Precipitation and temperature at specific growth stages influence soybean health-beneficial compounds concentration

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LIST OF ABBREVIATIONS

× g – Relative Centrifugal Force.

μg – Micro gram

μL – Micro litre

g – Gram

HPLC – High Pressure Liquid Chromatography

L – Litre

mol – Mole

mg – Milligram

mL – Millilitre

T– Temperature

cppt–cumulative precipitation

pptn-precipitation

α-toc–α-tocopherol

β-toc–β-tocopherol

γ-toc–γ-tocopherol

δ-toc–δ-tocopherol

T-toc– total tocopherol

ABSTRACT

Soybean [*Glycine max* (L.) Merr.] is an important source of health-beneficial compounds, including tocopherols, lutein and soyasaponin I. In this study, data from an 11-year field experiment was used to determine the effects of temperature and precipitation during different growth stage intervals on the concentration of these compounds in mature soybean seeds. Tocopherol concentrations in soybean was most influenced by air temperature changes, while lutein concentration was more sensitive to precipitation. Heat stress accumulation greater than 31°C from the mid-vegetative stage to physiological maturity (V3-R7) was significantly correlated (r^2= 0.94) with α-tocopherol concentration. Lutein concentration was negatively correlated with cumulative precipitation (cppt) during seed development to harvest (R5 to R8). Cultivars with higher tocopherol or lutein concentrations were less affected by climatic variables than cultivars with lower concentrations. Although soyasaponin I concentration response to air temperature and precipitation variables were observed at specific growth stage intervals for some cultivars, the responses were complex and cultivar specific. The air temperature of 20°C appeared to be a critical threshold in determining soybean soyasaponin I concentration response to air temperature. This research may be used to improve the production of health beneficial compounds by ensuring the soybean plants are exposed to the favorable climatic conditions during their most responsive stages.

RÉSUMÉ

Le soja [*Glycine max* (L.) Merr.] est une source importante de composés bénéfiques pour la santé, notamment des tocophérols, de la lutéine et de la soya-saponine I. Dans cette étude, les données d’une expérience terrain de 11 ans ont été utilisées pour déterminer les effets de la température et des précipitations pendant différents stades de croissance sur la teneur de ces composés dans des grains mature de soja. Les concentrations de tocophérol dans le soja sont plus influencées par les changements de température de l'air, tandis que la concentration de lutéine est plus sensible aux précipitations. L'accumulation de degrés de températures supérieures à 31 ° C depuis le stade mi-végétatif jusqu'à la maturité physiologique (V3-R7) était significativement corrélée (r^2 = 0,94) à la concentration en α-tocophérol. La concentration de lutéine était négativement corrélée avec la précipitation cumulative moyenne (cppt) pendant entre les stades R5 et R8. Les cultivars avec des concentrations plus élevées de tocophérol ou de lutéine étaient généralement moins affectés par les variables climatiques que les cultivars avec des concentrations plus faibles. Bien que la réponse de la concentration de soya-saponine I à la température de l'air et aux variables de précipitations ait été observée à des intervalles de stade de croissance spécifiques pour certains cultivars, les réponses étaient complexes et dépendaient selon le cultivar. La température de 20 ° C semble être un seuil critique dans la détermination de la réponse à la température de l'air de la concentration en soya- saponine I du soja en. Cette recherche peut être utilisée pour améliorer la production de composés bénéfiques pour la santé en s'assurant que les plants de soja sont exposés aux conditions climatiques favorables au cours de leurs phases les plus sensibles.

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CONTRIBUTION OF AUTHORS

This thesis has been written in the form of manuscripts. This research was designed by this candidate in cooperation with Dr. Philippe Seguin, thesis supervisor and Dr. Malcolm Morrison, co-supervisor. The candidate conducted the laboratory work [extractions, High Performance Liquid Chromatography (HPLC) analyses, etc.] for chapter 4, analyzed the data for all chapters, wrote both manuscripts and the thesis under the supervision of Dr. Philippe Seguin.

The first manuscript (Chapter 3) was co-authored by the candidate, Dr. Philippe Seguin (Department of Plant Science, McGill University), Dr. Malcolm Morrison, (Agriculture and Agri-Food Canada), and Ms. Shimin Fan (Department of Plant Science, McGill University). The candidate was the primary author of the manuscript, performed all data analysis, compiled and analyzed the results. Dr. Malcolm Morrison conducted the field experiments, and provided the samples for analysis. Dr. Philippe Seguin provided the funds and assistance for the research, including supervisory guidance and the reviewing of the manuscript. Ms. Shimin Fan extracted samples and analyzed samples with HPLC. Dr. Philippe Seguin and Dr. Malcolm Morrison also reviewed and edited several versions of the manuscript.

The second manuscript (Chapter 4) was co-authored by the candidate, Dr. Philippe Seguin (Department of Plant Science, McGill University), Dr. Malcolm Morrison (Agriculture and Agri-Food Canada), Dr. Elise Smedbol (Department of Plant Science, McGill University). The candidate performed all the laboratory work and data analyses, and was the primary author of the manuscript. Dr. Philippe Seguin provided the funds and assistance for the research, including supervisory guidance and reviewing of the manuscript. Dr. Malcolm Morrison conducted the field experiment and provided the samples for analysis. Dr. Elise Smedbol helped the candidate with the HPLC analyses and also helped for extractions of samples. All authors were involved in editing the manuscript.

CHAPTER 1

GENERAL INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is one of the oldest and most important crops known to humans. It is one of the ancient crops, which was introduced to the Western society two hundred years ago (Hymowitz and Harlan, 1983; Hymowitz and Shurtleff, 2005). Soybean is an important source of oil and protein and it is thus a major food and feed source. Soy-based food products are also believed to have health-promoting properties. As a good source of biologically active secondary metabolites, soybean has gained a considerable place in the nutraceutical market in recent years.

Tocopherols play an important role in preventing several chronic diseases, such as kidney (Tasanarong et al., 2013) and lung diseases (Hanson et al., 2016). Research also suggested tocopherols can promote meat quality when fed to livestock (Cheng et al., 2016). Alpha-tocopherol attracts most interests from scientists due to its greatest antioxidant activity. In plants, α-tocopherol might contribute to the prevention of oxidative damage in plants exposed to drought (Munné-Bosch et al. 1999). It can control the growth and development of plants by regulating the concentration of reactive oxygen species and plant hormones (Munné-Bosch et al. 2002).

Lutein is one of 600 known naturally occurring carotenoids. Its benefits to the human eye have been confirmed by several researchers (Roberts et al., 2009; Panova et al., 2017). Lutein protects macular pigments from oxidative damage induced by light and by the high rate of oxidative metabolism in the eye (Loane et al., 2008). It also has potential benefits for heart (Dwyer et al., 2001) and skin health (Heinrich et al., 2003). In plants, lutein plays an essential role in photosynthesis and it could minimize photooxidative damage to the photosynthetic apparatus.

Soyasaponin B is considered as a potentially bioactive phytochemical which is beneficial for human health (Hubert et al., 2005). It also has potential application in preventing kidney diseases (Philbrick et al., 2003) such as impeding kidney enlargement and cyst growth. In studies related to soyasaponin B in soybean, soyasaponin I is the compound that has attracted the most attention as it is found in the highest concentration relative to other soyasaponin B in mature soybean seeds (Seguin et al., 2014).

Health-beneficial compounds composition and concentration are influenced by numerous factors including genotype, environmental conditions and agronomic practices (Board et al., 1992; Frederick et al., 1998; Yin and Vyn, 2003; Vollmann et al., 2010). Tocopherol concentration is affected by environmental factors including temperature (Britz et al., 2007 and Carrao-Panizzi et al., 2007), water deficiency (Moran et al., 1994), UV-B light (Delong and Steffen, 1997; Malanga et al., 1997), salt (Gosset et al., 1994), and management factors like row spacing and seeding date (Seguin et al., 2010). Lutein concentration is determined by environmental factors including year, seeding date (Lee et al., 2009), and air temperature (Seguin et al., 2011). Soyasaponin B concentration has also been reported to be affected by temperature and rainfall (Seguin et al., 2014; Song et al., 2018).

While several studies have documented the effect of climatic and/or field management factors on the concentration of health-beneficial compounds in soybeans, there are limited field studies that have directly investigated the effects of air temperature and precipitation. Most studies have been conducting in controlled environments or using treatments that indirectly affect these two important climatic factors (e.g., changes in seeding date or field location). In addition, there is limited information available on the most responsive growth stages to environmental variables, such knowledge would help in tailoring recommendations to maximize the concentration of health-beneficial compounds in soybeans.

1.1 Objectives

- Identify the principle climatic factors affecting tocopherols, lutein and soyasaponin I concentrations in soybean seed.
- Develop regression models to predict tocopherols, lutein and soyasaponin I concentrations in mature soybean seeds based on seasonal climate factors.
- Identify the most responsive growth stage intervals (GSI) to climatic factors in terms of tocopherol, lutein and soyasaponin I concentrations.

1.2 Hypotheses

- Temperature and precipitation both affect tocopherols, lutein and soyasaponin I concentrations in soybean seeds.
- Environmental variables during the reproductive stages (R5-R8) have the largest effects on tocopherols, lutein and soyasaponin I concentration in mature soybean seeds.

CHAPTER 2

REVIEW OF LITERATURE

2.1 NUTRACEUTICALS AND FUNCTIONAL FOODS

The word "nutraceutical" is the combination of the words "nutrition" and "pharmaceutical" (Espin et al., 2007). It is often defined as dietary supplements with the potential to provide high and concentrated dosage of health-beneficial compounds. However, the definition of functional food is much looser and there is no explicit boundary between these two terms. Functional foods are similar in appearance to conventional foods but do contain biologically active components with potential health-benefits (Kim et al., 2007). They may be part of a normal diet but can enhance health or reduce the risk of certain diseases. Active ingredients in nutraceuticals and functional foods, which can contribute to people's health, include minerals, vitamins, dietary fiber, antioxidants and/or probiotics (Gul et al., 2016).

Along with the deeper understanding of links between diet and health, mostly driven by an aging population, functional food and nutraceutical markets are growing gradually in Canada. The Canadian nutraceutical market is expected to witness a growth rate of 5.62% during the period of 2019 to 2024. The rising demand can be attributed to the benefits of active ingredients to address health issues, like obesity, cholesterol and diabetes. Apart from that, products that may promote kids' health are becoming increasingly attractive for modern parents, such as omega-3 and DHA-enriched products for brain development.

2.2. SOYBEAN: A BRIEF INTRODUCTION

Soybean is an annual species, as well as a member of the Leguminosae family. As a self-pollinated plant, it has a relatively low natural crossing rate. For soybean, maturity group (MG) zones represent the most adaptable areas for a certain cultivar without implying that

MG-specific cultivars cannot be grown elsewhere. Maturity groups range from 000 to X, which, respectively, refers to very early maturing varieties and latest maturing ones. Besides, two critical determinants of maturity groups are photoperiod and temperature (Boerma et Specht, 2004). Optimum growing conditions are when temperature ranges between 21 to 32 °C. Temperatures below 14 °C will impede flowering while, high temperatures (above 30 °C) may lead to premature termination of growth (Singh, 2010). Although soybean can adapt to a variety of soil conditions, moist alluvial soils with high organic matter levels can best support soybean growth (Singh, 2010). In addition, similar to many other legumes, soybean can establish a symbiotic relationship with the bacterium *Bradyrhizobium japonicum* which can fix atmospheric nitrogen and make it available to the plant (Wilcox, 1987).

Soybean is one of the most important crops worldwide. Due to its wide range of industrial, feed, food, and pharmaceutical applications, the world production and productivity of soybean is increasing. Over the period of 2014-2016, the average global soybean yield was 2679 kg ha^{-1} and the overall production reached 321.49 million t. The United States contributes to more than one third (i.e., 110.35 million t) of the global soybean production, ranking No.1 among soybean-producing countries (FAO, 2018). Originating in East Asia, soybean has played a major role in the diet of many Asian people for thousands of years and is the raw material of numerous traditional Asian foods, including soymilk, tofu, soy sauce, etc. (Liu, 2005).

Today, the majority of soybean production are used as a source of protein and feed for livestock, after oil extraction, which is used as an edible oil source or used in industrial applications (Hartman et al., 2016). As one of the main oilseed crops, soybean accounts for 80% of the total annual consumption of edible oil and fat (Liu, 2004). Despite the aforementioned applications, soybean is still playing an important role in human diets and nutrition since it was domesticated due to its large content of protein and oil – approximately 40 and 20% respectively (Messina, 1999). Modern soy products, such as soy protein concentrate, soy flour/grits and textured soy protein are a low-cost and efficient alternative to animal protein. In addition, several health-beneficial properties of soybean have also increased their use as a food source.

2.3. HEALTH-BENEFICIAL COMPOUNDS IN SOYBEAN AND THEIR FUNCTIONS

Soybean contain a wide range of compounds with putative health benefits including isoflavones, tocopherols, lutein and soyasaponins. Large variations exist in term of concentrations of specific compounds. Perhaps the most studied health beneficial soybean compounds are the isoflavones daidzein, genistein, and glycitein (Figure 2.1). Over the last several decades, isoflavones have generated attention and interest among scientists and clinicians. Large amount of published research has proved the effectiveness of isoflavones in promoting people’s health, although, concerns regarding their safety have emerged in parallel (Messina, 2010). Evaluations of isoflavone safety have been undertaken by many countries and agencies worldwide since more and more people are concerned that consumption of soy infant formula may harm the long-term development of infants (Chen and Rogan, 2004).This project will focus on other compounds, namely tocopherols, lutein, and soyasaponin B, all of which have strong potential commercial value.

2.4. TOCOPHEROLS: SYNTHESIS AND IMPORTANCE

There are four types of tocopherols: α (alpha), β (beta), γ (gamma) and δ (delta), distinguished by the amount and position of methyl groups on the chromanol ring (Figure 2.2).

Tocopherols (α-, β-, γ-, and δ-tocopherol) are natural lipophilic antioxidants with vitamin E biological activity. The synthesis of tocopherol has only been observed in photosynthetic organisms.

In plants, the synthesis pathway of tocopherol begins with the precursor tyrosine, which can be catalysed by the enzyme tyrosine aminotransferase to p-hydroxyphenyl pyruvate (HPP). Homogentisic acid (HGA) is formed from p-hydroxyphenyl pyruvate (HPP) by the enzyme HPP dioxygenase (HPPD) (Norris et al., 1998). In plastids, another pathway originates from isopentenyl diphosphate, with geranylgeranyl diphosphate synthase 1 (*GGPS1*) for the synthesis of geranylgeranyl diphosphate (GGDP; Okada et al., 2000). GGDP is catalysed to form PDP through by GGDP reductase (GGDR; Keller et al., 1998). Then the condensation of HGA and PDP leads to 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ; Collakova and DellaPenna, 2003). MPBQ can be methylated to 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) by MPBQ methyltransferase (MPBQ MT; Shintani et al., 2002). MPBQ and DMPBQ can be catalyzed by tocopherol cyclase (TC) to form δ- and γ-tocopherol, respectively (Porfirova et al., 2002). Last, γ-tocopherol methyltransferase (γ-TMT), catalyzes methylation of γ- and δ-tocopherol to α- and β-tocopherol, respectively (Shintani and DellaPenna, 1998).

Tocopherols have the potential to prevent several chronic diseases, such as kidney (Tasanarong et al., 2013) and lung diseases (Hanson et al., 2016). Besides the putative health properties for humans, research also suggested tocopherols can promote meat quality when fed to livestock (Cheng et al., 2016). Most interests for tocopherol reside in α-tocopherol due to its relatively high antioxidant activity. In plants, Munné-Bosch et al. (1999) proposed that α-tocopherol might contribute to the prevention of oxidative damage in plants exposed to drought. In 2002, these scientists also found that α-tocopherol may affect intracellular signaling in plant cells, either in direct or indirect ways. In the latter case, α-tocopherol can control the growth and development of plants by regulating the concentration of reactive oxygen species and plant hormones (Munné-Bosch and Alegre, 2002).

2.5. LUTEIN: SYNTHESIS AND IMPORTANCE

Lutein is one of 600 known naturally occurring carotenoids which are synthesized within dark green leafy plants and widely considered to have photoprotective as well as antioxidant attributes (Cazzonelli, 2011). In plant, the first precursor of lutein synthesis pathway is isopentenyl pyrophosphate (IPP). First, IPP is isomerized into dimethylallyl pyrophosphate (DMAPP) by IPP isomerase, and then DMAPP is condensed with three molecules of IPP to form GGPP by GGPP synthase (GGPS) (Hirschberg 2001; Park et al. 2002). Two GGPP molecules produce phytoene in a condensation reaction catalyzed by phytoene synthase (PSY). Phytoene desaturase (PDO) catalyzes the dehydrogenation of phytoene and then further dehydrogenated to ζ-carotene. Zeta-carotene is dehydrogenated by ZDS catalysis into neurosporene, and further converts to lycopene (Breitenbach and Sandmann 2005). Lycopene is first catalyzed by lycopene ε-cyclase (LCYE) at one end to form a ε-ring, generating δ-carotene, and then catalyzed by lycopene β-cyclase (LCYB) at the other end to form a β-loop, generating α-carotene (Cunningham and Gantt 2001). Alpha-carotene generates lutein in conjunction with beta-cyclohydroxylase (BCH) and epsilon-hydroxylase (ECH) (Tian et al. 2004).

A large number of studies confirm the benefits of lutein to the human eye. For example, as one of the major causes of blindness in developed countries, age-related maculopathy could be effectively inhibited by sufficient lutein intake. Lutein protects macular pigments from oxidative damage induced by light and by the high rate of oxidative metabolism in the eye (Loane et al., 2008). Some other studies also indicated the potential benefits of lutein on heart (Dwyer et al., 2001) and skin health (Heinrich et al., 2003). Besides the aforementioned properties for human health, as the major carotenoid component in soybean (Figure 2.3), lutein also plays essential role in photosynthesis. When plants absorb excess light, the xanthophyll cycle can dissipate excess light energy in the form of heat and therefore help to minimize photooxidative damage to the photosynthetic apparatus. Without lutein, the light protection pathway will be blocked, and the leaves will be damaged when exposed to strong light. In severe cases, the leaves will die and photosynthesis will not occur (Niyogi et al., 2001).

