). Seedlings were grown in a greenhouse in Manhattan at Kansas State University for 3 weeks until they were transplanted to high tunnels and open field plots.

### ***Organic and conventional soil management***

Based on the analyses of soil sample (Kennebec silt loam) from K-State Horticulture Research and Extension Center, Olathe, KS, the amount of both organic and conventional fertilizers to be applied was determined. As an organic source, Hu-More 1N-0.4P-0.8K (composted cattle manure and alfalfa hay; Humalfa, Inc., Shattuck, OK, USA) was applied to each plot at  $224 \text{ kg} \cdot \text{ha}^{-1}$  N. Commercial synthetic fertilizer 13N-13P-13K (Greenskeeper Select Lawn and Garden Fertilizer, T and N, Inc., Foristell, MO, USA) was used for conventional cultivation at  $112 \text{ kg} \cdot \text{ha}^{-1}$  N. Fertilizers were incorporated into soil before 1 week of transplanting lettuce. The plots were irrigated through the drip tape line. Both organic and conventional plots in open field and high tunnel were divided into two groups; namely control (C) that did not receive any fertilizers and treated (F) that received either organic or synthetic fertilizer. Each group in open field and high tunnel had 24 plants in a row with a plant spacing of 30 cm.

### ***Total phenolic content***

For total phenolic content analysis, 3 leaf samples per treatment from 3 randomly selected plants were collected at the time of harvest. Samples were collected from fully expanded, just matured leaves and frozen immediately in liquid  $\text{N}_2$  before transferring them to the laboratory. Samples were stored at  $-20^\circ\text{C}$  until use. Total phenolic content of lettuce leaves was determined by a modified Folin-Ciocalteu reagent method (Pennycooke et al., 2005). About 1 g of fresh leaf tissue was macerated in liquid  $\text{N}_2$  with mortar and pestle and mixed with 3 mL 80% (v/v) acetone. The sample was placed into a 1.5 mL tightly covered micro-tube and incubated in darkness at  $4^\circ\text{C}$  overnight. Subsequently, the sample was centrifuged at 1000 rpm for 2 min and the supernatant was used as phenolic extract. A mixture of 135  $\mu\text{L}$  distilled water, 750  $\mu\text{L}$  1/10

dilution Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) and 600  $\mu\text{L}$  7.5% (w/v)  $\text{Na}_2\text{CO}_3$  was added to 50  $\mu\text{L}$  of phenolic extract in a 1.5 mL micro-tube. After vortexing for 10 s, the mixture was incubated at 45  $^\circ\text{C}$  in a water bath for 15 min. Samples were allowed to cool at room temperature before measuring the absorbance at 765 nm by a spectrophotometer (U-1100, Hitachi Ltd. Japan). A blank was prepared from 50  $\mu\text{L}$  80% (v/v) acetone. A gallic acid standard curve was prepared from a freshly made stock solution of 1  $\text{mg} \cdot \text{mL}^{-1}$  gallic acid (Acros Organics, Belgium) in 80% (v/v) acetone.

### ***Antioxidant capacity***

The sampling method and number for the measurement of antioxidant capacity were the same as those for the analysis of total phenolic content. A modified ABTS (Aminobenzotriazole) method (Awika et al., 2003; Miller and Rice-Evans, 1996; Pennycooke et al., 2005) was used to analyze the antioxidant capacity of lettuce leaves. Antioxidants were extracted by 5 mL extraction solution [acetone:water:acetic acid = 70:29.5:0.5, (v:v:v)] from about 1 g of lettuce frozen in liquid  $\text{N}_2$ . A 1 mL of the extract placed into a 1.5 mL tightly covered micro-tube was incubated in darkness at -20  $^\circ\text{C}$  overnight. Subsequently, the solution was centrifuged at 1000 rpm for 2 min. ABTS [(2.5 mM) (Roche Diagnostics, Indianapolis, IN, USA)] stock solution was prepared and 0.4 g of  $\text{MnO}_2$  (Acros Organics, Belgium) was added to 20 mL of stock solution to generate ABTS radical cations (ABTS\*), stirring the mixture occasionally for 30 min at room temperature. Excess  $\text{MnO}_2$  was removed by filtration first using a filter paper (No. 1, Whatman plc., UK) through a Buchner funnel, and then with a 0.2  $\mu\text{m}$  syringe-end filter (Millipore Corp., Bedford, MA, USA). The ABTS\* solution was incubated at 30  $^\circ\text{C}$  in a water bath during the analysis and was diluted to an absorbance 0.7 ( $\pm 0.02$ ) at 730 nm with 5 mM PBS [phosphate buffer saline, pH 7.4, ionic strength (150 mM NaCl)]. A 100  $\mu\text{L}$  of the extract was add to 1 mL

of ABTS\* solution. The solution was vortexed for 10 s and its absorbance was recorded at 730 nm by a spectrophotometer (U-1100, Hitachi Ltd. Japan) at the end of 1 min reaction time.

Trolox [(6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxyl acid) (Acros Organics, Belgium)] was used as a standard for quantification and the standard curve was prepared by 0.5 mM stock solution.

### ***High-performance liquid chromatography (HPLC) analyses***

#### ***Phenolic compounds***

The extraction of phenolic compounds from lettuce leaves was carried out as described by Nicolle et al. (2004) with minor modifications. Three leaves collected from different lettuce plants per treatment at the time of harvest were used. Frozen leaf sample (1 g) macerated in a mortar with a pestle using liquid N<sub>2</sub> was mixed with 50 mL of 70% methanol at 80 °C for 1 min. After stirring at room temperature for 1 h, the mixture was filtered through a filter paper (No. 1, Whatman plc., UK). The extract (25 mL) was evaporated to dryness by a rotary evaporator (Rotavapor R110, Brinkmann Instruments, Inc., Westbury, NY, USA) under reduced pressure at 50 °C and then resuspended in 5 mL of 70% methanol. The concentrated solution was filtered through a 0.45 µm filter ascrodisc (Millex, Millipore Corp., Bedford, MA, USA) before HPLC analysis. A 5 µL aliquot of the sample extract was injected into a HPLC system equipped with an autosampler (SpectraSYSTEM AS1000, Thermo Separation Products, San Jose, CA, USA), a pump (HP 1050, Hewlett Packard, Palo Alto, CA, USA), an integrator (HP 3396, Hewlett Packard, Palo Alto, CA, USA), and an UV/VIS detector (Acutect 500, Thermo Separation Products, San Jose, CA, USA). Phenolic compounds were separated from the extract using a column (Discovery BIO Wide Bore C-18, 15 cm × 4.6 mm, 5 µm, Supelco, Inc., Bellefonte, PA, USA) coupled to a guard column (Discovery BIO Wide Bore C-18, 2 cm × 4 mm, 5 µm, Supelco,



Inc., Bellefonte, PA, USA) at 60 °C. The sample extract were eluted with eluant A [H<sub>2</sub>O:CH<sub>3</sub>COOH = 338:1 (v/v)] and eluant B [H<sub>2</sub>O:C<sub>4</sub>H<sub>10</sub>O:CH<sub>3</sub>COOH = 330:8:1 (v/v/v)] at a flow of 1.8 mL · min<sup>-1</sup>. The gradient started from 20% B in A for 5 min, increasing 100% B in 20 min. After the equilibration for 2 min at 100% B, the composition of solution returned to the initial condition (20% B). Peaks from the sample extract were identified and quantified at 330 nm by comparing with standard compounds such as chlorogenic acid (Sigma-Aldrich, St. Louis, MO, USA), caffeic acid (Sigma-Aldrich, St. Louis, MO, USA), quercetin-3-O-glucoside (Sigma-Aldrich, St. Louis, MO, USA), chicoric acid (Indofine Chemical Co., Inc., Hillsborough, NJ, USA), and luteolin-7-O-glucoside (Indofine Chemical Co., Inc., Hillsborough, NJ, USA).

