Effect of Organic and Conventional Farming Practices on Quercetin Content in Spinach, *Spinacia oleracea*

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Abstract Positive stresses and limitations of nutrient accessibility inherent in organic systems may increase the biosynthesis of secondary metabolites relative to conventional systems. Since many secondary metabolites have antioxidant capabilities, the potential of agricultural conditions to increase their content in produce has implications for the health of individual consumers. In particular, the common antioxidant quercetin, present in spinach, *Spinacia oleracea*, has antihistamine and anti-tumor properties. Due to the beneficial properties of this compound, this study was designed to establish the influence of 5 treatments associated with organic and conventional farming on its content in spinach: 1) organic soil, 2) synthetic soil (with supplemental fertilizers), 3) synthetic pesticides, 4) simulated herbivory, and 5) synthetic herbicides. The content (mass per fresh weight) of quercetin and its glycocides rutin and quercitrin were quantified with high pressure liquid chromatography, HPLC. Rutin and quercitrin were not detectable in any of the samples. Quercetin was present with each of the treatments; however, its content did not differ significantly among them.

Introduction

Over the twentieth century, trends in agriculture have shifted production from local and small-scale farms to global and corporate businesses. Favored by economics of scale, large production systems have turned to chemical solutions to increase productivity and alleviate ecological problems -- a praxis often upsetting natural systems through the use of synthetic insecticides and herbicides (Francis 2004). Organic farming practices offer an alternative through the decreased use of chemical inputs. Consumers, driven by environmental and health concerns to select organic products, have encouraged this movement (Davies et al. 1995) and caused the market to increase 40-fold between 1986 and 1996 (Fisher 1999).

Organic policy varies throughout the world (Bourn and Prescott 2002), but generally includes a set of core regulations. The United States Department of Agriculture (USDA) requires certification to meet minimum standards prohibiting the use of nearly all chemical pesticides and herbicides and mandating practices that improve soil condition and minimize erosion (National Organic Program 2000). Studies have largely concluded that organic practices offer environmental benefits relative to conventional systems by increasing biodiversity, decreasing nutrient leaching (Hansen et al. 2001), and increasing nutrient recycling (Brandt and Mølgaard 2001). However, implications for human health are more equivocal.

Food Quality. Comprehensive reviews have reached some broad conclusions regarding food quality, although differences in study design and the variability inherent in agricultural systems contribute to ambiguous findings that are often difficult to interpret (Benbrook 2005). Research suggests that relative to their conventionally-produced counterparts, organically-produced crops generally have lower nitrate levels (Bourn and Prescott 2002, Brandt and Mølgaard 2001, Woese et al. 1997), higher dry matter content (Bourn and Prescott 2002, Woese et al. 1997), and fewer chemical residues (Baker et al. 2002, Woese et al. 1997). Conclusions regarding mineral content are more varied, with some studies stating no difference (Woese et al. 1997) and others reporting significantly higher levels of certain minerals in organic produce (Bourn and Prescott 2002, Worthington 2001). Likewise, studies addressing Vitamin C content report contrasting findings, ranging from higher levels to no difference or lower levels (Bourn and Prescott 2002, Woese et al. 1997).

Secondary Metabolites. The growing field of research focusing on levels of secondary metabolites may offer additional insights about food quality. Plants produce secondary metabolites primarily for defense; hence, many are stress-induced through wounding, pathogenic attack, exposure to high levels of ultraviolet light, the need for signaling, insufficient nutrients, or low temperatures (Dixon and Palva 1995). Relative to conventional systems, organic systems may increase the exposure of crops to such stresses, thus inducing the synthesis of secondary metabolites (Manach et al. 2004). Different cultivars display larger variation in secondary metabolite levels than those attributed to cultivation methods (Brandt and Mølgaard. 2001)

While crucial to plant health, some secondary metabolites like polyphenols and carotenoids may also offer benefits for humans by acting as antioxidants to prevent oxidative damage in cells and subsequent cancerous growth (Benbrook 2005). Radical-scavenging acids and enzymes are produced by the human body but dietary contributions augment the supply of antioxidants (Johns 1999).

