ENHANCING HUMAN HEALTH PROMOTING ACTIVITY THROUGH THE REGULATION OF THE METHYL JASMONATE MEDIATED GLUCOSINOLATE BIOSYNTHESIS IN *BRASSICA OLERACEA*

BY

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DISSERTATION

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ABSTRACT

Brassica oleracea vegetables are recognized as functional foods that contain various phytochemicals such as glucosinolates (GS) and flavonoids that have health-promoting bioactivity. Recent data suggest that methyl jasmonic acid (MeJA) can increase concentrations of GS and polyphenolics in *Brassica* plants. In Chapter 2 tissue/organ specific responses to MeJA treatments were investigated in five cultivars of broccoli and two cultivars of kale in field plots over two years, MeJA treatments significantly increased total phenolics and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) antioxidant activity of kale leaf tissues, but had no effect on phenolics of broccoli florets. Correlation of growing degree days, precipitation per day and solar radiation with phenolic concentrations suggest that these weather related factors are associated with the enhancement of phenolics and tissue ABTS antioxidant activity.

In order to evaluate if MeJA treatment can enhance induction of quinone reductase activity, an anticancer biomarker of broccoli floret extracts, MeJA treatments were applied to five broccoli cultivars in each of two years under field conditions (Chapter 3). Sulforaphane, phenethyl isothiocyanate, and hydrolysis products derived from neoglucobrassicin were significantly increased by MeJA treatment. Sulforaphane, N-methoxyindole-3-carbinol (NI3C), and neoascorbigen showed significant correlations with QR activity in hydrolysed broccoli extracts. Although sulforaphane is a known QR inducer, there is only one published report about QR activity of hydrolysis products of neoglucobrassicin (Haack et al., 2010). The concentration required for doubling specific QR activity (CD value) was calculated to be 35 and 38 μM for NI3C and neoascorbigen, respectively. The CD value of sulforaphane was previously estimated to be 0.2 μM. Given the QR inducing potency and increased amount of isothiocyanate hydrolysis product from glucoraphanin, sulforaphane is considered to be the major contributor to QR inductive activity of MeJA treated broccoli florets.

Chapter 4 reports that MeJA spray treatments were applied to the kale varieties ‘Dwarf Blue Curled Vates’ and ‘Red Winter’ in replicated field plantings in 2010 and 2011, to investigate alteration of the GS composition in the harvested leaf tissue. The MeJA treatment significantly increased gluconasturtiin (56%), glucobrassicin (98%), and neoglucobrassicin (150%) concentrations in the apical leaf tissue of these genotypes for both season. Induction of

quinone reductase (QR) activity was significantly increased by the extracts from the leaf tissue of these two cultivars. There were significant year and year by genotype interactions in the concentrations of GS and QR activity. To determine the relationship between GS hydrolysis products and QR activity, a range of concentrations of MeJA sprays were applied to kale leaf tissues of both cultivars in 2011. Correlation analysis of these results indicated that sulforaphane, NI3C, neoascorbigen, I3C, and diindolylmethane were all significantly correlated with QR activity. Thus, increased QR activity may be due to several hydrolysis products in kale leaves rather than individual products alone.

MeJA treatment can also increase ethylene production, which may be harmful for the maintenance of postharvest quality of broccoli. To increase health-promoting properties of broccoli while maintaining post-harvest storage quality, 1-methylcyclopropene (1-MCP, a competitive inhibitor of plant ethylene receptor proteins) was applied to control and MeJA treated broccoli (Chapter 5). The combination of 1-MCP with MeJA treatment maximized phytochemical content and QR activity while maintaining acceptable visual quality. In order to understand the mechanisms of response in broccoli to MeJA and 1-MCP treatments gene expression of GS biosynthetic, hydrolytic, chlorophyll catabolic, and pathogen related protein (PR) genes were measured by quantitative RT-PCR. MeJA treatment significantly increased transcript abundance of the indolyl GS biosynthesis genes *BoCYP79B2, BoCYP83B1,* as well as myrosinase, epithiospecifier protein modifier 1 (*BoESM1*), and epithiospecifier protein (*BoESP*) genes. Consequently, neoglucobrassicin and gluconasturtiin concentrations were significantly increased by MeJA treatment. In addition, increased sulforaphane, phenethyl isothicyanate, NI3C, and neoascorbigen were significantly correlated with QR inductive activity, indicating MeJA induced GS levels enhances potential cancer chemopreventive activity. MeJA treatment significantly increased ethylene production of broccoli floret at harvest date and reduced total chlorophyll content and visual quality during post-harvest storage. 1-MCP treatment significantly suppressed mRNA levels of the chlorophyll catabolism genes, *BoPaO* and *BoPPH*. As a result, the combined treatment of MeJA and 1-MCP provides enhanced QR inductive activity while maintaining post-harvest quality compared to MeJA treatment alone.