### ***Vitamin C***

Ascorbic acid was extracted from 1 g of lettuce leaf frozen in liquid N<sub>2</sub> as described by Wimalasiri and Wills (1983) with minor modifications. The sample ground in a mortar with a pestle was extracted with 10 mL of 3% metaphosphoric acid (HPO<sub>3</sub>) (Riedel-de Haën, Germany). Sample mixed with metaphosphoric acid solution was filtered through a filter paper (No. 1, Whatman plc., UK) and subsequently with a 0.45 µm filter ascrodisc (Millex, Millipore Corp., Bedford, MA, USA) under ice. The extract (5 µL) was immediately injected into a HPLC system equipped with a column (Discovery BIO Wide Bore C-18, 15 cm × 4.6 mm, 5 µm, Supelco, Inc., Bellefonte, PA, USA) and a guard column (Discovery BIO Wide Bore C-18, 2 cm × 4 mm, 5 µm, Supelco, Inc., Bellefonte, PA, USA) at 25 °C. The mobile phase was 10 mM ammonium dihydrogen phosphate [(NH<sub>4</sub>)H<sub>2</sub>PO<sub>4</sub>, pH 4] at a flow rate of 1 mL · min<sup>-1</sup>. Ascorbic acid was detected at 255 nm and was identified and quantified by ascorbic acid standard (Sigma-Aldrich, St. Louis, MO, USA).

### ***RNA gel blot analysis***

RNA gel blot analysis was conducted to determine the activation of key genes, phenylalanine ammonia-lyase (PAL),  $\gamma$ -tocopherol methyl transferase ( $\gamma$ -TMT), and L-galactose dehydrogenase (L-GalDH), responsible for the biosynthesis of phenolic compounds, vitamin C, and vitamin E, respectively (Bergmüller et al., 2003; Diallinas and Kanellis, 1994; Gatzek et al., 2002). The specific probes for L-GalDH (497 bp, accession number AJ417563) with primers 5'-AACTTCTTCGACACCTCCCC-3' and 5'-TCATCCCAACCAACACCGAC-3', and for  $\gamma$ -TMT (244 bp, accession number AF104220) with primers 5'-CATAGAAATCTATCTGCGGG-3' and 5'-CACTACGAAGCAGAGACACA-3', and for PAL (753 bp, accession number AF299330) with primers 5'-GCTTACAGTTTCTCAGGTGG-3' and 5'-TGATGCTTCAATTTGTGTGT-3' were amplified using genomic DNA from lettuce plants with the primer pairs designed by a primer design program (Primer3, Whitehead Institute, Cambridge, MA, USA). Amplification of probes was performed by PTC-100 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA) after initial denaturation at 94 °C for 5 min by 35 cycles of 1 min at 94 °C, 1 min at 51 °C ( $\gamma$ -TMT and PAL) or 54 °C (L-GalDH), and 1 min at 72 °C, followed by 5 min at 72 °C. The fragment size of each probe was checked by electrophoresis on 1.2% (w/v) agarose gels in 1 × Tris-Acetate-EDTA buffer. After electrophoresis, the fragments stained with ethidium bromide were checked by an UV transilluminator (FisherBiotech, Fairlawn, NJ, USA). The probes were stored at -20 °C until use.

Total RNA was extracted from lettuce leaves using a modified CTAB extraction method as described by Chang et al. (1993). Total RNA (9  $\mu$ g) was subjected to electrophoresis on 1% (w/v) agarose gel with 10% (v/v) formaldehyde. The RNA was transferred onto nylon membranes (Sigma-Aldrich, St. Louis, MO, USA) in a 20 × SSC solution (Sambrook and

Russell, 2001). The probes were labeled with  $^{32}\text{P}$  using random primer DNA labeling system (Sigma-Aldrich, St. Louis, MO, USA). During prehybridization, nylon membranes were incubated with 40 mL prehybridization buffer (1 mM EDTA, 0.5 M  $\text{NaH}_2\text{PO}_4$ , 7% SDS, pH 7.2) at  $50^\circ\text{C}$  for 6 h with Salmon Sperm DNA ( $10 \text{ mg} \cdot \text{mL}^{-1}$ ). The hybridization was performed as described by Sambrook and Russell (2001). Probes were hybridized to RNA on nylon membranes at  $50^\circ\text{C}$  for 12 h in 40 mL prehybridization buffer. The membranes were rinsed twice with wash I buffer (1 mM EDTA, 40 mM  $\text{NaH}_2\text{PO}_4$ , 5% SDS, pH 7.2) and then once with wash II buffer (1 mM EDTA, 40 mM  $\text{NaH}_2\text{PO}_4$ , 1% SDS, pH 7.2) at  $50^\circ\text{C}$  for 1 h. The membranes were then exposed in cassettes at room temperature for 1 d and the film was scanned by a gel and blot imaging system (Storm<sup>TM</sup>, GE Healthcare, Piscataway, NJ, USA).

### ***Plant growth characteristics***

Data on biomass accumulation in lettuce were collected at 2 and 4 weeks after transplanting. Fresh and dry weights of roots and shoots were determined using six lettuce plants per each treatment. Immediately after collecting samples, fresh weights of roots and shoots were measured in the field. The samples were dried at  $70^\circ\text{C}$  in an oven for 3 d for the determination of their dry weights.

### ***Statistical analysis***

Analysis of variance (ANOVA) was performed by the statistical analysis system (SAS) program. Duncan's multiple range test was used to compare means.

## **Results**

### ***Total phenolic content and antioxidant capacity***

There were no significant differences between the total phenolic content and antioxidant capacity of lettuce plants grown organically or conventionally in open field with or without fertilization (Fig. 6.1). Interestingly, under high tunnel conditions, lettuce plants consistently produced more total phenolics and had higher antioxidant capacity without any fertilization than with fertilization both in organically and conventionally managed plots (Fig. 6.2). Similar trend was also observed in open field, but the differences were not significant. Also, total phenolic content and antioxidant capacity did not vary significantly between organically and conventionally managed plants in both open field and high tunnel conditions, irrespective of fertilization.

### ***Phenolic compounds and vitamin C***

Comparisons of the compositions of phenolic acids in lettuce grown under different conditions revealed that in open field, organically managed crop produced higher amount of chlorogenic acid, chicoric acid, two major phenolics in lettuce, and quercetin-3-O-glucoside without any fertilization (Fig. 6.3). Furthermore, fertilization in organically managed lettuce significantly reduced levels of all of the above phenolic compounds, plus that of caffeic acid. However, such trend was not reflected in the results on the total phenolic content and antioxidant capacity (Fig. 6.1).