2.6. SOYASAPONIN I: SYNTHESIS AND IMPORTANCE

Saponins are one of the secondary metabolites in higher plants which take part in the defense response to different stresses (Wina et al., 2005). Saponins in soybean are classified into two major groups (Figure 2.4); soyasaponin A and B. Soyasaponin A, in part, accounts for the undesirable bitter taste in soy food products, while soyasaponin B is reported to have health-beneficial properties (Kudou et al., 1993). Soyasaponin B is considered a potentially bioactive phytochemicals which is beneficial for human health (Hubert et al., 2005). Philbrick et al. (2003) examined the potential application of soyasaponins B in preventing kidney diseases, and found that soyasaponin B can impede kidney enlargement and cyst growth in the mouse model of polycystic kidney disease.

Soyasaponin B include the 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP)- conjugated forms (i.e., αg, βg, βa, γg, and γa) and their corresponding non-DDMP forms (i.e., V, I, II, III, and IV, respectively) (Kudou et al.,1993). Although the DDMP-form predominates in unprocessed soybeans, these forms are more labile and easily degraded to their corresponding non-DDMP forms. In soybean, soyasaponin I concentration represents 68% of the total soyasaponin B (Seguin et al., 2014).

The knowledge about the biosynthesis pathway of soyasaponin I is currently limited. In few studies, soyasaponin I is supposedly biosynthesized by the successive additions of glucuronic acid, galactose, and rhamnose to soyasapogenol B (Shibuya et al., 2010; Wu et al., 2001).

2.7 FACTORS AFFECTING CONCENTRATION OF HEALTH-BENEFICIAL COMPOUNDS IN SOYBEAN.

2.7.1 Genetic Factors

2.7.1.1 Differences Among Cultivars of Soybean for Health-beneficial Compounds Concentrations

The genetic variability of the tocopherol concentration and composition has been extensively researched. In Japan, Ujiie et al., (2005) analyzed more than a thousand cultivated soybean cultivars as well as wild accessions. Compared to the standard tocopherol proportions of α-, γ- and δ-tocopherol in mature seeds of common soybean cultivars (approximately 5%, 65% and 30%, respectively), Kajimoto and Hasabe (1982) identified three varieties (Dobrofeance, Dobruoza 14 Pancevo and Keszthelyi Aproszemu Sarga) with relatively high α-tocopherol proportions and concentrations, of which the largest proportion was about 50% of the total tocopherol concentration. Furthermore, Carrera and Seguin (2016) indicated that in a given environment, the concentration of α-tocopherol among genotypes ranged between 2 and 127 μg g^{-1}. Three genotypes, including Heron, Venus and AC Orford had a consistently high α-tocopherol concentration across different environments, which means there is little environmental difference in tocopherols when they are high.

Compared to tocopherols, the information about genetic variability of lutein and soyasaponins I in soybean is far from abundant. Seguin et al. (2011) conducted a study about the variation and stability of lutein among 20 cultivars of soybeans. Large differences were observed between the 20 genotypes evaluated, with lutein concentrations ranging between 4.1 and 10.9 μg g^{-1}. Although this study focused on the interaction of genetics and the environment on lutein concentration, even in different environments genotypes with consistently high and stable lutein concentrations were identified. According to this study, selection and development of high-lutein cultivars in the future should be feasible.

Hu et al. (2002) determined that among 46 soybean varieties grown in Iowa in 1999, soyasaponin I concentration ranged between 0.05 and 0.62 μmol g^{-1}. Large differences have also been reported by Kim et al., (2006) who identified two Korean cultivars (i.e., Sojinkong and Daepungkong) where soyasaponin I concentration ranged between 0.71 and 0.91μmol g^{-1} in mature seeds.

2.7.1.2 Determinants of health-beneficial compounds concentrations in soybean

Since it is basic scientific knowledge that genotype (G), environment (E), and their interactions (G x E) are all vital determinants of phenotypes, it would be reasonable to assume these three factors will also influence the concentration of health-beneficial compounds in soybean. This field is intriguing because if we can determine the most influential factor or even quantitative confirmation about how much each of these factors contributes to health-beneficial compounds in soybean, it would contribute in determining the best breeding and field management strategies to increase health-beneficial compounds in soybeans.

Studies attempting to determine the importance of G, E, and G x E in determining health-beneficial compounds concentrations in soybeans provided conflicting results. For example, Whent et al. (2009) studied 24 varieties of soybean planted in Maryland, United States, and detected that E exerted greater impacts on tocopherols than the G x E interaction did. Carrera et al., (2014) examined tocopherol concentration differences over 23 environments and found the same conclusion.

Seguin et al., (2014) conducted an experiment about the effects of these three factors on soyasaponins I. This study indicated that the most influential factor is E which accounted for 62% of the total mean square while the G×E interaction only contributed to less than 1%.

In a study conducted in the United States, Lee et al., (2009) studied 15 genotypes planted in four different environments. They focused on three interactions including G × year, G × planting date and G × year × planting date, and all of them had significant impacts on lutein concentration. Although the responses across environments among each genotype were not distinctive, lutein concentration varied significantly across the four growing environments in 14 of the 15 genotypes evaluated. Seguin et al. (2011) studied 20 soybean genotypes and found that genotypes with the highest concentrations often ranked near the top in lutein concentration regardless of environment. They determined that the G, E and the G x E effect contributed to 55%, 29% and 15% of the total sum of squares, respectively.

2.7.2 The effect of environmental factors on soybean health-beneficial compounds

Due to its significant contribution to the variation in health-beneficial compounds observed in soybean as demonstrated in the previous section, the study of environmental factors attracts much attention. For soybean, many experiments were specifically conducted to determine the impact of environmental factors when plants are in their late reproductive stages (Stages R5-R7), since this period is in theory the most sensitive as many metabolites accumulate in seeds during this period (Carrera et Seguin, 2016). Also, agronomic factors such as fertilization and irrigation would also be considered as critical determinants affecting health-beneficial compounds in soybean in the way of having environmental factors changed.

2.7.2.1 Temperature

Air temperature has been reported to be one of the most important environmental factors affecting the concentration of health-beneficial compounds in soybean. Warm conditions are reported to favor tocopherol accumulation in soybean seeds. Britz and Kremer (2002) compared tocopherol concentration from soybean grown in greenhouses at 23 and 28 °C and found that seed from plants grown at 28 °C had greater α-tocopherol and lower δ-tocopherol concentrations. Chennupati et al. (2011) focused on the effects of high-temperature stress imposed at different growth stages of soybeans on the accumulation of tocopherols in seeds. After submitting two different cultivars to either stress conditions (i.e. 33/25 °C day/night T) or control conditions (i.e. 23/15 °C) in growth chambers, an increase of 752% was observed for α-tocopherol concentration under high temperatures.

Lutein, which is found in relatively low concentration in soybean, has been subject to limited studies in terms of its correlation with temperature. Seguin et al. (2011) indicated that lutein concentrations decreased from southern to northern sites, averaging 17.1 $\mu g\ g^{-1}$ in MTL, 9.3 $\mu g\ g^{-1}$ in SMB, and only 6.0 $\mu g\ g^{-1}$ in NOR, the northernmost site. Morrison et al. (2015) found that warmer year contributed to lowest lutein concentration in soybean seeds during the three-year experimental period.

According to Shiraiwa et al. (1991) and Tsukamoto et al. (1993), abiotic factors, such as T, had little influence on soyasaponins I concentration. High temperature stress applied during all growth stages resulted in large reductions in soyasaponins II, III, and IV, and when restricted to seed development and maturation (R5−R8) strongly reduced the concentration of soyasaponins III and IV (Seguin et al., 2014).

2.7.2.2 Precipitation

In Brazil, Carrão-Panizzi et al. (2007) found that lower than normal precipitation during the seed-filling period may have contributed to an increase in α-tocopherol concentration. However, there are limited studies specifically focusing on precipitation effects directly because researchers often rather studied the impact of soil moisture (Britz and Kremer, 2002; Caldwell et al., 2005). It is not difficult to understand because many of the experiments are conducted in greenhouses or growth chambers and soil moisture is the only parameter which can easily be modified whose effects on plants are representative to those of precipitation in natural conditions. As for lutein and soyasaponins I, the focus of related studies usually lies on other factors instead of precipitation or soil moisture such as seedling date or planting area (Lee et al., 2009; Seguin et al., 2014). Obviously, these factors will change precipitation or soil moisture faced by growing plants, but it would make it harder to directly determine the effects of precipitation or soil moisture.

2.7.2.3 Other Factors

In addition to these aforementioned factors, some other aspects such as carbon dioxide levels and agronomic management factors, including fertilization (Seguin et al., 2010; Carrera and Seguin, 2016; Shaw et al.,2016; Wang et al., 2019), seeding date and depth (Seguin et al., 2010; Morrison et al., 2015) may also exert impacts on health-beneficial compounds in soybean. However, further studies should emphasize on better descriptions of environmental effects on soybean health-beneficial compounds. On one hand, we can take more factors into account or take their interactions into account when designing experiments. On the other hand, we should also control variables more rigorously in order to analyze the impact of each factor separately and precisely.

2.8. RATIONALE OF THE PROJECT

Soybeans are becoming an increasingly important source of food for humans, and are of particular interest because of their content of health-beneficial compounds. Studies about factors affecting their concentration in seeds are essential since they could help better meet the needs of the nutraceutical and functional food markets.

As reviewed above, many previous studies have shown that health-beneficial compounds concentration and the type accumulated in seeds can be influenced both by genetic and environmental factors. Even if the development of cultivars with high concentrations of certain targeted compounds is possible, environmental factors still have been reported to have important impact on health-beneficial compounds accumulation in soybeans.

It is essential to determine which specific environmental factors affect the accumulation of health-beneficial compounds in soybeans and determine which developmental stages are most susceptible to these factors. Such understanding could help producers predict the concentration of specific compounds at harvest. Such predictive ability could help better meet the needs of the nutraceutical and functional food markets, which may require specific target concentrations.

Our experiment, under field conditions, will attempt to correlate abiotic factors to the concentration of targeted health-beneficial compounds in soybean. One specific aspect of this experiment is that it will also aim at identifying most responsive growth stage intervals (GSI). The result of our experiment will help us identify the most important abiotic factors as well as most responsive stages of development. This could help producers in achieving specific target concentrations by potentially modifying certain field practices such as planting dates, which for example can be used to expose specific stages of development to certain air temperatures or precipitations. Furthermore, predictive models could also be used to predict concentrations prior harvest.

Hence, in this project, our overall objective is to study the influence of temperature and precipitation during all developmental stages of soybeans. We expect to understand the impact of temperature and precipitation during specific growth stage intervals during plant growth on tocopherol, lutein and soyasaponin I concentrations in mature soybean seeds.

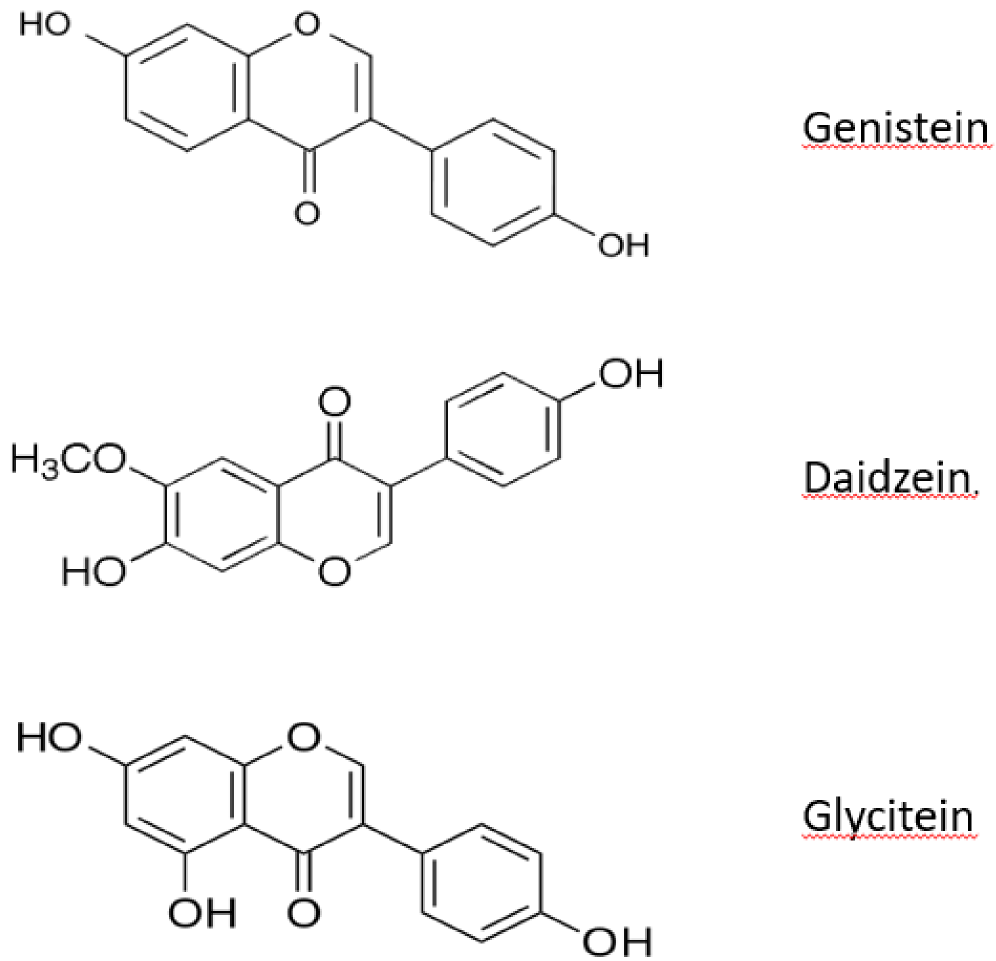


Figure 2.1. Chemical structure of isoflavones found in soybean seeds. Adapted from Masilamani et al., 2012.

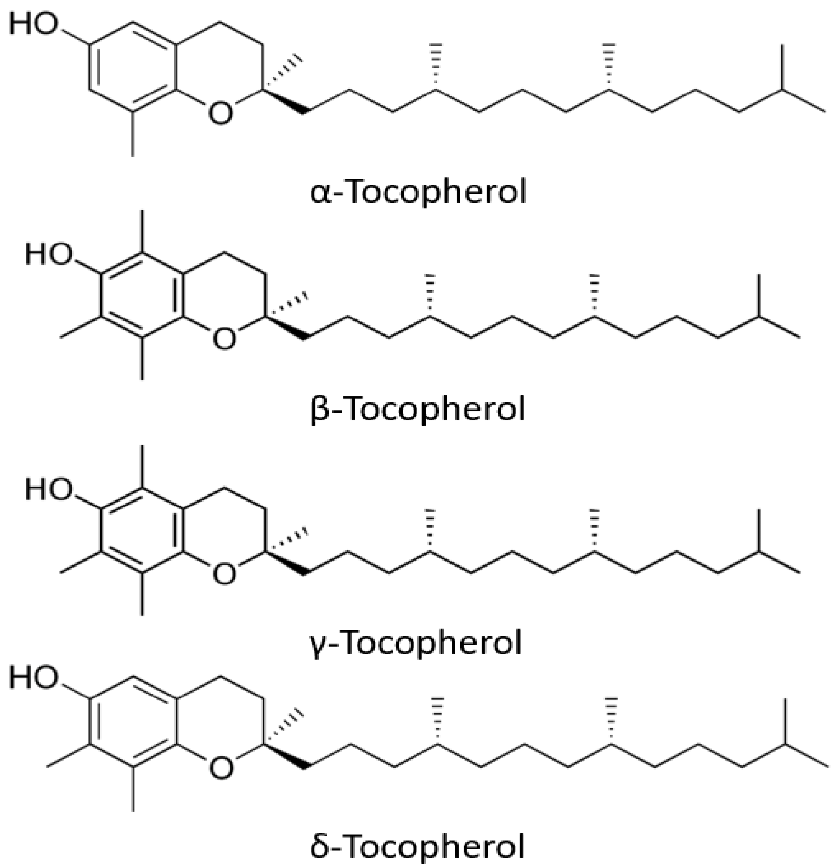


Figure 2.2. Chemical structure of tocopherols found in soybean seeds. Adapted from: Radhakrishnan et al., 2013.

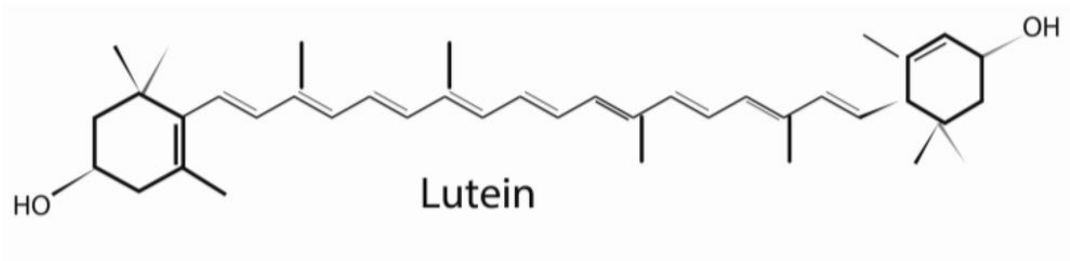
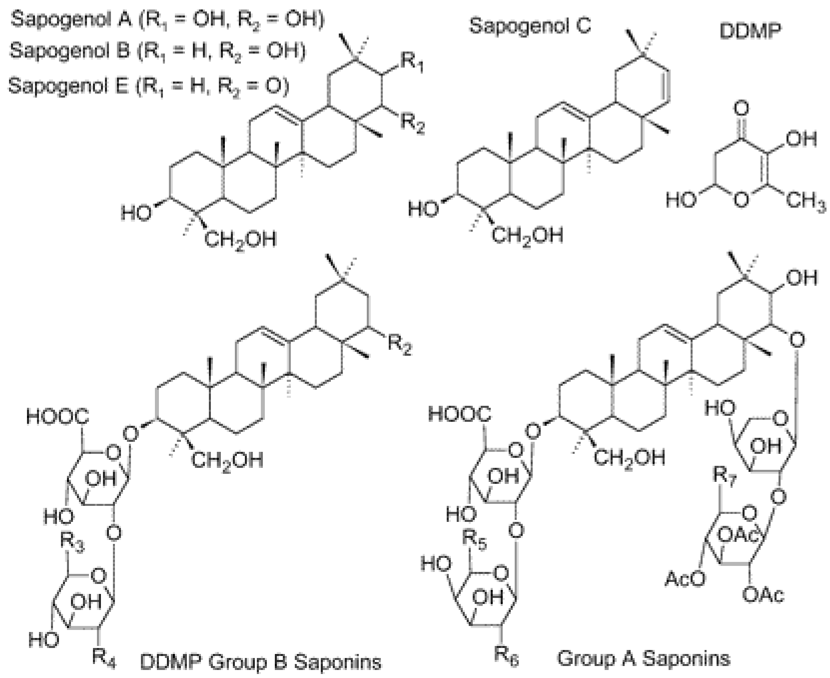


Figure 2.3. Chemical structure of lutein found in soybean seeds. Adapted from: Johnson, 2002



DDMP & Group B saponins		MW	R_2	R_3	R_4
Soyasaponin I	(Bb)	942	OH	CH_2OH	*O*-β-D-Glucose
Soyasaponin II	(Bc)	912	OH	H	*O*-α-L-Rhamnose
Soyasaponin III	(Bb')	796	OH	CH_2OH	OH
Soyasaponin IV	(Bc')	766	OH	H	OH
Soyasaponin V	(Ba)	958	OH	CH_2OH	*O*-α-L-Rhamnose
Soyasaponin	(Be)	940	O	CH_2OH	*O*-β-D-Glucose
Soyasaponin	(Bd)	956	O	CH_2OH	*O*-α-L-Rhamnose
Soyasaponin βg		1068	*O*-DDMP	CH_2OH	*O*-β-D-Glucose
Soyasaponin βa		1038	*O*-DDMP	H	*O*-α-L-Rhamnose
Soyasaponin γg		922	*O*-DDMP	CH_2OH	OH
Soyasaponin γa		892	*O*-DDMP	H	OH
Soyasaponin αg		1084	*O*-DDMP	CH_2OH	*O*-α-L-Rhamnose

Group A acetyl-saponins		MW	R_5	R_6	R_7
Soyasaponin aA_1	(Ab)	1436	CH_2OH	*O*-β-D-Glucose	CH_2OAc
Soyasaponin aA_2	(Af)	1274	CH_2OH	OH	CH_2OAc
Soyasaponin aA_3		1202	H	OH	CH_2OAc
Soyasaponin aA_4	(Aa)	1364	CH_2OH	*O*-β-D-Glucose	H
Soyasaponin aA_5	(Ae)	1202	CH_2OH	OH	H
Soyasaponin aA_6		1172	H	OH	H
Soyasaponin aA_7	(Ac)	1420	CH_2OH	*O*-α-L-Rhamnose	CH_2OAc
Soyasaponin aA_8	(Ad)	1406	H	*O*-β-D-Glucose	CH_2OAc

Figure 2.4. Molecular structure of groups A and B soyasaponins. Adapted from Berhow et al., 2006.