Hindered by a variety of confounding factors, studies linking health benefits to specific chemicals are difficult (Brandt et al. 2004). However, some studies have associated polyphenol consumption with a decrease in the occurrence of chronic diseases including cardiovascular disease (Kenkt et al. 2002) most notably in women (Mennen et al. 2004, Arai et al. 2000), some types of cancer, asthma, and type 2 diabetes (Kenkt et al. 2002). Quercetin, the most abundant flavonoid in food (Formica and Regelson 1995), is highly studied and has been linked to several health benefits attributed to its antihistamine (Middleton et al. 1981) and anti-tumor properties (Lamson and Brignall 2005, Lambert et al. 2005). Specifically, it has been associated with decreased rates of prostate cancer (McCann et al. 2005), possibly due to its inhibition of enzymes responsible for carcinogenesis (Chaudhary and Willett 2006). However, some animal models have shown no effect (Lambert et al. 2005).

Akin to research regarding nutrient levels, findings comparing antioxidant levels in conventionally and organically-cultivated crops are variable and subject to numerous confounding factors. Studies have reported a range of results including higher levels (Lombardi-Boccia et al. 2004, Asami et al. 2003, Carbonaro et al. 2002), no difference (Mitchell, unpublished data 2005), or lower levels (Lombardi- Boccia et al. 2004) of specific antioxidants in some organic fruits and vegetables.

Some research has specifically addressed the content of the antioxidant quercetin. One study reported higher levels in organic than conventional tomatoes but no difference in bell peppers (Mitchell and Chassy 2005). Another study quantifying quercetin content in five green vegetables (Qing-gen-cai, Chinese cabbage, spinach, green pepper, and Welsh onion) grown under conventional and organic conditions reported quercetin to be present only in organic Chinese cabbage (Ren et al. 2001). It also reported higher levels of quercitrin, a glycoside of quercetin, in all sampled vegetables. However, the interpretation of these findings is limited since this study failed to quantify the content of rutin, another glycoside of quercetin common in plants (Daniel et al. 1999).

While the breadth of literature addressing such issues is increasing, no resolute conclusions can be made regarding the relative antioxidant levels of conventional and organic produce. Lacking in this field is research concentrated on the effects of specific farming practices associated with conventional and organic systems (Benbrook 2005). The majority of existing studies employs complete farming systems and therefore cannot link specific practices to varying antioxidant levels.

This study concerns the effects of organic and conventional farming practices on quercetin levels in spinach, *Spinacia oleracea*, to identify potentially beneficial agricultural techniques. Quercetin, quercitrin, and rutin content has be quantified in identical cultivars of spinach grown under the treatments of synthetic soil conditions, organic soil conditions, synthetic pesticides, simulated herbivory, and synthetic herbicides.

The content of these compounds is expected to differ among the various treatments due to variation of conditions and stresses that may induce the synthesis of secondary metabolites. The presence of easily-accessible nitrogen in conventional soil may decrease the production of phenolic antioxidants as plants devote a larger proportion of resources to growth rather than the biosynthesis of non-essential secondary metabolites (Mitchell and Chassy 2005). Quercetin has been associated with defense against herbaceous insects and therefore simulated herbivory is expected to induce quercetin production (Horbowicz et al. 2004). In addition, herbicides that reduce carbon fixation may indirectly reduce secondary metabolite synthesis by limiting resources (Daniel et al. 1999).

Methods

Plant Materials and Preparation The whale *S. oleracea* cultivar was selected due to its availability to both home gardeners and commercial farmers in organic and conventional varieties. Organic seeds were purchased from Johnny’s Seeds (Winslow, ME). Organic

(Multipurpose Mix) and conventional soil (Ultra Potting Mix) was purchased from American Soil Products (Richmond, CA). Pots, fertilizer, pesticide, and herbicide were available from the cultivation facilities.

Cultivation proceeded at Oxford Tract Insectary and Greenhouse in Berkeley, CA from July, 15 2005 to August, 31 2005. Seeds were germinated in a greenhouse in each of 24 6-inch terra cotta seeding pots (available from Oxford Tract) at the rate of 10 seeds per pot. Hand irrigation occurred one to two times per day with sufficient frequency to avoid extremes of water availability and to minimize stress. At the development of the first set of true leaves, one seedling was transplanted to each 1-gallon pot. Cultivation continued outdoors on benches in a shaded area to minimize heat stress. Irrigation continued as specified above for germination.

Agricultural practices characteristic of organic and conventional systems and potentially influencing quercetin content were grouped into two soil condition treatments and four stress treatments. The soil and stress treatments were fully crossed to yield a total of eight treatment groups. Twelve individual plants were cultivated in each treatment group (Table 1). The plants were positioned on benches by treatment groups to minimize contamination. The soil condition treatments consisted of two conditions:

1. Organic Soil Conditions. Multipurpose Mix organic soil composed of 75% composed plant and animal materials and free of synthetic amendments, adhering to USDA regulations (National Organic Program) and consistent with the properties of organic soil, was used as the soil condition for seeding and 1-gallon pots.

