SMALL FRUIT PHENOLICS: PHENOLIC VARIATIONS AND RELATED HEALTH RELEVANCE

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SMALL FRUIT PHENOLICS:
PHENOLIC VARIATIONS AND RELATED HEALTH RELEVANCE

A Dissertation Presented

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ABSTRACT

SMALL FRUIT PHENOLICS,
PHENOLIC VARIATION AND RELATED HEALTH RELEVANCE

FEBRUARY 2019

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Berries, specifically, strawberries, raspberries, blackberries, and blueberries, are considered an integral part of a healthy plant-based diet. Berries contain high levels of phenolic compounds, which contribute to the health benefits of berry consumption. Many phenolic compounds exhibit high levels of antioxidant activity and other health related benefits, including anti-microbial, anti-inflammatory and cardio-vascular related functional capabilities.

Strawberry and raspberry fruit were screened and evaluated for potential in dietary management of type 2 diabetes and related hypertension using in vitro assay models. There were differences between cultivars within each crop related to antioxidant activity. Inhibition of α- amylase,
α-glucosidase, and ACE-1 differed significantly between crops and between cultivars within each crop.

Phenolic content of berry crops varies with fruit development. Although overall phenolic content may not change significantly, the phenolic profile of fruit changes developmentally from green to ripe. These differences in phenolic content and/or profile can result in different bioactive potentials relevant to type 2 diabetes and hypertension management.

Blueberry fruit at successive stages of development were investigated and screened for potential relevance in diabetes and hypertension management. Ripe blueberry had better potential for α- amylase and α-glucosidase inhibition, than green or pink fruit.

Through consumption of berry fruits, phenolic compounds provide human health benefits. Likewise, phenolic compounds present in fruit and leaves of berry plants are crucial to the overall health of plants themselves. Plants have a complex of protective metabolic responses that can be triggered by various types of biotic or abiotic stress. Elicitors are compounds that stimulate plant stress response via secondary metabolic pathways. Chitosan oligosaccharide (COS) is a water soluble derivative of chitin. Elicitation by chitosan has potential to improve plant growth by countering the negative effects of plant stress through stimulation of plant protective responses.
COS was evaluated as an elicitor of the plant protective response in field transplanted blackberry plugs as they transitioned from late summer field conditions through early autumn. Results indicate COS may have potential to improve adaptation of blackberry plug plants during summer transplanting and seasonal transition into autumn.

Keywords: berries, chitosan, diabetes, hyperglycemia, hypertension, phenolics
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CHAPTER 1

INTRODUCTION

Small fruits such as berries, including strawberry, raspberry and blueberry, are high value crops in many parts of the world. They are popular among consumers for eating fresh as well as being processed for juices and other products. Berries maintain a high profile among consumers not only because of their taste, but also, increasingly, for their well-documented health benefits.

**Berry production**

Strawberries, raspberries, and blueberries are popular fruits eaten fresh, frozen, or processed in many countries. The global harvest area for strawberries in 2015 was over 391,000 hectares. The dollar value for the harvest was 21,200 million U.S. dollars. China accounted for 39% of the production with around 3,319 metric tons. The United States was second with 1,272 metric tons and Mexico was third (World: strawberries, 2018). In 2014, the United States produced approximately 3 billion pounds of strawberries valued at over 2.9 billion dollars (AgMRC, 2018).

Raspberries and blackberries are grown in many countries throughout the world. Worldwide raspberry production was estimated to be around 590,000 metric tons in 2017. In 2015, the top five raspberry producing
countries, in descending order, were Russia, Poland, United States, Serbia and Mexico. In the United States, combined raspberry and blackberry production for 2015 was over 300 million pounds on 28000 acres, at an estimated value of over 240 million dollars (IRO, 2017: NARBA, 2017).

Global blueberry production in 2016 was approximately 655,000 metric tons. Lowbush production was at 206,500 metric tons in 2016. The United States, Chile, and Canada account for 76% of all highbush production. This was an increase of 24.5% over 2014, mainly due to increased planting in China. The United States produced 588.8 million pounds of cultivated blueberries at a dollar value of 720.2 million in 2016. In the United States, the wild blueberry harvest is also significant. Maine is the leading state producer at 101.6 million pounds valued at 27.7 million dollars (AgMRC Blueberries, 2018).

Berry production can be a profitable enterprise, not only for large agribusinesses but also for smaller “family” farm operations in many parts of the world (Demchak et al, 2014). Family farms make up 98% of all farms and 53% of all agricultural land worldwide. (Graueb et al, 2016) Small rural farms in many European countries grow berries not only for household consumption but also for local supermarkets (Toader and Roman, 2015). China is becoming a major producer of strawberries. The majority of strawberries grown there come from small family farms that grow a variety of crops (Carter et al, 2017).
Plant phenolics

Plant phenolic compounds are a diverse group of secondary metabolites that include the phenolic acids and flavonoids. Phenolic acids include caffeic acid, chlorogenic acid, ellagic acid, protocatechuic acid and resveratrol. Flavonoids include flavonols such as quercitin and kampferol, the anthocyanins and isoflavonoids such as genistein and diadezin. Plant phenolic metabolism begins with the shikimate pathway and continues with the phenylpropanoid pathway (Herrman, 1995).

Many plant phenolics have been widely studied for their antioxidant free radical quenching properties. These compounds work to counteract oxidation-induced free radicals and thereby maintain a homeostasis within the plant cell. Free radicals are a by-product of routine photosynthetic and respiratory metabolism. Environmental stresses such as temperature extremes or disease and insect attack can also cause an increase in free radical production and breakdown cellular balance. These stress events stimulate plant secondary metabolism and increase the synthesis of phenolic compounds to function as effective antioxidants (Apel and Hirt, 2004; Tripathy and Oelmuller, 2012).

In addition to their free radical scavenging properties, phenolic compounds are also vital to plant structure and development. They are important components of lignin biosynthesis and cell wall integrity (Vanholme et al, 2010). Plant anthocyanins are phenolic compounds that facilitate plant
pollination and seed dispersal by attracting pollinators due to their colorful pigmentation (Shi and Xie, 2014).

Plant phenolics and human health

Plant phenolics provide health benefits when included in a balanced human diet, via consumption of fruits and vegetables (Kahkonen et al., 1999; Shetty, 1999; Scalbert et al., 2005). The significance of plant phenolics as part of a healthy diet is due, in large part, to their antioxidant capabilities (Shetty, 1999; Scalbert et al., 2005; Lin et al., 2016). However, many phenolic compounds also have other human health-promoting activity. Phenolic acids have good anti-microbial activity as well as anti-inflammatory activity (Correia et al., 2004; Chun et al., 2005). They have been studied extensively for their anti-cancer properties including anti-proliferative and chemo-preventative effects (Han et al., 2006, Yang et al., 2015, Wahle et al., 2010). Research has linked phenolic compounds in many plant foods to beneficial potential in the management of type 2 diabetes (Kwon et al., 2007, Lin et al., 2016) and cardiovascular-related conditions (Baba et al., 2007, Basu et al., 2010, Kozuma et al., 2005).

Berry crops and plant phenolics

Berries are an excellent source of plant phenolic compounds. High levels of antioxidant activity due to phenolic content has been well-
documented in many berry crops including strawberries, raspberries, blueberries, cranberries and gooseberries (Hakkinen et al 2000, Maatta-Riihinen et al 2004). Strawberries and raspberries are high in ellagic acid and other phenolic compounds with anti-cancer potential (Kresty et al 2001; Meyers et al 2003; Vattem et al 2005). Procyanidins found at high levels in blueberries and cranberries are phenolic phytochemicals that possess good anti-microbial activity against *E. coli* and urinary tract infections (Foo et al 2000; Puupponen-Pimi et al 2005). Procyanidins and ellagitannins have been linked to improved cardiovascular health through their vaso-dilatory abilities (Mullen et al 2002; Kalea et al 2009). Berries, including raspberries, blueberries, and black currants show potential as effective dietary components for prevention of type 2 diabetes. Extracts of these fruits with high anthocyanin content exhibit good a-glucosidase and a-amylase inhibitory activities which can contribute to a reduction in postprandial blood glucose levels (Lin et al, 2016).

**Plant phenolics and plant stress**

Plant phenolics have agronomic value based upon their antioxidant free-radical scavenging abilities as well as their anti-microbial properties. Production of these secondary metabolites is stimulated when plants are under stress. Stress factors can be biotic, such as attack by a fungal or bacterial pathogen or they can be abiotic. Abiotic stress factors include
environmental events such as drought, unfavorable temperature, and UV light exposure (Dixon et al., 1994; Dixon and Paiva, 1995). The routine aerobic metabolism of photosynthesis and respiration in plants generates reactive oxygen species (ROS). An imbalance between ROS production and their detoxification results in oxidative stress that can lead ultimately to cell death. Antioxidants and anti-oxidative enzymes produced within plants can counteract ROS production and help to maintain a redox balance within plant cells (Sharma et al., 2012).

When a plant is exposed to stress, there is an increase in ROS production. Plants have a complex antioxidant defense system that helps them to combat this increase in ROS production. One component of their defense response is an increased production of plant phenolic compounds via stimulation of the phenylpropanoid pathway. These phenolic compounds function as effective free radical scavengers and stimulate the activity of antioxidant enzymes (Torras-Claveria et al., 2012).

Stimulation of the phenylpropanoid pathway means an overall increase in production of phenolic compounds, many of which play a role in the plant’s ability to stave off or survive an infection. Phytoalexins are isoflavonoids that have anti-microbial properties. They can disrupt pathogen metabolism or cellular structure (Freeman and Beattie, 2008)). Phytoalexins such as camalexin from Arabidopsis have anti-fungal and anti-bacterial properties (Dicko et al., 2005). The cell wall is the first line of defense against many
bacterial and fungal pathogens. Lignin is an integral part of the cell wall and increased lignification enhances its structure and increase resistance. Lignins are aromatic polymers built from phenolic compounds. Attack by a plant pathogen can lead to stimulation of the phenylpropanoid pathway and a resulting increase in cell wall lignification (Barber and Mitchell, 1997, Zhao et al, 2009).

**Objectives**

Based on relevance of plant phenolics in plant adaptive response and human health, the overall objective of this work was to study phenolic content and antioxidant related activity of different berry crops, including strawberry, raspberry, blackberry and blueberry. Specifically, phenolic content and antioxidant activity were evaluated as they relate to various aspects of human health and plant health.

The specific objectives were the following:

- To evaluate the phenolic content and antioxidant activity of different raspberry cultivars with regard to variation between cultivars and their potential in management of hyperglycemia and hypertension associated with type 2 diabetes.
- To evaluate the phenolic content and antioxidant activity of different strawberry cultivars with regard to variation between cultivars and their potential in management of hyperglycemia and hypertension associated with type 2 diabetes.
• To evaluate the phenolic content and antioxidant activity of blueberry fruit at specific developmental stages and investigate their potential during fruit development, for management of hyperglycemia and hypertension associated with type 2 diabetes.

• To evaluate chitosan oligosaccharide (COS) as an elicitor to stimulate the plant defense response in blackberry plants as it relates to phenolic content and antioxidant enzyme activity during stress related events.
CHAPTER 2

LITERATURE REVIEW

Strawberry

The modern strawberry (*Fragaria ananassa*) is a member of the *Rosaceae* family. It originated in France from a cross between *Fragaria virginiana*, an eastern North American species and *Fragaria chiloensis* from Chile. References to the strawberry and its medicinal value can be found dating back to the ancient Romans (Darrow, 1966).

Raspberry and Blackberry

Raspberry and blackberry belong to the *Rosaceae* family in the genus *Rubus*. Raspberries belong to the subgenus *Idaeobatus*. There are over 200 species of raspberries, the most common being the red raspberry (*Rubus idaeus*) and the black raspberry (*Rubus occidentalis*). Commercially grown red and black raspberry varieties are mainly *R. idaeus* or *R. occidentalis* crosses. Commercial blackberries today are complex multispecies plants belonging to the subgenus *Rubus*. Like the strawberry, the raspberry was prized for its medicinal value in ancient times. The Greeks referred to raspberries as “ida” fruits, named for Mount Ida where they were harvested (Jennings, 1988).
Blueberry

Blueberry (Vaccinium sp) belongs to the Ericaceae family, along with several other important berries including cranberries and lingonberries. Blueberry is native to North America and the wild -type is a low bush plant unlike many of the commercial high bush varieties grown today. Domestication of wild blueberry began around 1908 with the collaboration of a USDA breeder, F.V. Coville and a commercial cranberry grower from New Jersey, Elizabeth White (Grubinger, 1998).

Plant phenolic compounds

Plant phenolic compounds are a large and diverse group of phytochemicals produced through plant secondary metabolism. Phenolic compounds are aromatic compounds having one or more benzene rings with hydroxyl groups and their derivatives attached to the rings. These phytochemicals include phenolic acids and flavonoids. Chlorogenic acid, protocatechuic acid, gallic acid, and caffeic acid are examples of phenolic acids. The flavonoids consist of many different compounds including the flavonols, anthocyanidins, stilbenes and coumarins (Javanmardi et al, 2003).

Most plant phenolics are derived from phenylalanine or tyrosine. Synthesis of plant phenolic compounds begins with the shikimate pathway and continues into the phenylpropanoid pathway. The end product of the shikimate pathway is chorismate. Chorismate is a precursor of the aromatic
amino acids including tyrosine and phenylalanine. Deamination of phenylalanine and tyrosine leads into the phenylpropanoid pathway and the production of plant phenolic acids and flavonoids (Vogt, 2010).

**Plant phenolic compounds as antioxidants**

Many plant phenolic compounds have well documented antioxidant activity. The aerobic nature of plant photosynthetic and respiratory metabolism leads to production of reactive oxygen species (ROS). These ROS include free radicals like superoxide anion ($\text{O}_2^-$) and hydroxyl radical (\text{OH}) and non-radical molecules including singlet oxygen ($^1\text{O}_2$) and hydrogen peroxide ($\text{H}_2\text{O}_2$).

During photosynthesis, ROS are formed due to the leakage of electrons from electron transfer activities to molecular oxygen. This transfer results in production of superoxide radicals ($\text{O}_2^-$), which can then give rise to the production of secondary oxidants like hydrogen peroxide ($\text{H}_2\text{O}_2$). Excessive ROS production causes oxidative stress within cells due to lipid peroxidation, protein oxidation, and enzyme inhibition. This stress can ultimately lead to cell death (Tripathy and Oelmuller, 2012).

Phenolic antioxidants function as scavengers by donating hydrogen ions to free radicals making them more stable and less available to further oxidation (Kiokas *et al* 2008). Some phenolic compounds will bind pro-oxidant metals, such as copper and iron, to prevent the formation of free
radicals (Halliwell, 2007). Phenolic acids also stimulate the activity of antioxidant enzymes such as super oxidase dismutase (SOD), catalase and guaicol peroxidase (GPX) (Chiang et al, 2006, Ramos-Escudero et al., 2012).

**Plant phenolic compounds and human health**

Extensive investigation has been done on the benefits of plant phenolic compounds for overall human health. Phenolic phytochemicals found in fruits and vegetables exhibit strong antioxidant, free radical scavenging abilities and as such, they are recognized as integral components of a disease preventative diet (Shetty 1997, Scalbert et al, 2005).

Oxidative metabolism is essential for energy production from the human diet. By-products of this metabolism include oxygen containing free radicals or reactive oxygen species (ROS). These free radicals can cause oxidative breakdown as they search for stabilization at the expense of cellular components such as lipids, proteins and nucleic acids. The ability of phenolic phytochemicals to donate hydrogen to these free radicals can help stop the oxidative breakdown of cellular molecules (Pandey and Rizvi, 2009).

Health benefits from consumption of fruits and vegetables however, include not only acknowledged antioxidant activity, but related disease preventative properties. These include anti-microbial and anti-cancer characteristics as well as cardiovascular related effects (Castonguay et al 1997, Correia et al 2004, Mullen et al 2002, Puupponen-Pimia et al 2001).
Numerous studies have linked phytochemicals to the health benefits attributed to different plant based foods (de Pascual-Teresa et al. 2008, Hanhineva et al. 2010).

Research has shown an inverse relationship between chronic human disease and a diet rich in plant phenolic compounds. An increase in the antioxidative capacity of plasma following a diet high in phenolic rich plant based foods was attributed to either the presence of these compounds in the plasma or their effects upon pro-oxidative food elements such as iron (Scalbert et al 2005).

Evidence also supports the antioxidant role of specific phenolic phytochemicals such as resveratrol and capsaicin in preventing cellular damage from oxidative stress during cardiovascular related conditions (Bonnefont-Rousselot 2016, Pandey and Rizvi 2009). Quercetin and its metabolites, with their free radical scavenging abilities, prevented the accumulation of cholesterol in the vascular tissue of mice (Terao 2009).

Hypertension and related cardiovascular diseases are chronic diseases. LDL oxidation, endothelial dysfunction leading to plaque build-up in the arteries can lead to hypertension and related conditions including artherosclerosis, renal failure and increased risk of heart failure. Polyphenols can counter some of these actions, thereby exerting a protective effect against cardiovascular disease. Reduced levels of phosphatidylcholine oxidation were observed after consumption of green tea, which is high in
catechins and likewise, phenolic rich cocoa powder inhibited LDL oxidation (Kozuma et al, 2005, Pandey and Rizvi, 2009). The ability of the phenolic compounds to bind to LDL particles may lead to an increase in resistance of LDL to oxidation. There is also support for the role of flavonoids on the surface of LDL particles supplying hydrogen to lipophilic antioxidants (Baba et al 2007).

Much interest has focused on the anti-cancer properties of phenolic phytochemicals. Ellagic acid continues to be intensively investigated for both its therapeutic and preventative effects on various forms of cancer (Zhang et al. 2014). Strawberries, raspberries, and grapes are some of the fruit crops that have high levels of ellagic acid (Daniel et al. 1989, Hakkinen and Torronen 2000, Williner et al 2003). As a potent antioxidant, ellagic acid inhibited lipid peroxidation in cancer cells. In addition, the ellagic acid treated cells had higher levels of the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase (Han et al 2006).

Plant phenolics have enzyme inhibition properties. They can bind to various enzymes, forming an enzyme-inhibitor complex that renders the enzyme inactive. They can also bind to an enzyme substrate thereby reducing the activity of the enzymatic reaction. Prostate cancer is considered an aging disease and oxidative stress plays a major role in its development. Quercetin can inhibit the expression of certain enzymes involved in the development of prostate cancer and reduce the possibility of metastasis
(Yang et al, 2015). Flavonoids, including silybin, also have anti-proliferative effects on prostate cancer cells through enzyme inhibition (Agarwal et al 2013).

Many phenolic compounds possess good anti-microbial properties. These properties are based upon different modes of action, such as protein binding, enzyme inhibition and membrane disruption. Ellagitannins exhibit anti-microbial activity against bacteria, fungi, and viruses. They have shown anti-microbial action against intestinal pathogens *Salmonella* and *Staphylococcus* (Lipinska et al 2014, Puupponen-Pimi et al. 2001).