CONNECTING TEXT FOR CHAPTER 3

Soybeans are becoming increasingly important for the nutraceutical and functional food markets due to their high content of health-beneficial compounds. Studies aiming at increasing our understanding of environmental factors affecting their accumulation in soybeans will help better meet the needs of the nutraceutical and functional food markets. Few studies have examined the effects of environmental variables at different growth stage intervals on tocopherol and lutein concentrations in mature soybean seeds. Furthermore, in order to expand research in this area, it is important to quantify environmental variables and verify the correlation between them and tocopherol and lutein concentrations in soybean seeds. Regression models also may be developed to potentially ultimately predict the concentrations of compounds with knowledge of the environmental variables during plant growth.

The following chapter will be submitted to the Journal of Agricultural and Food Chemistry. The manuscript is co-authored by the candidate, Dr. Philippe Seguin and Ms. Shimin Fan, Department of Plant Science, Macdonald Campus of McGill University, as well as Dr. Malcolm Morrison, Agriculture and Agri-Food Canada. The candidate was the primary author of the manuscript, performed all data analysis, compiled and analyzed the results. Dr. Malcolm Morrison conducted the field experiments, and provided the samples for analysis. Dr. Philippe Seguin provided the funds and assistance for the research, including supervisory guidance and the reviewing of the manuscript. Ms. Shimin Fan extracted samples and analyzed samples with HPLC. Dr. Philippe Seguin and Dr. Malcolm Morrison also reviewed and edited several versions of the manuscript.

CHAPTER 3

Precipitation and temperature at specific growth stages influence soybean tocopherol and lutein concentrations

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3.1. ABSTRACT

Soybean [*Glycine max* (L.) Merr.] is an important source of health-beneficial compounds, including tocopherols and lutein. Seeds with elevated concentrations of these health-beneficial compounds may be required to meet the increasing needs of the functional food market. A study was conducted for a period of 11 years in Ottawa, ON, Canada to examine the relationship between temperature and precipitation during specific growth stage intervals (GSI) and the concentration of tocopherol (toc) and lutein in the seeds at harvest. Toc concentrations in soybean were most influenced by air temperature changes, while lutein concentration was more sensitive to precipitation. Heat stress accumulation of temperatures greater than 31°C during the mid-vegetative to physiological maturity GSI was positively correlated (r^2= 0.94) with α-toc concentration. Lutein concentration was negatively correlated (r^2= 0.61) with mean cumulative precipitation (cppt) during the seed development to maturation GSI. Overall, reproductive stages were more responsive to climatic variables than vegetative stages. In addition, the cultivars with higher toc or lutein concentrations were less affected by climatic variables than cultivars with lower concentrations. Climatic factors during specific growth stages may have significant impact on concentrations of health-beneficial compounds in mature seeds and this should be considered in the production of soybean for the functional food market.

KEYWORDS: soybean, abiotic factor, temperature, precipitation, tocopherol, lutein

3.2. INTRODUCTION

Soybean seeds contain several secondary metabolites that have health-beneficial properties. Isoflavones have generated considerable interest, but other compounds including tocopherols (toc) and lutein also have commercial value and potential. Tocopherols are natural lipophilic antioxidants with vitamin E biological activity, which are found widely in higher plants. They were reported to have potential in preventing several chronic diseases, including kidney and lung diseases(Hanson et al., 2016; Tasanarong, Vohakiat, Hutayanon, & Piyayotai, 2013). Tocopherols are classified into four different types: α-, β-, γ- and δ-toc, distinguished by the amount and position of methyl groups on the chromanol ring. While γ-toc represents the greatest proportion of toc in mature soybean seeds(Seguin, Turcotte, Tremblay, Pageau, & Liu, 2009), most interest for toc resides in α-toc as it can preferentially be absorbed and accumulated in humans, and thus has the greatest relative vitamin-E activity(Rigotti, 2007). Globally, soybean is an important source of vitamin E.

As one of the 600 known naturally occurring carotenoids synthesized within plants, lutein is considered to have photoprotective and antioxidant attributes(Cazzonelli, 2011). In humans, lutein protects macular pigments from oxidative damage induced by light and by the high rate of oxidative metabolism in the eye(Loane, Kelliher, Beatty, & Nolan, 2008). Other studies also indicated the potential benefits of lutein for heart and skin health(Dwyer et al., 2001; Heinrich et al., 2003). Although soybean is rarely considered a major lutein intake source for humans, it has been reported to have comparable contribution to some other important lutein sources(Seguin, Tremblay, Pageau, Liu, & Turcotte, 2011), such as sweet corn (*Zea mays* L.) and peas (*Pisum sativum* L.)(Holasova, Dostálová, Fiedlerová,

& Horáček, 2009; Scott & Eldridge, 2005). Along with an increasing demand for nutraceutical and functional foods, an in-depth understanding about factors affecting the accumulation of toc and lutein in soybean is required in order to produce soybeans with high and stable concentrations of targeted health-beneficial compounds.

Genotype, environment, and their interactions (G, E and G x E, respectively) all contribute in determining seed toc and lutein concentrations; although, there are conflicting reports on their relative importance. Dolde, Vlahakis, and Hazebroek (1999) determined from a field experiment conducted at five sites in the American Midwest, that the most important source of total toc (T-toc) variation in soybean could be attributed to G (82%), while the contribution of E and G x E was only 5 and 13%, respectively. In contrast, Carrera, Dardanelli, and Soldini (2014) reported from an experiment conducted in eight environments in Argentina that E contributed to 50% of T-toc concentration, while G and G x E contributed less (11 and 39%, respectively). Whent et al. (2009) in Maryland, USA, reported that G contributed to 79% of the variation in soybean lutein concentration, while E and G x E contributed 18 and 4%, respectively.

Large differences among cultivars have consistently been reported for both toc and lutein concentration. Dwiyanti, Ujiie, Thuy, Yamada, and Kitamura (2007) reported large variation (1.6 to 32.8 μg g^{-1}) in α-toc concentration among a Japanese collection of 490 soybean cultivars and 610 wild soybeans. Seguin et al. (2009) determined that among 20 cultivars evaluated in eastern Canada, α-toc concentration ranged between 9 and 33 μg g^{-1}, its proportion of T-toc concentration varied from 3 to 12%. Large differences in the proportion of the three main toc have been reported by Kajimoto and Hasebe (1982) who identified three varieties (i.e., Dobrofeance, Dobruoza Pancevo, and Keszthelyi

Aproszemu Sarga) where the proportion of α-toc to T-toc was approximately 50%. Large differences were also observed in lutein concentration among 20 soybean cultivars grown in eastern Canada, with concentrations ranging between 4.1 and 10.9 μg g^{-1}. (Seguin et al., 2011)

Specific environmental factors affect toc and lutein concentrations. An experiment conducted in controlled conditions to determine the effects of temperature (T) on toc concentrations reported that soybean that were exposed to high Ts (33°C/25°C, day/night) had much higher α-toc concentration (143 and 128%, in AC Proteina and OAC Champion, respectively) than when exposed to lower Ts (23°C /15°C, day/night).(B. P. Chennupati, 2013) The inverse relationship occurred between T and γ-, as well as δ-toc; high Ts reducing γ- and δ-toc by 34 and 44%, respectively. Interestingly, soybean that were exposed to higher Ts (33°C/25°C, day/night) only during the late reproductive stages (R5 to R8) (Fehr, Caviness, & Vorst, 1977) had α-toc concentrations in mature seeds that were 88% greater compared to plants grown at lower Ts (23°C /15°C, day/night). Such results demonstrated that T at specific growth stages can have a major impact on the accumulation of health beneficial compounds in soybean. Precipitation and soil moisture were also demonstrated to impact the concentration of health beneficial compounds in soybean. In a greenhouse experiment which subjected soybean to well-watered or drought conditions during the seed-filling period, low soil moisture (10−25% soil moisture capacity) resulted in higher seed α-toc but lower δ-toc concentrations. (S. J. Britz & Kremer, 2002)

Studies examining the relationship between lutein concentration and specific environment factors in the field are limited. A field trial conducted in Maryland, USA found that lutein concentration was negatively correlated with air T and positively correlated with precipitation. (Whent et al., 2009) Other studies reported that lutein concentration was affected by seeding date, which resulted in the exposure of soybean plants at specific stages of development to different environmental conditions, including air T and precipitation(Lee et al., 2009; Seguin et al., 2011).

In field trials, it is difficult to establish direct relationships between environmental and climatic factors and the concentration of specific health-beneficial compounds in soybean. However, when experiments are conducted over many years or locations, the degree of association between climatic parameters and the concentration of health beneficial compounds can be investigated. The objectives of this study were to examine the effects of T and precipitation during specific growth stages intervals on toc and lutein concentrations in mature soybean seeds. Specifically, the goal was to determine the most responsive growth stage intervals to specific environmental variables.

3.3. MATERIALS AND METHODS

3.3.1. Field Experiment Description.

A field experiment was conducted at the Central Experiment Farm of Agriculture and Agri-Food Canada in Ottawa, ON, Canada (45°23′N 75°43′W) from 2001 to 2004 and from 2007 to 2013 for a total of 11 years. Three short-season cultivars with contrasting lutein concentration and three with contrasting toc concentrations, were selected from a long-term soybean cultivar evaluation experiment.(Morrison, Cober, Frégeau-Reid, & Seguin, 2015) All cultivars were grown in a randomized complete block design with four replications. Local recommendations for weed control and fertilization were used during the course of experimentation (OMAFRA, 2018). This experiment was part of a larger, long-term research program investigating variation in seed yield and composition among a range of soybean cultivars. Description of specific management practices and site used were previously provided in detail by Morrison et al. (2010) At maturity, the plots were harvested and a 500 g subsample was taken from each plot and stored in plastic bags at 4ºC prior toc and lutein extraction and quantification.

3.3.2. Data Collection and Laboratory Analyses

Approximately 50 g of seeds from each plot were dried and ground in a coffee grinder (Black & Decker, Towson, MD, USA). Tocopherols extraction and quantification were conducted using a modified version of the protocol of Dwiyanti et al. (2007) as described by P. Chennupati, Seguin, and Liu (2011). The extraction and quantification of lutein were conducted following the protocol of Kanamaru, Wang, Abe, Yamada, and Kitamura (2006), with slight modifications as previously described in Seguin et al. (2011).

Climate data including daily maximum (Tmax) and minimum T (Tmin), and precipitation (ppt) were collected from a nearby weather station. From planting to harvest, the phenological development was monitored using the Fehr et al. (1977) growth stage determination system. Seven phenological growth stages were determined: seeding, emergence, three trifoliate leaves, first flower, beginning seed filling, physiological maturity and ripe (i.e., Sd, Ve, V3, R1, R5, R7 and R8, respectively). The duration between two growth stages was regarded as a growth stage interval (GSI). The GSI were not only limited to successive stages, but was also obtained by adding consecutive GSI together, resulting in a total of 21 GSI. The duration of each GSI was then used to determine a range of climate data for each specific GSI. Since each of the six soybean cultivars had different growth rates, the climate parameters for each GSI were distinct for each specific cultivar. The parameter determined included:

Cumulative precipitation (cppt) which was obtained by adding up the daily precipitation for each day in one GSI:

$$Cppt = \sum (\text{daily precipitation})$$

For a given day, average T (T_{avg}) was determined as the average of the Tmax and Tmin:

$$T_{avg} = \frac{Tmax+Tmin}{2}$$

For each GSI, daily average T (DTavg) was determined as the sum of T_{avg} divided by the number of days in the given GSI. Daily average maximum T (DTmax) and minimum T (DTmin) were determined similarly.

$$DTavg = \frac{\sum (Tavg)}{number\ of\ days}$$

In order to identify the T most influential in affecting the accumulation of toc or lutein, the concepts of heat- and cold-stress days were developed (later referred to as HSD and CSD, respectively). A stress day was the summation of T above a baseline T during a GSI (T_h and T_c, for heat- and cold-stress baseline Ts, respectively). For HSD T_h evaluated ranged between 20 and 36°C and for CSD T_c ranged between 5 and 20°C. If Tmax was less than base T_h, the difference was set to 0. Similarly, if base T_c was less than Tmin, the difference was set to 0.

$$HSD=\sum (\text{Tmax} - \text{baseT}h)$$

$$CSD=\sum (\text{baseT}c - \text{Tmin})$$

3.3.3. Statistical Analyses

Correlations between toc or lutein concentrations and GSI climate parameters were determined using SAS (PROC CORR, version 9.3, Cary, NC, USA) with the objective of finding the highest significant correlation coefficient (*r*); only these are presented and discussed. To examine the general trend, correlations with climate parameters were determined for the average response across the three cultivars. Then, because the cultivars were chosen to represent high, medium, and low concentrations of the compounds, individual cultivar correlations provided insight on the effect of initial concentration on the response to the climate parameter and differences in cultivar response. Linear regression between toc or lutein concentrations and climate parameters during each GSI were conducted to examine the linear response of the compound to changes in the climate parameter. The level of significance was set at $P<0.05$ with only significant effects being discussed.

3.4. RESULTS

The wide variation in Tmax and Tmin over the growing seasons observed during the period of experimentation (2001 to 2013) ensured that soybeans were exposed to a wide range of temperatures during each GSI (Figure 3.1a). For example, the largest temperature variation for a given date across the 11 years was 17.3 ℃ on 26 May and the lowest one was 5.7 ℃ on 11 August. Such variation also translated in the specific GSI being exposed to a large range of temperatures. For example, the mean Tave during the seed filling stages (R5-R8) ranged between 17.3 ℃ and 21.4 ℃. Similar variation in the average weekly cppt was also observed during the course of experiment for all GSIs. (Figure 3.1b)

Although there were differences in toc concentrations among years for the three cultivars, their ranking in each year was relatively stable (Figure 3.2). AC Orford consistently had the highest α-toc concentration, the differences between McCall and AC Orford were small, and Comet had the lowest α-toc concentration. Over the 11 years of the experiment the average α-toc concentrations were 27, 23 and 10 μg g^{-1} for AC Orford, McCall and Comet, respectively (Figure 3.2a). The average concentration of γ-toc and T-toc was greater in McCall (265 and 449 μg g^{-1}, respectively than AC Orford (121 and 361 μg g^{-1}, respectively and Comet last (99 and 323 μg g^{-1}, respectively (Figure 3.2c, d). Differences among three cultivars were overall consistent across years. This consistency combined with a lack in any specific trend in concentration across years suggests long-term seed storage at 4°C did not influence compounds concentrations adversely. All of the samples were analyzed at the end of the field experimentation.

Across cultivars, the mean cumulative precipitation (cppt) during the R1-R8 GSI was negatively correlated with α-toc concentration but positively correlated with δ-toc concentration, while there was no significant correlation between cppt and γ-toc or T-toc concentration during any GSI (Table 3.1). As cppt increased between the R1 and R8 stages, α-toc concentration decreased but δ-toc concentration increased, with significant coefficients of determination (r^2) of 0.39 and 0.44, respectively. The regression equations were $y = -0.07x + 32.20$ and $y = 0.27x + 82.50$ (Figure 3.3a, b), respectively. The α-toc concentration decreased by 0.07 ug g^{-1} while δ-toc concentration increased by 0.27 ug g^{-1} for every increase in millimeter of precipitation. When examined for each cultivar separately, α-toc concentration decreased as cppt increased during the Sd-Ve GSI for Comet and during the R1-R8 GSI for McCall, respectively. In addition, for these two cultivars, γ-toc concentration was also negatively correlated with cppt during the R1-R7 GSI for Comet and R7-R8 GSI for McCall. Comet was the only cultivar that had significant correlations between cppt and δ-toc concentration, as well as T-toc concentrations. The δ-toc and T-toc concentrations increased as cppt increased during the R1-R8 and R7-R8 GSI, respectively. In contrast to the other two cultivars, no correlation was significant for any toc during any GSI for AC Orford.