2. Conventional Soil Conditions. Ultra Potting Mix conventional soil composed of coconut coir with synthetic mineral amendments was used as the soil condition for seeding and 1-gallon pots. To minimize nutrient deficiencies due to leaching, Scotts Peters Formula General Purpose 20-20-20 nitrogen-phosphorous-potassium water soluble fertilizer (available from Oxford Tract, Marysville, OH) was applied. Approximately 45 mL fertilizer was diluted in 20 L water. Approximately .5 L of solution was supplied to plants via a hand watering can.

The stress treatments consisted of four treatments:

1. Control. No modifications were made to the soil conditions to control for the effects of mineral availability and soil texture.

2. Simulated Herbivory. Herbivory by the diamondback moth, a pest known to infest California spinach crops (USDA 1999) and possibly repelled by quercetin gycosides (Horbowicz

et al. 2004), was simulated with leaf clipping. Six hours prior to harvest, 1 cm cuts were made with scissors in the edges of all leaves to simulate attack by the diamondback moth and maximize the metabolic response of the plant (Pontoppidan et al. 2005). Cuts were placed approximately every 5 cm along the periphery of leaves.

3. Synthetic Pesticides. Marathon II (imidacloprid) (available from Oxford Tract, Olympic Horticultural Products, Mainland, PA) was applied to plants in a concentration equivalent to 1.7 oz per 100 gallons water, the maximum dosage for vegetable plants (Olympic Horticultural Products n.d.). Approximately .5 mL Marathon II was diluted in .50 L water. Using a hand-held pressure sprayer, the solution was applied to the surface of leaves until droplets formed. Imidacloprid is an insecticide used to combat aphids in spinach crops (USDA 1999).

4. Synthetic Herbicides. After the emergence of 4-6 true leaves, Poast (sethoxydim) (available from Oxford Tract, BASF, Florham Park, NJ) was applied to pots adjacent to spinach plants to simulate the effects of drift of herbicide spot application to weeds. The equivalent concentration to the maximum dosage for spinach was used, approximately .45 ml diluted in .50 L water (BASF). Sethoxydim is a post-emergence herbicide selective for grassy weeds and approved for use on spinach crops.

Upon reaching maturity and before bolting, the edible portion of developed leaves was removed by cutting at the leaf base. Leaves for each plant were subsequently weighed, placed in one gallon freezer bags and stored at -18° C.

Table 1: Cultivation Schedule: 2005 dates of cultivation and treatment application for the eight treatment groups listed by soil and stress treatment.

Soil Treatment	Organic				Conventional			
Stress Treatment	Control	Simulated Herbivory	Pesticide	Herbicide	Control	Simulated Herbivory	Pesticide	Herbicide
Seed Planted	7-15	7-15	7-15	7-15	7-15	7-15	7-15	7-15
Transplanted (first set of true leaves)	8-31	8-31	7-29	8-31	7-29	7-29	7-29	7-29
Herbicide Application				8-9				8-9
Pesticide Application 1			8-9				8-9	
Pesticide Application 2			8-15				8-15	
Pesticide Application 3			8-22				8-22	
Synthetic Fertilizer Application 1			8-22		8-22	8-22	8-22	8-22
Synthetic Fertilizer Application 2			8-29		8-29	8-29	8-29	8-29
Simulated Herbivory		8-31				8-31		
Harvest	8-31	8-31	8-31	8-31	8-31	8-31	8-31	8-31

Quercetin Extraction and Content Analysis HPLC grade methanol for extraction and standard preparation and HPLC grade acetonitrile for the mobile phase solvent were purchased from Fisher Scientific (Fair Lawn, NJ). Phosphoric acid 85% was purchased from Mallinckrodt, Inc. (Paris Kentucky). The standards quercetin dihydrate, rutin trihydrate 95%, and quercitrin hydrate 85% were obtained from Sigma Chemical Company (St. Louis, MO).