Under high tunnel conditions, fertilization in conventionally managed crop significantly reduced the amounts of chlorogenic acid, luteolin-7-O-glucoside, and quercetin-3-O-glucoside (Fig. 6.4). Contrary to the results in open field, fertilization in high tunnel had no effect on the phenolic composition of organically managed crop. Nevertheless, the increase in major phenolic compounds due to no fertilization in conventionally managed crop was consistent with the

results on the total phenolic content and antioxidant capacity. Regarding vitamin C, no significant differences were observed among all the treatments.

### ***Gene expression***

In open field, compared to fertilization, no fertilization appeared to activate the PAL, L-GalDH, and  $\gamma$ -TMT in lettuce crop in both organically and conventionally managed plots. However, conventionally managed crop was more sensitive to fertilization than the organically managed crop (Fig. 6.5). Contrastingly, in high tunnel, fertilization in organically managed crop seemed to increase the transcript levels of all these genes. In addition, these results also conflict with the results on the changes in the levels of several phenolic compounds induced by fertilization in high tunnel (Fig. 6.6).

### ***Growth characteristics***

Biomass accumulations in lettuce (on fresh weight basis) grown in open field were similar in organically managed plots with or without fertilization (Fig. 6.7). It was slightly higher than that in conventionally managed crop with fertilization. However, the poorest shoot growth was in conventionally managed plants with no fertilization. Based on root fresh weights at the time of harvest, root growth was highest in both organically and conventionally managed crops with fertilization. Similar to shoot growth, conventionally managed crop with no fertilization had the poorest root system.

Under high tunnel, shoot fresh weights at the time of harvest were similar in conventionally managed crop with or without fertilization and in organically managed fertilized crop (Fig. 6.8). The shoot and root growth were adversely affected in conventionally managed crop that did not receive any fertilization.

Based on dry matter, the shoot biomass accumulation for organically managed crop was consistently higher in open field than in high tunnel. Also, organically managed crops in open field and high tunnel were similar whether they were fertilized or not (Table 6.1). However, there was a large difference in shoot biomass in conventionally managed crop depending on whether it was fertilized or not. The lowest biomass was consistently in conventionally managed crop not receiving any fertilization in open field and high tunnel. Moreover, shoot biomass accumulations did not significantly vary whether the crop was managed organically or conventionally in open field and high tunnel as long as it received fertilization. Furthermore, the changes in root dry matter accumulation in lettuce were similar to those in the shoot.

## **Discussion**

Under open field conditions, organically managed lettuce accumulated more of the major phenolic compounds such as chicoric acid and quercetin-3-O-glucoside compared to conventionally managed crop. However, these increases were not reflected in the total phenolic content or antioxidant capacity. The response of plants in high tunnel was different in that no difference was observed between organically and conventionally managed crops. The plant responses with regard to phytochemical composition were different in open field and high tunnel because of large differences in growing conditions between these two methods of culture. Comparison of phenolic compounds in lettuce with regard to growing conditions indicates that the content of most of the phenolic compounds examined were higher in lettuce growing in open field than that grown in high tunnel (Figs. 6.3 and 6.4), although this trend was not clear with the total phenolic content. However, it is worth noting that fertilization consistently reduced the content of several key phenolic compounds in organically managed plants, especially chlorogenic acid, chicoric acid, caffeic acid, and quercetin-3-O-glucoside. Similarly, fertilization

under high tunnel conditions also decreased the content of chlorogenic acid and quercetin-3-O-glucoside in conventionally managed crop. There was no effect of fertilization in organically managed crop in high tunnel.

Both in open field and in high tunnel, the amount of some phenolic compounds was reduced by fertilization. In fact, this is reflected in the decreased total phenolic content and antioxidant capacity in response to fertilization in both organically and conventionally managed crops in high tunnel. Nutrient deficiency, especially nitrogen was found to induce phytochemicals such as ascorbic acid, flavanoids, and flavonols in *Arabidopsis* and tomato (Bongue-Bartelsman and Phillips, 1995; Kandlbinder et al., 2004; Stewart et al., 2001). Conversely, nitrogen over fertilization had a negative effect on crop quality, decreasing polyphenol content (Fernández-Escobar et al., 2006; Leser and Treutter, 2005). Furthermore, the deficiency of other nutrients such as phosphorous, sulfur, and zinc also had an impact on the phytochemicals and related enzymes in several plant species (Candan and Tarhan, 2003; Kandlbinder et al., 2004; Stewart et al., 2001). It is interesting that in open field, organically managed plants, not receiving any fertilizers, showed elevated levels of key phytochemicals. Application of organic matter to soil increases microbial mass and microbial activity (Burger and Jackson, 2003; Tu et al., 2006). The microbial activity may be at elevated levels in an open field, which may result in fixation of plant nutrients including nitrogen. Similar scenario may also exist in high tunnel, causing nutrient deficiency in unfertilized plots. Thus, the total phenolic content and antioxidant capacity were higher in unfertilized organic plot, compared to fertilized plots. Similar trend observed in conventionally managed crop may also be due to nutrient deficiency caused by lack of fertilization.

Also, the increase in phytochemicals in organically managed plots can be attributed to favorable soil environment. Addition of organic matter improves soil flora which may interact with roots (Walker et al., 2003). Studies have found that vesicular-arbuscular mycorrhiza (VAM), the most common symbiotic fungus in higher plants, induced accumulation of secondary metabolites in barley and legume roots (Harrison and Dixon, 1993; Peipp et al., 1997; Walker et al., 2003). The plots used in our study have been managed organically for at least the last 5 years and thus, it is reasonable to expect favorable root-microflora interaction, promoting phytochemical accumulation.

Comparison of the activation of key genes involved in the biosynthesis of phenolics and other antioxidants in lettuce under different managements and growing conditions showed that the unfertilized lettuce plants regardless of whether they were in open field or in high tunnel tended to activate PAL, L-GalDH, and  $\gamma$ -TMT. This is consistent with the general observation that the phenolic content and antioxidant capacity were higher in unfertilized lettuce. However, if we examine the gene activation with respect to management practices, there were inconsistencies with results on the total phenolic content and antioxidant capacity. Similarly, under high tunnel conditions, organically managed plants appeared to have higher transcripts of these genes compared to those managed conventionally, but fertilization did not suppress their activation. A clear correlation between gene activation and phytochemical accumulation was not observed. Thus, these results point to the complexity of factors under field conditions, which is further compounded by our imposed different management practices and growing conditions.

With regard to plant growth, both organically managed and conventionally managed crops performed well under fertilization regardless of growing conditions. However, in unfertilized crops, organic management appeared to do slightly better with regard to biomass



accumulation than their counterpart in conventionally managed crops. The better performance of organically managed plots can be attributed better long-range fertility in these plots, as the soil organic matter content is built up over the years, which can provide better nutrition to the crop.

The overall results indicate that elevated higher accumulation of phenolics occurs in lettuce grown in open field than in high tunnel. Also, higher soil fertility may actually hinder the accumulation of health-promoting phytochemicals in lettuce while it favors better crop growth and yield.