When averaged across the three cultivars, the mean daily average temperature (DTavg) during the R1-R7 and R7-R8 GSI were positively correlated with α- and γ-toc concentrations, respectively (Table 3.1). In contrast, a negative correlation was observed between DTavg during the R5-R7 GSI and δ-toc concentration, while no significant correlation was observed between DTavg at any GSI and T-toc concentration. As the DTave increased during the R1-R7 and R7-R8 GSI, the α-toc and γ-toc concentration increased, respectively, with the coefficients of determination for the linear regression (r^2) being 0.67 and 0.61, respectively. The regression equations were y = 5.62x - 95.15 and y =10.96x + 39.82, respectively (Figure 3.4a, b). The α-toc concentration increased by 5.62 ug g^{-1} and γ-toc concentration increased by 10.96 ug g^{-1} for every 1 C° increase. In contrast, as the DTave during the R5-R7 GSI increased the δ-toc concentration decreased, by 16.29 ug g^{-1} for every 1 C° increase (r^2 = 0.83) and the regression equation was y = -16.29x + 454.81 (Figure 3.4c). On a cultivar basis, significant positive correlations between DTavg during the reproductive stages (R5-R7 and R5-R8, respectively) and α-toc concentration were observed for McCall and AC Orford. The α-toc concentration increased as DTavg increased during the entire growing season (Sd-R8 GSI) for Comet. The γ-toc concentration in Comet and McCall increase as DTavg increased during the R7-R8 and R1-R8 GSIs, respectively.

Identical to the mean of cultivars, as the DTave during the R5-R7 GSI increased the δ-toc concentration decreased for all of the three cultivars and none of them showed significant correlation between T-toc concentration and DTavg for any GSI.

When averaged across cultivars, the concentration of α- and γ-toc were positively correlated with DTmax during the R1-R8 GSI (Table 3.1). As DTmax increased during this GSI, the α- and γ-toc concentration increased with significant r^2 of 0.79 and 0.56, respectively. The regression equations were y = 4.67x -99.87 and y = 10.98x -52.05, respectively (Figure 3.5a, b). When averaged across cultivars, the concentration of α- and γ-toc were positively correlated with DTmax during the R1-R8 GSI. As DTmax increased during the R5-R7 GSI, however, the δ-toc concentration decreased by 12.99 ug g^{-1} for every 1 C° increase (Figure 3.5c). None of the correlation between T-toc concentration and DTmax at any GSI were significant. On a cultivar basis, α-toc concentration increased as DTmax increased during the R1-R7 GSI for McCall and AC Orford, and during the R1-R5 GSI for Comet. For McCall, as for the mean of the three cultivars, γ-toc concentration increased with rise DTmax during the R1-R8 GSI. There was a positive correlation for Comet during late reproductive stages (R7-R8 GSI). In contrast, δ-toc concentration decreased while DTmax during the R5-R8, R1-R7 and R5-R7 GSIs for Comet, McCall and AC Orford increased, respectively. No correlations between DTmax and T-toc concentration were found.

Across cultivars, positive correlations were observed between mean DTmin during the R5-R8 GSI and α-toc concentration, and DTmin during the R7-R8 GSI and γ-toc concentration (Table 3.1), As mean DTmin during the GSI increased, the α-toc and γ-toc increased (r^2 = 0.52 and 0.48, respectively. The regression equations were y = 4.69x - 45.03

and y = 8.12x + 137.04, respectively (Figure 3.6a, b). The α-toc concentration increased by 4.69 ug g^{-1} and γ-toc concentration by 8.12 ug g^{-1} for every 1 C° increase. In contrast, the mean DTmin was negatively correlated with δ-toc concentration during the R5-R7 GSI. As DTmin increased concentration decreased significantly (r^2 = 0.71), by 18.57 ug g^{-1} for every 1 C° increase. The regression equation was y = -18.57x + 397.04 (Figure 3.6c). On an individual cultivar basis, α-toc concentration increased as DTmin increased during the R1-R8 and R5-R8 GSIs, for Comet and McCall, respectively. The γ-toc concentration was also positively correlated with DTmin for these two cultivars (R1-R7 and R7-R8 GSI, respectively). For the three cultivars, as DTmin during the R5-R7 GSI increased, the δ-toc concentration decreased. McCall was the only cultivar that had a significant positive correlation between DTmin (R1-R5 GSI) and T-toc concentration.

Given the strong impact of T at specific GSI, the concepts of heat- and cold-stress days were used (i.e., HSD and CSD, respectively) in order to identify T most affecting the accumulation of health-beneficial compounds of interest. Both consisted in summing all °C above base stress T (T_h and T_c, for heat- and cold-stress, respectively); with T_h evaluated ranging between 20 and 36°C for HSD and T_c ranging between 5 and 20°C for CSD.

Across the cultivars there was a positive correlation between α-toc concentration and HSD at temperatures greater than 31°C during the V3-R7 GSI (Table 3.2). As HSD above 31°C increased during this GSI, the α-toc concentration increased, (r^2 = 0.94 Figure 3.7a). The regression equation was y = 0.59x + 13.12. The largest correlation coefficients were observed during the R1-R7 GSI for Comet and AC Orford with T_h of 27°C and 31°C, respectively. Correlation coefficient was maximal for McCall during the V3-R7 GSI with the T_h of 31°C.

Heat-stress days greater than 22°C, during the R1-R8 GSI was positively correlated with γ-toc concentration across cultivars (Table 3.2). As accumulation of HSD above 22°C increased during this GSI the γ-toc concentration increased. The regression model was y = 0.17x + 183.93 with a r^2 of 0.48 (Figure 3.7b). The response of individual cultivars varied, with no correlation between HSD and γ-toc concentration being observed for AC Orford, and positive correlations during the R7-R8 GSI with a T_h of 22°C for Comet and R1-R8 GSI with a T_h of 25°C for McCall (Table 3.2). For δ- and T-toc concentration, correlations with HSD were all negative, correlation coefficients being maximal for the R5-R7 GSI with a T_h value of 27°C for δ-toc concentration, and Ve-R1 GSI with a T_h value of 21°C for T-toc concentration The regression equations were y = -1.05x + 158.65 and y= -1.14 + 514.72, respectively (Figure 3.7c, d). The response of individual cultivars was highly variable for both δ -toc and T-toc concentration with largest significant correlations being observed at a range of GSI and T_h, which ranged from Ve-R1 GSI to R5-R8 GSI and T_h of 24 to 35°C.

Mean cultivar α-toc concentration was negatively correlated with CSD with T_c less than 15°C during the R5-R8 GSI (Table 3.3). As the CSD increased during the R5-R8 GSI, the α-toc concentration decreased. The regression equation was y = -0.17x + 35.11 with a r^2 of 0.52 (Figure 3.8a). On a cultivar basis, Comet had a significant negative correlation between α-toc concentration and CSD with T_c less than 14°C during the R5-R8 GSI, while McCall also had a significant correlation during the R5-R7 GSI with CSD with a T_c of 17°C, and there was no significant correlation observed for AC Orford.

The concentrations of γ-toc and T-toc were negatively correlated with CSD with the T_c lower than 11 and 12°C, respectively, during the R7-R8 GSI. In contrast, mean δ-toc had its highest significant positive correlation during the R5-R7 GSI with a T_c of 18°C (Table 3.3). The coefficient of determination of the linear regression was 0.67, 0.40 and 0.73 for γ-toc, T-toc and δ-toc concentrations, respectively. The regression equations were y = -2.96x + 258.93, y = -1.89x + 403.42 and y = 0.59x + 55.77, respectively (Figure 3.8b, d, c). Response of individual cultivars was somewhat consistent for γ-toc and δ-toc concentration, with significant correlations occurring at the late reproductive stages in most cases (Table 3.3). On a cultivar basis, only one positive correlation was observed for T-toc concentration in Comet at the Sd-R7 GSI which contrasted with the response across cultivar, being positive.

Lutein is a health-beneficial compound found in relatively low concentration in soybean. The variation among the three cultivars evaluated across years remained consistent. AC Bravor had the highest average lutein concentration (9.78 $\mu g g^{-1}$) followed by Maple Glen (7.62 $\mu g g^{-1}$) and Pagoda (4.29 $\mu g g^{-1}$) (Figure 3.9).

Across cultivars, lutein concentration was most affected by cumulative precipitation (cppt) during the R5-R8 GSI (Table 3.4). As cppt increased, lutein concentration decreased, with an r^2 of 0.61. The regression equation was y=-0.02x+9.22 (Figure 3.10a). Lutein concentration decreased by 0.02 ug g^{-1} each mm increase in cppt. The response of individual cultivars varied significantly. Lutein concentration increased as the cppt during the Ve-V3 GSI increased for Pagoda while the compound concentration decreased as the cppt increased during the R5-R8 GSI for Maple Glen and AC Bravor.

There were, no significant correlations between DTmax and lutein concentration in any GSI (Table 3.4). As DTave and DTmin increased, lutein concentration increased with r^2 of 0.40 and 0.44, respectively. The regression equations were y=0.33x+0.39 and y=0.33x+2.20, respectively (Figure 3.10b, c). Across cultivars, lutein concentration increased as DTave/DTmin increased during the V3 to R1 GSI, by 0.33 ug g^{-1} for every °C increase. When examined for each cultivar individually, only Pagoda showed significant positive correlation for both DTave and DTmax during late reproductive and late vegetative stages, respectively. The response of cultivars to DTmin varied greatly, a positive correlation being observed for Pagoda during the Ve-V3 GSI, a negative one for Maple Glen during early reproductive stages (R1-R5 GSI), while no correlation was significant at any GSI for AC Bravor.

Mean lutein concentration was positively correlated with HSD with a T_h greater than 29°C during the R5-R8 GSI (Table 3.5). As it increased, lutein concentration increased with r^2 of 0.37. The regression equation was y=0.05x+6.52. On a cultivar basis, Pagoda was the only cultivar which had a significant correlation between lutein concentration and HSD with a T_h greater than 28°C during the R5-R8 GSI (Table 3.5). There was no significant correlation between lutein concentration and CSD neither for mean nor for any cultivar in any GSI.

3.5. DISCUSSION

Tocopherol

The response of toc concentration to precipitation was overall smaller than for T variables, with only α- and δ-toc concentrations responding both during the R1-R8 GSI. There was a significant negative correlation between cppt and α-toc concentration (r^2 =0.39), and a positive correlation with δ-toc concentration (r^2 =0.44) (Figure 3.3b). The linear regression demonstrated that each additional 100 mm of precipitation during reproductive stages led to a 23% decline of α-toc concentration in seeds at harvest, but also to a concomitant 33% increase in δ-toc concentration. The responses of cultivars to cppt were variable, AC Orford not responding at all, while the most responsive stages in the other cultivars varied, although in most cases it was restricted to reproductive stages. Our results are in agreement with those from a greenhouse experiment where soybean grown at low soil moisture (10 to 25% of water holding capacity) had higher α-toc than when grown at higher soil moisture (80 to 90% of water holding capacity) (Britz et Kremer, 2002). The authors reported difference among cultivars, but all followed a similar trend, the reverse being observed for δ-toc and γ-toc concentrations. In Brazil, Carrão-Panizzi et Erhan (2007) reported that lower cppt was associated with greater α-toc and T-toc concentrations, while higher cppt was associated with greater δ- and γ-toc concentrations. These results are in agreement with those from our experiment, which demonstrated that, across the three cultivars evaluated, toc concentrations, especially α-toc concentration, were more responsive to pptn during reproductive compared to vegetative stages.

We found that Comet α-toc was only affected by cppt during germination (Sd to Ve). It is difficult to understand how cppt prior emergence could have an influence on toc in mature seeds. Experiments in controlled environments should be conducted to determine the if the response was physiological in nature.

There is overwhelming evidence that warm T during seed development resulted in an increase in α-toc concentration and γ-toc concentration and a decrease in δ-toc concentration. This can be seen by the changes in these compounds with the increase in DTave which was a product of both the increase in DTmax and DTmin. Further research to characterize the T and GSI of importance showed that, across cultivars, HSD greater than 31°C during the V3-R7 GSI was positively correlated with α-toc concentration

(r^2=0.94) while HSD greater than 22°C during the R1-R8 GSI was positively correlated with γ-toc concentration (r^2=0.48). Conversely, δ- and T-toc concentration were negatively correlated with HSD greater than 27°C between stages R5 and R7 (r^2=0.85) and HSD greater than 21°C between Ve and R1 (r^2=0.66), respectively (Figure 3.7).

The linear relationships between HSD and toc could be used to forecast toc concentrations in seeds prior harvest, especially α-toc. Trends were similar for the three cultivars studied, but the response of a greater number of cultivars would need to be evaluated to determine if the response observed to HSD is universal. Previous studies generally reported positive responses of α-toc to higher T during seed development(Steven J. Britz, Kremer, & Kenworthy, 2008), but never identified the specific T and stages of developments to which plants are the most responsive.

The increase in α- and γ-toc concentration in warmer environments could be attributed in part to a stimulus effect of T on the key enzyme *MPBQ methyl transferase* (MPBQ MT) which converts 2-methyl-6-phytyl-1,4-benzoquinone (MPBQ) to 2,3-dimethyl-6-phytyl-1,4-BQ (DMPBQ) (Sattler et al., 2003) (Supplemental figure 3.2). According to the synthesis pathway, the increase in 2,3-dimethyl-6-phytyl-1,4-BQ (DMPBQ) can contribute to higher α- and γ-toc concentration at the same time. This hypothesis could also explain the concurrent decrease in δ-toc concentration caused by high temperature. The role of MPBQ-MT on toc concentration has been studied in other plants (Naqvi et al., 2011; Tang et al., 2016), but not for soybean. Further research is needed to verify this hypothesis because to our knowledge previous studies mainly focused on the role of γ-TMT (Tavva et al., 2007; Dwiyanti et al., 2011) and there is currently limited information on the effect of T on key enzymes and/or the expression of genes involved in toc synthesis.

Correlation between T and T-toc concentration were not significant, which is in agreement with several previous studies, but this is not the case for the response we observed for γ-toc concentration. In our experiment, we observed a positive correlation between T and γ-toc concentration. An experiment conducted by Britz and Kremer (2002) in controlled environments indicated that soybean grown at high T (i.e., 28°C) after flowering led to very little increase in the T-toc concentration and a decrease in γ-toc concentration compared to plants grown at cooler T (i.e., 23°C). In another study, however, γ- or T-toc concentrations were not greatly impacted by T (Chennupati et al., 2011).

The CSD explained less of the variation compared to other variables, especially HSD, for all toc except γ-toc. Concentration increased with accumulated minimum T lower than 11 °C during late reproductive stages (i.e., R7-R8 GSI), the coefficient of determination r^2= 0.67) was the largest observed for γ- toc concentration (Figure 3.8).

Lutein

For lutein, results showed that precipitation was more influential than T. A negative correlation was observed between lutein concentration and cppt during the R5-R8 GSI with a significant r^2 of 0.61 implying that as cppt increased during seed filling, lutein synthesis and/or accumulation decreased.

Mean lutein concentration was positively correlated with DTavg and DTmin during the V3-R1 GSI, with significant r^2 of 0.40 and 0.44, respectively. The response of individual cultivars varied. Positive correlations were observed for Pagoda between lutein concentration and DTavg, DTmax and DTmin during specific GSI, and the lutein concentration of Maple Glen was negatively correlated with DTmin during early reproductive stages. No correlation was observed for AC Bravor. While studies on the influence of the environment on lutein concentration are limited, Whent et al. (2009) reported that soybean lutein concentration was negatively correlated with average daily T (r = -0.24) but positively correlated with precipitation (r = 0.31), which is the opposite to our observations. However, in Whent's experiment, average climate data during soybean growth from seeding to harvest was selected and used for the computation of correlations, they did not look at specific GSI. Thus, comparison between both studies should be done for the Sd-R8 GSI in our experiment. Although the r^2 and general statistics have not been shown in table 4, since the correlation between climatic variables during Sd-R8 GSI and lutein concentration were lower than those observed at other GSI, the results were still the opposite of those of Whent et al. (2009). Tavg, Tmax and Tmin during Sd-R8 GSI were positively correlated with lutein concentration (r = 0.30, r = 0.33, r = 0.22, respectively, $P < 0.05$) and precipitation was negatively correlated (r = -0.21, $P < 0.05$). Since both are field experiments, such contradictory results possibly could be attributed to different experimental sites and the use of different cultivars.

Warm T had a positive effect on lutein concentration, but the effects were only observed before the initiation of flowering. This phenomenon may be possibly due to abiotic factors influencing ε-carotene hydroxylase, the key enzyme in the lutein synthesis pathway. In a review of the literature, we did not find studies examining the effects of T on lutein concentration. In future studies, the impact of abiotic factors on expression of genes involved in lutein synthesis should be investigated to determine how T and cppt may influence lutein concentration in mature soybean seeds.

Across cultivars, lutein concentration was positively correlated with the accumulation of HSD greater than 29°C during the R5-R8 GSI. On a cultivar basis, however, only Pagoda showed this trend while there was no correlation for the other two cultivars. As mentioned before, the positive effects of warm average T on lutein concentration were only observed during V3-R1 GSI. The inconsistency may be possibly because high T stress which is relatively rare in field conditions during reproductive stages would have significant effects on lutein concentration. Meanwhile, daily T far from heat stress during V3-R1 GSI would have more effects on lutein concentration than HSD. There was no significant correlation between lutein concentration and CSD; neither for mean across cultivars nor for any cultivar at any GSI. On the basis of our current results and limited previous experiments, further investigation of the relationship between climate parameters and lutein concentration will be needed, especially in controlled environments to confirm the impact of high temperature.

When contrasting cultivar response for toc concentration, the least responsive to cppt was the high-toc cultivar AC Orford while the most responsive was the low-toc one, Comet. It is possible that lower response of high-toc cultivars illustrates that toc rich cultivars may have reached a physiological maximum level of toc concentration, while low-toc cultivars may have potential to synthesize more once they are exposed to abiotic stimulus. Response to T also followed the same principle; the high-toc cultivar, AC Orford was always the least responsive cultivar to DTavg, DTmin and DTmax, while the low-toc one (i.e., Comet) was the most responsive. Similar observations were also made for lutein, as the high-lutein cultivars, Maple Glen and AC Bravor had negative correlations with cppt during seed filling stages, while the response of Pagoda (a cultivar with lower lutein concentration) was positive. This could possibly reflect that for low lutein cultivars, precipitation stimulates the accumulation of lutein, while for high lutein cultivars, too much precipitation could become an adverse condition since lutein concentration is already near its maximum. There were no significant correlations between T parameters and lutein concentration at any GSI for the high lutein cultivar AC Bravor, but the low lutein cultivar Pagoda was responsive. Experimentation with a larger number of cultivars with contrasting concentrations of health-beneficial compounds will be required to confirm that cultivars with inherently low concentrations are generally more responsive to climatic variables.