A stock solution of quercetin, rutin, and quercitrin was prepared in 50:50 methanol:deionized water and subsequently diluted in the same solvent to obtain four standards. HPLC analysis for calibration curves proceeded on an Agilent 4 channel quarentary pump (Agilent, Palo Alto, CA) equipped with a UV-VIS diode array detector and Shiseido Capcell (Tokyo, Japan) C_{18} 250mm x 4.6mm reverse phase column. A 1.25 mL/min flow rate and 5 µL injections were used and absorption was recorded at 372 nm. The mobile phase consisted of solvent A, .2% phosphoric acid, and solvent B, HPLC grade acetonitrile, with a gradient of 80% solvent A and 20% solvent B for 15 minutes, 5% solvent A and 95% solvent B for 7 minutes, and 80% solvent A and 20% solvent B for 3 minutes. Data collection and integration was performed with ChemStation software (Agilent).

Individual plants in each treatment group were combined to yield a total of 8 samples. Harvested leaves were lyophilized, pulverized, and weighed and 2.00 g of the resulting samples were removed, diluted with 15 mL MeOH, shaken vigorously by hand for 5 minutes, and sonicated for 5 minutes. Five mL of solution was removed, diluted with 5 mL deionized water and filtered with Acrodisc .45 µm PVDF filters (Gelman Sciences, Ann Arbor, MI)

The dilutions were injected onto HPLC to determine the concentrations of quercetin, rutin, and quercetrin. The content of the compounds (mg per 100 g fresh weight) were subsequently determined.

Results

While this study provides a quantitative comparison of the quercetin content among the treatments, qualitative observations are instructive for the interpretation of results. Throughout cultivation, the appearance of the spinach provided an external measure of stresses that potentially influence quercetin content.

From 0 to 4 weeks plants cultivated in organic and conventional soils were visually equivalent. Plants cultivated in organic soil required more frequent watering due to a more porous soil structure. At 4 weeks, plants grown in conventional soil were noticeably less vigorous presumably as a result of nutrient and nitrogen leaching. Synthetic fertilizer was applied weekly to this treatment group until maturity was reached. However, plants cultivated in conventional soil failed to reach the size and mass of those cultivated in organic soil. Within soil treatments, plants grown under control, simulated herbivory, pesticide application, and herbicide application treatments displayed similar growth. No natural pests or weeds were present in any of the treatments.

HPLC analysis showed no detectable rutin or quercitrin in any samples (Figure 1). However, all samples exhibited quercetin content ranging from 0.0024 to 0.0060% dry weight as determined from peak area. This is equivalent to 22 to 88 mg per 100 g fresh weight (Table 2). Two-way analysis of variance (ANOVA) without replication showed no significant differences in quercetin content by percent dry weight ($p = .20$) or mg per 100 g fresh weight ($p = .16$) among the samples.

Table 2: Content of quercetin (mg per 100 g fresh weight) for the eight treatments

	Control	Simulated Herbivory	Pesticide Application	Herbicide Application

Organic Soil Conditions	54.19	37.90	37.92	27.66
Conventional Soil Conditions	88.43	54.65	25.21	22.11

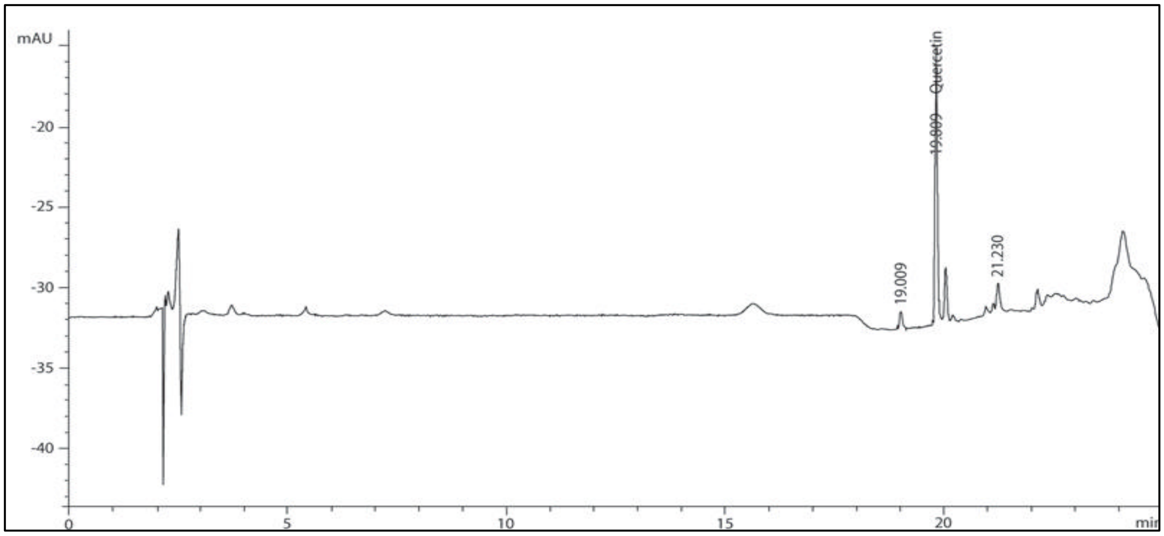

Figure 1: Sample chromatogram for organic soil conditions, control sample.