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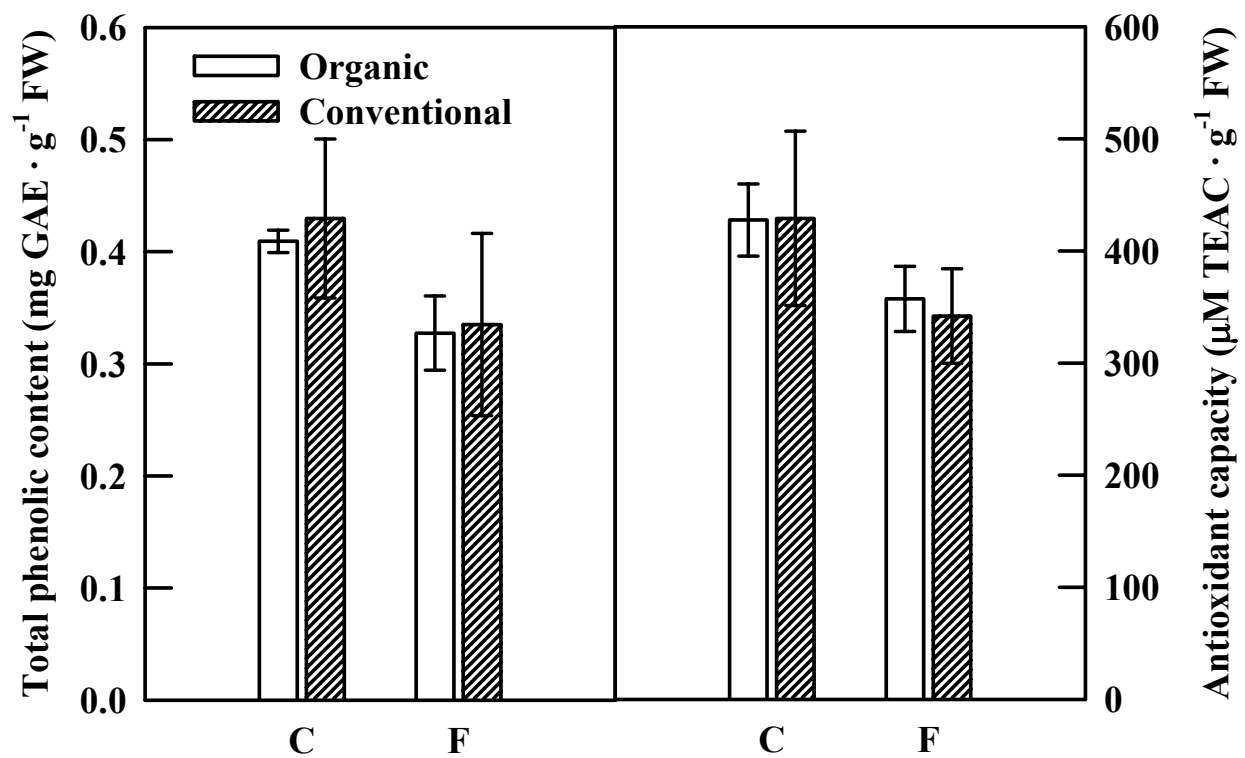
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## Figures and Tables

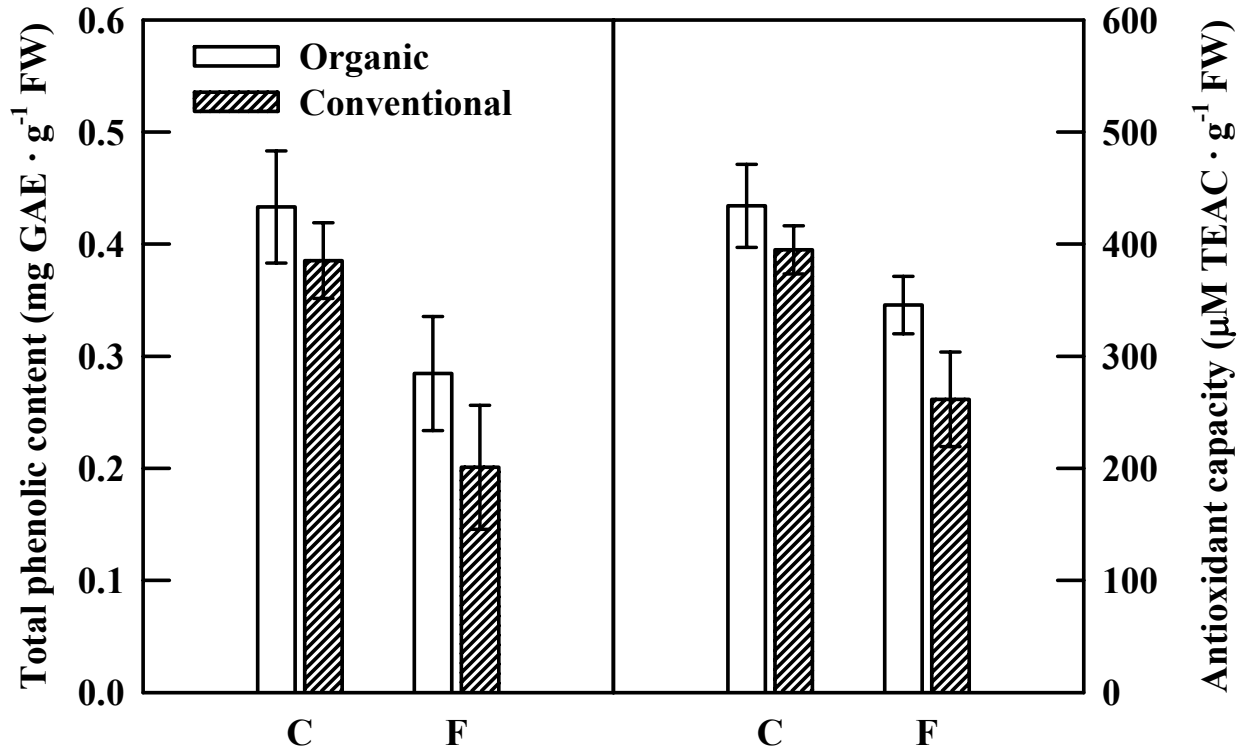
**Figure 6.1. Total phenolic content and antioxidant capacity of organically (open bars) and conventionally (shade bars) managed lettuce grown in open field at the time of harvest.**

Lettuce plants were grown at K-State Horticulture Research and Extension Center, Olathe, KS after transplanting for 4 weeks. Total phenolic content and antioxidant capacity were measured using three lettuce plants per treatment. The bars represent standard errors. C and F indicate control group and fertilizer group, respectively.