3.6. CONCLUSIONS

Tocopherol concentrations in soybean is most influenced by air T changes, while lutein concentration is more sensitive to precipitation. Specifically, accumulated HSD greater than 31°C during the V3-R7 stages influences α-toc concentration in soybean seeds the most, while accumulation of HSD greater than 27°C during the R5-R7 stages is the most influential for δ-toc concentration. Lutein concentration was negatively correlated with cppt during the R5-R8 GSI. Overall, reproductive stages were the most responsive to environment variables compared to vegetative stages, cultivars with low concentrations of toc and lutein also being more responsive. Our results suggest that models could be used to predict toc and lutein concentrations prior harvest. For example, based on the HSD at temperatures greater than 31°C during V3-R7 GSI, farmers could foresee α-toc concentration in soybean seeds with the equation $y = 0.59x + 13.12$ ($r^2 = 0.94$, x: HSD with T_h at 31°C during V3-R7, y = α-toc concentration). Likewise, $y = -1.05x + 158.65$ could potentially be used to predict the δ-toc concentration in soybean seeds with the information of HSD value during R5-R7 GSI with a T_h value of 27°C. Lutein concentration could be potentially predicted with y = -0.02x + 9.22 (x: cumulative precipitation during R5-R8, y = lutein concentration). Trials conducted in controlled environment are needed to confirm the correlations we observed between climatic variables and concentrations of targeted health-beneficial compounds, while molecular and metabolomics studies should be conducted to provide a mechanistic understanding of the responses we observed. Models developed in the present study could become valuable tools for agricultural producers and the industry to produce soybean with high and stable concentrations of health-beneficial compounds.

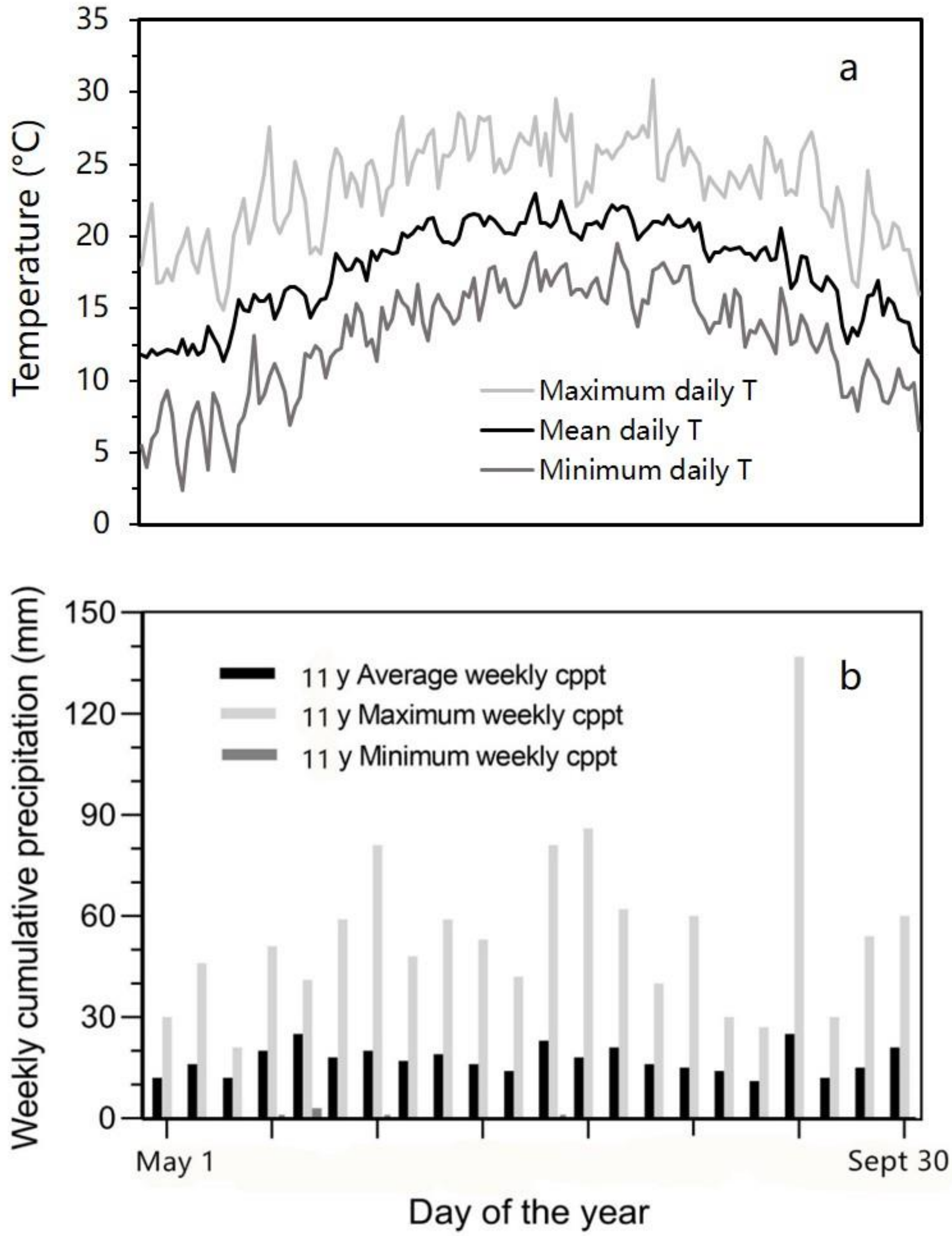


Figure 3.1. (a) Maximum daily temperature (T), mean daily temperature and minimum daily temperature across 11 years of experimentation in Ottawa, ON, Canada (2001 to 2004 and 2007 to 2013) from May 1 to September 30. (b) Maximum weekly cumulative precipitation (cppt), average weekly cumulative precipitation and minimum weekly precipitation across the 11 years of experimentation.

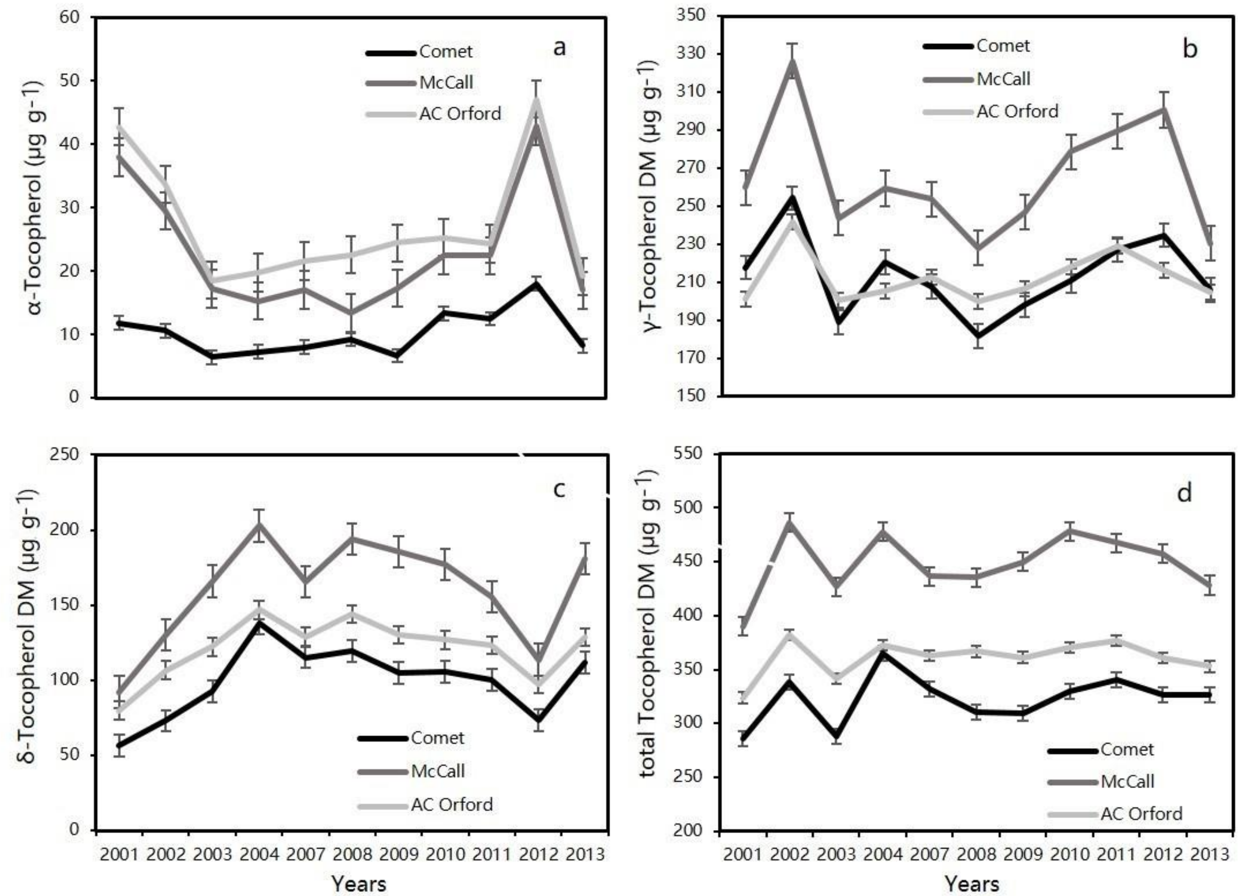
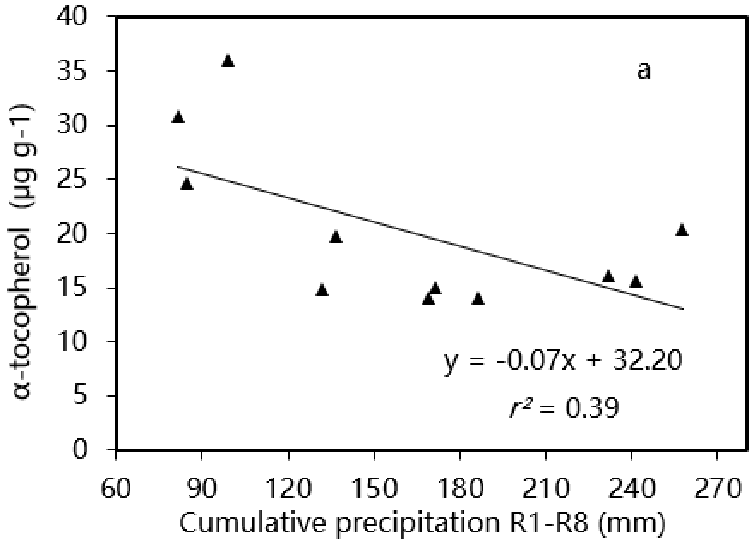


Figure 3.2. Seed tocopherol concentration for three cultivars (Comet, McCall and AC Orford) grown at Ottawa, ON, Canada during 11 years, (a) α-tocopherol, (b) γ-tocopherol (c) δ-tocopherol concentration, and(d) total-tocopherol concentration. Vertical bars indicate standard variation.

Table 3.1. Highest significant (p≤0.05) correlation coefficient (r) and corresponding soybean growth stage interval (GSI) between the concentration of α-tocopherol (α-toc), γ-tocopherol (γ-toc), δ-tocopherol (δ-toc) and total-tocopherol (T-toc) and cumulative precipitation, daily average T (DTave), daily average maximum T(DTmax) and daily average minimum T(DTmin) for the means of all cultivars and by cultivar.

Name		Cumulative precipitation				Daily Tave				Daily average Tmax				Daily average Tmin			
		α-toc	γ-toc	δ-toc	T-toc	α-toc	γ-toc	δ-toc	T-toc	α-toc	γ-toc	δ-Toc	T-toc	α-toc	γ-toc	δ-toc	T-toc
Means of cultivars	GSI	R1-R8	-	R1-R8	-	R1-R7	R7-R8	R5-R7	-	R1-R8	R1-R8	R5-R7	-	R5-R8	R7-R8	R5-R7	-
	r	-0.63		0.66		0.82	0.78	-0.91		0.89	0.75	-0.91		0.72	0.70	-0.84	
Comet	GSI	Sd-Ve	R1-R7	R1-R8	R7-R8	Sd-R8	R7-R8	R5-R7	-	R1-R5	R7-R8	R5-R8	-	R1-R8	R7-R8	R5-R7	-
	r	-0.81	-0.63	0.79	0.64	0.87	0.72	-0.92		0.92	0.70	-0.91		0.84	0.72	-0.92	
McCall	GSI	R1-R8	R7-R8	-	-	R5-R7	R1-R8	R5-R7	-	R1-R7	R1-R8	R1-R7	-	R5-R8	R1-R7	R5-R7	R1-R5
	r	-0.62	-0.61			0.74	0.77	-0.82		0.88	0.78	-0.86		0.65	0.68	-0.68	0.62
AC Orford	GSI	-	-	-	-	R5-R8	-	R5-R7	-	R1-R7	-	R5-R7	-	-	-	R5-R7	-
	r					0.75		-0.86		0.81		-0.88				-0.74	

† Growth stage interval (GSI), Sd, Ve, V3, R1, R5, R7, R8 correspond to seeding, emergence, three trifoliate leaves, first flower, beginning of seed development, beginning of ripening, and ripe, respectively.
‡ - Correlations not significant (p>0.05).



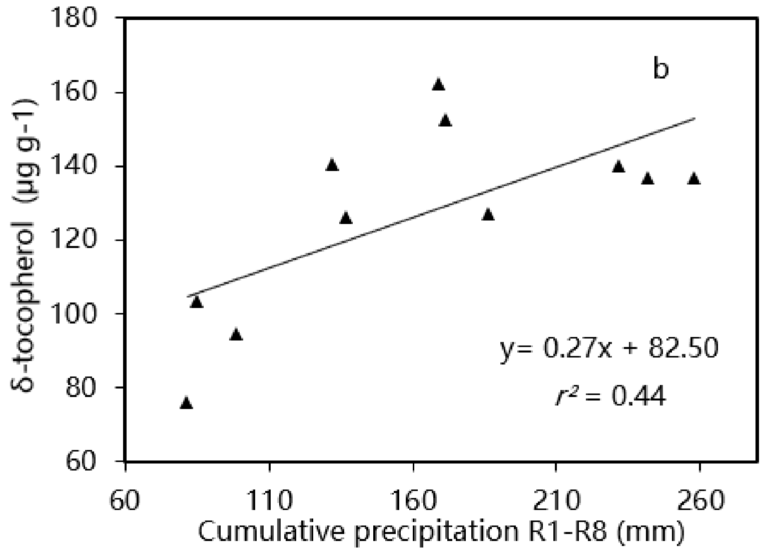


Figure 3.3. Linear regression relationships between: (a) mean cumulative precipitation (mm) during reproductive stages (R1to R8) and mean α-tocopherol concentration (µg g^{-1}). (b) mean cumulative precipitation (mm) during reproductive stages (R1 to R8) and mean δ-tocopherol concentration (µg g^{-1}).

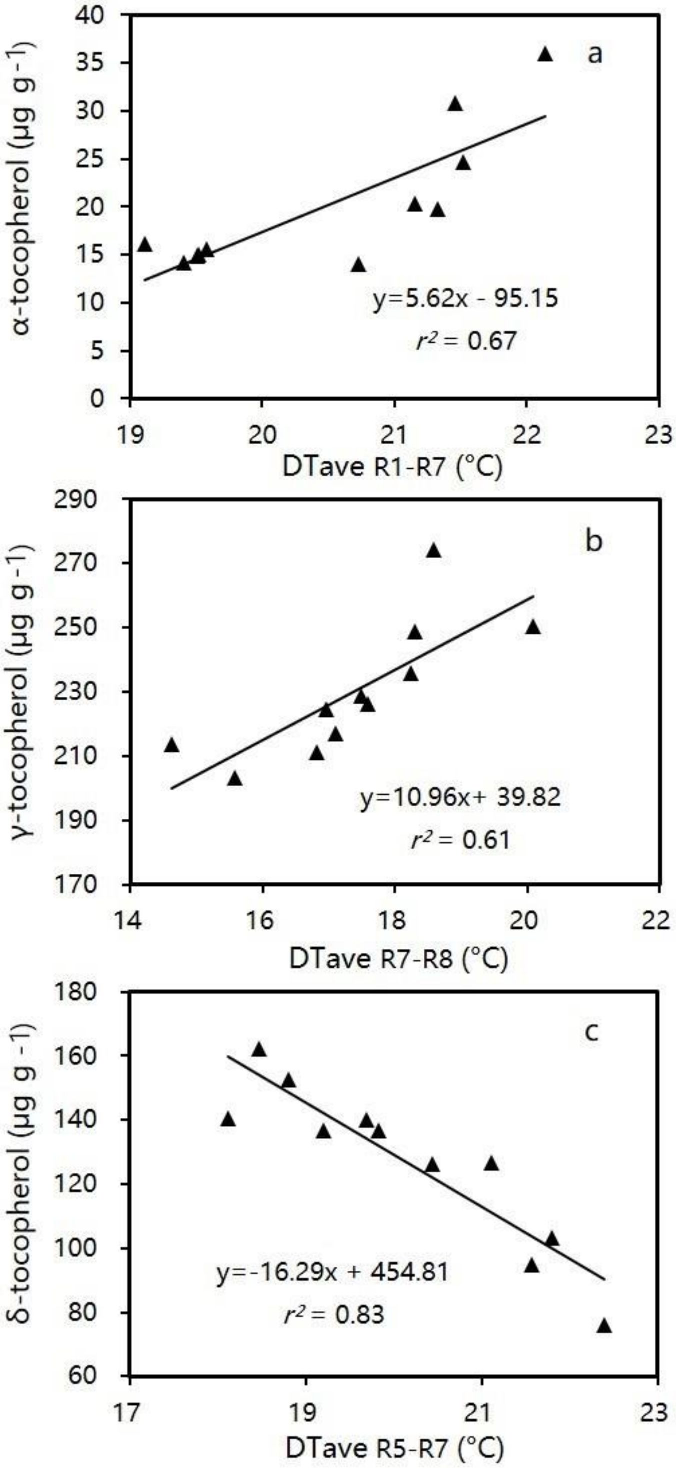


Figure 3.4. Linear regression relationships between: (a) DTave (°C) during R1 to R7 and mean α-tocopherol concentration (μg g^{-1}); (b) DTave (°C) during R7 to R8 and mean γ-tocopherol concentration (μg g^{-1}); (c) DTave (°C) during R5 to R7 and mean δ-tocopherol concentration (μg g^{-1}).