Discussion

As an unreplicated study with inherent confounding factors, low power is expected and limited inferences may be concluded from results. While simulating farming conditions, this study differs in a number of aspects including time scale and external factors. The influence of some conditions, like the accumulation of pesticidal chemicals in soil, may only be observed over a longer period of time. Similarly, conditions like soil medium could not fully simulate those observed under larger-scale agricultural systems. However, this type of study is valuable to provide preliminary information for further research by indicating which factors may have a large influence on quercetin biosynthesis.

During cultivation, the reduced vigor as evidence of nutrient leaching and nitrogen deficiencies in conventional soil compounds the difficulties of extrapolating findings to apply to complete agricultural systems. Literature cites higher nitrogen availability in conventional soils as increasing growth and decreasing the allocation of resources to secondary metabolite metabolism (Mitchell and Chassy 2005). However, in this study, a converse relationship resulted in the decreased growth of the plants grown under conventional soil conditions. Presumably, these conditions would lead to a higher content of the secondary metabolite quercetin in the conventional soil samples. Nevertheless, no significant differences were observed between the two soil treatments.

Simulated herbivory did not significantly induce quercetin synthesis. However, these findings should not nullify the hypothesis that insect herbivory induces secondary metabolite synthesis, specifically that of quercetin and its glycosides. In this study simulated herbivory did not replicate the mechanical stimuli of all types of spinach pests nor the chemical stimuli in insect saliva. Therefore, further research must consider multiple mechanisms of herbivory as well as chemical stimuli.

The application of pesticides likewise did not affect quercetin synthesis. Existing literature suggests the role of pesticides in secondary metabolite synthesis as decreasing the presence of herbaceous insects and hence stress caused by herbivory (Brandt and Mølgaard 2001). Since none of the treatments suffered from pest attack, the application of pesticides did not vary stress levels due to herbivory. These conditions make findings regarding the role of pesticides in modulating the level of herbaceous stress inconclusive.

The application of herbicides also had no significant effect on the level of quercetin in samples although levels were the lowest for both the organic soil and conventional soil treatments. One reason for these findings may be the limited application of the herbicide (one time near the beginning of cultivation) in accordance to guidelines and to avoid adversely damaging plants. This dose may have been too small to significantly affect carbon fixation and similarly secondary metabolite synthesis. Although it is difficult to avoid damage or mortality of spinach plants with the application of herbicides, especially those that target herbaceous weeds, future research should address various rates of application and the potential for significant differences. Herbicide application may also influence secondary metabolite synthesis by limited stress caused by the presence of weeds (Brandt and. Mølgaard). Since no weeds were present in any of the treatments, weed stress was not a variable considered.

This study is limited to a single cultivar of spinach and therefore its findings cannot be extrapolated to all vegetables or food in general. In addition, the influence of each of these treatments may differ significantly in the commercial agricultural setting and therefore conclusions should not be applied to commercially available spinach. In interpreting these findings, this study makes no claims regarding the health benefits of quercetin or the merit of increasing its concentration in foods. Current research has broadly concluded its beneficial qualities as an antioxidant and antihistamine; however, some studies also note its pro-oxitive properties (Kawanishi et al. 2005) and potential to damage DNA at high concentrations (Watjen et al. 2005). No threshold or upper limit for the benefits from the dietary intake of quercetin has been established. Therefore, the implications for human health of varying quercetin consumption cannot be concluded.

This study provides an important framework for further research regarding the content of secondary metabolites, namely antioxidants with potential health implications, in food produced under conventional and organic conditions. Future studies should focus on extending this work to include other crops, antioxidants, and modes of stress. In addition, antioxidant content in food available for individual consumers to purchase in supermarkets should also be considered. This growing field of research has broad implications for farmers, consumers, and the future of course of food production and policy.

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