Ellagitannin extracts have fungistatic activity against *Geotrichum candidum*, an opportunistic fungus that can cause infection in immune compromised patients (Klewicka et al. 2016). Proanthocyanidins found in cranberry fruit exhibit anti-microbial activity against *E. coli* and *H. pylori* (Howell et al. 2005, Vattem et al. 2005).

Diabetes mellitus is a chronic disease caused by a glucose imbalance resulting in hyperglycemia. Type 1 diabetes is due to failure to produce insulin. Insulin, produced by the pancreas, is vital to regulating the glucose balance in the body. Type 2 diabetes results from the body’s inability to progressively produce enough insulin to maintain glucose homeostasis following elevated increase. Increased risk of type 2 diabetes is associated with diets high in soluble carbohydrates, which result in hyperglycemia, an excessive increase in post-prandial blood glucose levels (DiCarli et al 2003).
The consumption of phenolic rich foods has been linked to management of type 2 diabetes based upon their ability to counter oxidative stress due to hyperglycemia (Wisweidel et al. 2004). Alpha-amylase and α-glucosidase are enzymes that facilitate the breakdown of carbohydrates to glucose and inhibition of these enzymes can benefit control hyperglycemia and subsequent oxidation breakdown (Kwon et al., 2007; Sarkar and Shetty, 2014). Inhibition of these enzymes can help to regulate glucose absorption by the body (DiCarli et al 2003, Lin et al 2016). Proanthocyanidins for example found in persimmon fruit inhibited α-amylase and ellagic acid and anthocyanidins in raspberry fruit had α-glucosidase inhibitory activity (Kawalami et al 2010, Zhang et al, 2011). Similar strategies have been extended to other small fruits (Da Silva Pinto et al, 2010).

**Berries and plant phenolic compounds**

Small fruits, or berries, include a range of horticulturally diverse crops. Blueberries and cranberries are berries, whereas, strawberries, raspberries, and blackberries are not true berries. Rather, they are classified as aggregate fruits, meaning the individual fruits develop from multiple ovaries on the same flower. Collectively though, all of the above are commonly referred to as berries.

Berries are popular consumer fruits. They contain significant amounts of phenolic phytochemicals with antioxidant properties as well as other health

Ellagitannins found in red raspberries exhibited good vasodilation capabilities (Mullen et al 2002). High levels of tannins were also believed to be responsible for $\alpha$-amylase inhibition in raspberry extracts (McDougall et al 2005).

Likewise, blueberry fruit contain high levels of flavonoids and phenolic acids (Kalt et al. 2007, Puupponen-Pimi et al 2005). Other members of the Ribes family, cranberries and bilberries, are also very good sources of phenolic compounds including anthocyanidins and phenolic acids (Moyer et al 2002, Zheng and Wang 2003). Phenolic acids found in blueberry fruit, have anti-microbial and anti-inflammatory properties, which can potentially contribute to colon health (Russell et al. 2007). The phenolic acids in the fruit of other Vaccinium species including cranberry fruit (Vaccinium macrocarpon) and bilberry fruit (Vaccinium myrtillus) also have anti-microbial activity against

**Plant phenolic compounds and plant stress**

Plant phenolic compounds are a significant component of plant response to stress. A stress event may be due to environmental factors such as extreme temperature, drought, or nutrient deficiency. The plant defense response may also be triggered by attack from a pathogen or pest, such as a fungal or bacterial infection or insect feeding (Tripathy and Oelmuller, 2012).

These stress events are, by nature, oxidative and can result in an increase in ROS production (Dixon et al. 1994, Lamb and Dixon 1997). Environmental stress can often cause inhibition of photosynthesis where the absorption of light energy becomes excessive relative to the photosynthetic activity. In order to dissipate the excess energy, electrons are transferred to molecular oxygen and ROS are produced (Ksas et al. 2015).

Many phenolic compounds produced in response to stress events can neutralize ROS, due to their antioxidant abilities. They can also induce an antioxidant enzyme response in the form of enzyme scavenging systems. Catalase is a main H$_2$O$_2$ scavenging enzyme, and superoxide dismutase (SOD) catalyzes the dismutation of O$_2^-$ to H$_2$O$_2$ and O$_2$ (Zhao et al. 2016).

Attack by a pathogen or pest can elicit a defense response in the plant under attack (Tripathy and Oelmuller, 2012). Phenolic compounds are
integral in protecting plants from insects and disease. Phytoalexins are isoflavonoids produced in response to a pathogen onset. Phytoalexins are pathogen specific and they work by disrupting pathogen metabolism or cellular structure (Freeman and Beattie 2008). Medicarpin for example in alfalfa and rishtin in tomato and potato are two examples of phytoalexins particular to certain crops. Some tannins are water soluble flavonoid polymers. They are toxic to insects as they can bind to salivary proteins and digestive enzymes resulting in protein inactivation (van Loon et al. 2006).

Lignins are another example of polymeric phenolic compounds providing a plant defense against pathogens. Lignins are branched polymers composed of phenolic monomers. They are rigid and insoluble and cannot be digested. As part of the cell wall, they constitute a physical barrier to pathogen invasion. Biosynthesis of lignins can be stimulated after a pathogen infection or wound as well as other types of metabolic stress (Cano-Delgado et al. 2003).

**Plant phenolic compounds, proline and plant stress**

Proline is an amino acid that is integral to the plant stress response. Under stressful conditions, proline has been found to accumulate in certain plants at high levels. Initially proline was thought to function mainly as an osmolyte for the protection of cellular membranes. Subsequent research
supports an additional regulatory role for proline in plant metabolism (Hare et al, 1999, Kavi Kishor et al, 2005, Shetty, 1999).

Proline synthesis can generate reducing power to maintain the cellular NADPH/NADP ratio and help prevent ROS formation as well as provide additional energy for a plant to utilize during times of stress. NADP produced during proline synthesis can up-regulate the pentose phosphate pathway via G6PDH. In turn, an elevated pentose phosphate pathway can stimulate the shikimate and phenylpropanoid pathways for the increased production of phenolic compounds to be utilized in stress-related plant cellular responses. (Hare et al, 1999, Shetty and Wahlqvist, 2004).
CHAPTER 3

CLONAL VARIATION IN RASPBERRY FRUIT PHENOLICS AND
RELEVANCE FOR DIABETES AND HYPERTENSION MANAGEMENT

Abstract

The potential functionality of fruit extracts from twelve *Rubus* cultivars was evaluated for diabetes and hypertension management. Inhibition of $\alpha$-amylase, $\alpha$-glucosidase, and ACE-1 activity was evaluated in conjunction with phenolic content and antioxidant activity. Black raspberries, Jewel and MacBlack had the highest overall phenolic content and antioxidant activity. Red raspberry cultivars, Nova, Heritage, and K-81-6, showed the overall highest $\alpha$-amylase inhibition in water and ethanol extracts. Jewel, black raspberry, was the least effective against $\alpha$-amylase with inhibitory activity of 40% or lower. Ethanol extracts from fruit of all twelve cultivars showed high inhibitory activity (above 85%) against $\alpha$–glucosidase. The yellow raspberry, KCB-1, was the most effective at inhibiting $\alpha$-glucosidase in both water and ethanol extracts. Fruit extracts of the yellow raspberries, KCB-1 and Kiwi Gold, showed the most potential for ACE-1 inhibition. Jewel and the red raspberry, Caroline, also had good activity against ACE-1.

Introduction

The beneficial effects of consuming fruits and vegetables are well known. These foods are considered essential to maintaining a balanced healthy diet. Many of their health-promoting characteristics are due to the presence of an array of phenolic phytochemicals including phenolic acids and flavonoids (Kahkonen et al. 1999, Shetty 1999). Phenolic phytochemicals exhibit strong antioxidant, free radical scavenging activity and their role in promoting a healthy, disease-preventive diet is attributed in large part to this capability (Shetty 1997, Scalbert et al, 2005). Health benefits from consumption of fruits and vegetables however, include not only acknowledged antioxidant activity, but related disease preventative properties. These include anti-microbial and anti-cancer characteristics as well as cardiovascular related effects (Castonguay et al 1997, Correia et al 2004, Mullen et al 2002, Puupponen-Pimia et al 2001).

Diet as a means of disease prevention is becoming increasingly important today. Type 2 diabetes is a major health concern world wide. Elevated risk of type 2 diabetes is associated with diets high in carbohydrates, which result in hyperglycemia, an excessive increase in post-prandial blood glucose levels (DiCarli et al 2003).

α–Glucosidases are enzymes that catalyze the absorption of digested glucose from dietary polysaccharides in the small intestine. Inhibition of α-
glucosidase is considered one of the more effective measures for regulating type II diabetes by controlling glucose uptake (Puls et al 1977).

α-Glucosidase inhibitors currently in use, such as acarbose and vogliobose, control hyperglycemia but may have unwanted side effects due to increased inhibition of α-amylase, which leaves undigested starch in the colon. (Bischoff et al 1985).

Phenolic phytochemicals potentially provide a natural source of α-glucosidase inhibitors, including α-amylase inhibition, therefore, a plant-based diet high in phytochemicals can effect post-prandial glucose management. Acylated anthocyanins inhibited α-glucosidase enzymes and reduced glucose uptake in rats (Matsui et al 2001). α-Glucosidase inhibition by small fruit extracts was related to their anthocyanin content. Blueberry and black currant extracts, with the highest anthocyanin content, were more efficient inhibitors of α-glucosidase than strawberry and red raspberry extracts (McDougall et al 2005). Tea extracts containing catechin inhibited α-amylase in rats resulting in lowered blood glucose levels (Matsumoto et al 1993).

Development of cardiovascular disease is a significant complication often associated with type II diabetes. The oxidative stress caused by post-prandial hyperglycemia can damage the vascular endothelium, leading to diabetic neuropathy as well as hypertension. (Sies, H. et al 2005). Angiotensin I converting enzyme plays a significant role in hypertension. ACE activity converts angiotensin I into angiotensin II, which is a very powerful
vasoconstrictor (Skeggs, L.T. 1956). Consequently, ACE-1 inhibition is an effective means of controlling hypertension (Johnston, J.I. 1992). Phenolic phytochemicals contained in different plant foods can have inhibitory effects upon ACE-I. Procyanidins, which are found at high levels in most berry crops, have shown ACE-1 inhibition (Actis-Goretta et al 2003) and diets rich in procyanidins reduced blood pressure in elderly people (Taubert, D. et al 2003). Kiwifruit extracts were also capable of ACE-1 inhibition in vitro (Jung et al 2005).

Berries are an excellent source of phytochemicals. High phenolic content and antioxidant activity has been well documented for many berry crops including strawberries, raspberries, blueberries and cranberries (Hakkinen et al 2000, Maatta-Riihinen et al 2004). Raspberries belong to the Rosaceae family and include popular consumer berries such as red raspberries (Rubus idaeus), black raspberries (Rubus occidentalis) and blackberries (Rubus idaeobatus). They exhibit excellent antioxidant activity along with other health related characteristics. Raspberries, particularly black raspberries are a good source of ellagic acid and raspberry extracts have shown cancer chemopreventative and anti-proliferative abilities (Kresty et al 2001, Liu et al 2002). Ellagitannins found in red raspberries exhibited good vasodilation capabilities (Mullen et al 2002). High levels of tannins were also believed to be responsible for α-amylase inhibition in raspberry extracts (McDougall et al 2005).
Phenolic content and phenolic profile can vary widely between *Rubus* species as well as between cultivars within a species. Black raspberries and blackberries have a higher overall phenolic content than red raspberries (Wang *et al* 2001). Likewise, the total anthocyanin content is higher in black raspberries than in red raspberries and anthocyanin profiles varied between the red and black berries (Wada *et al* 2002).

The objective of this research therefore, was to investigate the clonal variation in phenolic content and antioxidant activity among several established *Rubus* cultivars and determine their potential for type II diabetes associated hypertension management using *in vitro* enzyme model systems.

**Materials and Methods**

**Fruit Samples and Extract Preparation**

Raspberry fruit was harvested when ripe from a local commercial raspberry field and frozen at -20°C.

*Water Extracts* 100ml of distilled water was added to 40g of raspberry fruit and homogenized for 1 min using a Waring blender. Next the mixture was centrifuged at 15,000g and 4°C for 20 min. The supernatant was then filtered through a Whatman No.1 filter paper.

*Ethanol Extracts* 100ml of 95% ethanol was added to 40g of raspberry fruit and homogenized for 1 min using a Waring blender. Next the
mixture was centrifuged at 15,000g and 4°C for 20 min. The supernatant was then filtered through a Whatman No.1 filter paper.

**Total Phenolics Assay**

Total phenolic content of the water and ethanol extracts was measured using an assay modified by Shetty *et al* (1995). 1ml of extract was added to a test tube along with 1ml of 95% ethanol and 5ml of distilled water. 0.5ml of 50% (v/v) Folin-Ciocalteau reagent (Sigma) was added to the test tube, mixed and left to sit for 5 min. Then 1ml of 5% Na₂CO₃ was added to the mixture, mixed and left for 60 min in the dark. Absorbance was read at 725nm. Standard curves were created using increasing concentrations of gallic acid in 95% ethanol. Absorbance values were converted to total phenolics and expressed as µg equivalents of gallic acid per gram fresh weight of fruit.

**HPLC Analysis of Phenolics**

2.5ml of sample extract was vacuum concentrated and brought to 0.5ml with distilled water. This 0.5ml sample was filtered through a 0.2mm filter. 5ml of this sample was used for HPLC analysis as previously described (Kwon *et al* 2006). Pure standards of protocatechuic acid, chlorogenic acid, caffeic acid, coumaric acid, rosmarinic acid, ellagic acid, and quercetin (Sigma Chemical Co., St. Louis, MO) in 100% methanol were used to
calibrate the standard curves and retention times. Ellagic acid equivalents were used as a measure to include all peaks related to ellagic acid.

**Antioxidant Activity Assay (DPPH inhibition)**

Antioxidant activity was measured using a modified DPPH (1,1-diphenyl-2-picrylhydrazyl, Sigma Chemical Co.) radical inhibition assay (Cervato *et al* 2000). 0.25 ml of extract was added to 1.25 ml of 60 mM DPPH in 95% ethanol. The samples were vortexted and after 2 minutes their absorbance was measured at 517 nm. Control samples contained 0.25 ml of 95% ethanol instead of sample extracts. The antioxidant activity of the extracts was expressed as % inhibition of DPPH radical formation and was calculated using the following formula:

\[
% \text{ inhibition} = \left( \frac{A_{517\text{control}} - A_{517\text{sample}}}{A_{517\text{control}}} \right) \times 100
\]

**Antioxidant Activity Assay (ABTS inhibition)**

Antioxidant activity was measured using the ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay (Pellegrini *et al* 2002). ABTS radical cation was prepared by mixing 5 ml of 7 mM ABTS solution with 88 mls of 140 mM K$_2$S$_2$O$_4$ solution. This mixture is then stored in the dark at room temperature for 12 – 16 hours before using. Prior to the assay, this mixture is diluted with ethanol at an approximate ratio of 1:88 (ABTS: ethanol) and adjusted to yield an absorbance at 734 nm of
0.70 ± 0.02. 1ml ABTS was added to 50ml of extract and the mixture was vortexed for 30s. The sample was incubated at room temperature for 2.5 minutes and then the absorbance was measured at 734 nm. The antioxidant activity of the extracts was expressed as % inhibition of ABTS radical formation and was calculated using the following formula:

\[
\% \text{ inhibition} = \left( \frac{A_{734\text{control}} - A_{734\text{sample}}}{A_{734\text{control}}} \right) \times 100
\]

**α-Amylase inhibition disc assay**

Prior to performing the assay, 2.0ml of each ethanol extract sample was vacuum concentrated to remove the ethanol and then reconstituted to 2.0ml with distilled water. All ethanol and water samples were adjusted to pH 6.00. 800ml of each sample (adjusted phenolic content of 5, 10, and 25µg/ml) was added to 200ml of porcine pancreatic α-amylase (Sigma Chemical Co.) solution (1000 U / ml 20mM sodium phosphate buffer, pH 6.9). After 15 minutes, 100ml of this solution was added to a 13mm sterile paper disc placed at the center of a starch agar Petri plate (1% potato starch, 1% agar). Petri plates were allowed to stand at room temperature for 2 days, after which 3ml of iodine stain solution (5mM iodine in 3% potassium iodide) was added to each plate. The excess stain was drained after 15 minutes and the diameter of the cleared zone was measured. Control plates had discs inoculated with PPA solution only. Results were expressed as % inhibition of α-amylase and calculated using the following formula:
% inhibition = [(Control diameter – Sample diameter) / Control diameter] x 100

**α-Glucosidase inhibition assay**

Prior to performing the assay, 2.0ml of each ethanol extract sample was vacuum concentrated to remove the ethanol and then reconstituted to 2.0ml with distilled water. All ethanol and water samples were adjusted to pH 6.00. α-Glucosidase was purchased from Sigma Chemical Co. The α-glucosidase inhibition assay was performed following the method of Kwon et al (2006). Results were expressed as % inhibition of α-glucosidase and calculated using the following formula:

% inhibition = ((DA$_{405}$ Control - DA$_{405}$ Sample)/ DA$_{405}$ Control) x 100

**Angiotensin converting enzyme inhibition assay**

Prior to performing the assay, 2.0ml of each ethanol extract sample was vacuum concentrated to remove the ethanol and then reconstituted to 2.0ml with distilled water. All ethanol and water samples were adjusted to pH 6.00. The ACE inhibition assay was a modification of the method developed by Cushman and Cheung (1971). The hippuryl-histidyl-leucine (HHL) used for the substrate and the angiotensin-1-converting enzyme (ACE-1) were purchased from Sigma Chemical Co. The assay was performed as previously described (Kwon et al 2006). Results were expressed as % inhibition of ACE-1 and calculated using the following formula:
\[
\text{% inhibition} = \left( \frac{(E_{\text{control}} - E_{\text{sample}})}{(E_{\text{control}} - E_{\text{blank}})} \right) \times 100
\]

**Statistical Analysis**

All data were analyzed for standard deviation using Microsoft Excel XP to determine general trends in various metabolite factors.

**Results**

**Total Phenolic Content**

The total phenolic content differed between raspberry cultivars and between water and ethanol extracts of each cultivar (Fig. 1). The black raspberries, Jewel and MacBlack, had the highest phenolic content in both water and ethanol extracts. The red raspberries Magana and Caroline had the lowest phenolic content.
A phenolic profile of the raspberry extracts was created using HPLC analysis. The individual phenolics identified were caffeic acid, coumaric acid, protocatechuic acid, ellagic acid, and quercetin (Tables 1,2). Due to overlapping of peaks during HPLC analysis, we considered pure ellagic acid and its close analogs as total “ellagic acid equivalents” (Table 3). Protocatechuic acid and ellagic acid were the major phenolic metabolites in the fruit. The black raspberry cultivars, Jewel and MacBlack, had the highest levels of protocatechuic and ellagic acids. The purple raspberry, Royalty, and the red raspberry K81-6 also had high levels of protocatechuic and ellagic
acids. Jewel and MacBlack were highest in quercetin whereas the yellow cultivars, Kiwi Gold and KCB-1 had no detectable amounts of quercetin.