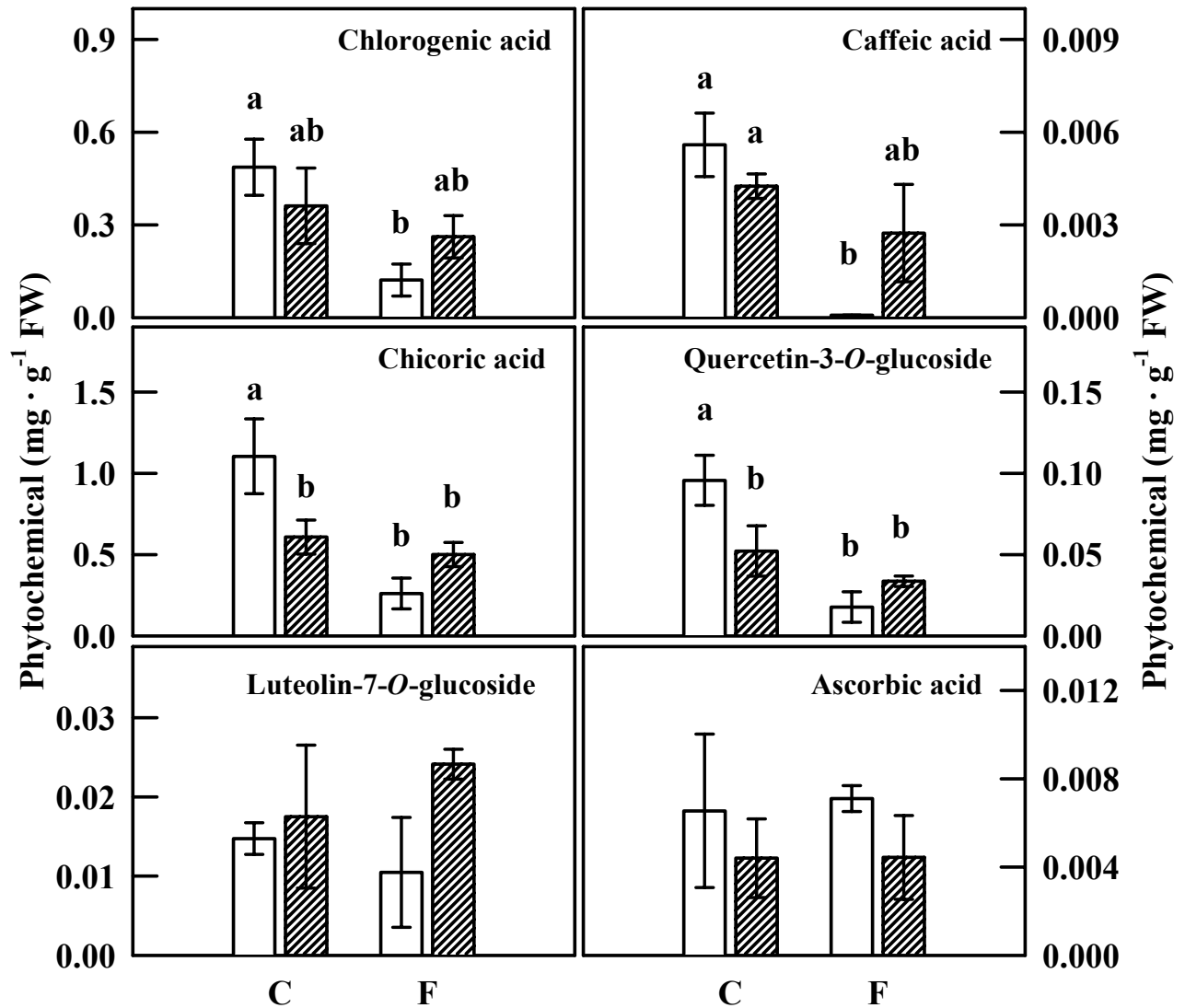
**Figure 6.2. Total phenolic content and antioxidant capacity of organically (open bars) and conventionally (shade bars) managed lettuce grown under high tunnel at the time of harvest.**

Lettuce plants were grown at K-State Horticulture Research and Extension Center, Olathe, KS after transplanting for 4 weeks. Total phenolic content and antioxidant capacity were measured using three lettuce plants per treatment. The bars represent standard errors. C and F indicate control group and fertilizer group, respectively.



**Figure 6.3. Phenolic compounds and vitamin C of organically (open bars) and conventionally (shade bars) managed lettuce grown in open field at the time of harvest.**

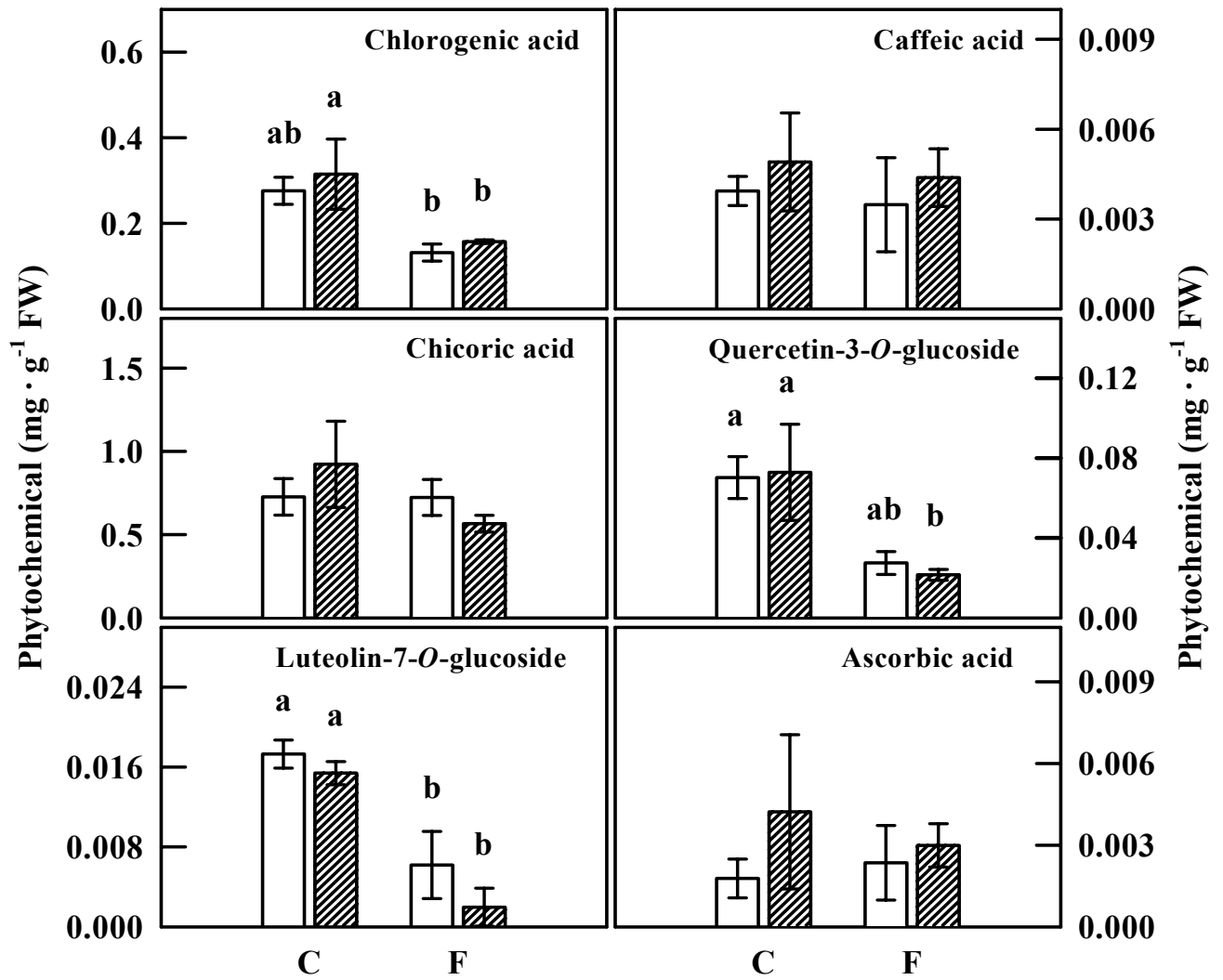
Lettuce plants were grown at K-State Horticulture Research and Extension Center, Olathe, KS after transplanting for 4 weeks. Phenolic compounds and vitamin C were measured using three lettuce plants per treatment. C and F indicate control group and fertilizer group, respectively. Significant differences ( $P=0.05$ ) between the treatments are indicated by different letters.