In Chapter 6, the effect of MeJA treatments were investigated in a cauliflower (*B. oleracea* L. var. *botrytis*) cultivar. Visual quality, ethylene production, GS compositional changes, and QR inductive activity of cauliflower curd extracts were examined during post-harvest storage at 4 °C. There was no significant ethylene production or visual quality loss with the MeJA treatment. Unlike broccoli, MeJA significantly increased glucoraphanin, glucobrassicin, and neoglucobrassicin, implying that GS compositional changes associated with MeJA treatment maybe species-specific. Increased GS concentrations were significantly correlated with QR inductive activity. In conclusion, MeJA treatment to cauliflower significantly enhanced QR inductive activity without a loss in post-harvest quality.

Several studies to determine application protocols that maximize accumulation of GS and other phytochemicals in broccoli florets were discussed in Chapter 7. We investigated the effect of solvents and varying MeJA application concentrations, application number, and application date in days prior to harvest of broccoli florets of the cultivar 'Green Magic'. MeJA application four days prior to harvest generated broccoli florets with the highest concentrations of GS. Although a single application of 250 μM MeJA significantly increased GS concentrations in broccoli florets, two consecutive days of treatment (four and three days prior to harvest) of 250 μM MeJA further increased total GS concentrations (primarily neoglucobrassicin) and QR activity four days prior to harvest. With increasing treatment concentrations of MeJA to broccoli florets gluconasturtiin, neoglucobrassicin, and glucoraphanin floret concentrations and QR inductive and nitric oxide production inhibitory activity were gradually increased. These application protocols were found to maximize GS concentrations and putatively enhance the health promoting properties of broccoli florets.

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CHAPTER 1

Literature Review

1.1. Rationale

Increased life span has made people pay more attention to the quality of their lifestyles. Diseases and health problems have become a major concern in our aging population. A nutritious diet can prevent cancer and other diseases (Abdulla and Gruber, 2000). Hence, functional plant foods that promote health have received a great deal of attention from scientists and other consumers. *Brassica oleracea* vegetables including broccoli, cabbage, cauliflower, kohlrabi, Brussels sprouts, and kale are commonly consumed around the world. These vegetables are recognized as functional foods that contain various phytochemicals such as glucosinolates (GS), flavonoids, carotenoids, vitamins, and minerals that have health-promoting bioactivity. Research suggests that it is possible to improve beneficial phytochemicals in *B. oleracea* vegetables by both cultivation and genetic manipulation (Brown et al., 2002).

Although health-promoting compounds can be improved by breeding, these programs are costly and can require 8-12 years to develop improved cultivars. In contrast, developing new production practices that enhance vegetable health-promoting bioactivity can accelerate the potential delivery of nutritionally enhanced foods to consumers. Recent data suggest that the plant-signaling hormone, methyl jasmonic acid (MeJA), can increase concentrations of GS and polyphenolics in broccoli florets and sprouts, which are putative health-promoting compounds (Kim and Juvik, 2011; Pérez-Balibrea et al., 2011).

Although MeJA has previously been shown to enhance GS in broccoli florets and sprouts, these studies were conducted in greenhouse environments. It is important to evaluate the effect of MeJA treatments on broccoli under field conditions as a potential cultivation practice. Field environments experience heat, drought, and exposure to pests, factors which can attenuate or magnify MeJA mediated phytochemical enhancement. Since most commercial *Brassica* vegetable production is in the field, cultivation methods to enhance their health promotion must be tested in field environments.

Health-promoting compounds in *Brassica oleracea* can be degraded and lost during post-harvest storage because these vegetables are perishable commodities. MeJA treatment can increase endogenous ethylene production and respiration of vegetable products, which can be harmful to the maintenance of post-harvest quality and phytochemical content. However, the effect of pre-harvest treatment of MeJA on post-harvest *B. oleracea* vegetable quality has yet to be examined. Inhibition of ethylene production or blocking the ethylene receptor is an effective way to improve shelf life and quality. It has been reported that application of 1-MCP (1-methylcyclopropene) increases shelf life of broccoli by competitive inhibition of ethylene receptor proteins. Thus, hypothetically, 1-MCP application to MeJA treated broccoli or cauliflower may optimize post-harvest maintenance of MeJA enhanced phytochemical content.