Table 3.1. HPLC analysis of individual phenolic compounds in water extracts of raspberry fruit

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Caffeic Acid (µg/g FW)</th>
<th>Coumaric Acid (µg/g FW)</th>
<th>Protocatechuic Acid (µg/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K81-6</td>
<td>ND</td>
<td>3.3 ± 0.27</td>
<td>28.9 ± 0.10</td>
</tr>
<tr>
<td>Magana</td>
<td>ND</td>
<td>2.8 ± 0.83</td>
<td>50.2 ± 3.00</td>
</tr>
<tr>
<td>Nova</td>
<td>3.7 ± 0.88</td>
<td>2 ± 0.45</td>
<td>14.5 ± 2.46</td>
</tr>
<tr>
<td>Autumn Britten</td>
<td>ND</td>
<td>4.2 ± 0.56</td>
<td>25.2 ± 1.26</td>
</tr>
<tr>
<td>Caroline</td>
<td>ND</td>
<td>1.9 ± 0.22</td>
<td>36 ± 1.08</td>
</tr>
<tr>
<td>Heritage</td>
<td>ND</td>
<td>2.1 ± 0.001</td>
<td>32.7 ± 0.96</td>
</tr>
<tr>
<td>Polana</td>
<td>6.8 ± 1.2</td>
<td>3.3 ± 0.56</td>
<td>29.2 ± 2.16</td>
</tr>
<tr>
<td>Kiwi Gold</td>
<td>3.5 ± 1.01</td>
<td>1.2 ± 0.12</td>
<td>ND</td>
</tr>
<tr>
<td>KCB-1</td>
<td>3.3 ± 0.94</td>
<td>2 ± 0.16</td>
<td>25 ± 3.64</td>
</tr>
<tr>
<td>Jewel</td>
<td>ND</td>
<td>ND</td>
<td>71.8 ± 2.00</td>
</tr>
<tr>
<td>MacBlack</td>
<td>ND</td>
<td>ND</td>
<td>47.7 ± 2.28</td>
</tr>
<tr>
<td>Royalty</td>
<td>9.4 ± 1.9</td>
<td>12.5 ± 0.05</td>
<td>42.2 ± 2.58</td>
</tr>
</tbody>
</table>

* Not Detected
Table 3.2. HPLC analysis of individual phenolic compounds in ethanol extracts of raspberry fruit

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Protocatechuic Acid (µg/g FW)</th>
<th>Quercetin (µg/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K81-6</td>
<td>37.6 ± 7.41</td>
<td>3.32 ± 0.89</td>
</tr>
<tr>
<td>Magana</td>
<td>19.5 ± 1.55</td>
<td>5.12 ± 1.31</td>
</tr>
<tr>
<td>Nova</td>
<td>ND</td>
<td>6.61 ± 1.68</td>
</tr>
<tr>
<td>Autumn Britten</td>
<td>ND</td>
<td>3.57 ± 0.41</td>
</tr>
<tr>
<td>Caroline</td>
<td>ND</td>
<td>3.6 ± 0.82</td>
</tr>
<tr>
<td>Heritage</td>
<td>23 ± 3.28</td>
<td>4.79 ± 0.75</td>
</tr>
<tr>
<td>Polana</td>
<td>31.2 ± 1.49</td>
<td>5.89 ± 0.94</td>
</tr>
<tr>
<td>Kiwi Gold</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>KCB-1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Jewel</td>
<td>68.4 ± 4.60</td>
<td>18.42 ± 1.20</td>
</tr>
<tr>
<td>MacBlack</td>
<td>46.7 ± 1.09</td>
<td>10.12 ± 2.40</td>
</tr>
<tr>
<td>Royalty</td>
<td>36.5 ± 3.00</td>
<td>4.37 ± 0.15</td>
</tr>
</tbody>
</table>

* Not Detected
Table 3.3. HPLC analysis of ellagic acid “equivalents” in water and ethanol extracts of raspberry fruit

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Water</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>K81-6</td>
<td>10.4 ± 0.67</td>
<td>33.8 ± 1.15</td>
</tr>
<tr>
<td>Magana</td>
<td>13.5 ± 2.97</td>
<td>24.3 ± 1.15</td>
</tr>
<tr>
<td>Nova</td>
<td>3.1 ± 0.85</td>
<td>14.4 ± 1.10</td>
</tr>
<tr>
<td>Autumn Britten</td>
<td>ND^*</td>
<td>12.7 ± 1.19</td>
</tr>
<tr>
<td>Caroline</td>
<td>5 ± 0.21</td>
<td>12.2 ± 1.13</td>
</tr>
<tr>
<td>Heritage</td>
<td>4.2 ± 0.99</td>
<td>11.7 ± 2.52</td>
</tr>
<tr>
<td>Polana</td>
<td>13.8 ± 0.56</td>
<td>17.9 ± 1.51</td>
</tr>
<tr>
<td>Kiwi Gold</td>
<td>3.5 ± 1.20</td>
<td>10.3 ± 1.47</td>
</tr>
<tr>
<td>KCB-1</td>
<td>2.8 ± 0.24</td>
<td>18.1 ± 2.68</td>
</tr>
<tr>
<td>Jewel</td>
<td>27.4 ± 1.75</td>
<td>49.1 ± 2.44</td>
</tr>
<tr>
<td>MacBlack</td>
<td>20.1 ± 2.38</td>
<td>29.7 ± 2.28</td>
</tr>
<tr>
<td>Royalty</td>
<td>20.7 ± 0.09</td>
<td>22.2 ± 0.84</td>
</tr>
</tbody>
</table>

^* Not Detected  
^"Sum of all peaks related to ellagic acid

The total phenolic content was greatest for Jewel and Mac Black as measured by the Folin-ciocalteu assay as well as determined by total peak area in the HPLC analysis. Kiwi Gold, on the other hand, had the lowest measurable peak area in HPLC analysis, but total phenolic content as determined by the Folin-Ciocalteu assay was greater than for Caroline and Magana red raspberries (Fig. 3.2 and 3.3).
Figure 3.2. Total soluble phenolic content versus HPLC total peak area of water extracts of raspberry fruit
Figure 3.3. Total soluble phenolic content versus HPLC total peak area of ethanol extracts of raspberry fruit

**Antioxidant activity**

The antioxidant activity as measured by the DPPH radical scavenging assay was very high for all samples (70% and higher) except for the black raspberries, Jewel and MacBlack. These two cultivars measured inhibition between 25% and 47% at total phenolic content (Fig. 3.4). However, as phenolic content was adjusted downward for these two cultivars, their antioxidant activity as measured by the assay increased (Fig 3.5). At 50ug/ml and 25ug/ml adjusted phenolic content, Jewel and MacBlack had the highest rate of DPPH inhibition (Fig 3.6). These results were due to the very dark
color of the Jewel and MacBlack extracts being used in an assay that measures antioxidant activity based upon sample decolorization.

Figure 3.4. DPPH radical scavenging activity of water and ethanol extracts of raspberry fruit
Figure 3.5. DPPH radical scavenging activity of water and ethanol extracts of raspberry fruit adjusted to 100µg/ml total phenolic content
One of the challenges of the DPPH assay is the confounding effects of the darker pigments of the Jewel and Mac Black extracts at higher concentrations. Therefore, at the lower concentrations the effects are clearer. Consequently, comparisons are more accurate at lower doses. Alternatively, other compatible antioxidant assays will need to clarify the differences. Therefore, ABTS assay helps to clarify the relevance of antioxidant potential.

The ABTS radical cation decolorization assay also measures antioxidant ability via free radical scavenging. Overall, Jewel and Mac Black exhibited the highest activity (Fig. 3.7). When phenolic content of the extracts was adjusted to 20\(\mu\)g/ml, the yellow raspberries, KiwiGold and KCB-1, along
with the purple cultivar Royalty, had activity intermediate between the black and red cultivars (Fig.3.8).

Figure 3.7. ABTS radical inhibition activity of water and ethanol extracts of raspberry fruit @ 20x dilution
Figure 3.8. ABTS radical inhibition activity of water and ethanol extracts of raspberry fruit adjusted to 20µg/ml total phenolic content

α-Amylase inhibition

Water extracts of all cultivars at total phenolic content gave complete inhibition of α-amylase. When phenolic content was adjusted to 25µg/ml, we began to see variability between cultivars. Water extracts exhibited better overall amylase inhibition than ethanol extracts. Nova red raspberry showed very high activity in both water and ethanol. Along with Nova, water extracts of two other red raspberries K81-6 and Heritage were more effective at inhibiting α-amylase than the other cultivars. Overall, Jewel black raspberry showed the lowest α-amylase inhibitory activity (Figs. 3.9,3.10).
Figure 3.9. Differences in α-amylase inhibition based upon dose dependency in water extracts of raspberry fruit, (water extracts evaluated at 5, 10, and 25µg/ml adjusted phenolic content)
Fig. 3.10. Differences in $\alpha$-amylase inhibition based upon dose dependency in ethanol extracts of raspberry fruit, (ethanol extracts evaluated at 5, 10, and 25 $\mu$g/ml adjusted phenolic content)

$\alpha$-Glucosidase inhibition

Ethanol extracts were more effective in inhibiting $\alpha$-glucosidase than were the corresponding water extracts. Ethanol extracts of all cultivars, at total phenolic content, had $\alpha$-glucosidase inhibition above 85%. KCB-1 yellow raspberry had the best overall inhibition of $\alpha$-glucosidase with inhibition percentages of 77% and 97% in water and ethanol extracts, respectively. Jewel, Mac Black and the purple raspberry Royalty, followed KCB-1 with inhibitory activity between 55% and 60% for water extractions (Fig. 3.11).
Evaluation of α-glucosidase inhibition potential based upon dose dependency showed that Royalty ethanol extracts and KCB-1 water extracts were the most promising samples with. Royalty showed α-glucosidase inhibition of 23% at adjusted phenolic content of 10µg/ml to 96% enzyme inhibition at 100µg/ml adjusted phenolic content. Jewel and Mac Black also exhibited enzyme inhibition over a soluble phenolic range from 16% inhibition @ 10µg/ml adjusted phenolic content to 57% inhibition @ 100µg/ml (Figs. 3.12, 3.13).

Figure 3.11. α-Glucosidase inhibitory activity of water and ethanol extracts of raspberry fruit
Figure 3.12. Differences in $\alpha$-glucosidase inhibition based upon dose dependency in water extracts of raspberry fruit, (water extracts evaluated at 10, 50, and 100 $\mu$g/ml adjusted phenolic content)
Figure 3.13. Differences in $\alpha$-glucosidase inhibition based upon dose dependency in ethanol extracts of raspberry fruit, (ethanol extracts evaluated at 10, 50, and 100 $\mu$g/ml adjusted phenolic content)

**ACE-1 Inhibition**

ACE-1 inhibition varied widely between cultivars. At total phenolic content, Kiwi Gold, KCB-1, Caroline, Jewel, and Mac Black all exhibit ACE-1 inhibition above 70%. However, water extracts of KCB-1 were active against ACE-1 but ethanol extracts showed no effect. Nova red raspberry had no inhibitory effect upon ACE-1. The red raspberries, K81-6 and Autumn Britten had limited inhibition (20%) of ACE-1 (Fig. 3.14). Kiwi Gold and KCB-1 were the only two cultivars to show any ACE-1 inhibition when the total phenolic content was adjusted to 100 $\mu$g/ml (Fig. 3.15).
Figure 3.14. ACE-1 inhibitory activity of water and ethanol extracts of raspberry fruit
Antioxidant activity, as measured by DPPH and ABTS inhibition assays, appears linked to total phenolic content. Jewel and Mac Black black raspberry have the highest overall phenolic content as measured by the Folin-Ciocalteau assay and HPLC analysis. They also exhibit the highest antioxidant activity in both DPPH and ABTS assays. In the ABTS assay, Jewel and MacBlack antioxidant activity is almost twice as high as most of the red raspberry fruits.
High antioxidant activity did not however predict good \(\alpha\)-amylase inhibition. Jewel, black raspberry, was one of the least effective at inhibiting \(\alpha\)-amylase but it had overall the highest phenolic content and antioxidant activity as measured. It also contained the highest levels of ellagic acid. \(\alpha\)-Amylase inhibition may be due to the structure of specific phenolic compounds. Previous research with strawberry and red raspberry fruit extracts suggested that \(\alpha\)-amylase inhibition by these berries might be due to high ellagitannin content. However, ellagic acid has little to no inhibition against \(\alpha\)-amylase (McDougall et al. 2005). Likewise, \(\alpha\)-amylase inhibition by bioprocessed pineapple waste did not appear to be linked to high antioxidant activity but was correlated with changes in structure of the existing phenolics (Correia et al. 2004).

Likewise, high antioxidant activity does not predict good \(\alpha\)-glucosidase inhibition. Jewel and Mac Black exhibit the highest inhibition of DPPH at 100 and 50 mg/ml adjusted phenolic content, as well as highest inhibition of ABTS, however, Royalty and KCB-1 have higher \(\alpha\)-glucosidase inhibition at 100 and 50 \(\mu\)g/ml adjusted phenolic content than either Jewel or Mac Black. Overall, the darker pigmented (black, purple) raspberries had higher \(\alpha\)-glucosidase inhibition than the red raspberries. Studies have shown that anthocyanins are effective inhibitors of \(\alpha\)-glucosidase with acylated anthocyanins being much more efficient than deacylated ones (Matsui et al. 2001b). HPLC analysis detected caffeic acid in the extracts of Royalty and
KCB-1 and caffeic acid shows high $\alpha$-glucosidase inhibition. Also, acylation of anthocyanins with caffeic acid was shown to be important for $\alpha$-glucosidase inhibition (Matsui et al. 2001a).

ACE-1 inhibition does not appear linked to total phenolic content or antioxidant activity either. Kiwi Gold and Caroline have lower total phenolic content as well as lowered antioxidant activity but have good inhibitory activity against ACE-1. Conversely, Jewel with the highest measurable total phenolic content and antioxidant activity shows good ACE-1 inhibition as well.

Water extracts of the two yellow raspberries, Kiwi Gold and KCB-1, exhibit good inhibition of ACE-1. As mentioned earlier, their inhibitory activity does not appear to be linked to either high phenolic content or antioxidant activity. The efficient inhibition of ACE-1 may be dependent upon specific plant phenolics rather than the total phenolic content of the berry extract. Procyanidins are effective inhibitors of ACE-1, however, their efficacy is dependent upon the flavonoid structure (Ottavini et al. 2006).

Additionally, inhibitory activity may be due to factors other than phenolic content. ACE-1 inhibition in herbal extracts did not appear to be linked to phenolic content either but possibly dependent upon non-phenolic factors (Kwon et al. 2006). Certain seed proteins, for example, the storage proteins of soybean, are well-known ACE-1 inhibitors (Mallikarjun Gouda et al. 2006) as are small peptides such as those found in milk and cheese (Gobbetti et al. 2000, Pripp et al. 2005). It is possible that some unknown protein factor
is influencing enzyme inhibition in the fruit extracts. An ACE-1 inhibitory peptide was isolated from broccoli and water-soluble extractions of broccoli had higher ACE-1 inhibition than other organic solvent extractions (Lee et al. 2006). The ACE-1 inhibition exhibited by the water extractions of Kiwi Gold and KCB-1 might be due to a water soluble peptide as well.

In terms of diabetes management, moderate $\alpha$-amylase inhibition and high $\alpha$-glucosidase inhibition are the most desirable combination of characteristics. Excessively high $\alpha$-amylase inhibition leads to undigested starch in the intestines and consequent side effects such as stomach distention, flatulence and diarrhea (Bischoff et al. 1985). KCB-1 shows moderate $\alpha$-amylase inhibition but high $\alpha$-glucosidase inhibition. Also, MacBlack and Jewel exhibit moderate to low $\alpha$-amylase inhibition combined with good $\alpha$-glucosidase inhibition.

Royalty purple raspberry, on the other hand, has the highest inhibitory activity against $\alpha$-amylase in water extracts. This characteristic combined with high $\alpha$-glucosidase inhibition make Royalty an undesirable candidate for diabetes management due to the likelihood of undigested starch causing side effects. Likewise, the summer-bearing red raspberries, K81-6, Nova and Magana, have low $\alpha$-glucosidase inhibition in water extracts and high $\alpha$-amylase inhibition making them less favorable for diabetes management as well.
Conclusion

Type 2 diabetes is often associated with hypertension. Several of the raspberry fruit extracts had good inhibitory activity against ACE-1 in combination with effective $\alpha$-amylase and $\alpha$-glucosidase inhibition. KCB-1, Jewel and Mac Black, with their high ACE-1, moderate $\alpha$-amylase and high $\alpha$-glucosidase inhibition could provide a dietary supplement to a population at risk for type II diabetes and/or hypertension.
CHAPTER 4

PHENOLIC-LINKED VARIATION IN STRAWBERRY CULTIVARS FOR
POTENTIAL DIETARY MANAGEMENT OF HYPERGLYCEMIA AND
RELATED COMPLICATIONS OF HYPERTENSION

Abstract

Fruit extracts of different strawberry cultivars were evaluated for their potential to contribute to the dietary management of hyperglycemia-linked to type 2 diabetes and related hypertension. In vitro inhibition of α-amylase, α-glucosidase, and angiotensin-1-converting enzyme (ACE) activity was evaluated using fruit extracts and correlated to phenolic content and antioxidant activity. There were significant differences between cultivars in both phenolic-linked antioxidant activity and inhibitory activity for the targeted disease relevant enzymes. Honeoye, Idea, and Jewel cultivars exhibited moderate α-amylase inhibition. Strawberry cultivars, in general, exhibited good uniform α-glucosidase inhibition with Ovation being the most effective cultivar. Water extracts of Jewel and Ovation cultivars had moderate ACE inhibition compared to low inhibition observed in other cultivars. Strawberry cultivars with combined inhibitory potential against α-glucosidase and ACE
and with moderate or low $\alpha$-amylase inhibitory potential could be targeted for potential management of hyperglycemia-linked type 2 diabetes and related complication of hypertension.

Introduction

Fruits and vegetables are fundamental components of a well-balanced, healthy diet. They contain a diverse assortment of protective phenolic phytochemicals. These phenolic compounds make a significant contribution to the role of fruits and vegetables as health-promoting foods (Kahkonen et al. 1999, Shetty 1999). Numerous studies support the antioxidant potential of these phenolic phytochemicals (Shetty 1997, Scalbert et al., 2005). Phenolic compounds, including phenolic acids and flavonoids, are also recognized for other disease-preventative characteristics such as antimicrobial properties, anti-cancer activity and cardiovascular-related effects (Castonguay et al 1997, Chun et al 2005, Correia et al 2004, Mullen et al 2002, Puuopponen-Pimia et al 2001).