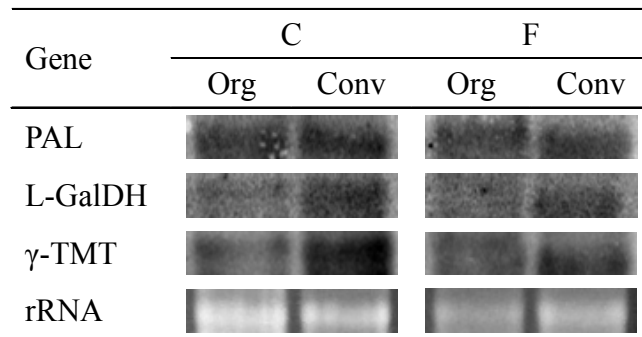
**Figure 6.4. Phenolic compounds and vitamin C of organically (open bars) and conventionally (shade bars) managed lettuce grown under high tunnel at the time of harvest.**

Lettuce plants were grown at K-State Horticulture Research and Extension Center, Olathe, KS after transplanting for 4 weeks. Phenolic compounds and vitamin C were measured using three lettuce plants per treatment. C and F indicate control group and fertilizer group, respectively. Significant differences ( $P=0.05$ ) between the treatments are indicated by different letters.



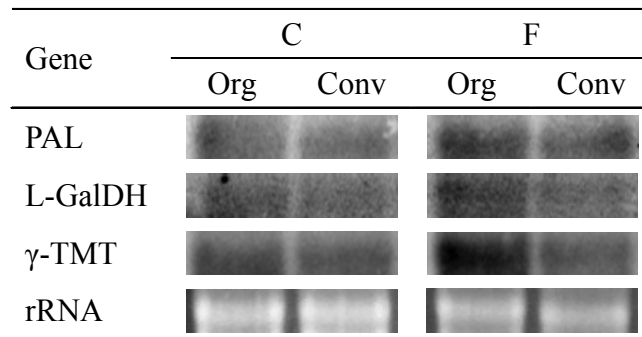
**Figure 6.5. The gene expression of PAL, L-GalDH, and  $\gamma$ -TMT in lettuce from organic (Org) and conventional (Conv) cultivation in open field at the time of harvest.**

Lettuce plants were harvested at 4 weeks after transplanting. C and F indicate control group and fertilizer group, respectively. rRNA was used as a loading standard.



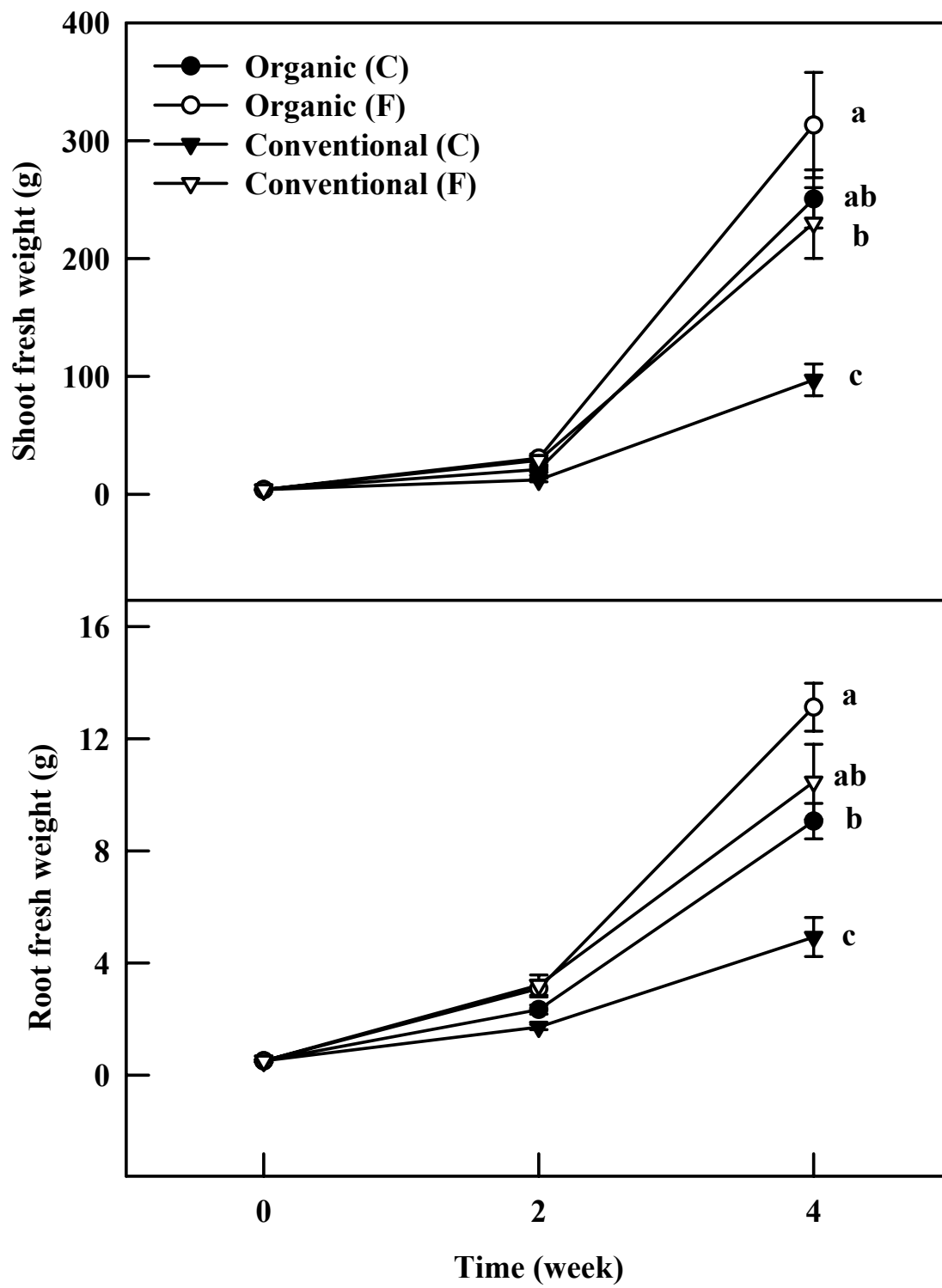
**Figure 6.6. The gene expression of PAL, L-GalDH, and  $\gamma$ -TMT in lettuce from organic (Org) and conventional (Conv) cultivation under high tunnel at the time of harvest.**

Lettuce plants were harvested at 4 weeks after transplanting. C and F indicate control group and fertilizer group, respectively. rRNA was used as a loading standard.