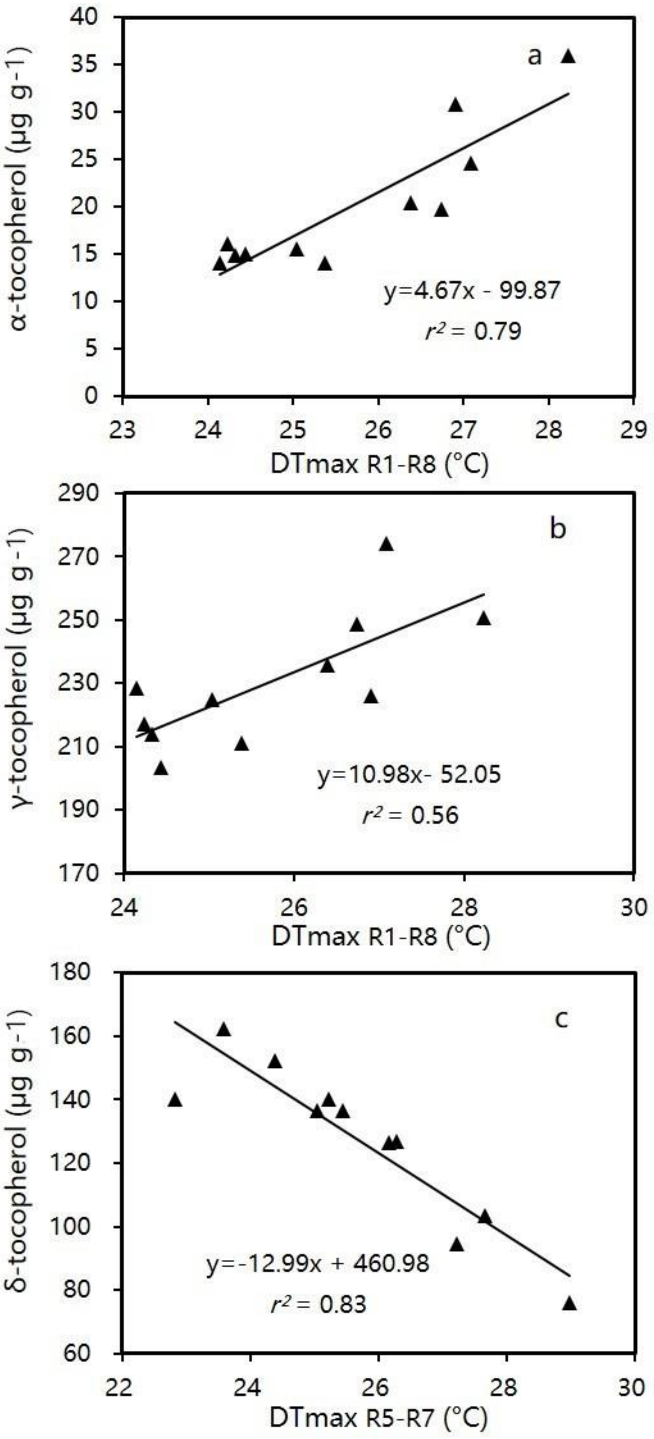
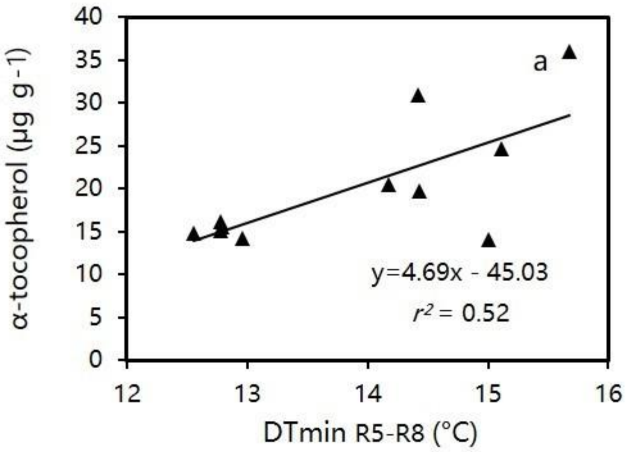
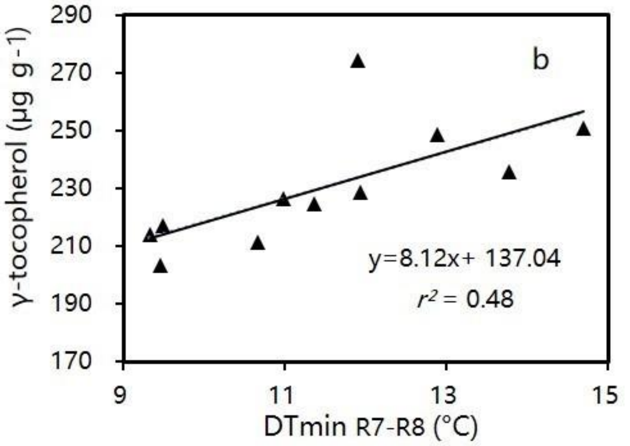


Figure 3.5. Linear regression relationships between: (a) DTmax (°C) during reproductive stages (R1 to R8) and mean α-tocopherol concentration (µg g^{-1}); (b) DTmax (°C) during reproductive stages (R1 to R8) and mean γ-tocopherol concentration (µg g^{-1}); (c) DTmax (°C) during R5 to R7 and mean δ-tocopherol concentration (µg g^{-1}).





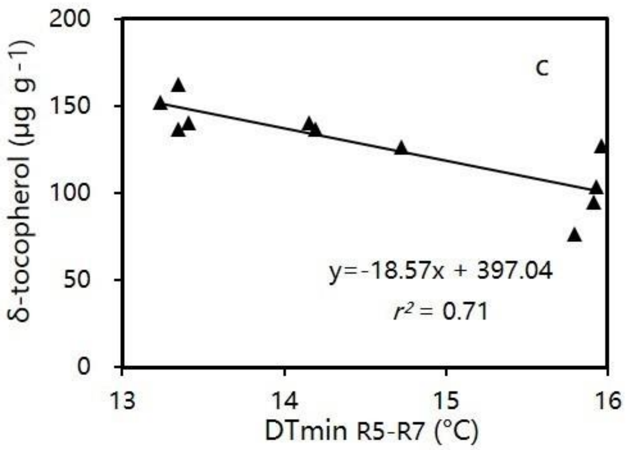


Figure 3.6. Linear regression relationships between: (a) DTmin (°C) during R5 to R8 and mean α-tocopherol concentration (µg g^{-1}); (b) DTmin (°C) during R7 to R8 and mean γ-tocopherol concentration (µg g^{-1}); (c) DTmin (°C) during R5 to R7 and mean δ-tocopherol concentration (µg g^{-1}).

Table 3.2. Highest significant (p≤0.05) correlation coefficient (r), corresponding growth stage interval (GSI) and heat stress base T (T_h) between α-tocopherol (α-toc), γ-tocopherol (γ-toc), δ-tocopherol (δ-toc) and total-tocopherol (T-toc) concentration and heat stress days (HSD) for the means of all cultivars and by cultivar.

	α-toc			γ-toc			δ-toc			T-toc		
	GSI	r	T_h	GSI	r	T_h	GSI	r	T_h	GSI	r	T_h
Means of cultivars	V3-R7	0.97	31	R1-R8	0.70	22	R5-R7	-0.92	27	Ve-R1	-0.81	21
Comet	R1-R7	0.94	27	R7-R8	0.70	22	R5-R8	-0.91	24	Ve-V3	-0.83	29
McCall	V3-R7	0.98	31	R1-R7	0.76	25	Ve-R7	-0.92	28	Ve-R1	-0.73	25
AC Orford	R1-R7	0.95	31	-	-	-	Ve-R7	-0.91	31	R5-R7	-0.74	35

† Growth stage interval (GSI), Sd, Ve, V3, R1, R5, R7, R8 correspond to seeding, emergence, three trifoliate leaves, first flower, beginning seed development, beginning ripe, ripe, respectively.
‡ - Correlations not significant (p>0.05).

Table 3.3. Highest significant ($p \leq 0.05$) correlation coefficient (r), corresponding growth stage interval (GSI) and cold stress base T (T_c) between α-tocopherol (α-toc), γ-tocopherol (γ-toc), δ-tocopherol (δ-toc) and total-tocopherol (T-toc) concentration and cold stress days (CSD) for the means of all cultivars and by cultivar.

	α-toc			γ-toc			δ-toc			T-toc		
	GSI	r	T_c	GSI	r	T_c	GSI	r	T_c	GSI	r	T_c
Means of cultivars	R5-R8	-0.72	15	R7-R8	-0.82	11	R5-R7	0.86	18	R7-R8	-0.63	12
Comet	R5-R8	-0.78	14	R5-R8	-0.66	13	R5-R8	0.85	18	Sd-R7	0.60	5
McCall	R5-R7	-0.69	17	R1-R7	-0.70	20	R5-R7	0.78	18	-	-	-
AC Orford	-	-	-	R7-R8	-0.70	12	R5-R7	0.73	17	-	-	-

† Growth stage interval (GSI), Sd, Ve, V3, R1, R5, R7, R8 correspond to seeding, emergence, three trifoliate leaves, first flower, beginning seed development, beginning ripe, ripe, respectively.
‡ - Correlations not significant ($p>0.05$).

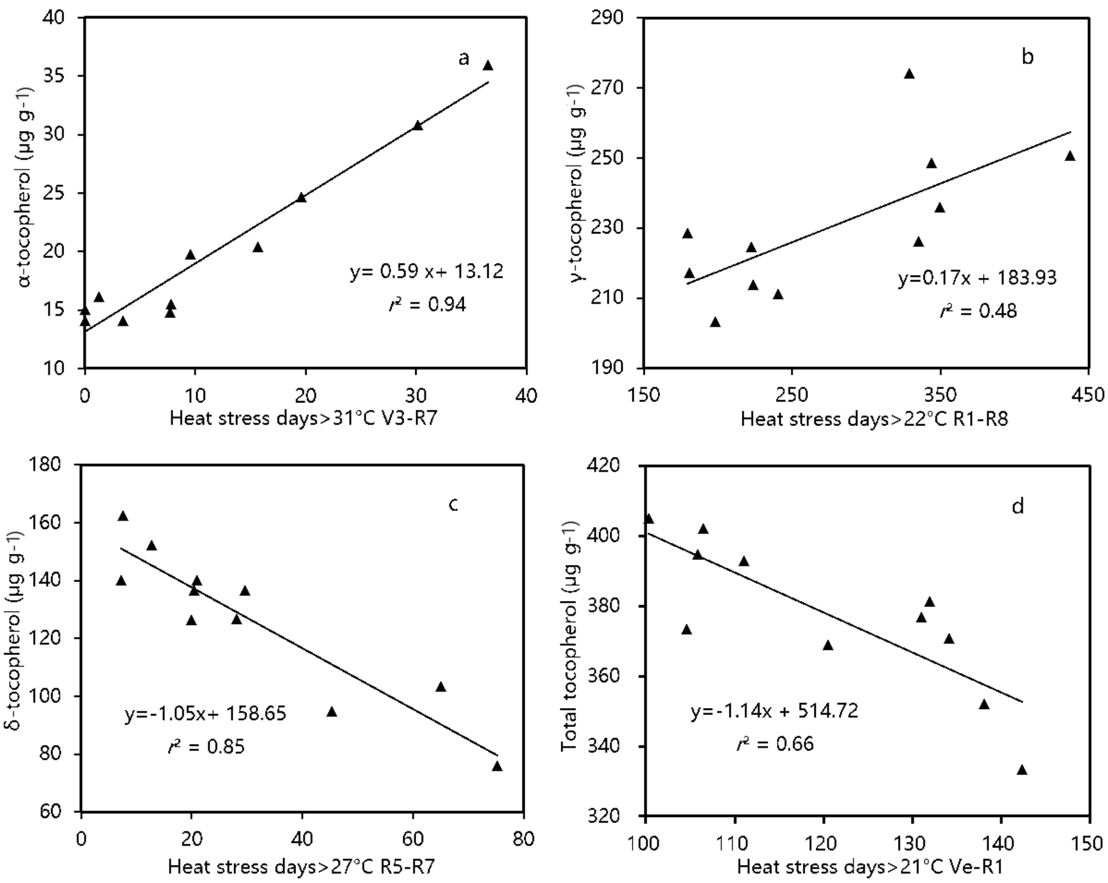


Figure 3.7. Linear regression relationships between: (a) heat stress days (HSD) above 31°C during V3 to R7 and mean α-tocopherol concentration (μg g^{-1}); (b) heat stress days (HSD) above 22°C during reproductive stages (R1 to R8) and mean γ-tocopherol concentration (μg g^{-1}); (c) heat stress days (HSD) above 27°C during R5 to R7 and mean δ-tocopherol concentration (μg g^{-1}); (d) heat stress days (HSD) above 21°C during Ve to R1 and mean total-tocopherol concentration (μg g^{-1})

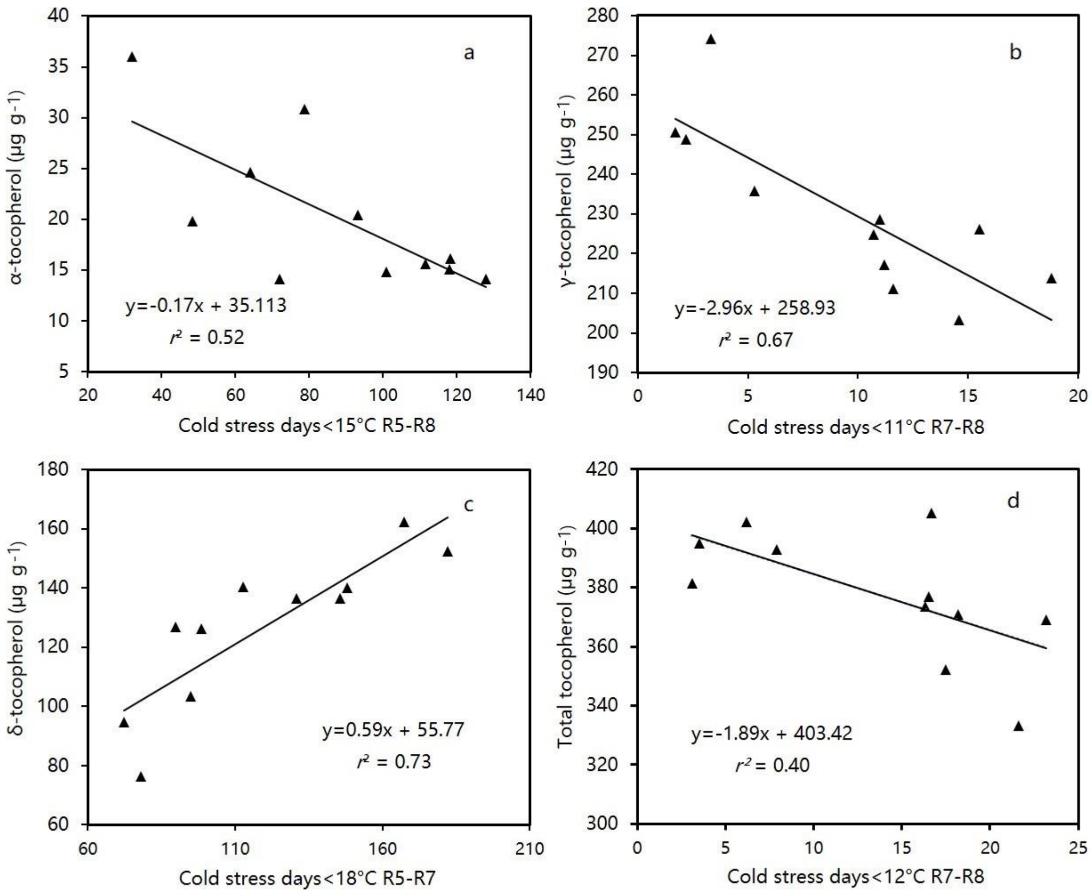


Figure 3.8. Linear regression relationships between: (a) cold stress days (CSD) below 15°C during R5 to R8 and mean α-tocopherol concentration (µg g^{-1}); (b) cold stress days (CSD) below 11°C during R7 to R8 and mean γ-tocopherol concentration (µg g^{-1}); (c) cold stress days (CSD) below 18°C during R5 to R7 and mean δ-tocopherol concentration (µg g^{-1}); (d) cold stress days (CSD) below 12°C during R7 to R8 and mean total-tocopherol concentration (µg g^{-1}).

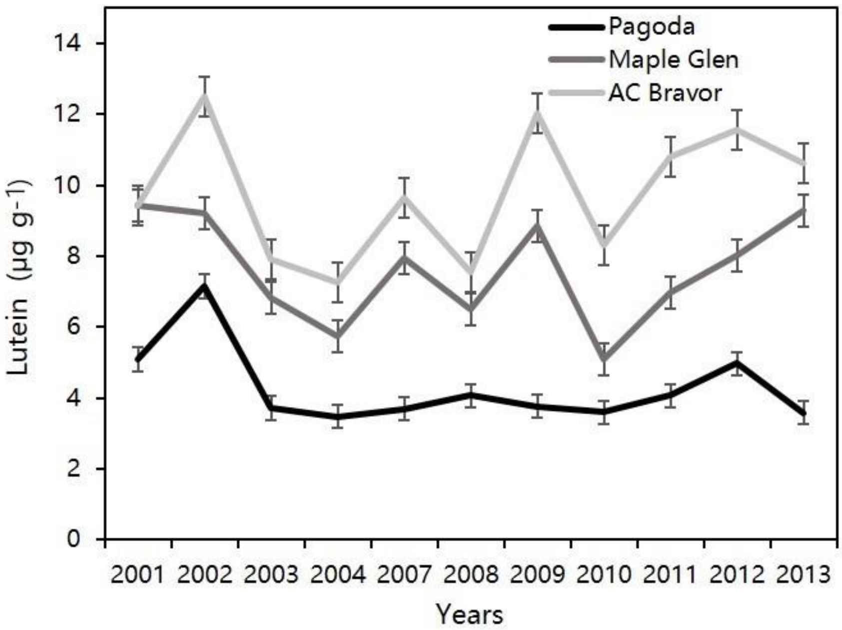


Figure 3.9. Lutein concentration in soybean seeds of three cultivars (Pagoda, Maple Glen and AC Bravor) grown at one site in Ottawa, ON, Canada during 11 years.