As the world’s population continues to increase, diet as a means of chronic disease prevention becomes increasingly more important. Type 2 diabetes has become one of the world’s leading chronic diseases. Diet is known to impact both the cause and prevention of type 2 diabetes (Scalbert et al 2005). Diets high in soluble carbohydrates can result in hyperglycemia, leading to elevation of post-prandial glucose and increasing the risk of type 2 diabetes (DiCarli et al 2003). Cellular damage caused by the oxidative stress from hyperglycemia-linked type 2 diabetes can lead to macular degeneration and kidney diseases (Vincent et al 2005).
One of the control measures for type 2 diabetes is the management of hyperglycemia via inhibition of α-glucosidases. These are enzymes that control the breakdown and absorption of glucose precursors into the small intestine (Puls et al 1977). Hyperglycemia can be managed successfully using drugs such as acarbose, which is a α-glucosidase inhibitor that delays glucose absorption. However, there may be side effects from these drugs, due to undigested starch in the colon resulting from excessive α-amylase inhibition (Bischoff et al 1985).


Hyperglycemia associated with type 2 diabetes often leads to hypertension, a macrovascular complication in which angiotension-1-converting enzyme (ACE) is stimulated (Sies, H. et al 2005). ACE is a significant enzyme that converts angiotensin I into angiotensin II, which is a very powerful vasoconstrictor (Skeggs 1956). Inhibition of ACE activity is an effective means of regulating hypertension (Johnston 1992). Synthetic inhibitors of ACE, such as the widely used lisinopril, are very successful in controlling high blood pressure (Ondetti et al 1997). Likewise, certain foods have been shown to have a therapeutic effect on hypertension and this may
be due to the presence of naturally occurring ACE inhibitors. Some food proteins contain ACE inhibitory peptides including soy seed storage proteins and milk proteins (Gobbetti et al 2000, Mallikarjun Gouda et al 2006, Pripp et al 2005). Flavonoid rich foods, including red raspberries and cocoa, have also been cited for their cardiovascular benefits through regulation of hypertension (Actis-Goretti et al 2003, Mullen et al 2002, Taubert et al 2003).

Phenolic phytochemicals can be targeted for managing both hyperglycemia and hypertension associated with type 2 diabetes. In addition, phenolic phytochemicals have antioxidant properties that can be targeted against microvascular complications from oxidative stress, such as kidney disease and impaired wound healing (Suresh Babu et al 1998) Within this context, various fruits, including berries, have potential. Berries contain significant amounts of phenolic phytochemicals with potential health benefits (Hakkinen et al 2000, Maatta-Riihinen et al 2004, Shetty 1997, 1999). Cranberries and blueberries have high levels of procyanidins with antimicrobial activity against E. coli and urinary tract infections (Foo et al 2000). Phenolic extracts of strawberry and raspberry, with high levels of ellagic acid and other phytochemicals, have been studied for their anti-cancer properties, (Kresty et al 2001, Liu et al 2002, Maas et al 1991, Meyers et al 2003, Vattem et al 2005).
Phenolic content can differ between cultivars within a given berry crop, including strawberry, raspberry, and blackberry (Kosar et al 2004, Olsson et al 2004, Siriwoharan et al 2004) The health relevant functional benefits of raspberries can vary based upon differences in phenolic content that exist between cultivars (Cheplick et al 2007).

The objectives of this study were to evaluate the fruit of different strawberry cultivars for their phenolic content and antioxidant activity and determine the correlation to α-amylase, α-glucosidase, and ACE inhibitory activities using in vitro enzyme models. These findings could provide the biochemical rationale to include specific strawberry cultivars as a part of dietary support and clinical studies for managing hyperglycemia and hypertension linked to type 2 diabetes.

**Materials and Methods**

**Fruit Samples and Extract Preparation**

Strawberry fruit was harvested when ripe (approximately 40 days past bloom) from a local strawberry field during their commercial picking operation and frozen at -20°C.

*Water Extracts* A volume of 100 ml of distilled water was added to 40 g of strawberry fruit and homogenized for 1 min using a Waring blender. The mixture was centrifuged at 15,000g and 4°C for 20 min. The supernatant was then filtered through a Whatman No.1 filter paper.
**Ethanol Extracts** A volume of 100 ml of 95% ethanol was added to 40 g of strawberry fruit and homogenized for 1 min using a Waring blender. Next the mixture was centrifuged at 15,000g and 4°C for 20 min. The supernatant was then filtered through a Whatman No.1 filter paper.

**Total Phenolics Assay**

Total phenolic content of the water and ethanol extracts was measured using an assay modified by Shetty et al (1995). 1 ml of extract was added to a test tube along with 1 ml of 95% ethanol and 5 ml of distilled water. A volume of 0.5 ml of 50% (v/v) Folin-Ciocalteau reagent (Sigma) was added to the test tube, mixed and left to sit for 5 min. Then 1 ml of 5% Na$_2$CO$_3$ was added to the mixture, mixed and left for 60 min in the dark. Absorbance was read at 725 nm. Standard curves were created using increasing concentrations of gallic acid in 95% ethanol. Absorbance values were converted to total phenolics and expressed as µg equivalents of gallic acid per gram fresh weight of fruit.

**HPLC Analysis of Phenolics**

A volume of 2.5 ml of sample extract was vacuum concentrated and brought to 0.5 ml with distilled water. This 0.5 ml sample was filtered through a 0.2 mm filter. 5 ml of this sample was used for HPLC analysis as previously described (Kwon et al 2006). Pure standards of protocatechuic acid,
chlorogenic acid, caffeic acid, coumaric acid, rosmarinic acid, ellagic acid, and quercetin (Sigma Chemical Co., St. Louis, MO) in 100% methanol were used to calibrate the standard curves and retention times.

**Antioxidant Activity Assay (DPPH inhibition)**

Antioxidant activity was measured using a modified DPPH (1,1-diphenyl-2-picrylhydrazyl, Sigma Chemical Co.) radical inhibition assay (Cervato et al. 2000). A volume of 0.25 ml of extract was added to 1.25 ml of 60 mM DPPH in 95% ethanol. The samples were vortexed and after 2 min the absorbance was measured at 517 nm. Control samples contained 0.25 ml of 95% ethanol instead of sample extracts. The antioxidant activity of the extracts was expressed as % inhibition of DPPH radical formation and was calculated using the following formula:

\[
% \text{ inhibition} = \left(\frac{A_{517}\text{control} - A_{517}\text{sample}}{A_{517}\text{control}}\right) \times 100
\]

**Antioxidant Activity Assay (ABTS inhibition)**

Antioxidant activity was measured using the ABTS (2, 2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation de-colorization assay (Pellegrini et al. 2002). ABTS radical cation was prepared by mixing 5 ml of 7 mM ABTS solution with 88 ml of 140 mM K₂S₂O₄ solution. This mixture is then stored in the dark at room temperature for 12 – 16 hours before use. Prior to the assay, this mixture is diluted with ethanol at an approximate ratio
of 1:88 (ABTS:ethanol) and adjusted to yield an absorbance at 734 nm of 0.70 ± 0.02. A volume of 1 ml ABTS was added to 50 ml of extract and the mixture was vortexed for 30s. The sample was incubated at room temperature for 2.5 min and then the absorbance was measured at 734 nm. The antioxidant activity of the extracts was expressed as % inhibition of ABTS radical formation and was calculated using the following formula:

\[
\% \text{ inhibition} = \left( \frac{A_{734\text{control}} - A_{734\text{sample}}}{A_{734\text{control}}} \right) \times 100
\]

**α-Amylase Inhibition Disc Assay**

Prior to performing the assay, 2.0 ml of each ethanol extract sample was vacuum concentrated to remove the ethanol and then reconstituted to 2.0 ml with distilled water. 800 ml of each sample (adjusted phenolic content of 10, 50 and 100 µg/ml) was added to 200 ml of porcine pancreatic α-amylase (Sigma Chemical Co.) solution (1000 U / ml 20mM sodium phosphate buffer, pH 6.9). After 15 minutes, 100 ml of this solution was added to a 13 mm sterile paper disc placed at the center of a starch agar Petri plate (1% potato starch, 1% agar). Petri plates were allowed to stand at room temperature for 3 days, after which 3 ml of iodine stain solution (5 mM iodine in 3% potassium iodide) was added to each plate. The excess stain was drained after 15 min and the diameter of the cleared zone was measured. Control plates had discs inoculated with PPA solution only. Results were
expressed as % inhibition of α-amylase and calculated using the following formula:

% inhibition = \[\frac{(\text{Control diameter} - \text{Sample diameter})}{\text{Control diameter}}\] x 100

**α-Glucosidase Inhibition Assay**

Prior to performing the assay, 2.0 ml of each ethanol extract sample was vacuum concentrated to remove the ethanol and then reconstituted to 2.0 ml with distilled water. All ethanol and water samples were adjusted to pH 6.00. α-Glucosidase was purchased from Sigma Chemical Co. The α-glucosidase inhibition assay was performed following the method of Kwon et al (2006). Results were expressed as % inhibition of α-glucosidase and calculated using the following formula:

% inhibition = \[\frac{(\text{DA}_{405} \text{ Control} - \text{DA}_{405} \text{ Sample})}{\text{DA}_{405} \text{ Control}}\] x 100

**Angiotensin-1- converting Enzyme Inhibition Assay**

Prior to performing the assay, 2.0 ml of each ethanol extract sample was vacuum concentrated to remove the ethanol and then reconstituted to 2.0 ml with distilled water. All ethanol and water samples were adjusted to pH 6.00. The ACE inhibition assay was a modification of the method developed by Cushman and Cheung (1971). The hippuryl-histidyl-leucine (HHL) used for the substrate and the angiotensin-1-converting enzyme (ACE) were purchased from Sigma Chemical Co. The assay was performed as previously
described (Kwon et al 2006). Results were expressed as % inhibition of ACE and calculated using the following formula:

\[ \% \text{ inhibition} = \left( \frac{E_{\text{control}} - E_{\text{sample}}}{E_{\text{control}} - E_{\text{blank}}} \right) \times 100 \]

**Statistical Analysis**

All experiments were performed in triplicate. Statistical analysis of data was determined by ANOVA single factor test using Microsoft Excel XP.

**Results**

**Total Phenolics**

Water extracts of all the strawberry cultivars evaluated had phenolic content between 400 and 600 mg/g FW, except for Eros (383 μg/g FW) and Idea (236 μg/g FW). Ethanol extracts had a higher overall phenolic content than the water extracts between 595 μg/g FW for Eros and 694 μg/g FW for the cultivar Sparkle. Only ethanol extracts of Idea were below this range at 419 μg/g FW (Fig. 4.1).
Figure 4.1. Total soluble phenolics in water and ethanol extracts of strawberry fruit cultivars. Variation among water extracts is significant at $P < 0.05$ by ANOVA. Variation among ethanol extracts is significant at $P < 0.05$ by ANOVA.

### HPLC Analysis

HPLC analysis was used to obtain a general trend of the phenolic profile of the strawberry extracts (Table 4.1). Chlorogenic acid was an important specific phenolic found in all cultivars except Idea and Tristar.

Honeoye and Sparkle contained the highest amounts of ellagic acid in ethanol extracts at 44 and 53 µg/g FW, respectively. Sparkle, with 20 µg/g FW, also contained the highest amount of measurable ellagic acid of all water
extracts followed by DarSelect with 19 μg/g FW. Ellagic acid was not detected in the Honeoye, Idea, Jewel, Ovation and Seneca water extracts.

Protocatechuic acid was detected in only 7 of the 14 cultivars evaluated. The highest level of protocatechuic acid was found in water extracts of Eros. Seneca, Honeoye, Mira and DarSelect all contained similar amounts of protocatechuic acid in the range of 20 to 30 μg/g FW. Lesser amounts were found in Florence and Lambada. Quercetin was found in ethanol extracts of all cultivars. Cavendish (12.9 μg/g FW) had the highest and Idea (1.7 μg/g FW) had the lowest measurable amounts (Table 4.1).

HPLC analysis indicated that Idea extracts had the lowest overall total peak area representing all phenolic signatures for both water and ethanol extracts. This was also confirmed by the Folin-Ciocalteu assay. Idea berries had no detectable amount of chlorogenic acid or protocatechuic acid. Likewise, Idea fruit had the least amount of ellagic acid and quercetin.
Table 4.1. HPLC analysis of individual phenolic compounds in water and ethanol extracts of strawberry fruit.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Chlorogenic Acid (µg/g FW)</th>
<th>Ellagic Acid (µg/g FW)</th>
<th>Protocatechuic Acid (µg/g FW)</th>
<th>Quercetin (µg/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>Water</td>
<td>Ethanol</td>
<td>Water</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Honeoye</td>
<td>67.3 ± 9.7</td>
<td>nd*</td>
<td>44 ± 10.7</td>
<td>23.8 ± 3.9</td>
</tr>
<tr>
<td>Jewel</td>
<td>47.4 ± 15.5</td>
<td>nd*</td>
<td>19 ± 1.7</td>
<td>nd*</td>
</tr>
<tr>
<td>Seneca</td>
<td>83.3 ± 27.3</td>
<td>nd*</td>
<td>28.5 ± 13.3</td>
<td>27.1 ± 3.6</td>
</tr>
<tr>
<td>Sparkle</td>
<td>102.3 ± 21.0</td>
<td>20.3 ± 9.1</td>
<td>52.5 ± 4.6</td>
<td>nd*</td>
</tr>
<tr>
<td>N'Easter</td>
<td>151.5 ± 23.3</td>
<td>13.1 ± 3.6</td>
<td>27 ± 4.6</td>
<td>nd*</td>
</tr>
<tr>
<td>Ovation</td>
<td>57.9 ± 3.6</td>
<td>nd*</td>
<td>28.2 ± 2.2</td>
<td>nd*</td>
</tr>
<tr>
<td>Tristar</td>
<td>nd*</td>
<td>7.6 ± 1.4</td>
<td>35.5 ± 1.4</td>
<td>nd*</td>
</tr>
<tr>
<td>Cavendish</td>
<td>152.2 ± 9.1</td>
<td>14.9 ± 3.6</td>
<td>29.9 ± 6.8</td>
<td>nd*</td>
</tr>
<tr>
<td>Mira</td>
<td>101.7 ± 19.3</td>
<td>5.8 ± 0.1</td>
<td>28.2 ± 3.8</td>
<td>23.4 ± 5.9</td>
</tr>
<tr>
<td>Eros</td>
<td>100.2 ± 10.1</td>
<td>7.9 ± 2.5</td>
<td>26.4 ± 8.9</td>
<td>46.4 ± 4.1</td>
</tr>
<tr>
<td>Florence</td>
<td>78.7 ± 21.2</td>
<td>8.1 ± 4.1</td>
<td>30 ± 5.3</td>
<td>16.2 ± 4.2</td>
</tr>
<tr>
<td>DarSelect</td>
<td>65.4 ± 7.3</td>
<td>18.8 ± 7.3</td>
<td>30 ± 6.3</td>
<td>21.9 ± 6.6</td>
</tr>
<tr>
<td>Idea</td>
<td>nd*</td>
<td>nd*</td>
<td>15.6 ± 6.1</td>
<td>nd*</td>
</tr>
<tr>
<td>Lambada</td>
<td>51.1 ± 9.1</td>
<td>5.1 ± 0.6</td>
<td>24.9 ± 4.8</td>
<td>11 ± 5</td>
</tr>
</tbody>
</table>

*nd = not detected
Antioxidant Activity

DPPH Inhibition

All of the strawberry cultivars exhibit antioxidant activity in both water and ethanol extracts as measured by the DPPH radical scavenging assay. When phenolic content was adjusted to 100 µg/ml, many of the cultivars show DPPH inhibition above 80% and maximum activity saturation was observed above this average phenolic content. At the adjusted level of 100 µg/ml, Northeaster had the lowest overall antioxidant activity while Sparkle and Lambada had the highest activity (Fig. 4.2).
Figure 4.2. DPPH radical scavenging activity of water and ethanol extracts of strawberry fruit cultivars adjusted to 100µg/ml total phenolic content. Variation among water extracts is significant at P < 0.05 by ANOVA. Variation among ethanol extracts is significant at P < 0.05 by ANOVA.

**ABTS Inhibition**

The ABTS radical cation de-colorization assay also measures antioxidant ability via free radical scavenging. At an adjusted phenolic content of 20 µg/ml, Idea, Tristar, Sparkle, Ovation and Cavendish have ABTS radical inhibition of 80% or higher in both water and ethanol extracts. The lowest overall activity was found in water extracts of Northeaster (50% ABTS radical inhibition) (Fig. 4.3).
Figure 4.3. ABTS radical inhibition activity of water and ethanol extracts of strawberry fruit cultivars adjusted to 20 µg/ml total phenolic content. Variation among water extracts is significant at P < 0.05 by ANOVA. Variation among ethanol extracts is significant at P < 0.05 by ANOVA.

\[ \alpha \text{-Amylase Inhibition} \]

Water extracts of strawberry fruit had higher \( \alpha \)-amylase inhibition than ethanol extracts (Figs. 4.4, 4.5). Evaluation of the water extracts based upon dose dependency, showed that Honeoye, Idea, Jewel and Tristar cultivars had the most effective inhibitory activity. Honeoye, Idea, and Jewel exhibited \( \alpha \)-amylase inhibition around 50% at an adjusted phenolic content of 100 mg/ml. Water extracts of Sparkle with 15% \( \alpha \)-amylase inhibition were the least effective at inhibiting \( \alpha \)-amylase (Fig. 4.4). There was very little variability in inhibitory activity among the strawberry ethanol extracts (Fig. 4.5)
Figure 4.4. Differences in α-amylase inhibition based upon dose dependency in water extracts of strawberry fruit cultivars, (water extracts evaluated at 10, 50 and 100 µg/ml adjusted phenolic content, pH of extracts @ 6.00)
Figure 4.5. Differences in \( \alpha \)-amylase inhibition based upon dose dependency in ethanol extracts of strawberry fruit cultivars, (ethanol extracts evaluated at 10, 50 and 100 \( \mu \)g/ml adjusted phenolic content, pH of extracts @ 6.00)

**\( \alpha \)-Glucosidase Inhibition**

Ethanol extracts of strawberry fruit had higher \( \alpha \)-glucosidase inhibitory activity than water extracts. (Fig. 4.6). The lowest overall inhibitory activity was observed in Idea and Tristar extracts. Water extracts of Idea had 14% \( \alpha \)-glucosidase inhibition while ethanol extracts of Tristar had 46% inhibition. The highest overall inhibitory activity was found in Ovation (71% water, 97% ethanol), Florence (75% water, 92% ethanol), and Sparkle (63% water, 90% ethanol).
α-Glucosidase inhibition as based upon dose dependency showed that Ovation was the most effective cultivar overall. Ethanol extracts of Cavendish, Florence, Honeoye, Idea and Mira were also effective inhibitors (Figs. 4.7, 4.8).

Figure 4.6. α-Glucosidase inhibitory activity of water and ethanol extracts of strawberry fruit cultivars (pH of extracts @ 6.00)
Figure 4.7. Differences in $\alpha$-glucosidase inhibition based upon dose dependency in water extracts of strawberry fruit cultivars, (water extracts evaluated at 10, 50, and 100$\mu$g/ml adjusted phenolic content, pH of extracts @ 6.00)
ACE Inhibition

Strawberry cultivars showed limited inhibition of ACE. Water extracts of Ovation and Jewel had the highest inhibitory activity of 38% and 34% inhibition, respectively. Ethanol extracts of Jewel had inhibitory activity of 28% followed by 22% inhibition of ACE by Lambada ethanol extracts (Fig. 4.9).
Figure 4.9. ACE-1 inhibitory activity of water and ethanol extracts of strawberry fruit cultivars (pH of extracts @ 6.00). Variation among water extracts is significant at P < 0.05 by ANOVA. Variation among ethanol extracts is significant at P < 0.05 by ANOVA.