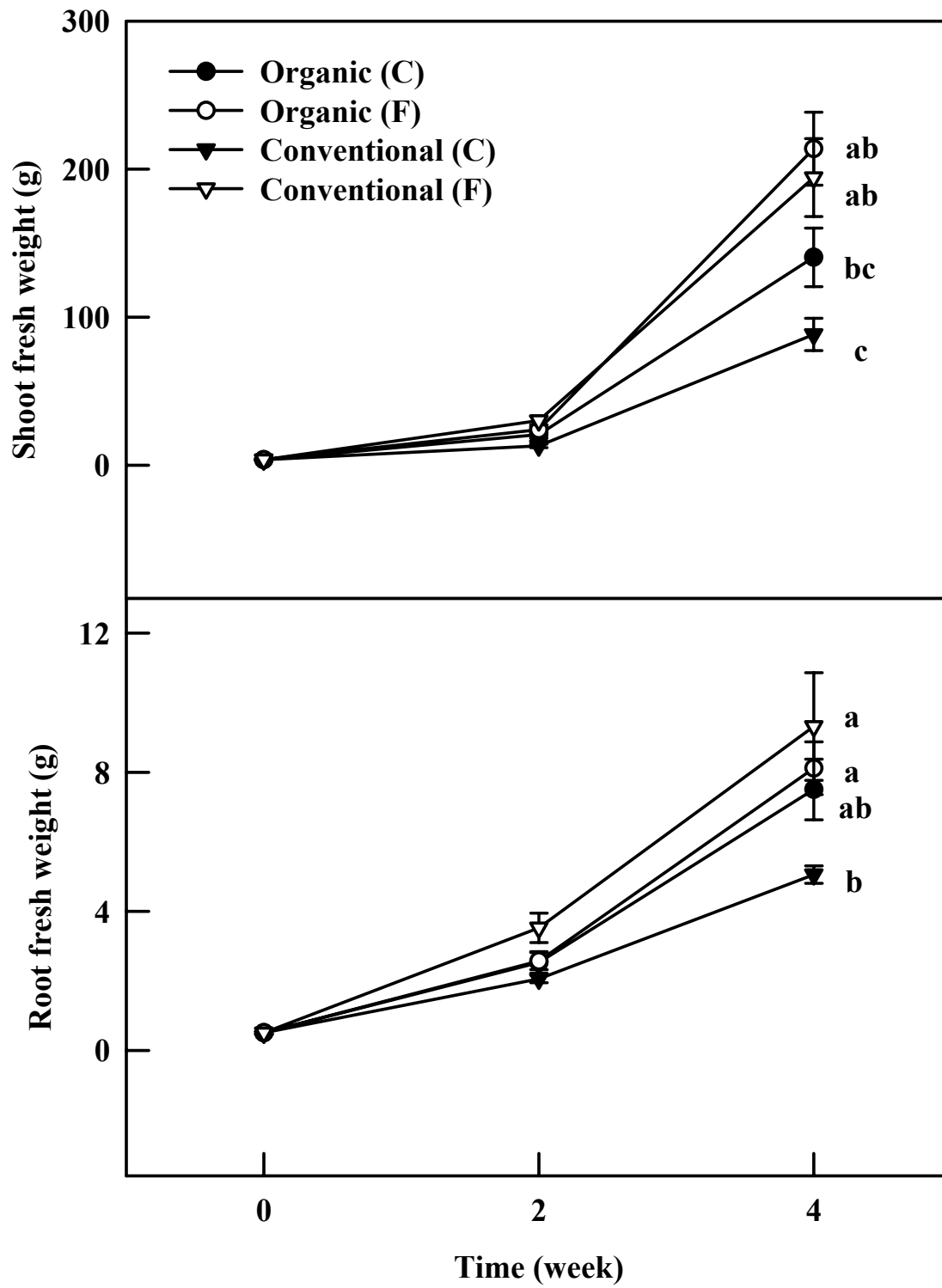
**Figure 6.7. Changes in fresh weights of shoots and roots of organically and conventionally managed lettuce in open field.**

Growth characteristics were measured using six lettuce plants per treatment at 2 and 4 weeks of transplanting. C and F indicate control group and fertilizer group, respectively. The bars represent standard errors. Significant differences ( $P=0.01$ ) between the treatments are indicated by different letters.



**Figure 6.8. Changes in fresh weights of shoots and roots of organically and conventionally managed lettuce under high tunnel.**

Growth characteristics were measured using six lettuce plants per treatment at 2 and 4 weeks of transplanting. C and F indicate control group and fertilizer group, respectively. The bars represent standard errors. Significant differences ( $P=0.01$ ) between the treatments are indicated by different letters.



**Table 6.1. Shoot and root dry weights of organically and conventionally managed lettuce plants both in open field and under high tunnel.**

Growth characteristics were measured at 2 and 4 weeks of transplanting. C and F indicate control group and fertilizer group, respectively. The values are the means of six lettuce plants per treatment.

Growing condition	Soil management	Fertilization	2 weeks		4 weeks	
			Shoot (g)	Root (g)	Shoot (g)	Root (g)
Open field	Organic	C	1.7 bc <sup>z</sup>	0.15 bc	15.7 ab	0.85 bc
		F	2.4 a	0.19 ab	18.6 a	1.19 a
	Conventional	C	1.1 d	0.12 c	6.8 d	0.42 d
		F	2.3 a	0.19 ab	14.5 ab	0.80 bc
High tunnel	Organic	C	1.6 cd	0.17 abc	9.0 cd	0.60 cd
		F	1.8 abc	0.19 ab	11.7 bc	0.62 cd
	Conventional	C	1.2 cd	0.18 abc	5.7 d	0.42 d
		F	2.3 ab	0.23 a	12.8 bc	0.96 ab
Significance			***	*	***	***

<sup>z</sup> Mean separation within columns by Duncan's multiple range test.

\* Significant at  $P=0.05$ .