Table 3.4. Highest significant (p≤0.05) correlation coefficient (*r*) and corresponding growth stage interval (GSI) between lutein concentration and various environment variables (cumulative precipitation, daily average T, daily average maximum T and daily average minimum T) in seeds of soybean cultivars grown for 11 years at one site in Ottawa, ON, Canada.

Name		Cumulative precipitation	Daily average T	Daily average maximum T	Daily average minimum T
Means of cultivars	GSI	R5-R8	V3-R1	-	V3-R1
	r	-0.78	0.64		0.66
Pagoda	GSI	Ve-V3	R5-R8	V3-R1	Ve-V3
	r	0.76	0.75	0.77	0.67
Maple Glen	GSI	R5-R8	-	-	R1-R5
	r	-0.71			-0.81
AC Bravor	GSI	R5-R8	-	-	-
	r	-0.62			

† Growth stage interval (GSI), Sd, Ve, V3, R1, R5, R7, R8 correspond to seeding, emergence, three trifoliate leaves, first flower, beginning seed development, beginning ripe, ripe, respectively.

‡ - Correlations not significant (p>0.05).

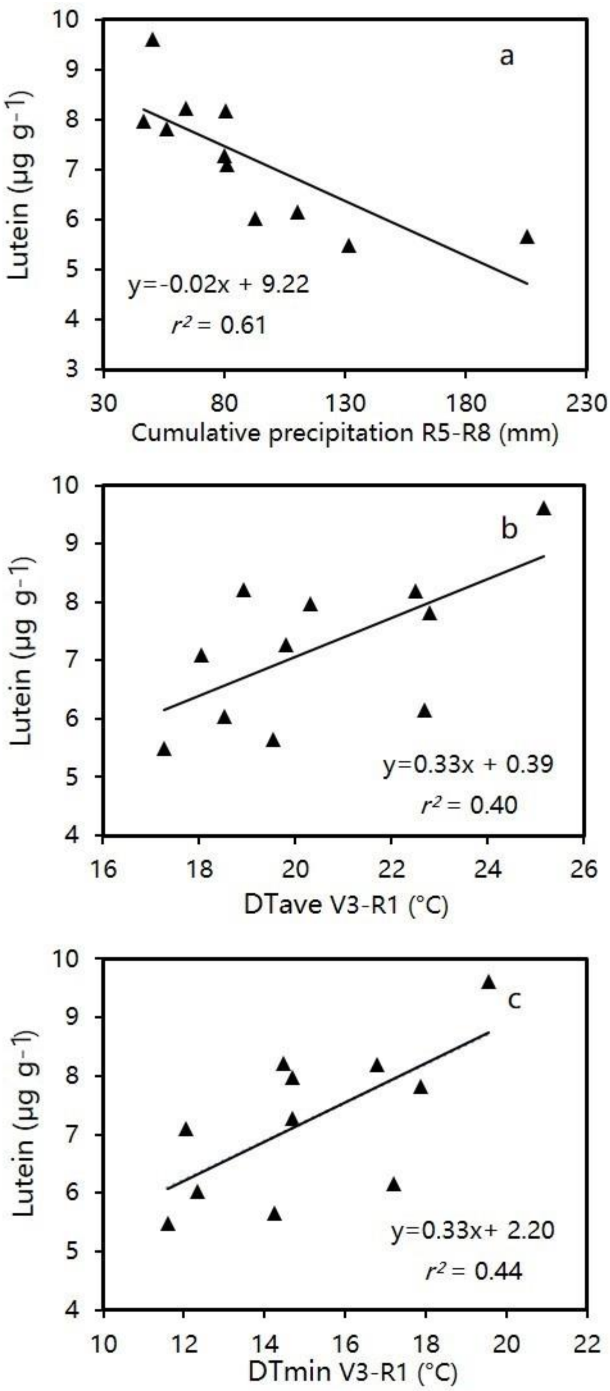


Figure 3.10. Linear regression relationships between: (a) cumulative precipitation (mm) during R5 to R7 and lutein concentration (μg g^{-1}) in mature soybean seeds; (b) DTave (°C) during V3 to R1 and mean lutein concentration (μg g^{-1}); (c) DTmin (°C) during V3 to R1 and mean δ-tocopherol concentration (μg g^{-1}). Results are the mean of 3 cultivars grown at one site in Ottawa, ON, Canada for 11 years.

Table 3.5. Highest significant (p≤0.05) correlation coefficient (*r*), corresponding growth stage interval (GSI) and heat stress base T (T_h) between lutein concentration and heat stress days (HSD) for the means of all cultivars and by cultivar.

Name	lutein		
	GSI	r	T_h
Means of cultivars	R5-R8	0.61	29
Pagoda	R5-R8	0.79	28
Maple Glen	-	-	-
AC Bravor	-	-	-

† Growth stage interval (GSI), Sd, Ve, V3, R1, R5, R7, R8 correspond to seeding, emergence, three trifoliate leaves, first flower, beginning seed development, beginning ripe, ripe, respectively.
‡ - Correlations not significant (p>0.05).

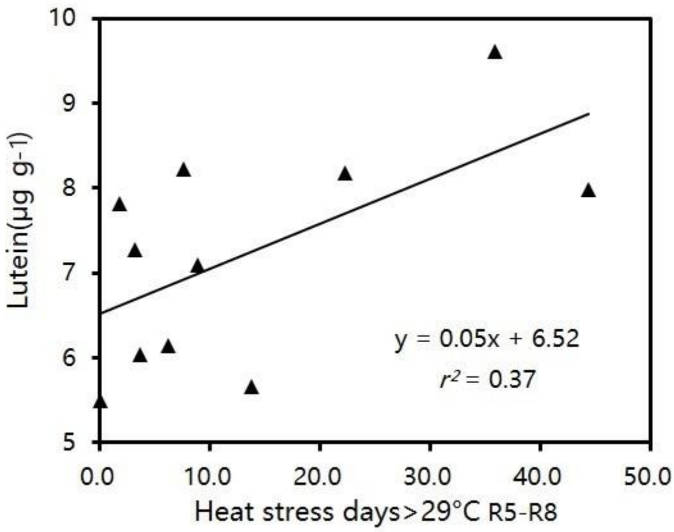


Figure 3.11. Linear regression relationships between heat stress days (HSD) above 29°C during R5 to R8 and mean lutein concentration (μg g^{-1}).

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CONNECTING TEXT FOR CHAPTER 4

In the previous chapter, we studied the effects of temperature and precipitation at different GSI on soybean tocopherol and lutein concentrations. Results observed demonstrated that tocopherol concentrations in soybean are more responsive to temperature changes, while lutein concentrations are more responsive to precipitation. Accumulation of HSD greater than 31°C during the V3-R7 stages had the most effects on α-tocopherol concentration, while accumulation of HSD greater than 27°C during the R5-R7 GSI impacted δ-tocopherol concentration the most. Lutein concentration was negatively correlated with mean cumulative precipitation (cppt) during the GSI R5-R8.

Limited research has examined the effects of climate variables on soyasaponin I concentration, which is also an important health-beneficial compound of soybean. In the following study, we investigated the effects of temperature and precipitation at different GSI on soybean soyasaponin I concentration. The following chapter has been published in Canadian Journal of Plant Science (Published on the web 10 July 2020, https://doi.org/10.1139/CJPS-2020-0066). This manuscript is co-authored by the candidate, Dr. Philippe Seguin (Department of Plant Science, McGill University), Dr. Malcolm Morrison (Agriculture and Agri-Food Canada), Dr. Elise Smedbol (Department of Plant Science, McGill University). The candidate carried out the extraction, HPLC and data analyses and was the primary author of the manuscript. Dr. Philippe Seguin provided funds and assistance for this research, including supervisory guidance and reviewing of the manuscript. Dr. Malcolm Morrison conducted the field experiment and provided the samples for analysis. Dr. Elise Smedbol helped the candidate with the HPLC analyses and also helped for extractions of samples. All authors were involved in editing the manuscript.

CHAPTER 4

Impact of climatic factors at specific growth stages on soybean soyasaponin I concentration

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4.1. ABSTRACT

Soybean [*Glycine max* (L.) Merr.] is an important source of health-beneficial compounds, including soysaponin I. A field study was conducted for 11 years in Ottawa, ON to assess the impacts of temperature and precipitation during specific growth stage intervals on soyasaponin I concentration in three soybean cultivars. Soyasaponin I concentration response to air temperature and precipitation variables were observed at specific growth stage intervals for some cultivars. The response was complex and cultivar specific. Overall, reproductive stages were more responsive and 20°C appeared to be a critical threshold in determining soybean soyasaponin I concentration response to air temperature.

Keywords: soybean, abiotic factor, temperature, precipitation, soyasaponin

4.2. INTRODUCTION

Soybean is an important source of health-beneficial compounds, including isoflavones, tocopherols, and soyasaponins (Liu et al., 2004). While considerable research has been conducted to increase our understanding of abiotic and biotic factors affecting the concentration of isoflavones and tocopherols in soybean seeds (Seguin et al., 2007; Carrera et Seguin, 2016), limited information is currently available for soyasaponins. Soyasaponins can be divided into two main groups based on their aglycone structures and properties. Soyasaponin A are reported to be responsible in part for the undesirable bitter and astringent taste of soybean or soy-related products (Kitagawa et al., 1988; Okubo et al., 1992; Lasztity et al., 1998). In contrast, soyasaponin B have been reported to have hypocholesterolemic, anticarcinogenic, antiviral, and hepatoprotective properties (Oakenfull et Sidhu., 1990; Potter., 1995; Berhow et al., 2000). Soyasaponins B can be further classified into two groups, the 2,3- dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP)-conjugated forms (i.e., αg, βg, βa, γg, and γa) and their corresponding non-DDMP forms (i.e., V, I, II, III, and IV, respectively) (Kudou et al., 1993). The DDMP-forms are easily degraded to their corresponding non-DDMP forms during processing. Consequently, several quantification procedures degrade DDMP-forms into their corresponding non-DDMP-forms which, with this approach represent a sum of both DDMP and non-DDMP forms (e.g., Hubert et al., 2005). Among all the non-DDMP soyasaponin B, soyasaponin I is found in the highest concentration representing approximately 70% of total soyasaponin B in mature soybean seeds (Seguin et al., 2014), it is also the most abundant in commercial soy products (Salyer et al. 2013).

Significant differences among genotypes have been reported for soyasaponin I concentration. Hu et al. (2002), for example, reported that among 46 soybean cultivars grown in one environment in Iowa (United States), soyasaponin I concentration (i.e., I + βg) ranged between 1.86 and 4.53 μmol g^{-1}. Differences have also been reported by Seguin et al. (2014) among 20 short-season genotypes in a study conducted in Quebec (Canada), the concentrations across four environments ranging between 1.82 and 3.86 μmol g^{-1}. Specific environmental factors or field management strategies have also been reported to impact soybean soyasaponin concentration, although research remains limited. Song et al. (2018) reported that total saponin B concentration in soybean seeds was negatively correlated with seasonal mean daily temperature and positively correlated with seasonal rainfall. In addition, saponin I was negatively correlated with mean daily temperature and rainfall, while a positive correlation with rainfall was observed for saponin βg. Seguin et al. (2014) reported that earlier seeding dates (i.e., May vs. June) resulted in the highest soyasaponin I concentration for two cultivars grown in Quebec (Canada). This response was hypothesized to be associated with the prevailing air temperature, as different seeding dates expose developing seeds to different temperatures. A study was conducted in controlled environments to confirm this hypothesis. High temperature stress during late reproductive stages (i.e., R5-R8; Fehr and Caviness, 1977) resulted in a significant increase in soyasaponin I concentration in AC Proteina, but only a slight increase in OAC Champion. However, the same stress during early reproductive stages (i.e., R1-R4) led to slightly higher soyasaponin I concentration in AC Proteina, but lower concentration in OAC Champion. The response to different temperatures during the reproductive stages was complex and varied with cultivars (Seguin et al., 2014).

The objective of this study was to examine the effects of temperature and precipitation during specific growth stages intervals on soyasaponin I concentration in mature soybean seeds of different cultivars. Specifically, the goal was to identify the most responsive growth stage intervals to specific environmental variables. Our hypothesis was that climatic factors will affect soyasaponin I concentration and that reproductive stages will be the most responsive.

4.3. MATERIALS AND METHODS

A field experiment was conducted at the Central Experiment Farm of Agriculture and Agri-Food Canada in Ottawa, ON, Canada (45°23′N 75°43′W) from 2001 to 2004 and from 2007 to 2013 for a total of 11 years. This experiment was part of a larger long-term project investigating the impact of abiotic factors on soybean traits with nutraceutical value. Data for isoflavones were previously reported in Morrison et al. (2010), which also presented in detail the experimental site, field management, and the general experimental approach; they will thus only be briefly presented herein. Three short-season cultivars (i.e., AC Orford, Comet, and McCall) with contrasted soyasaponin concentrations, according to preliminary data, were grown in a randomized complete block design with four replications for 11 field seasons. Plots were managed following local recommendations (OMAFRA, 2017). At maturity, the plots were harvested, and seed subsampled and stored at 4°C until processing for soyasaponin B extraction and quantification.

Approximately 50 g of seeds from each plot were ground in a coffee grinder (Black & Decker, Towson, MD, USA). Soyasaponin extraction and quantification were conducted using established procedures (Seguin et al., 2014). The procedure used for extraction includes an alkaline degradation step that converts DDMP-conjugated soyasaponin B into their corresponding non-DDMP forms. As previous studies demonstrated that in those conditions soyasaponin I represents approximately

70% of all soyasaponin B quantified in soybean seeds (Hubert et al., 2005; Seguin et al., 2014), only this form was quantified herein. A stock solution of soyasaponin I was made by dissolving the analytical standard (Sigma-Aldrich, Oakville, ON) in 100% methanol HPLC grade, followed by serial dilutions (ranging from 10 to 640 μg ml^{-1}). A standard curve with high linearity ($r^2 = 0.99$) was obtained by plotting soyasaponin concentration as a function of peak area under curve.

Climatic variables during specific growth stage intervals (GSI) used to establish correlations with soyasaponin I concentration in mature soybean seeds were described in detail in Morrison et al. (2010). Briefly, climate variables used included daily average, maximum and minimum temperatures, and cumulative precipitation which were collected from a nearby weather station (Government of Canada, 2020). The concept of heat- and cold-stress days (later referred to as HSD and CSD, respectively) was used to identify the temperature threshold from which crop response is observed. Briefly, a stress day was defined as the summation of temperature above or below a baseline temperature during a specific GSI (T*h* and T*c*, for heat- and cold-stress baseline temperatures, respectively); see Morrison et al. (2010) for details. A total of 21 GSI were used in the study and included all possible growth stage combinations between seven stages as defined by Fehr and Caviness (1977) including: seeding, emergence, three trifoliate leaves, first flower, beginning seed filling, physiological maturity and ripe (i.e., Sd, Ve, V3, R1, R5, R7 and R8, respectively).

Correlations between soyasaponin I concentrations and climate parameters during specific GSI were determined using PROC CORR in SAS (version 9.3, Cary, NC, USA) with the objective of finding the highest correlation coefficients (*r*). The statistical significance of each correlation was also determined. Correlations with climate parameters were determined for each cultivar and across cultivars.

Differences between cultivars was also determined using PROC MIXED in SAS using years and replicates as random factors and cultivars as fixed factors. The threshold used for all analyses was $P<0.05$, only significant results being discussed.

4.4. RESULTS AND DISCUSSION

During the 11 years of experimentation, wide variations in climatic conditions were observed ensuring that soybeans were exposed to a wide range of conditions (data not shown). For example, the largest temperature variation for a given day across the 11 years was 17.3 °C on May 26th and the lowest one was 5.7 °C on August 11th. Such variation resulted in specific GSI being exposed to a large range of temperatures. For example, the mean daily temperature (T_{ave}) observed during the seed filling stages (i.e., R5- R8) ranged between 17.3 and 21.4 °C. Similar variation in other variables, including cumulative precipitation was also observed during experimentation for all GSIs.

Differences in soyasaponin I concentration between cultivars remained small across the 11 years of experimentation. The cultivar McCall had the highest concentration with an average of 1.27 μmol g^{-1} across years, and values ranging from 0.87 to 1.73 μmol g^{-1} for individual years. Comet and AC Orford had lower concentrations with an average of 1.06 μmol g^{-1}, and values ranging from 0.66 to 1.64 μmol g^{-1} (data not shown). AC Orford was also reported as being a low soyasaponin I cultivar in an evaluation of 20 cultivars in a multi-environmental trial previously conducted in Quebec (Canada) (Seguin et al., 2014).

There was no significant correlation observed between soyasaponin I concentration and any temperature nor precipitation variable at any GSI when data were averaged for the three cultivars (Table 4.1 and 4.2). However, some significant correlations were observed for specific cultivars, illustrating that response to climatic variables varied depending on the cultivar. More significant correlations were observed for McCall, the cultivar with the highest soyasaponin I concentration across years of experimentation (i.e., for 4 out of 6 variables studied), while none was observed for AC Orford the cultivar with the lowest overall concentration.

A significant negative correlation between soyasaponin I and cumulative precipitation was observed for the cultivar Comet (Table 4.1). This correlation was observed during the entire reproductive period (between stages R1 and R8) and was overall the highest correlation we observed (r = -0.71). The most significant correlation for McCall (r = -0.67) was between soyasaponin I concentration and daily average maximum temperature between stage V3 and R1. In contrast, for the cultivar Comet a positive correlation (r = 0.63) for this climatic variable was observed for the entire growing season (Ve to R8). Significant correlations between soyasaponin I, daily average temperature and daily average minimum temperatures were only observed for the cultivar McCall. A significant negative correlation (r = -0.61) between soyasaponin I concentration and the mean daily average temperature during the V3-R1 GSI was observed, while a positive correlation (r = 0.63) for daily average minimum temperature was observed during late reproductive stages (R7 to R8).

Previous studies have also directly or indirectly reported correlations between air temperature, precipitation, and soyasaponin B concentrations in soybeans. For example, Song et al. (2018) reported that saponin I was negatively correlated with mean daily air temperature and precipitation, while a positive correlation with rainfall was observed for saponin βg. Given that correlations for both soyasaponin were opposite it is hard to compare these results to ours given that our methodology quantified both soyasaponin I and βg together. Seguin et al. (2014) reported that earlier seeding dates (i.e., May vs. June) resulted in the highest soyasaponin I concentration for two cultivars grown in Quebec (Canada). Authors hypothesized that this response could be explained by differences in prevailing air temperature, seeding dates exposing developing seeds to different temperatures, with earlier seeding exposing developing seeds to overall higher air temperatures.