**Discussion**

Phenolic – linked antioxidant activity based upon free radical scavenging in strawberry fruit has implications for anti-hyperglycemia and anti-hypertensive potential. Among the strawberry cultivars used in this study, free radical scavenging-linked antioxidant activity was not associated solely with total phenolic content. The DPPH inhibition assay results indicate a similar activity profile for all the cultivars. Idea strawberry fruit had the lowest overall phenolic content of all the cultivars in this study as determined by both
the Folin-Ciocalteu assay and HPLC analysis based on total peak area. However, antioxidant activity represented by DPPH inhibition showed Idea fruit to have inhibitory activity in ethanol extracts equal to the other fruit samples.

Unlike previous results with different cultivars of raspberry, strawberry did not show distinct variability in antioxidant activity (expressed as % DPPH inhibition) linked to phenolic content (data not shown, Cheplick et al 2007). This uniformity may reflect less genetic divergence of these cultivars overall for water-soluble antioxidant activity linked to free radical scavenging. This result may also reflect more uniformity in the quality of the soluble phenolics found in strawberry cultivars.

Conversely, with the ABTS assay, when all cultivar extracts were evaluated at a 1:15 dilution (data not shown), results indicate Idea had the lowest antioxidant activity and Cavendish and Ovation, with higher phenolic content, showed the highest activity. The ABTS assay appears better able to distinguish strawberry cultivar variation than the DPPH assay. The ABTS assay tends to measure more lipid labile phenolics than the DPPH assay where radical scavenging abilities of water-soluble phenolics dominate. Consequently, there may be constancy among the water-soluble phenolics but some lipophilic phenolics or those effective at the hydrophilic/lipophilic interface may vary.
α-Amylase inhibition was not linked directly to antioxidant activity. Water extracts of Idea cultivar along with Honeoye and Jewel cultivars had the highest and best inhibition overall, based upon dose dependency (Fig. 4). However, antioxidant capacity, as determined by the ABTS assay, was much higher for Idea then for either Honeoye or Jewel cultivars. Previous studies comparing raspberry cultivars also suggested that α-amylase inhibition was not linked to high antioxidant activity. Black raspberry fruit exhibited the highest overall antioxidant activity and the lowest α-amylase inhibitory activity (Cheplick et al 2007). α-Amylase inhibition may be due to the structure of specific phenolics. α-Amylase inhibition by bioprocessed pineapple wastes was suggested to be the result of alterations in the structure of existing phenolics rather than the overall phenolic content or antioxidant activity of the pineapple wastes (Correia et al 2004). Ellagic acid derivatives and tannins in general are reported to be effective α-amylase inhibitors (Zhang and Kashket 1997). Many fruits including strawberry, raspberry, and grape are known to contain high levels of tannins and these fruits have exhibited α-amylase inhibitory properties (McDougall et al 2005).

High antioxidant activity was not a predictor of good α-glucosidase inhibition. Likewise, high phenolic content did not correlate to α-glucosidase inhibition either. Idea fruit had the lowest inhibitory activity against α-glucosidase from water and ethanol extracts as well as the lowest overall phenolic content. Tristar, on the other hand, had low α-glucosidase inhibition
but similar phenolic levels to Florence, which exhibited some of the highest inhibitory activity against $\alpha$-glucosidase. Fruit extracts, including blueberry, currant, raspberry, and strawberry, containing high levels of anthocyanins, show good \textit{in vitro} inhibition of $\alpha$-glucosidase (McDougall \textit{et al} 2005). Additionally, fruit extracts containing higher levels of acylated anthocyanins appear to be more efficient inhibitors than other fruit extracts (Matsui \textit{et al} 2001a, Matsui \textit{et al} 2001b). $\alpha$-Glucosidase inhibition by raspberry fruit extracts was better for the darker pigmented purple raspberry than the lighter colored red cultivars. However, yellow-pigmented raspberry fruit had better inhibitory activity than red raspberry fruit (Cheplick \textit{et al} 2007). Therefore, inhibition of $\alpha$-glucosidase may be influenced more by specific anthocyanins rather than the actual amount of the overall plant phenolics.

Jewel and Ovation cultivar extracts were the most effective at inhibiting ACE, with 34% and 38% inhibition, respectively. The inhibition of ACE did not correlate to either high phenolic content or antioxidant activity. Raspberry and pepper fruit extracts assayed for ACE inhibition under similar conditions were not linked to high phenolic content or antioxidant activity either (Cheplick \textit{et al} 2007, Kwon \textit{et al} 2007). Also, raspberry and pepper fruit extracts, with similar or lower phenolic content than the strawberry extracts based upon the Folin-Ciocalteu assay, had higher ACE inhibition, up to 80%, than the strawberry extracts (Cheplick \textit{et al} 2007, Kwon \textit{et al} 2007). ACE inhibition may be based upon specific phenolic compounds or other water-soluble compounds.
Inhibition of ACE by cocoa procyanidins has been shown, but inhibitory efficacy results from their specific structure (Ottavini et al. 2006). Water-soluble extracts of green coffee bean, whose main component was chlorogenic acid, lowered blood pressure in mildly hypertensive patients (Kozuma et al. 2005). Chlorogenic acid and its derivatives have lowered blood pressure in hypertensive rats and improved vasodilation (Suzuki et al. 2002). In this study, chlorogenic acid was a major phenolic found in every strawberry cultivar except Idea and Tristar, however, higher levels of this phenolic acid did not correlate to higher levels of ACE inhibition (Fig. 9).

Dietary management of hyperglycemia linked to type 2 diabetes can be targeted through foods that have high $\alpha$-glucosidase and moderate $\alpha$-amylase inhibition. Excessive $\alpha$-amylase inhibition can lead to undigested starch in the intestines and consequent stomach distention and discomfort (Puls et al., 1977). Water extracts of Honeoye and Jewel exhibited moderate $\alpha$-amylase inhibition and good $\alpha$-glucosidase inhibition making them potential candidates for dietary designs to manage early stages of hyperglycemia linked to type 2 diabetes.

Hypertension is a related long-term complication of type 2 diabetes. Inhibition of ACE by strawberry fruit may be an additional benefit of including specific strawberry cultivars in a diet regimen. For example, Jewel strawberry fruit with moderate $\alpha$-amylase inhibition, good $\alpha$-glucosidase inhibition and moderate ACE inhibitory capacity, could provide additional health benefits to
a population at risk for type 2 diabetes and its complications. Further, when combined with good free radical linked antioxidant activity, it could also be targeted to counter oxidative stress-linked complications of hyperglycemia such as macular and kidney dysfunction.

**Conclusion**

These results indicate that specific strawberry cultivars have the potential to enhance a healthy diet through their capacity to inhibit $\alpha$-glucosidase as well as, moderately inhibit $\alpha$-amylase. Additionally, the ACE inhibitory capacity for improved anti-hypertension potential and antioxidant protection function against cellular oxidative stress-linked complications further supports the potential use of specific strawberry cultivars as an important addition to a healthy disease-preventive diet.
CHAPTER 5

PHENOLIC BIOACTIVES FROM DEVELOPMENTAL STAGES OF Highbush Blueberry (Vaccinium corymbosum) for Hyperglycemia Management Using In Vitro Models

Abstract

Blueberry is a rich source of soluble phenolics as well as human health relevant antioxidants. Phenolic-linked bioactive functionality of blueberry for type 2 diabetes management was screened during fruit maturation, especially from green to ripening stages using in vitro assays. Green fruit showed highest total soluble phenolic content, whereas all three developmental stages of blueberry fruit exhibited high total antioxidant activity. Overall, ripe fruit had higher α-amylase and α-glucosidase inhibitory activity than green or green/pink fruit, and showed significant potential to improve glucose metabolism through in vitro assays. High phenolic-linked antioxidant activity along with moderate to high α-amylase and α-glucosidase inhibitory activity in ripe blueberry indicated its potential relevance as part of diet-based prevention and management of early stages of hyperglycemia associated with development of type 2 diabetes. This in vitro screening study provides a biochemical rationale and dietary strategy to develop the right blueberry
cultivar and stage of fruit development for further validation in animal and clinical studies.

Introduction

High bush blueberry (*Vaccinium corymbosum*) is a popular fruit that has been extensively studied for its potential human health benefits. Research in neuroscience, cancer prevention, and cardiovascular diseases has established blueberry fruit as a functional health-promoting food (Basu et al. 2010, Goyarzu et al. 2004, Joseph et al. 2004, Seeram 2008). In addition, blueberry fruit has strong antioxidant properties which contribute to its role as a beneficial component of a healthy diet, especially to combat oxidative stress-linked non-communicable chronic diseases (NCDs) (Ghosh *et al.* 2007, Joseph *et al.* 2004, Kalt *et al.* 2007).

Blueberry fruit contains high levels of phenolics, including flavonoids and phenolic acids (Kalt *et al.* 2007, Puupponen-Pimi *et al.* 2005). Numerous studies have linked these phytochemicals to the health benefits attributed to different plant based foods (de Pascual-Teresa *et al.* 2008, Hanhineva *et al.* 2010). Blueberry fruit is a good source of anthocyanins and proanthocyanidins with potential to promote cardiovascular health (Kalea *et al.* 2009, Shaugnessy *et al.* 2009,). Phenolic acids, in blueberry fruit, have anti-microbial and anti-inflammatory properties that can potentially contribute to colon health (Russell *et al.* 2007). The phenolic acids in the fruit of other *Vaccinium* species including cranberry fruit (*Vaccinium macrocarpon*) and bilberry fruit (*Vaccinium myrtillus*) have anti-microbial activity against intestinal

A diet rich in plant-based foods can become an integral part of managing NCDs such as type 2 diabetes and hypertension (Hanhineva et al. 2010, Knekt et al. 2002, Scalbert et al. 2005). Management of type 2 diabetes includes suppressing post-prandial hyperglycemia by reducing the amount of glucose absorbed into the bloodstream from the diet (Puls et al. 1997). Inhibition of α-amylase and α-glucosidase can reduce starch breakdown and concurrent absorption of glucose in the intestine. Pharmaceutical drugs currently used in diabetes management, can cause gastrointestinal side effects due to excessive α-amylase inhibition (Bischoff et al. 1985). Many phenolic metabolites from plant-based food showed α-amylase and α-glucosidase inhibitory activity in in vitro studies, and offer better strategy to manage type 2 diabetes without potential harmful side effects (Grussu et al. 2011, Johnson et al. 2011, McDougall et al. 2005). Foods naturally rich in these phenolics have the potential to provide simple dietary strategies to regulate early stages of hyperglycemia.

Individuals with type 2 diabetes are at increased risk for cardiovascular disease including hypertension (Am Diabetes Assoc. 2003). Hypertension is a macrovascular complication in which angiotensin – converting enzyme (ACE
– I) is stimulated (Sies et al. 2005). ACE catalyzes the conversion of angiotensin I into angiotensin II which is a potent vasoconstrictor. Angiotensin II also stimulates the production of aldosterone, which contributes to hypertension by increasing sodium retention. Inhibition of ACE – I is a therapeutic approach to managing hypertension (Johnston 1992). Extracts of different fruits, including raspberry, strawberry, cranberry, pepper, and kiwi, have ACE – I inhibitory activity in in vitro assays (Cheplick et al. 2007, 2010, da Silva Pinto et al. 2010, Kwon et al. 2007, Jung et al. 2005).

The phenolic composition of fruit changes as it ripens. In many cases, as fruit development proceeds, the total phenolic content remains relatively constant while the overall specific phenolic profile changes (Forney et al. 2012). These changes in phenolic profile as well as overall content, do not always affect the measured antioxidant activity. Anthocyanin content increased substantially as blackberry fruit ripened, however, the total phenolic content and antioxidant activity remained unchanged (Siriwoharn et al. 2004). The total phenolic content and antioxidant activity of sweet cherry fruit decreased as the fruit developed from green to pink and then increased back to green levels as the fruit continued to ripen (Serrano et al. 2005). An increase in total anthocyanin levels in ripening cherry fruit however, did not necessarily correlate to a measured increase in antioxidant capacity (Chaovanalikit and Wrolstad, 2004). In apples, total phenolic content based on fresh weight, decreased in the skin and pulp as fruit ripened and
antioxidant activity was relatively unchanged (Kondo et al. 2002). In strawberry, ellagic acid content increased as fruit ripened from green to pink but then decreased to its lowest level at the fully ripened stage (de O.Pineli et al. 2011, Olsson et al. 2004). However, chlorogenic acid, coumaric acid, and ascorbic acid content were higher in green strawberry fruit than ripe strawberry fruit. Water soluble total antioxidant activity increased in strawberry fruit from green to ripe stage but water insoluble antioxidant activity was highest in green fruit and lowest in ripe strawberry fruit. Ellagic acid content correlated to water insoluble antioxidant activity and ascorbic acid was linked to the water soluble activity (Olsson et al. 2004). Blueberry fruit had a decrease in total phenolic content as it ripened from green to blue and a change in phenolic composition as anthocyanin levels increased and hydroxycinnamic acids decreased (Castrejon et al. 2008). Antioxidant activity as measured by the TEAC and ESR assays also decreased as fruit development progressed (Castrejon et al. 2008).

The objectives of this study were to evaluate the phenolic-linked health relevance of blueberry fruit at specific developmental stages and to investigate the potential anti-hyperglycemic and anti-hypertension properties of blueberry phenolics through in vitro assays.
Materials and Methods

Fruit Samples and Extract Preparation

Blueberry fruit, var. Bluecrop, was harvested at three different developmental stages from a local commercial blueberry field in Whatley, Massachusetts, (42.4333° N, 72.6333° W and 290 ft elevation) USA and then frozen at -20°C. We selected Bluecrop cultivar due to its wide use as commercial mid-season cultivar in Massachusetts, New Jersey, and Michigan. Bluecrop is winter hardy, high yielding and is known for vigor and consistent production. Regular cultivation practices were followed to grow this cultivar in the field. The three stages of ripening investigated in this study were hard green, green/pink, and ripe.

Water and Ethanol Extracts

A total volume of 100 mL of distilled water or 95% ethanol was added to 40 g of blueberry fruit and homogenized for 1 min using a Waring blender. The mixture was centrifuged at 15,000g and 4°C for 20 min. The supernatant was filtered through a Whatman No.1 filter paper.
Total Phenolics Assay

Total phenolic content of the water and ethanol extracts was measured using an assay modified by Shetty et al. (1995). A 1 mL volume of extract was added to a test tube along with 1 mL of 95% ethanol and 5 mL of distilled water. A 0.5 mL volume of 50% (v/v) Folin-Ciocalteau reagent (Sigma) was added to the test tube, mixed and left to sit for 5 min. A 1 mL volume of 5% Na₂CO₃ was added to the mixture, mixed and left for 60 min in the dark. Absorbance was read at 725 nm. Standard curves were created using increasing concentrations of gallic acid in 95% ethanol. Absorbance values were converted to total phenolics and expressed as µg equivalents of gallic acid per gram fresh weight of fruit.

HPLC Analysis of Phenolics

A volume of 2.5 mL of sample extract was vacuum concentrated and brought to 0.5 mL with distilled water and filtered through a 0.2 mm filter. A volume of 5 mL of this sample was used for HPLC analysis as previously described (Kwon et al. 2006). Pure standards of protocatechuic acid, chlorogenic acid, caffeic acid, coumaric acid, rosmarinic acid, ellagic acid, and quercetin (Sigma Chemical Co., St. Louis, MO) in 100% methanol were used for calibration.
Antioxidant Activity Assay (DPPH inhibition)

Antioxidant activity was measured using a modified DPPH (1,1-diphenyl-2 picrylhydrazyl, Sigma Chemical Co., D9132-5G) radical inhibition assay (Cervato et al. 2000). A volume of 0.25 mL of extract was added to 1.25 mL of 60 mM DPPH in 95% ethanol. The samples were vortexed and after 2 minutes their absorbance was measured at 517 nm. Control samples contained 0.25 mL of 95% ethanol instead of sample extracts. Antioxidant activity with DPPH inhibition assay was also carried out after adjusting total phenolic content to 100 µg/ml. The adjustment represented the antioxidant activity in reference to the phenolic content of blueberry fruit. The antioxidant activity of the extracts was expressed as % inhibition of DPPH radical formation and calculated using the following formula:

\[
\% \text{ inhibition} = \frac{(A_{517}^{\text{control}} - A_{517}^{\text{sample}})}{A_{517}^{\text{control}}} \times 100
\]

Antioxidant Activity Assay (ABTS inhibition)

Antioxidant activity was measured using the ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid, Sigma Chemical Co., A1888-1G) radical cation decolorization assay (Pellegrini et al. 2002). ABTS radical cation was prepared by mixing 5 mL of 7 mM ABTS solution with 88 mL of 140 mM K$_2$S$_2$O$_4$ solution, and stored in the dark at room temperature for 12 – 16 hours. Prior to the assay, this mixture is diluted with ethanol at an approximate ratio of 1:88 (ABTS : ethanol) and adjusted to yield an
absorbance at 734 nm of 0.70 ± 0.02. A volume of 1 mL ABTS was added to 50 mL of extract and the mixture vortexed for 30s. The sample was incubated at room temperature for 2.5 min and the absorbance was measured at 734 nm. Blueberry extracts were diluted to @ 1:30 as without dilution showed very high inhibition (90-100%). Similar to DPPH, ABTS inhibition also carried out with adjusted total phenolic content (50µg/ml). The antioxidant activity of the extracts was expressed as % inhibition of ABTS radical formation and was calculated using the following formula:

\[
\% \text{ inhibition} = \left( \frac{A_{734\text{control}} - A_{734\text{sample}}}{A_{734\text{control}}} \right) \times 100
\]

**α-Amylase Inhibition Disc Assay**

Ethanol fruit extractions were vacuum concentrated to remove the ethanol and reconstituted to 2.0 mL with distilled water. The water and reconstituted ethanol samples were then adjusted to pH 6.00 prior to running the assay, after which 800 mL of each sample (adjusted phenolic content of 10, 50, and 100 µg/mL) was added to 200 mL of porcine pancreatic α-amylase (PPA) (Sigma Chemical Co. A3176-5MU) solution (1000 U / mL 20 mM sodium phosphate buffer, pH 6.9). After 15 minutes, 100 mL of this solution was added to a 13 mm sterile paper disc at the center of a starch agar Petri plate (1% potato starch, 1% agar). Petri plates were allowed to stand at room temperature for 3 days, after which 3 mL of iodine stain solution (5 mM iodine in 3 % potassium iodide) was added. The excess stain was
drained after 15 minutes and the diameter of the cleared zone measured. Control plates had discs inoculated with PPA solution only. Results were expressed as % inhibition of α-amylase and calculated using the following formula:

\[
\% \text{ inhibition} = \frac{(\text{Control diameter} - \text{Sample diameter})}{\text{Control diameter}} \times 100
\]

**α-Glucosidase Inhibition Assay**

Water and ethanol fruit extraction samples were prepared as described above for the α-amylase inhibition assay. α-Glucosidase was purchased from Sigma Chemical Co (G0660-750UN). The α-glucosidase inhibition assay was performed following the method of Kwon et al (2006). α-Glucosidase assays were carried out in both water and ethanol extracts after adjusted to different phenolic contents (10, 50, 100 µg/ml adjusted phenolic content, and on the basis of total phenolic content). Results were expressed as % inhibition of α-glucosidase and calculated using the following formula:

\[
\% \text{ inhibition} = \frac{(\text{DA}_{405} \text{ Control} - \text{DA}_{405} \text{ Sample})}{\text{DA}_{405} \text{ Control}} \times 100
\]

**Angiotensin–1–converting enzyme Assay**

Water and ethanol fruit extraction samples were prepared as described previously for the α-amylase inhibition assay. The ACE inhibition assay was a modification of the method developed by Cushman and Cheung (1971). The hippuryl-histidyl-leucine (HHL) used for the substrate and the
angiotensin-I-converting enzyme (ACE-I.) were purchased from Sigma Chemical Co (A6778-.25UN). The assay was performed as previously described (Kwon et al. 2006). Results were expressed as % inhibition of ACE-I and calculated using the following formula:

\[
\text{% inhibition} = \frac{(E \text{ control} - E \text{ sample})}{(E \text{ control} - E \text{ blank})} \times 100
\]

Statistical Analysis

All experiments were performed in triplicate with six replications. Statistical analysis of data was determined by ANOVA single factor test using Microsoft Excel 2011.