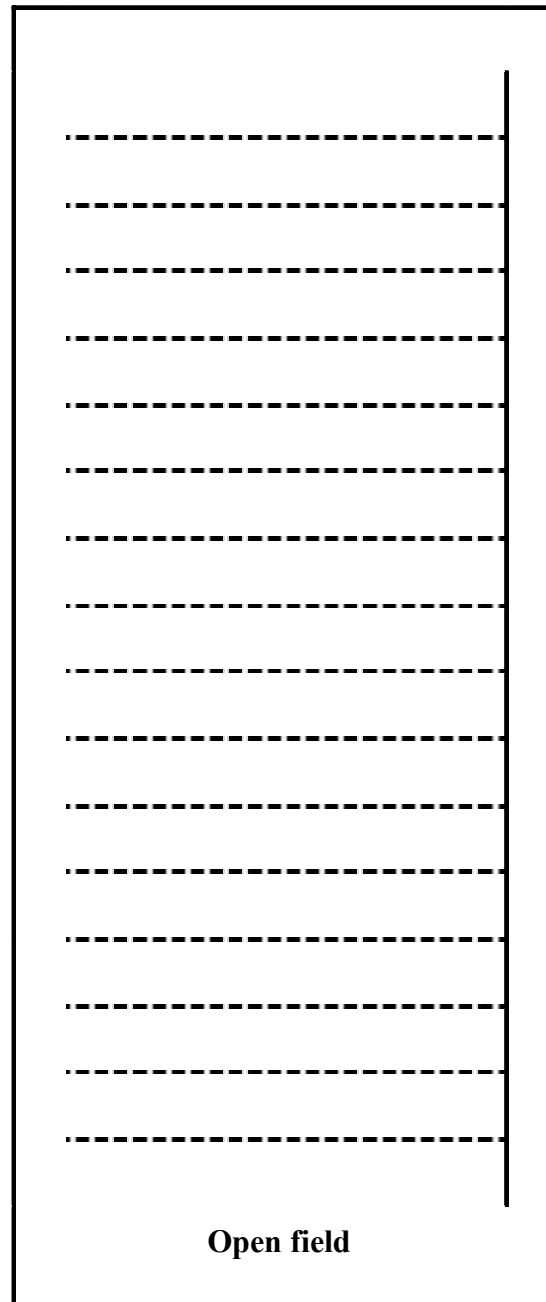
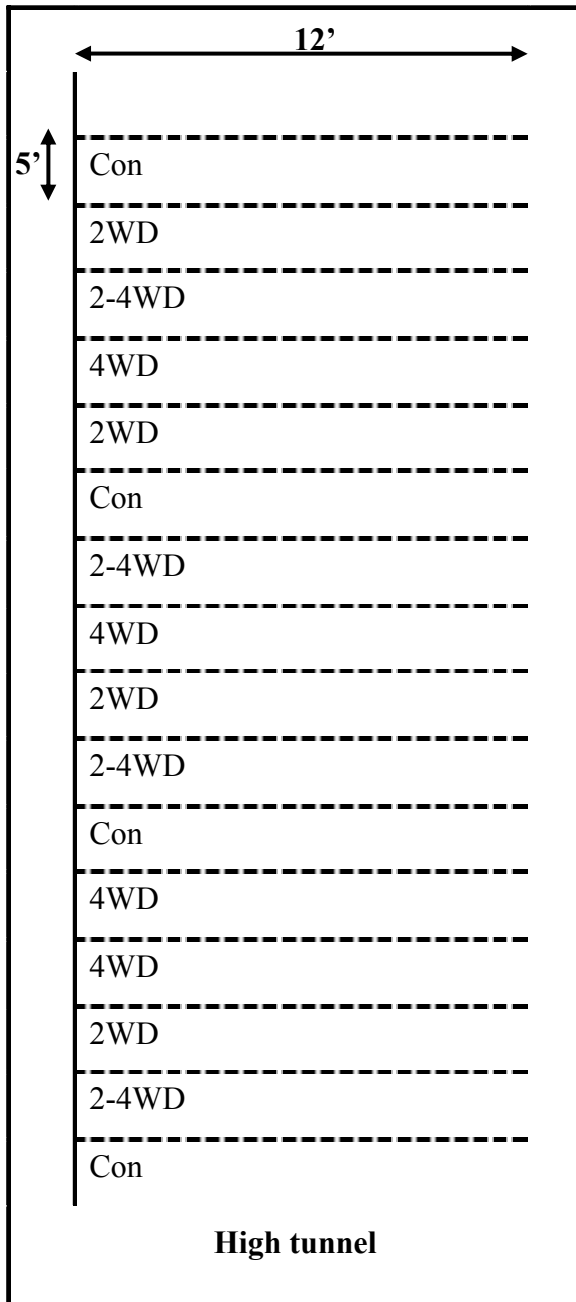
\*\*\* Significant at  $P=0.001$ .



## **Appendix A - Additional Details on Materials and Methods**

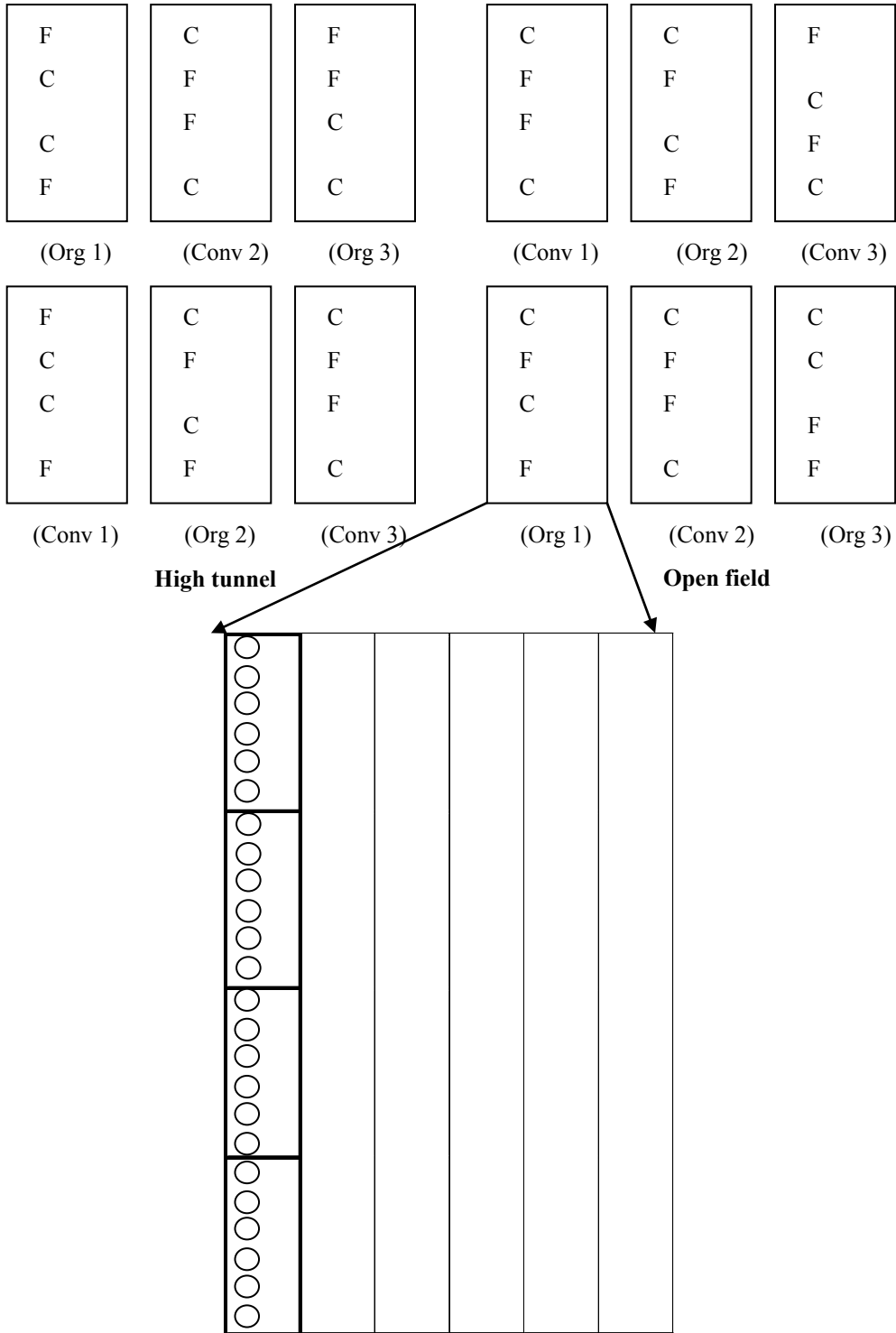
**Figure A.1. Experimental layout for drought stress trial in lettuce in open field and high tunnel (chapter 5).**

The dotted lines indicate the centers of rows where drip tape was laid. There was 5' between the rows, and two lettuce varieties, 'Red Sails' and 'Baronet', were planted on either side of the drip tape (random order for these subplots). Drip lines were connected to a header line (solid line) with valves to control flow to the individual plots.



**Figure A.2. Experimental layout of the study to compare between organically and conventionally managed lettuce (chapter 6).**

C- Control; F-Fertilized



**Table A.1. Soil analysis data of plots for drought stress experiment (chapter 5).**

Sampling place	Mehlich-3 P (ppm)	K (ppm)	Ca (ppm)	NO <sub>3</sub> -N (ppm)
High tunnel	35	152	2224	12.1
Open field	21	133	2331	11.0
QC1-1	32	141	2148	12.1

QC's are duplicates of highlighted samples for quality control checks.