The HSD and CSD concepts suggest that 20°C is a critical temperature, deviations from which can affect soybean soyasaponin I concentration, response depending on the cultivar. In the case of the high-soyasaponin I cultivar McCall, HSD accumulation above 20°C during late reproductive stages (R7 to R8) was positively correlated (r = 0.66) with soyasaponin I. This illustrates that temperature events above 20°C during this GSI were associated with higher soyasaponin concentrations. In contrast, for the low-soyasaponin I cultivar Comet, a negative correlation (r = -0.70) between CSD during seed maturation (R5 to R8) and soyasaponin was observed, illustrating that events with temperatures below 20°C during this GSI were associated with lower soyasaponin concentrations.

Reproductive stages, overall, appeared to be more responsive to climatic factors as five of the seven significant correlations observed were during some GSI including late reproductive stages. Reproductive stages, in particular the seed development stages, were also often reported to be the most responsive to climatic factors for other health-beneficial compounds including isoflavones and tocopherols (Morrison et al., 2008; Carrera et Seguin., 2016). All the climatic variables studied were significantly correlated with soyasaponin I concentration for at least one of the three soybean cultivars. However, the response of soyasaponin I to temperature and precipitation appeared, overall, to be complex and highly cultivar specific. Indeed, there was no significant correlation between soyasaponin I concentration in AC

Orford and any climate variable for any GSI, while correlations were observed for four of the six climatic variables studied in McCall. Consequently, no correlation was significant when determined across cultivars. A study was previously conducted in controlled environments to determine the effect of high temperature on soyasaponin B concentration in two soybeans cultivars (AC Proteina and OAC Champion) with contrasted concentrations. Differences in the cultivars' response were reported, with a positive response to higher temperature during seed development being observed for only one of the two cultivars (Seguin et al., 2014). While other studies have reported on differences in total soyasaponin B or soyasaponin I concentrations of different cultivars (e.g., Hu et al., 2002), to our knowledge, none other have investigated the response of different cultivars to climatic factors. We could hypothesize that differences in cultivars concentration and response to climatic factors could be due to the presence of specific genes or alleles associated with greater soyasaponin I synthesis. Indeed, some studies have identified the presence of specific quantitative trait loci (QTLs) associated with greater total soyasaponin or soyasaponin I concentrations in soybean (Huang et al., 2012; MacDonell and Rajcan, 2018).

Another possibility to explain the impact of climatic factors on soyasaponin I concentrations we observed could be a reflection of an effect on seed weight. Indeed, it is possible that the absolute soyasaponin I per single seed is not affected or moderately affected by specific climatic factors and that these rather affect single seed weight, which in turn would result in changed concentrations on a per gram basis. Further studies should thus examine the relative contributions of the effect of climatic factors on seed weight versus absolute soyasaponin I content per seed on the resulting concentrations observed in mature seeds.

In conclusion, soyasaponin I concentration in mature soybeans is influenced by climatic factors. Overall, reproductive stages appeared more responsive and 20°C appeared to be a critical temperature influencing soybean soyasaponin I concentration; our hypotheses were thus confirmed. The response to temperature and precipitation is, however, complex and cultivar specific. This variation between cultivars suggests that response to climatic factors could be linked to genotypic factors and genomic studies should be conducted to identify genes associated with soybean response. Our results demonstrate that climatic factors should be a consideration if soybean is to be produced specifically for the nutraceutical and functional markets as a source of soyasaponin I. In addition, it is essential to understand the genetic source of the differential response of cultivars to climatic factors, if soybeans are to be produced with specific targeted soyasaponin I concentrations.

Table 4.1. Highest correlation coefficient (*r*) and corresponding growth stage interval (GSI) for the relation between various environment variables (cumulative precipitation, daily average T, daily average maximum T and daily average minimum T) and soyasaponin I concentration in mature seeds of soybean cultivars grown for 11 years at one site in Ottawa, ON.

Cultivar		Cumulative precipitation	Daily average T	Daily average maximum T	Daily average minimum T
AC Orford	GSI	Sd-R5	Ve-V3	Ve-V3	V3-R1
	r	0.23	0.45	0.45	-0.32
	P-value	NS	NS	NS	NS
Comet	GSI	R1-R8	Ve-R7	Ve-R8	Ve-R7
	r	-0.71	0.58	0.63	0.44
	P-value	0.01	NS	0.04	NS
McCall	GSI	Sd-V3	V3-R1	V3-R1	R7-R8
	r	-0.54	-0.61	-0.67	0.63
	P-value	NS	0.04	0.02	0.04
Mean of cultivars	GSI	Sd-R8	Ve-V3	Ve-V7	Sd-V3
	r	-0.26	0.39	0.46	0.32
	P-value	NS	NS	NS	NS

Growth stages Sd, Ve, V3, R1, R5, R7, and R8, correspond to seeding, emergence, three trifoliate leaves, first flower, beginning of seed development, beginning ripe, ripe, respectively according to Fehr and Caviness (1977).

Table 4.2 Highest correlation coefficient (*r*) and corresponding growth stage interval (GSI) for the relation between heat (HSD) and cold (CSD) stress days and soyasaponin I concentration in mature seeds of soybean cultivars grown for 11 years at one site in Ottawa, ON. The heat (T_h) and cold (T_c) stress base temperatures in °Celsius are also indicated. The HSD and CSD refers to the accumulation of ° Celsius above the corresponding base temperature during a given growth stage interval.

Cultivar		HSD	CSD
AC Orford	T_h or T_c	20	20
	GSI	Sd-V3	Ve-V3
	r	0.35	-0.55
	P-value	NS	0.08
Comet	T_h or T_c	20	20
	GSI	R1-R8	R5-R8
	r	0.50	-0.70
	P-value	NS	0.02
McCall	T_h or T_c	20	20
	GSI	R7-R8	Ve-V3
	r	0.66	0.48
	P-value	0.03	NS
Mean of cultivars	T_h or T_c	21	20
	GSI	V3-R1	Ve-V3
	r	-0.40	-0.43
	P-value	NS	NS

Growth stages Sd, Ve, V3, R1, R5, R7, and R8, correspond to seeding, emergence, three trifoliate leaves, first flower, beginning of seed development, beginning ripe, ripe, respectively according to Fehr and Caviness (1977).

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CHAPTER 5

GENERAL CONCLUSIONS

Soybeans have been drawing attention in recent years due to their potential beneficial effects on human health. They contain several compounds including tocopherols, lutein and soyasaponin I that have putative health-beneficial properties (Liu, 2004). The objectives were to determine the effects of temperature and precipitation during different growth stage intervals on soybean tocopherol, lutein and soyasaponin I concentrations.

The first objective was to determine the effect of temperature and precipitation during different growth stage intervals on tocopherols and lutein concentration in mature soybean seeds. The overall results suggested that we could use accumulated HSD greater than 31°C during the V3-R7 stages to forecast α-tocopherol concentration and accumulated HSD greater than 27°C during the R5-R7 GSI to predict the potential δ-tocopherol concentration in field production. The reproductive stages are more responsive to environment variables than the vegetative stages. Cultivars with high concentration of tocopherol were generally less affected by environment variables than cultivars with low concentrations. The second objective was to determine the effect of temperature and precipitation during different growth stage intervals on soyasaponin I concentration in mature soybean seeds. Soyasaponin I concentration in mature soybeans is influenced by both temperature and precipitation. Overall, reproductive stages appeared more responsive and 20°C appeared to be a critical temperature influencing soybean soyasaponin I concentration. The response to temperature and precipitation is, however, complex and cultivar specific. This variation between cultivars suggests that response to climatic factors could be linked to genotypic factors.

CHAPTER 6

RECOMMENDATIONS FOR FUTURE RESEARCH

The results of this project highlighted the importance of more in-depth research related to the effects of environmental factors on the concentration of health-beneficial compounds in soybean. Further research could explore other stresses including soil pH, light duration and quality, heavy metals, etc. to determine whether they have impacts on health-beneficial compounds concentration in soybean.

Furthermore, in order to have a better understanding regarding the inner mechanisms of how the environmental factors affect the compounds concentration in soybean, gene expression studies of the compounds biosynthetic pathway are required.

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APPENDICES

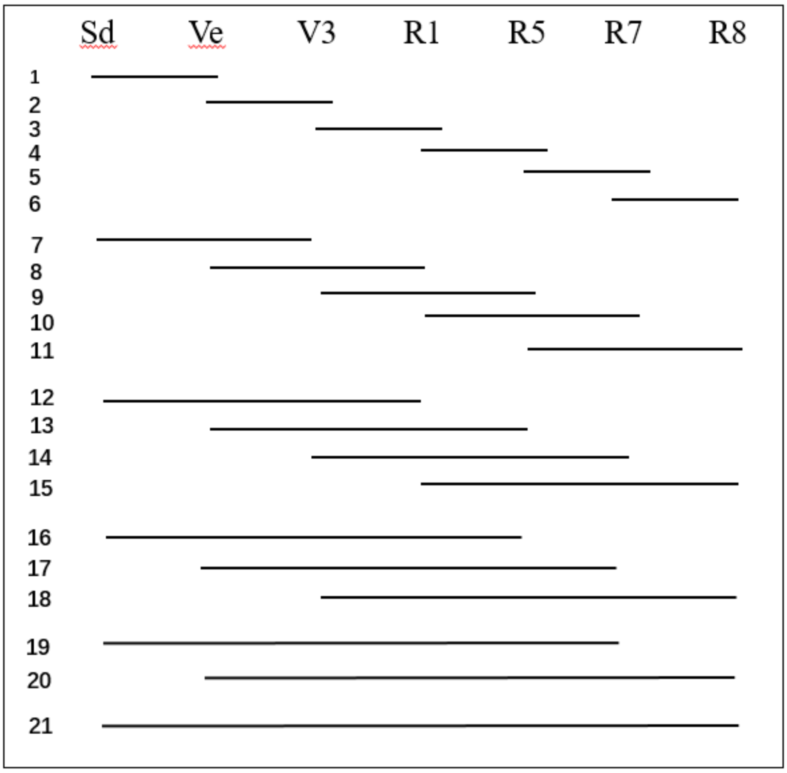

Figure A.1. Growth stage interval (GSI) (The duration between two growth stage of soybean) and the twenty-one GSI defined in this experiment (1, 2, 3, 4…, 21 corresponding to Sd-Ve, Ve-V3, V3-R1, R1-R5, R5-R7, R7-R8, Sd- V3, Ve-R1, V3-R5, R1-R7, R5-R8, Sd-R1, Ve-R5, V3-R7, R1-R8, Sd- R5, VE-R7, V3-R8, Sd- R7, Ve-R8, Sd-R8).

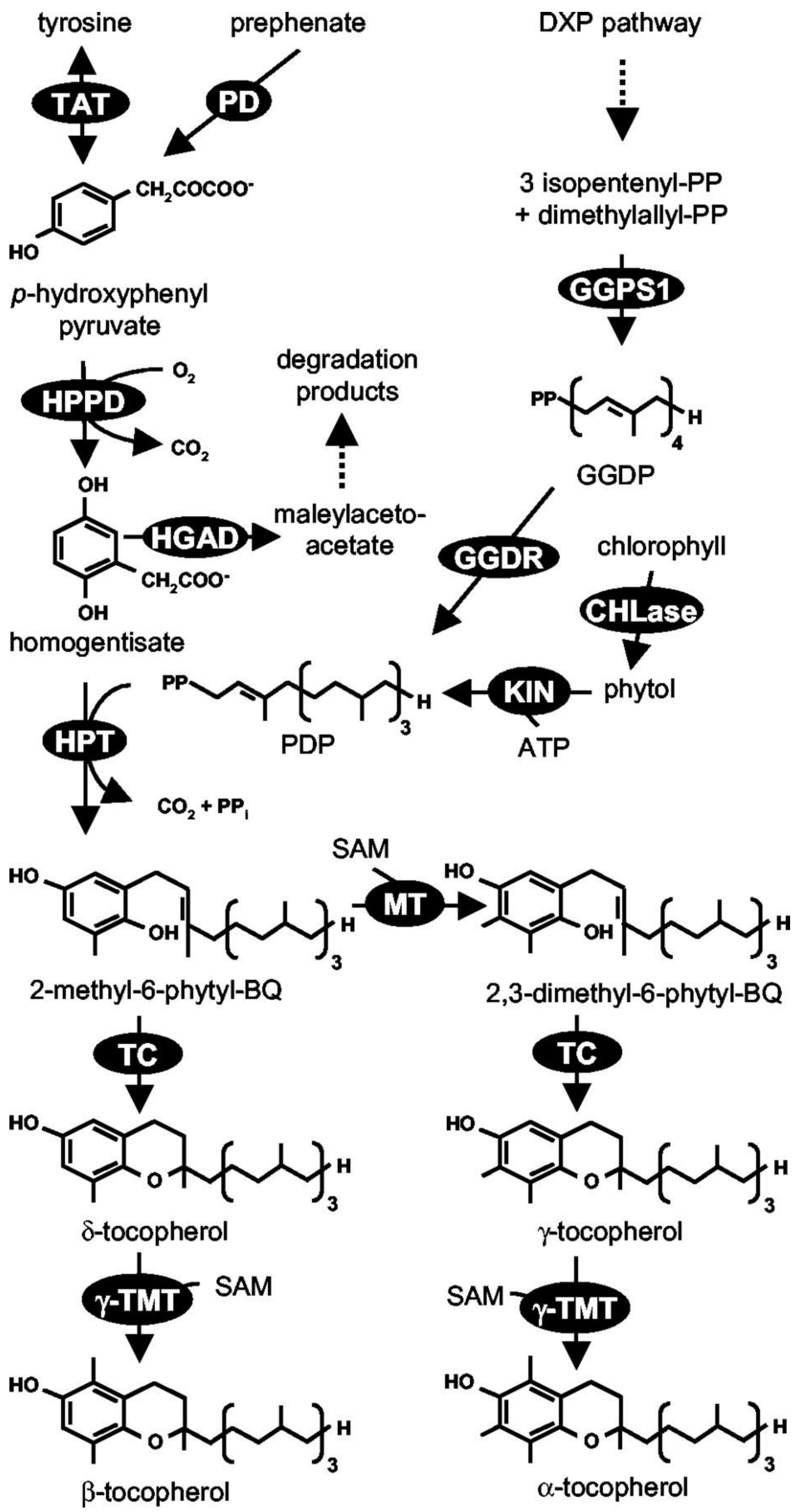

Figure A.2. Tocopherol Biosynthetic Pathway (Collakova et DellaPenna, 2003)

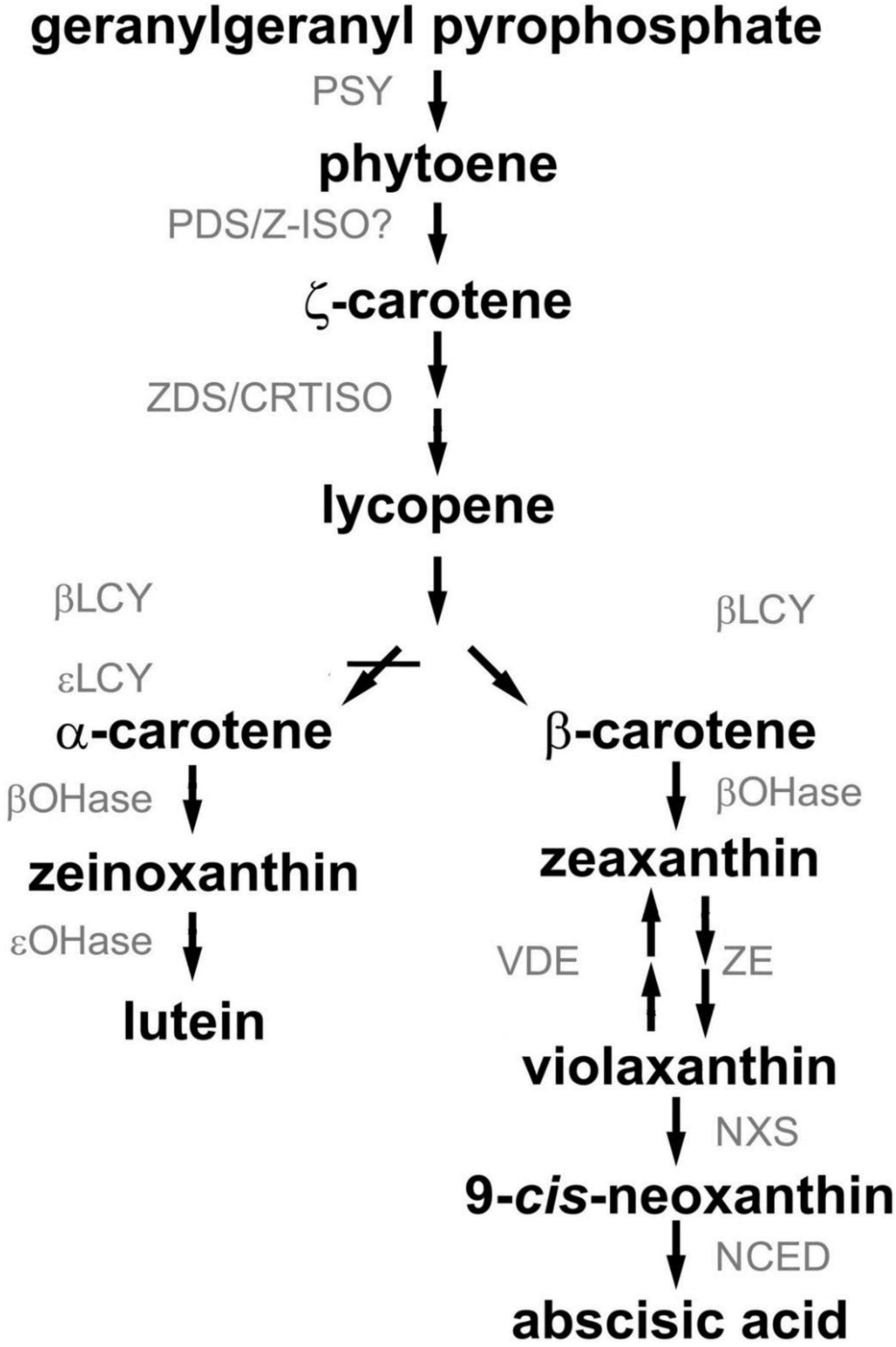

Figure A.3. Lutein Biosynthetic Pathway. Adapted from Matsubara et al., 2003