Results

Total Phenolic Content

Green fruit had the highest total soluble phenolic content, and ethanol extracts of green fruit at 1002 µg/g FW had almost twice the total soluble phenolic content of both green/pink (638 µg/g FW) and ripe fruit (626 µg/g FW). Ethanol extracts had higher total soluble phenolic content than water extracts at all three developmental stages of ripening. The total soluble phenolic contents of green/pink and ripe blueberry fruit were similar and remained unchanged in these growth stages (Fig 5.1).
Figure 5.1. Total soluble phenolics in water and ethanol extracts of different developmental stages of blueberry fruit. Variation among water extracts is significant at P< 0.05 by ANOVA. Variation among ethanol extracts is significant at P<0.05 by ANOVA.

HPLC Analysis of Phenolic Content

The two main phenolics found in blueberry fruit were chlorogenic acid and quercetin. Chlorogenic acid at 1012 µg/g FW, was twice as high in green fruit than in green/pink fruit at 540 µg/g FW. Ripe fruit had the lowest detectable amount of chlorogenic acid at 160 µg/g FW. Green fruit also had the highest detectable amount of quercetin at 139 µg/g FW for ethanol extracts, followed by green/pink fruit with 124 µg/g FW and ripe fruit with 104 µg/g FW (Table 5.1). Two detected phenolic acids, chlorogenic acid and quercetin were low in water extracts when compared with ethanol extracts. Similar to ethanol extracts, higher concentration of chlorogenic acid and quercetin were observed in green fruit (515µg/g FW and 49.09µg/g FW
respectively) followed by green/pink (109 µg/g FW and 21 µg/g FW respectively) and ripe fruit (27 µg/g FW and none).

Table 5.1. Changes in chlorogenic acid and quercetin (µg/g FW of blueberry fruit) content in V. corymbosum at three maturity stages of blueberry fruit development.

<table>
<thead>
<tr>
<th></th>
<th>Chlorogenic Acid</th>
<th>Quercitin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Green</td>
<td>515.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1012.44&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gr/Pink</td>
<td>108.98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>539.67&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ripe</td>
<td>27.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>159.86&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Antioxidant Activity

DPPH Inhibition

Ethanol extracts of all three developmental stages had similar DPPH inhibition despite variations in total soluble phenolic content. Water extracts of green fruit at undiluted total soluble phenolic content showed higher DPPH inhibition at 86% than green/pink and ripe fruit. DPPH inhibition of water extracts of green/pink and ripe fruit at undiluted total soluble phenolic content were 67% and 63%, respectively (Fig 5.2). When the phenolic content is
adjusted to 100 $\mu$g/mL, the antioxidant activity is similar for the three stages of fruit development in both water and ethanol extracts (Fig 5.3).

Figure 5.2. DPPH radical scavenging activity of water and ethanol extracts of different developmental stages of blueberry fruit. Variation among water extracts is significant at $P<0.05$ by ANOVA. Variation among ethanol extracts is significant at $P<0.05$ by ANOVA.
Figure 5.3. DPPH radical scavenging activity of water and ethanol extracts of different developmental stages of blueberry fruit adjusted to 100 µg/mL total phenolic content. Variation among water extracts is significant at P< 0.05 by ANOVA. Variation among ethanol extracts is significant at P<0.05 by ANOVA.

**ABTS Inhibition**

In all developmental stages ethanol extracts of blueberry fruits had higher ABTS inhibitory activity than water extracts. Green fruit ethanol extracts had higher ABTS inhibition than corresponding green/pink and ripe fruit extracts (Fig 5.4). When the total phenolic content was adjusted to 50 µg/mL, green fruit extracts had the highest DPPH inhibitory activity in both water and ethanol extracts. Green/pink fruit extracts had the lowest activity in water and ethanol extracts (Fig 5.5).
Figure 5.4. ABTS radical inhibition activity of water and ethanol extracts of different developmental stages of blueberry fruit @ 30x dilution. Variation among ethanol extracts is significant at P<0.05 by ANOVA.
Figure 5.5. ABTS radical inhibition activity of water and ethanol extracts of different developmental stages of blueberry fruit adjusted to 50 mg/mL total phenolic content. Variation among water extracts is significant at $P<0.05$ by ANOVA. Variation among ethanol extracts is significant at $P<0.05$.

**α-Amylase Inhibition Assay**

Extracts of ripe fruit had the highest α-amylase inhibitory activity followed by green fruit extracts and then green/pink fruit extracts. Water extracts had higher inhibition than ethanol extracts in ripe fruit. Green and green/pink fruit extracts had higher α-amylase inhibition at an adjusted phenolic content of 10 µg/mL than at 50 and 100 µg/mL adjusted phenolic content (Figs 5.6, 5.7).
Figure 5.6. Differences in $\alpha$–amylase inhibition based upon dose dependency in water extracts of different developmental stages of blueberry fruit, (water extracts evaluated @ 10, 50, and 100 $\mu$g/mL adjusted phenolic content). Variation among water extracts with adjusted phenolic content is significant at $P<0.05$ by ANOVA.
Figure 5.7. Differences in $\alpha$-amylase inhibition based upon dose dependency in ethanol extracts of different developmental stages of blueberry fruit, (water extracts evaluated @ 10, 50, and 100 $\mu$g/mL adjusted phenolic content). Variation among water extracts with adjusted phenolic content is significant at $P<0.05$ by ANOVA.

$\alpha$-Glucosidase Inhibition Assay

Inhibition of $\alpha$-glucosidase was higher in ethanol extracts than in water extracts. Ethanol extracts of green fruit based on total phenolic content had the highest measured inhibitory activity against $\alpha$-glucosidase (Figs 5.8, 5.9). Among water extracts, ripe fruit had the highest inhibitory activity, followed by green/pink fruit extracts and green fruit extracts respectively (Fig 5.8). Ripe
fruit extracts had the highest inhibition of $\alpha$-glucosidase based upon a dose dependent response for both water and ethanol extracts (Figs 5.8, 5.9).

Figure 5.8. Differences in $\alpha$-glucosidase inhibition based upon dose dependency in water extracts of different developmental stages of blueberry fruit, (water extracts evaluated @ 10, 50, and 100 $\mu$g/mL adjusted phenolic content and TP-total phenolic content). Variation among water extracts with adjusted phenolic content is significant at $P< 0.05$ by ANOVA.
Figure 5.9. Differences in α-glucosidase inhibition based upon dose dependency in ethanol extracts of different developmental stages of blueberry fruit, (water extracts evaluated @ 10, 50, and 100 µg/mL adjusted phenolic content and TP-total phenolic content). Variation among water extracts with adjusted phenolic content is significant at P< 0.05 by ANOVA.

**ACE–I Inhibition Assay**

Blueberry fruit extracts (water and ethanol) from different developmental stages exhibited no ACE–I inhibition.

**Discussion**

Antioxidant activity measured by free radical scavenging is not based solely upon total soluble phenolic content. When antioxidant activity is evaluated based upon DPPH radical scavenging, ethanol extracts of all three fruit
development stages had similar inhibitory activity, even though green fruit extracts had a total soluble phenolic content almost twice as high as the green/pink and ripe fruit extracts (Fig 5.1, 5.2). Antioxidant activity was similar for green and ripe fruit when total soluble phenolic content was adjusted to 100 µg/mL (Fig 5.3). Antioxidant activity of strawberry fruit measured by DPPH assay had similar results with no significant difference in antioxidant activity between green, pink, and ripe strawberry fruit, even though pink fruit had the highest phenolic content (de O. Pineli et al. 2011). Likewise in apples, the total soluble phenolic content decreased as fruit ripened from green to ripe however, antioxidant activity remained unchanged (Kondo et al. 2002). This maintenance of antioxidant activity, at the three stages of fruit development, may be due to the overall specific composition of the phenolic profile or the presence of a minimum concentration of phenolics sufficient to sustain the level of antioxidant activity (Kondo et al. 2002).

Antioxidant activity, as measured by the free radical scavenging of the ABTS assay, is highest overall in green fruit, which had the highest phenolic content as well (Fig 5.4). However, at 50 µg/mL adjusted phenolic content, green fruit extracts also had higher antioxidant activity than green/pink and ripe fruit extracts (Fig 5.5). Results showed significant positive correlation between total phenolic content and total antioxidant activity in blueberry extracts. The change in phenolic compounds, as fruit develops from green to ripe, could result in a phenolic profile for green fruit that is more relevant to
the ABTS inhibition assay than that of ripe fruit. Chlorogenic acid was identified as the main phenolic contributor to the high antioxidant activity in blueberry and cranberry fruit as measured by the ORAC assay (Prior et al. 2001). Chun et al (2003) reported that in plum cultivars, anthocyanins had the highest overall antioxidant capacity followed by chlorogenic acid and then quercetin in ABTS assays. However, the major antioxidant activity was attributed to chlorogenic acid due to its high concentration in plum fruit (Chun et al. 2003). In this study, the levels of chlorogenic acid were significantly higher in green fruit than in green/pink or ripe fruit. Quercetin levels were also higher in green fruit (Table 5.1). Specific phenolic acids like chlorogenic acid may be contributing to the higher antioxidant activity in green fruit. Wang et al. (2012) also found significant cultivar variations in phenolic content and antioxidant activity of peel and flesh in 33 blueberry cultivars. The change in phenolic compounds, as fruit develops from green to ripe, could result in a phenolic profile for green fruit that is more sensitive to the ABTS inhibition assay than that of ripe fruit.

Blueberry fruit extracts had moderate α-amylase inhibitory activity and the ripe fruit extracts had the highest overall inhibition (Figs 5.6, 5.7). The increased α-amylase inhibitory activity in ripe fruit extracts as compared to green and green/pink fruit extracts may be due to specific phenolic compounds and not linked to the total soluble phenolic content. Previous research with strawberry and red raspberry fruit extracts suggested that α-
amylase inhibitory activity in these berries could be due to high ellagitannin content (McDougall et al. 2005). However, ellagitannins could not solely account for the α-amylase inhibitory activity of raspberry fruit in later studies (Grussu et al. 2011). Increased levels of anthocyanins do not always link to increased α-amylase inhibitory activity. Dark colored fruits such as black currants and black raspberries had higher anthocyanin levels when compared to lighter colored red currants and red raspberries (Wu et al. 2006). Extracts of red and yellow raspberry fruit had higher inhibition of α-amylase than extracts of black raspberry fruit while red currants were more effective inhibitors than black currants (Cheplick et al. 2007, da Silva Pinto et al. 2010).

Green fruit extracts with α-amylase inhibitory activity intermediate between ripe and green/pink fruit, may have particular specific phenolic compounds that are more effective at α-amylase inhibition than those present in green/pink and ripe fruit. Chlorogenic acid and its derivatives are known inhibitors of α-amylases. In this study, HPLC analysis detected chlorogenic acid in green fruit extracts to be over twice as high as the detectable levels in both green/pink and ripe fruit (Table 5.1). Blueberry ripening results in a shift of phenolics toward anthocyanin synthesis and a concurrent decrease in other phenolic compounds, including hydroxycinnamic acids and flavonols (Castrejon et al. 2008, Kalt et al. 2003). Therefore, α-amylase inhibitory activity in ripe fruit could be due to specific proanthocyanidins and anthocyanins which were not detectable in green blueberry fruit, while
enzyme inhibitory activity in green fruit was due to a phenolic profile that changes as fruit ripening advances.

When α-glucosidase inhibition was measured based upon the total soluble phenolic content of the fruit extracts, ethanol extracts of green fruit had the highest inhibition of α-glucosidase (Figs 5.8, 5.9). However, enzyme inhibition by water extracts based upon total soluble phenolic content result in ripe fruit extracts having the highest inhibitory activity and green fruit extracts having the lowest α-glucosidase inhibitory activity (Fig 5.8). Phenolic content in ethanol extracts, therefore, is more significant in the inhibition of α-glucosidase than in water extracts. α-Glucosidase inhibition by water extracts, however, may be the result of several factors, including phenolic content as well as sugar analogs which increase with fruit ripening. Glycosylation of flavonoids results in their increased water solubility and therefore, may make them more available in water extracts than other compounds (Hostel, 1981).

Cyanidin and related glycosides are effective inhibitors of α-glucosidase. In raspberry, cyanidin-diglucoside along with other anthocyanins were effective α-glucosidase inhibitors (Zhang et al. 2010). Cyanidin-3-glucoside was a more effective α-glucosidase inhibitor than cyanidin and this may be due to structural differences between the two compounds (Akkarachiyasit et al. 2010).

Extracts of ripe blueberry fruit had the best overall α-glucosidase inhibitory activity based upon dose dependent response study (Figs 5.8, 5.9).
Anthocyanins and procyanidins are well-known inhibitors of α-glucosidase. These phenolic compounds increase dramatically as fruit develops from green to ripe. Previous research has shown that specific anthocyanins may be more effective at α-glucosidase inhibition than simply overall anthocyanin content. Fruit extracts containing higher levels of acylated anthocyanins appear to be more efficient inhibitors than deacylated ones (Matsui et al. 2001a, b). α-Glucosidase inhibition by raspberry fruit extracts was better for a purple raspberry variety than the lighter colored red varieties. However, yellow raspberry fruit had better inhibitory activity than red raspberry fruit (Cheplick et al. 2007). Blueberry proanthocyanidins had higher α-glucosidase inhibitory activity than blueberry anthocyanins (Johnson et al. 2011). Ellagic acid and catechin were identified as active inhibitors of α-glucosidase along with several specific anthocyanins in ripe raspberry fruit (Zhang et al. 2010). Therefore, inhibition of α-glucosidase may be influenced more by specific phenolic compounds rather than the actual amount of the overall plant phenolics. High α-glucosidase inhibitory activity was observed in blueberry peel and flesh in correlation with higher phenolic content and high antioxidant activity (Wang et al. 2012).

Blueberry fruit extracts did not exhibit any ACE-I inhibitory activity. This is in agreement with McAnulty et al. (2005), where daily intake of blueberry fruit for 3 weeks did not have any effect on ACE–I inhibition or blood pressure. However, it is not clear how in vitro assays may correlate to in vivo
Regulation of post-prandial hyperglycemia is a crucial part of type 2 diabetes management. Reducing glucose release from the final steps of starch breakdown and thereby reducing absorption in the intestines by inhibition of $\alpha$-glucosidases is a key component of this management. Synthetic inhibitors are very effective at inhibiting these enzymes and regulating glucose absorption. However, dietary regulation through everyday foods is potentially an efficient and cost-effective tool for managing type 2 diabetes in an ever expanding global population of at risk individuals especially in poor communities. The moderate $\alpha$-amylase inhibition coupled to high $\alpha$-glucosidase inhibitory activity, and good antioxidant potential of the blueberry fruit extracts, are a desirable profile for a food design that can contribute to a balanced diet to potentially manage type 2 diabetes. High $\alpha$-amylase inhibitory activity can result in physical discomfort due to an excess of undigested starch in the intestines. Therefore, ripe blueberry fruit with a potential for moderate $\alpha$-amylase inhibitory activity coupled to high $\alpha$-glucosidase inhibitory activity would be a positive addition to a diet targeted towards the management of type 2 diabetes without the negative effects of excessive $\alpha$-amylase inhibition.

Type 2 diabetes is commonly linked to cardiovascular complications such as hypertension as well as such oxidative stress-linked micro vascular
conditions such as macular degeneration and kidney failure. Blueberry fruit with good free radical linked antioxidant activity has the potential to help counter the effects of oxidative cellular damage. Previous research on blueberry consumption showed a significant reduction in lipid hydroperoxides among chronic smokers who consumed a daily diet of blueberries (McAnulty et al. 2005). Research suggests that plasma levels of lipid hydroperoxides increase after consumption of oxidized lipids and antioxidant ingestion can counter the resulting postprandial oxidative stress (Wisweidel et al. 2004).

Attenuation of insulin resistance was observed in high fat-fed mice with whole blueberry powder diet (DeFuria et al. 2009). Blueberry anthocyanins can modulate stress signaling pathways and have potential to counter oxidative stress through anti-inflammatory mechanisms (DeFuria et al. 2009).

Conclusion

This study indicates that the ripened stage of blueberry fruit has potential for diet-based management of hyperglycemia and associated oxidative complications, especially in the early stage of disease development. Moderate a-amylase inhibition, in conjunction with high α-glucosidase inhibition found in ripe blueberry fruit would be beneficial additions to a dietary approach for management of type 2 diabetes. This in vitro screening study provides a biochemical rationale and sound strategy to develop the right
blueberry cultivar and stage of fruit development for further validation in animal and clinical studies.
CHAPTER 6

IMPROVED RESILIENCE AND METABOLIC RESPONSE OF
TRANSPLANTED BLACKBERRY PLUGS USING CHITOSAN
OLIGOSACCHARIDE ELICITOR TREATMENT

Abstract

Transplanting of micro-propagated blackberry plugs in the field during late summer and early fall is a common nursery practice for commercial blackberry production. During this field transition, blackberry plants generally experience different stresses in addition to transplanting shock coupled with sudden exposure to low night temperature in the fall. Improving stress resilience of field transplanted blackberry plants by recruiting plant endogenous protective metabolic responses has significant merit. Therefore, the aim of this study was to improve stress resilience of newly transplanted “Chester Thornless” blackberry in the field during fall transition through stimulation of phenolic antioxidant and proline-linked metabolic responses by using bioprocessed chitosan oligosaccharide (COS) as an elicitor treatment. Fourteen weeks old blackberry plugs were transplanted in the field in late July and COS was sprayed weekly to run-off for a 6 week period after transplanting. Total soluble phenolic content, total antioxidant activity, total
proline content, proline-dehydrogenase (PDH), and succinate
dehydrogenase (SDH) enzyme activity of blackberry shoots were evaluated
weekly during COS application. Improvement in total soluble phenolic content
and antioxidant activity based on ABTS free radical scavenging assay was
observed in field transplanted blackberry at 6 and 7 weeks with COS
elicitation treatment.

**Introduction**

Blackberry (*Rubus spp.*) is an important edible berry crop in the family *Rosaceae*. Due to its growing popularity among consumers commercial blackberry production is increasing rapidly in the United States and around the world. Overall, the current market value of the blackberry production in the United States is more than $50 million, while the value of imported fresh blackberries is around $207 million (AMRC 2015; NASS 2015). North America (USA, Mexico, and Canada) is the leading producer of blackberry in the world and its production is expected to increase significantly in the future to satisfy the growing consumer demand of blackberry either as a fresh berry or as a processed and canned product (Kaume *et al*. 2012). The sustainability and improvement of blackberry production in the future will largely depend on the development of new stress resilient cultivars along with improvement in production practices and post-harvest preservation strategies. Commercial blackberry cultivars are mostly classified according to their growth habits as erect, semi-erect, and trailing types and also as thorny and thornless cultivars (Clark and Finn 2011). As a temperate fruit crop, blackberry is generally tolerant to cold temperature, however semi-erect thornless cultivars are mostly cold sensitive. Among thornless semi-erect blackberry cultivars, “Chester Thornless” is the most stress-resilient cultivar with significant cold tolerance, and also resistant to cane blight (Weber 2013). However, at critical growth stages and during seasonal transitions (especially from late
summer to autumn) blackberry cultivars including “Chester Thornless” are exposed to different abiotic and biotic stresses that are variable and unpredictable. Further, cultivation practices such as harrowing, transplanting, and pruning can mechanically damage root or shoot tissues and subsequent wound exposure can make plants more susceptible to other external stresses (Close et al. 2005).

Developing field-ready blackberry plugs in the greenhouse from micropropagation is a common nursery practice, and this strategy is now being widely used for commercial and home-grown blackberry production (Najaf-Abadi and Hamidoghli 2009). After growing in the greenhouse, blackberry plugs are commonly transplanted in the field during late summer and early fall. During this transplanting process, blackberry plants generally experience some degree of transplanting shock (Demchak 2009). At this re-establishment phase, transplanting shock coupled with other environmental factors such as seasonal transition (late summer) potentially affect the growth, survival, and fitness of blackberry crop in the field. Therefore, improving resilience of the blackberry plant during this transplanting phase by recruiting the plant’s protective defense responses is important, especially to ensure better and subsequent improvement in vigor, fitness, and productivity for the coming fruiting year.

In general, plants’ adaptive response against environmental and other external stresses including transplanting shock coupled with seasonal
transition, potentially involves several physiological, metabolic, and structural adjustments (Kozlowski and Pallardy 2002). Biosynthesis of secondary metabolites, such as phenolic bioactives in plants under stress is part of an important metabolic response and also associated with other critical metabolic regulations, including partition and allocation of carbon between different catabolic and anabolic needs and redox-linked cellular energy balance (Shetty 1997; Shetty and Wahlqvist 2004). Such redox- linked metabolic regulation involving the protective function of phenolics is important to counter higher concentrations of reactive oxygen species (ROS) in the cells and to mitigate stress- induced oxidative breakdowns, which have significant relevance for improving establishment and recovery of plants after transplanting shock in the field (Choudhury et al. 2013). Phenolic metabolites with high antioxidant potentials can neutralize ROS or can induce endogenous antioxidant enzyme response and thus can help plants to withstand stress-induced oxidative pressure associated with transplanting shock (Rice-Evans et al. 1997; Shetty 1997; Shalaby and Horwitz 2014)

Further, protective function of phenolics and antioxidant enzymes is also associated with other metabolic responses of plants, such as higher accumulation of proline in the cytosol and its potential role as an active metabolic regulator in the mitochondria to support energy (ATP) synthesis under stress (Hare et al. 1999; Shetty and Wahlqvist 2004; Ben Rejeb et al. 2014).
Proline is the most abundant amino acid in plant cells and significantly accumulates far beyond cellular protein needs under abiotic and other external stresses (Hare et al. 1999). The protective function of proline under such stresses was earlier considered as an osmolyte to maintain solute balance in the cytosol and to protect cellular membrane (Kavi Kishor and Sreenivasulu 2014). However, the potential role of proline as an active metabolic regulator under external stresses in plants was not investigated and not explained until recently (Hare et al. 1999; Shetty and Wahlqvist 2004). In the cytosol, proline is synthesized from glutamate by a series of reduction reactions (Rayapati and Stewart 1991). During the respiration process, oxidation reactions produce hydride ions, which help reduction of pyrroline-5-carboxylate (P5C) to proline in the cytosol (Rayapati and Stewart 1991; Hare et al. 1999; Shetty and Wahlqvist 2004). The accumulated proline in the cytosol could then enter into the mitochondria and can act as a reducing equivalent by donating electron instead of NADH to support oxidative phosphorylation for energy (ATP) synthesis (Hare et al. 1999; Shetty and Wahlqvist 2004). This energy generating metabolic role of proline potentially helps plants to generate ATP by supporting oxidative phosphorylation without driving the energy expensive TCA/Krebs cycle and NADH dependent respiration under induced stress conditions such as transplanting shock and seasonal transition (Hare et al. 1999; Shetty and Wahlqvist 2004). Several redox-linked metabolic strategies have been developed to improve
resilience of plants against external stresses by recruiting protective metabolic functions of proline and phenolic antioxidants (Shetty and Wahlqvist 2004; Sarkar and Shetty 2014). One such innovative metabolic strategy is the use of natural elicitors as seed, foliar, or root zone treatments to up-regulate endogenous protective defense related pathways in plants to counter external stresses including transplanting shock.

Chitosan oligosaccharide (COS) as a natural bioprocessed elicitor has shown diverse functions including improvement of biotic and abiotic stress resilience in plant (Agrawal et al. 2002; Fan et al. 2010; Sarkar et al. 2010). Being a compound made from fungal cell walls or equivalent from marine sources, COS can trigger critical host adaptive protective responses in plants at very low doses without causing harmful effects (Kim and Rajapakse 2005; El Hadrami et al. 2010). Bioprocessed soluble COS as seed or foliar treatments has shown effective protection against different fungal pathogens, microbes, cold stress and can potentially stimulate secondary metabolite synthesis in plants for improving both biotic and abiotic stress tolerances (Khan et al. 2003; Prapagdee et al. 2007; Sarkar et al. 2010). Therefore use of COS as a natural elicitor to improve resilience of blackberry plugs in the field during transplanting and seasonal transition has significant merit.

The objectives of this study were to evaluate the impact and efficacy of COS as an elicitor treatment to improve resilience of newly transplanted
“Chester Thornless” blackberry plugs during seasonal transition from late summer through early autumn after field transplanting. Further phenolic antioxidant and proline- linked metabolic responses were evaluated to determine the COS induced improvement in critical endogenous defense responses in newly transplanted blackberry plugs.

Materials and Methods

Plant Material and Chitosan Oligosaccharide (COS) Treatments

Micro-propagated blackberry plants, var. “Chester Thornless” (*Rubus fruticosus* ‘Chester’), were planted into plug trays (cell size 1.5” x 2.75”) filled with a soilless peat-based potting mix in the greenhouse (in Whatley, MA-01093, USA) on April 23, 2007. The size of the blackberry plug was 3.8 cm x 7 cm with top growth range between 7.5 to 10 cm. A modified method from Broome and Zimmerman, (1978) was used for the micro-propagation of blackberry. For greenhouse study, 14 weeks after planting, blackberry plants (var. “Chester Thornless”) were sprayed (mostly adaxial layer was exposed to spray) weekly with soluble bioprocessed COS (4 g/ L of water) from August 4th to September 16th. Chitosan oligosaccharide (COS-C) with 400~2,000 molecular weight and 80 mesh size (95%) (derived from shells of marine crustaceans) cross linked with ascorbic acid, obtained from Kong Poong Bio (Jeju, South Korea). For field experiment 14 weeks old blackberry plugs (var. “Chester Thornless”) were transplanted out to a nursery field on
July 27\textsuperscript{th} and sprayed weekly with soluble bioprocessed COS (4 g/ L of water), from August 4\textsuperscript{th} to September 16\textsuperscript{th}. The plants were sprayed to run off using a hand-held pressure sprayer (Solo 450 series hand held sprayer) with 15 psi approximate pressure. Control plants were sprayed with water. The average mean temperature was 20\textdegree C and total rainfall was 6.96 cm during the time of the experiment. However, field plants were also irrigated as needed. The experiment was carried out both in the greenhouse and in the field to evaluate the impact of transplanting in the field by comparing with the greenhouse grown plugs. Blackberry shoot sample were collected from both greenhouse and field experiment just before and one week after each COS application (from July 31\textsuperscript{st} to September 23\textsuperscript{rd}). Blackberry leaf sample was collected just one day before the COS spray application. Biological replicates representing individual plants were used in triplicates and leaf samples were collected randomly from the individual plants of the respective treatment block. Newest fully expanded leaf just below shoot tip was collected and immediately extracted following grinding on the same day (within 3 hours). From the randomly mixed leaf sample 200 mg and 50 mg of leaf sample were separated for the extraction.

**Total Phenolics Assay**

The total soluble phenolic content of blackberry shoot samples was determined by an assay modified from Shetty et al. (1995). A quantity of 50
mg (fresh weight-FW) blackberry leaf tissue was immersed in 2.5 mL of 95% ethanol and kept in the freezer for 48-h. After 48-h, the sample was homogenized and centrifuged at 12,225 g for 10 min. Then 0.5 mL of sample supernatant was diluted with 0.5 mL of distilled water and transferred into a test tube with 1 mL of 95% ethanol and 5 mL of distilled water. Then 0.5 ml of 50% (v/v) Folin-Ciocalteau reagent (Sigma-Aldrich, St Louis, MO, USA) was added to the test tube, mixed and left to sit for 5 mins. After that 1mL of 5% Na₂CO₃ was added and mixed and incubate for 60 mins in the dark. Absorbance was read at 725nm. Standard curves were created using increasing concentrations of gallic acid in 95% ethanol. Absorbance values were converted to total soluble phenolic content and expressed as μg equivalents of gallic acid per gram fresh weight (FW) of shoot sample.

**Antioxidant Activity Assay (DPPH inhibition)**

The same extraction from the total soluble phenolic content assay was used to determine antioxidant activity by DPPH free radical scavenging assay (Kwon et al. 2009). To 1.25 mL of 60 μM DPPH (2,2- Diphenyl-1-picrylhydrazyl, 1898-66-4, Sigma-Aldrich, St. Louis, MO, USA) in ethanol, 0.250 mL of shoot culture extract in 95% ethanol was added, the decrease in absorbance was monitored at 517 nm until a constant reading was obtained. The readings were compared with the controls, which contained 0.250 mL of 95 % ethanol instead of the extract. The % inhibition was
calculated by:

\[
\text{% inhibition} = \left( \frac{A_{517}^{\text{control}} - A_{517}^{\text{sample}}}{A_{517}^{\text{control}}} \right) \times 100
\]

**Antioxidant Activity Assay (ABTS inhibition)**

Total antioxidant activity was also measured using the ABTS radical cation decolorization assay and with 10 times dilution of blackberry leaf sample (Re et al. 1999). ABTS radical cation was prepared by mixing 5 ml of 7 mM ABTS (2,2-azinobis(3- ethylbenzothiazoline-6-sulfonic acid), 30931-67-0, Sigma-Aldrich, St. Louis, MO, USA) solution with 88 μL of 140 mM K2S2O4 solution. This mixture is then stored in the dark at room temperature for 12 – 16 hours before using. Prior to the assay, this mixture is diluted with ethanol at an approximate ratio of 1:88 (ABTS : ethanol) and adjusted to yield an absorbance at 734nm of 0.70 + 0.02. A volume of 1 mL ABTS was added to 50 μL of extract and the mixture was vortexed for 30s. The sample was incubated at room temperature for 2.5 minutes and then the absorbance was measured at 734 nm. The antioxidant activity of the extracts was expressed as % inhibition of ABTS radical formation and was calculated using the following formula:

\[
\text{% inhibition} = \left( \frac{A_{734}^{\text{control}} - A_{734}^{\text{sample}}}{A_{734}^{\text{control}}} \right) \times 100
\]

**Enzyme Extraction**

A cold pestle and motor was used to thoroughly grind 100 mg of the
blackberry leaf tissue in 2 mL of cold enzyme extraction buffer [0.5 % polyvinylpyrrolidone (PVP), 3 mM EDTA. 0.1 M potassium phosphate buffer (prepared by mixing monobasic- to dibasic- with pH 7.5]. The sample was then centrifuged at 12,000 x g for 15 min at 2-5°C and stored on ice. The supernatant was used for further biochemical analysis.

**Total Protein Assay**

Protein content was measured by the method of Bradford assay (Bradford 1976). The dye reagent concentrate (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was diluted 1:4 with distilled water. A volume of 5 mL of diluted dye reagent was added to 100 μL of the leaf tissue extract. After vortexing and incubating for 5 min, the absorbance was measured at 595 nm against a 5 mL reagent blank and 100 μL buffer solutions using a UV-VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY).

**Analysis of Proline**

The proline content of the blackberry leaf tissue was determined using high performance liquid chromatography (HPLC) method (Sarkar et al. 2009). The HPLC analysis was performed using an Agilent 1100 liquid chromatograph equipped with a diode array detector (DAD 1100). The analytical column was reverse phase Nucleosil C18, 250 nm x 4.6 mm with a
packing material of 5 μm particle size and at temperature 24°C. The leaf tissue extracts were eluted out in an isocratic manner with a mobile phase consisting of 20 mM phosphoric acid (pH 2.5 by phosphoric acid) at a flow rate of 1 mL min\(^{-1}\) and detected at 210 nm. L-Proline (Sigma chemicals, St. Louis, MO) dissolved in the 20 mM potassium phosphate solution was used to calibrate the standard curve (Kwon et al. 2009). The amount of proline in the sample was reported as mg of proline per milliliter and converted to μg mg\(^{-1}\) FW.

**Proline Dehydrogenase (PDH) Assay**

A modified method described by Costilow and Cooper (1978) was used to assay the activity of proline dehydrogenase. The enzyme reaction mixture containing 100 mM sodium carbonate buffer (pH 10.3), 20 mM L-proline solution and 10 mM NAD (β-nicotinamide adenine dinucleotide hydrate, 53-84-9, Sigma-Aldrich, St. Louis, MO, USA) was used. To 1 mL of this reaction mixture, 200 μL of extracted enzyme sample was added. The increase in absorbance was measured at 340 nm for 3 min, at 32 °C. The absorbance was recorded at zero time and then after 3 min. In this spectrophotometric assay, one unit of enzyme activity is equal to the amount causing an increase in absorbance of 0.01 per min at 340 nm (1.0 cm light path).
Succinate Dehydrogenase (SDH) Assay

A modified method described by (Bregman 1987) was used to assay the activity of succinate dehydrogenase. The tissue extract suspension was diluted with 2.0 mL of enzyme extraction buffer. The enzyme sample was then assayed at room temperature for succinate dehydrogenase activity. The assay mixture consisted of the following: 1.0 mL of 0.4 M potassium phosphate buffer (pH 7.2); 40 μL of 0.15 M sodium succinate (pH 7.0); 40 μL of 0.2 M sodium azide; and 10 mL of 6.0 mg/mL DCPIP (Dichlorophenolindophenol). This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 600 nm wavelength. To 1.0 mL of this mixture, 200 μL of the enzyme sample was added. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction coefficient of DCPIP (19.1 mM\(^{-1}\) cm\(^{-1}\)).

Statistical Analysis

All biochemical analysis was performed in triplicates. Means, standard errors and standard deviations were calculated using Microsoft Excel 2010. Analysis of variance (ANOVA) for the data was performed using the Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC). Based on the initial ANOVA results, significant differences in phenolics, proline, antioxidant, and enzyme (PDH and SDH) activities between elicitor treatments (water and COS) were determined using the Tukey’s least mean square test at a
confidence level of 95% (p < 0.05) separately for all time points (week 1 through week 7).

Results and discussion

Total Soluble Phenolic Content

Exposure to environmental stress or wounding of tissues potentially results in an increase in oxidative stress, which subsequently leads to redox-linked metabolic breakdown in plant cells (Walter et al. 2013). Therefore, restoring redox homeostasis is essential for post-stress recovery and to determine overall fitness and resilience of plants against biotic and abiotic stresses (Kapoor et al. 2015). Such redox-linked metabolic response associated with the stress-recovery process potentially involves stimulation of phenolic antioxidants and up-regulation of other critical protective defense related anabolic pathways such as gateway pentose phosphate pathway (PPP) (Shetty and Wahlqvist 2004). Therefore, the soluble phenolic content of plant tissues is an effective bio-marker to understand the overall redox-linked metabolic responses of plant under external stresses such as transplanting shock coupled with seasonal transition. Based on this scientific rationale, the total soluble phenolic content of leaf and shoot tissues from newly transplanted blackberry plugs was evaluated before and after spraying with a bioprocessed natural elicitor (COS). Shoot samples of blackberry plugs both from the field and the greenhouse were evaluated and compared to
determine the potential impact of COS elicitor treatment for improving stress resilience after transplanting in the field.

Overall, both in the greenhouse and in the field, total soluble phenolic content of blackberry shoots increased steadily with spraying and highest total soluble phenolic content was observed after 3 weeks of spraying (irrespective of the spraying treatments-control and COS) (Figure 6.1A & 6.1B). Further, from 3 weeks to 6 weeks after transplanting, higher total soluble phenolic content was observed in the field grown blackberry when compared with the greenhouse grown blackberry plugs (not-transplanted). Transplanting shock along with changes in macro-and micro-climate in the field potentially resulted in higher total soluble phenolic content in transplanted blackberry. Such metabolic response involving stimulation of biosynthesis of protective secondary metabolites might have relevance for improving resilience and fitness of blackberry plants after transplanting. Not only increased concentration of phenolics, but also the subsequent polymerization of phenolics potentially have significant relevance for structural adjustments, which are essential for improving stress resilience during recovery and re-establishment phase after transplanting (Randhir et al. 2002; Shetty and Wahlqvist 2004). Alteration in secondary metabolism and increased concentration of related terpenoids after transplanting was previously reported in pine seedlings (Sallas et al. 1999).
Figure 6.1A. Total soluble phenolic content (μg/mg fresh weight) of blackberry shoots from the field during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). Different alphabets indicate significant differences in phenolic content between elicitor treatments for each time point at p < 0.05.
In this study, under greenhouse conditions, higher total soluble phenolic content of blackberry shoots was observed with COS elicitor treatment after 4 and 5 weeks (p ≤ 0.05) of spraying, while after 6 weeks no significant differences in phenolic content between treatments (control & COS) was observed. However in the field, COS elicitor treatment resulted in significantly higher (p ≤ 0.05) total soluble phenolic content in transplanted blackberry plugs at 4, 6, and 7 weeks. Higher phenolic content in transplanted blackberry after COS spraying has potential relevance for using COS as a
natural elicitor to stimulate phenolic biosynthesis and to subsequently improve resilience against abiotic and biotic stresses after transplanting.

Improved resilience against abiotic and biotic stresses through up-regulation of phenolic biosynthesis was observed previously with COS application (Khan et al. 2003; Prapagdee et al. 2007; Sarkar et al. 2010). Therefore, application of COS as a run-off or foliar treatment to improve resilience of blackberry after transplanting, especially during the seasonal transition, has significant merit. However, this novel elicitation strategy with COS has to be further evaluated with other blackberry cultivars and in different hardiness zones of the United States in the future.

**Total Antioxidant Activity**

The stress and wounding response of plants, in part, also involves other enzymatic and non-enzymatic antioxidant mobilization. These antioxidant-linked metabolic responses eventually help plants to mitigate the stress-induced breakdown of redox-homeostasis (Foyer and Noctor 2005). Therefore, antioxidant activity is an important indicator to understand overall stress response of a plant and its potential resilience against external stresses such as transplanting shock coupled with seasonal transition. Total antioxidant activity of blackberry plugs with and without COS elicitor treatments after transplanting were evaluated using two different free radical scavenging assays (12,12-diphenyl-21-picrylhydrazyl-DPPH & 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)- ABTS). Overall, very high
antioxidant activity (70-90% inhibition) was observed in blackberry shoots both from the greenhouse and from the field. No significant differences in total antioxidant activity based upon DPPH free radical scavenging assay was observed between spraying treatments and between growing conditions (transplanted in field and greenhouse) (Figure 6.2A & 6.2B). Even when phenolic content was adjusted to 50µg/mL, no significant differences in total antioxidant activity (from week 2 through week 7) between treatments were observed with DPPH free radical scavenging assay (Figure 6.3A & 6.3B). A high baseline value of DPPH % inhibition might have contributed to not observing any significant further increases and differences between spraying treatments or with transplanting shock in “Chester Thornless” blackberry.
Figure 6.2A. Total antioxidant activity (DPPH % inhibition based on total phenolic content) of blackberry shoots from the field during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). No significant differences were observed in DPPH based antioxidant activity between treatments.
Figure 6.2B. Total antioxidant activity (DPPH % inhibition based on total phenolic content) of blackberry shoots from the greenhouse during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). No significant differences were observed in DPPH based antioxidant activity between treatments.
Figure 6.3A. Total antioxidant activity (DPPH % inhibition based on the phenolic content adjusted to 50 μg/mL) of blackberry shoots from the field (3A) during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). No significant differences was observed in DPPH based antioxidant activity between treatments.
Figure 6.3B. Total antioxidant activity (DPPH % inhibition based on the phenolic content adjusted to 50 μg/mL) of blackberry shoots from the greenhouse during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). No significant differences was observed in DPPH based antioxidant activity between treatments.

However, significant differences (p < 0.05) in total antioxidant activity of blackberry shoots among spray treatments and growing conditions were observed with the ABTS free radical scavenging assay (Figure 4A & 4B) (from week 2 through week 7). Total antioxidant activity based on the total phenolic content and measured by the ABTS free radical scavenging assay was highest at week 4 (after 3 weeks of spraying) for both control and COS treated blackberry plugs. Such high total antioxidant activity also coincided
with an increase in total phenolic content of blackberry shoots with both spraying treatments. Based on the ABTS free radical scavenging assay, higher total antioxidant activity was also observed in field transplanted blackberry plugs when compared with the greenhouse grown blackberry plugs (based on total phenolic content and when phenolic content was adjusted to 20 μg/mL) (Figures 6.4A, 6.4B, 6.5A, & 6.5B). In field transplanted blackberry, COS elicitor treatment resulted in higher (p < 0.05) total antioxidant activity (ABTS % inhibition) at 4, 5, 6, and 7 weeks when compared to the control. There was a decline in antioxidant activity for control plants from week 5 through week 6 whereas, COS treated blackberry exhibited a sustained antioxidant response from week 4 through week 7. Higher antioxidant activity and improved cold tolerance in creeping bentgrass (Agrostis spp.) after COS foliar application was also observed previously (Sarkar et al. 2010). Improvement in total antioxidant activity also positively correlated with total soluble phenolic content after COS spraying, and might have significant relevance for improving stress resilience in blackberry after field transplanting and especially during seasonal transition of fall, when day and night temperature vary significantly. The differences between the DPPH and ABTS based assays are potentially linked to the type of phenolics associated with radical scavenging and where water soluble phenolics are more responsive in the ABTS assay (Re et al. 1999).
Figure 6.4A. Total antioxidant activity (ABTS % inhibition based on total phenolic content) of blackberry shoots from the field during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). Different alphabets indicate significant differences in ABTS based antioxidant activity between elicitor treatments for each time point at p < 0.05.
Figure 6.4B. Total antioxidant activity (ABTS % inhibition based on total phenolic content) of blackberry shoots from the greenhouse during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). Different alphabets indicate significant differences in ABTS based antioxidant activity between elicitor treatments for each time point at $p < 0.05$. 
Figure 6.5A. Total antioxidant activity (ABTS % inhibition based on the phenolic content adjusted to 20 μg/mL) of blackberry shoots from the field during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). Different alphabets indicate significant differences in ABTS based antioxidant activity between elicitor treatments for each time point at p < 0.05.
In general, blackberry plants continue to grow during fall (September and October), as long as temperatures do not drop below freezing. However, as canes elongate, begin to trail, and reach the soil, the shoot tips will stop growing and begin to root. The onset of root initiation could trigger an increase in phenolic content and antioxidant activity to counter higher levels of rooting and transplanting shock-induced oxidative stress. Weekly applications of COS to field grown “Chester Thornless” blackberry plants could have stimulated the stress response to increase phenolic biosynthesis.
and associated antioxidant activity to counter such oxidative pressure. On the contrary, in the greenhouse, blackberry plugs continue to grow until the temperatures are lowered to slow and eventually cease shoot growth. The shoot tips generally do not root in the greenhouse and therefore do not need to stimulate stress related metabolic responses including phenolic-linked antioxidant response. Therefore increased phenolic content along with increased antioxidant activity in field transplanted blackberry after COS treatment would have more relevance for improving resilience of blackberry during seasonal transition of fall. Such metabolic response involving phenolic and associated antioxidant activity can potentially help blackberry to recover from overall stress and to improve vigor and fitness after transplanting shock in the field.

**Total Proline Content and Proline Dehydrogenase (PDH) Activity**

In general under biotic and abiotic stress, stimulation of proline synthesis in the cytosol is a common stress-induced metabolic response of plants (Fabro *et al.* 2004; Claussen, 2005). This higher accumulation of proline, either as an osmolyte in the cytosol or as a potential reducing equivalent instead of the NADH in the mitochondria for energy synthesis (ATP) is, in part, a plant endogenous defense response to counter stress-induced metabolic breakdowns and protect against subsequent damage of cellular organelles (Hare *et al.* 1999; Shetty and Wahlqvist 2004). Further, synthesis of proline in the cytosol is also associated with the gateway pentose
phosphate pathway (PPP), which potentially drives carbon flux towards phenolic biosynthesis via shikimate and phenylpropanoid pathways (Shetty and Wahlqvist 2004). Therefore, the phenolic-linked antioxidant enzyme response of a plant under stress is also potentially associated with the synthesis of proline (Shetty and Wahlqvist 2004). Based on this scientific rationale, we hypothesized that COS elicitor treatment would stimulate proline synthesis in the cytosol which would then drive carbon flux towards phenolic biosynthesis through up-regulation of gateway PPP in field transplanted blackberry plants (Shetty and Wahlqvist 2004). Further, the subsequent increase in oxidation of proline in the mitochondria with the help of proline dehydrogenase (PDH) would eventually help to conserve energy by replacing NADH to support oxidative phosphorylation of respiration, which is an energy expensive process and not an ideal metabolic response during stress-recovery (Hare et al. 1999; Shetty and Wahlqvist 2004).

In this study, field grown “Chester Thornless” blackberry plugs had high levels of proline throughout the 8 week period irrespective of the spraying treatments (Figure 6.6A). Even in the greenhouse proline content of blackberry plugs remained steady from week 1 (August 1) through week 7 (September 23) (Figure 6.6B). Just after transplanting, slightly higher proline content was observed in field grown blackberry plugs when compared with the greenhouse grown blackberry plugs. This result potentially indicates a slight stimulation of proline synthesis in blackberry to counter transplanting
shock in the field. However, COS spraying treatment did not result in any further stimulation of proline in the blackberry shoots when compared with the control. Higher baseline proline content may have contributed for not finding any significant differences between treatments both in the greenhouse and in the field. No proline was detected in blackberry plants from the greenhouse with COS application at week 4. Although increased accumulation of proline may indicate a positive response to a stressful condition in a plant, maintaining a constant level of proline may also be indicative of a plant’s adaptation to stress. Proline metabolism at a sustained high level with its link to gateway PPP will drive alternative energy production and biosynthesis of secondary metabolites, which is potentially essential for the maintenance of cellular redox balance after transplanting shock especially during seasonal transition.
Figure 6.6A. Total proline content (μg/mg fresh weight) of blackberry shoots from the field during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). No significant differences were observed in proline content between treatments.
Figure 6.6B. Total proline content (μg/mg fresh weight) of blackberry shoots from the greenhouse during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). No significant differences were observed in proline content between treatments.

While proline content remained steady, the PDH activity of the blackberry plugs varied significantly during the 8 week period (Figure 6.7A & 6.7B). In the field transplanted blackberry plugs, the highest PDH activity was observed at week 5 (September 8) for both control and COS treated plants. Proline content did not correlate with the PDH activity for COS treated blackberry plants. However in the greenhouse PDH activity was highest at week 5 (September 8) for control plants and at week 4 (September 1) for COS treated plants (p <0.05). Significant variations and higher PDH activity at week
4 and 5 potentially indicate oxidation of proline in the mitochondria. However, changes in PDH activity did not correlate with the proline content of the blackberry plugs. The fluctuations of PDH activity might be due to variations in the environmental conditions (especially night temperature) and response of the blackberry plants to the changing environment. Additionally, the oxidation of proline in the mitochondria at specific stages and its relevant metabolic flux during transition and recovery of the blackberry plants might have resulted in these wide variations in PDH activity. Therefore, the proline-linked metabolic response of field transplanted “Chester Thornless” blackberry plants after COS spraying is not conclusive in this study and needs further evaluation with other blackberry cultivars and under different stress conditions.
Figure 6.7A. Proline dehydrogenase (PDH) activity (Units/mg protein) of blackberry shoots from the field during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). Different alphabets indicate significant differences in PDH activity between elicitor treatments for each time point at p < 0.05.
Figure 6.7B. Proline dehydrogenase (PDH) activity (Units/mg protein) of blackberry shoots from the greenhouse during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). Different alphabets indicate significant differences in PDH activity between elicitor treatments for each time point at p ≤ 0.05.

**Succinate Dehydrogenase (SDH) Activity**

Succinate dehydrogenase is an important enzyme of the Tricarboxylic Acid Cycle-TCA/Kreb’s cycle and activity of SDH enzyme can indicate the rate of the energy expending catabolic respiration process and active growth in plants (Cooley and Vermaas 2001). Overall, similar to the total soluble phenolic content, SDH activity of blackberry shoots both from the greenhouse and the field increased steadily from week 1 to week 4 after initiation of the
spraying treatment (Figure 6.8A & 6.8B). This result suggests a potential increase in respiration rate and active growth in “Chester Thornless” blackberry during this seasonal transition and with spraying. There is a usual reduction in SDH activity with decreasing temperatures and cold acclimated plants generally exhibit lower SDH activity than actively growing plants (Atkin and Tjoelker 2003). In field transplanted “Chester Thornless” blackberry, COS elicitor treatment resulted in significantly higher SDH activity (p < 0.05) when compared to the control at week 7, suggesting a potential stimulation of the TCA cycle and respiration rate. However, unlike the COS treated blackberry, SDH activity of the control plants began to decline at week 7 which indicated a reduction in active growth with lowering night temperature. Higher SDH activity in field transplanted blackberry after COS elicitor treatment suggest a continuation of active growth and potential resistance against rooting and winter dormancy. Similarly, in the greenhouse grown blackberry plugs, SDH activity remained steady at week 4 through week 8 suggesting a continuation of active growth with higher respiration rate. Higher rates of respiration in blackberry plugs during this seasonal transition, especially with COS elicitation treatment, might be due to the increasing demand for energy (ATP) synthesis through oxidative phosphorylation, which has significant relevance for different metabolic adjustments to support active growth during seasonal transition of fall.
Figure 6.8A. Succinate dehydrogenase (SDH) activity (nmol/mg protein) of blackberry shoots from the field during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). Different alphabets indicate significant differences in SDH activity between elicitor treatments for each time point at p < 0.05.
Figure 6.8B. Succinate dehydrogenase (SDH) activity (nmol/mg protein) of blackberry shoots from the greenhouse during 6 weeks of spraying with chitosan oligosaccharide (COS) and water (Control). Different alphabets indicate significant differences in SDH activity between elicitor treatments for each time point at p < 0.05.

**Conclusion**

Blackberry is a widely cultivated small fruit crop, which continues to grow during the shortening days of autumn. Transplanting of micro-propagated blackberry plugs in the field during late summer and early fall is a common cultivation practice which potentially exposes blackberry plant to several external stresses including transplanting shock coupled with rapid variations in day and night temperature. Therefore, improving resilience of
field-transplanted blackberry plugs during seasonal transition is essential for recovery and better establishment of blackberry plants prior to the winter. Although this is a one year study, a novel elicitation strategy with soluble bioprocessed COS was used to improve resilience of field transplanted blackberry through stimulation of phenolic antioxidant-linked metabolic response, which is part of the stress-induced adaptive response of plants. This metabolic rationale has merit for further deeper study. Higher phenolic content and total antioxidant activity based on ABTS free radical scavenging assay was observed in field transplanted blackberry with COS elicitation treatment at 6 and 7 weeks after transplanting. Similar to the soluble phenolic content, higher SDH activity was also observed with COS elicitation treatment at week 7. The proline content and PDH activity of blackberry after transplanting varied significantly and its potential role to induce stress resilience was not conclusive in this study. Overall results suggest that COS elicitation strategy might have relevance to improve resilience of field transplanted blackberry through stimulation of phenolic biosynthesis and mitochondrial energy (ATP) synthesis by up-regulation of TCA cycle driven respiration process. However, other primocane and florican blackberry cultivars with COS elicitation treatments must be evaluated in the future to further prove this metabolically driven concept, especially under different abiotic and biotic stresses and with a multi-year study. Further, metabolic responses involving gateway pentose phosphate,
and phenylpropanoid pathway and activity of individual antioxidant enzymes such as superoxide dismutase, catalase, guaiacol peroxidase, glutathione synthase, and NADPH oxidase should also be evaluated for better understanding of the adaptive response of blackberry cultivars under stress including transplanting shock coupled with seasonal transition.
CHAPTER 7

CONCLUSION

Berry crops, including strawberry, raspberry, blackberry, and blueberry are small fruit crops that are particularly popular with consumers. Over the years, berries have been bred and selected for many attributes, including flavor, color, post harvest shelf life and disease resistance. Within each berry crop, there are now many different varieties.

Strawberries, raspberries, and blueberries are valued for their human health relevant bioactive properties. They are high in phenolic content that is in part related to their health related antioxidant activities and also provide fiber and they are low in calories.

Different raspberry varieties have different phenolic profiles, with the largest distinction being differences between red, black, purple and yellow varieties. The raspberry varieties that were evaluated in this study showed potential for dietary management of type 2 diabetes using in vitro models based upon moderate α-amylase and good α-glucosidase inhibition, as well as very good ACE-1 inhibition. However, there were significant differences in enzyme inhibition between varieties. A purple-fruited raspberry had high α-amylase and high α-glucosidase inhibition and would therefore, be less desirable than some of the other red varieties exhibiting high α-glucosidase
but lower $\alpha$-amylase inhibition. Excessive $\alpha$-amylase inhibition can lead to undigested starch and individual metabolic discomfort. The yellow fruited raspberry varieties were the most effective at ACE-1 inhibition, despite lower phenolic content and antioxidant activity. These results suggest that ACE-1 inhibition may be due to specific phenolic compounds or other non-phenolic factors, rather than overall phenolic content.

Different strawberry varieties also have different phenolic profiles. Differences between strawberry varieties were not as pronounced as those between raspberry varieties. The narrow genetic base of commercial strawberry varieties today may be a contributing factor in these results. However, like raspberry fruit, strawberry fruit had moderate $\alpha$-amylase and good $\alpha$-glucosidase inhibition along with ACE-1 inhibition and high antioxidant activity. These findings support the inclusion of strawberry fruit in a recommended dietary plan for individuals at risk for type 2 diabetes.

The phenolic profile of fruit changes as the fruit develops from green to ripe. In evaluating the development of blueberry fruit, green fruit had the highest overall phenolic content and antioxidant activity. Ripe fruit extracts, however, had the highest $\alpha$-amylase and $\alpha$-glucosidase inhibition. These results support related fruit crops research that suggests inhibition of $\alpha$-glucosidase is not dependent upon total phenolic content, but rather appears to be influenced by specific phenolic compounds.
High levels of phenolic compounds benefit the overall health of plants, just as they benefit human health through dietary consumption. A plant’s metabolic response to stress is multi-faceted. One component of the stress response is an increased synthesis of phenolic phytochemicals. These compounds work to counter stress induced oxidative damage, provide structural defenses to withstand biotic and abiotic attacks, and contribute anti-microbial and anti-fungal properties.

Elicitors are compounds used in agriculture to trigger a defense response in plants and resultant increase in plant protectant compounds, such as phenolic metabolites. Chitosan oligosaccharide (COS) is a natural compound derived from marine sources and similar to that found in fungal cell walls. It can initiate a plant defense response at low concentration and not cause plant injury. COS showed potential to be used as an elicitor for improving the transition of transplanted blackberry plugs to a field setting coupled with the seasonal shift from late summer to fall. COS was effective at increasing antioxidant activity in treated plugs relative to untreated control plugs. However, treatment effects on related response activity including proline content and enzyme activity was not conclusive and requires further study.

Evaluating the differences between varieties within a strawberry, raspberry, or blueberry crop contributes to the understanding of how phenolic compounds link to dietary human health benefits. This research supports the
premise that high phenolic content yields good antioxidant benefits. It also adds to past and advances future studies targeting specific phenolic compounds for specific health targeted bioactivity. In addition, with the understanding that these phenolic compounds benefit plant health via many similar and related mechanisms that confer dietary human health benefits, the stimulation of phenolic biosynthetic pathways through COS elicitation has potential to promote plant health as well. Consequently, examining the benefit of plant phenolics to a plant-based diet in conjunction with their value to overall plant health can contribute to an integrated understanding of the importance of plant phenolic compounds for all eukaryotes from plant systems in production agriculture and to their health benefits when part of a healthy diet.
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