This research has been designed to evaluate how to optimize the putative health-promoting bioactivity of *B. oleracea* vegetables by pre-harvest MeJA treatments and during post-harvest storage.

1.2. History of the Species *Brassica oleracea*

Brassica is a genus of plants in the mustard family (*Brassicaceae*), which contains important agricultural and horticultural crops. Certain parts of these crops have been developed for food, such as roots (e.g. rutabaga), stems (e.g. kohlrabi), leaves (e.g. cabbage, kale), immature flowers (e.g. cauliflower, broccoli), and seeds (e.g. mustard seed and oil-producing rapeseed).

B. oleracea vegetables are consumed around the world. Leafy kales and branching, thin-stemmed kales were the earliest cultivated *Brassicas*. These *B. oleracea var. acephala* types have given rise to the forage kales, fed mainly to livestock. *B. oleracea var. capitata*, the cabbage group evolved in Germany. Both red and white cabbages were known to be cultivated from about A.D. 1150 in Germany and in England by the fourteenth century. Cauliflower, in its present form, was unknown before the early Middle Ages. It was not mentioned by the earlier German herbalists and was only described in 1576 as *Brassica florida botrytis* and in 1583 as *Brassica cauliflora*. It was grown for seed in the area surrounding the Gulf of Naples in Italy. At the beginning of the seventeenth century, cauliflower reached Germany, France and England. Sprouting broccoli, *B. oleracea var. italica*, came from the Levant, Cyprus or Crete to Italy. It was not mentioned until 1660 and was referred to as ‘sprouting colli-flower’ or ‘Italian Asparagus’ in Miller’s Gardner’s Dictionary of 1724. Brussels sprouts, *B. oleracea var. germmifera*, in its present form, appeared about 1750 in Belgium as a ‘sport’, but by 1820 was known as ‘Chou de Bruxelles’ (Simmonds, 1976).

1.2.1. Glucosinolates

GS are sulfur-containing secondary metabolites found primarily in the *Brassicaceae*, although other GS-containing plant families occur within the *Capparaceae* and *Caricaceae* (Fahey et al., 2001). In the past few decades, research on GS in crop plants has increased following the discovery of their putative role as cancer-prevention agents, crop-protection compounds, and bio-fumigants in crop production fields (Halkier and Gershenzon, 2006).

There are approximately 120 described GS sharing a chemical structure consisting of a β-D-glucopyranose residue linked via a sulfur atom to a (Z)-*N*-hydroximinosulfate ester, plus a variable R group (Figure 1.1). GS are classified according to their amino acid precursor (Halkier and Gershenzon, 2006). GS derived from alanine, leucine, isoleucine, methionine, or valine are called aliphatic GS, those derived from phenylalanine or tyrosine are called aromatic GS, and those derived from tryptophan are called indolyl GS.

1.2.1.1. Glucosinolates Biosynthesis

GS biosynthesis genes have been intensively studying in *Arabidopsis* with biochemical assays. There is high homology of gene sequences in GS biosynthesis between *Arabidopsis* and *Brassicaceae* (Bak et al., 1998). GS biosynthesis consists of three independent steps: (i) chain elongation of selected precursor amino acids, (ii) formation of the core glucosinolate structure, and (iii) secondary modifications of the amino acid side chain. A side chain amino acid precursor proceeds through a series of chain elongations prior to entering the pathway. Two genes, *methylthioalkylmalate*

synthase 1 (*MAM1*) and *MAM2*, have been identified in *Arabidopsis*, where *MAM1* regulates the first two methionine elongation cycles, whereas *MAM2* results in only one round of elongation (Kroymann et al., 2003; Textor et al., 2004) (Figure 1.2).

The second step is the GS core structure formation from the precursor amino acids by reaction with various cytochrome P450 enzymes (CYP) (Sønderby et al., 2010), CYP79 genes catalyze the conversion of the amino acid to aldoximes. *CYP79F1* and *CYP79F2* genes are responsible for aldoxime production leading to aliphatic GS derived from chain-elongated methionine derivatives, whereas *CYP79B2* and *CYP79B3* have distinct functions for indolyl GS biosynthesis derived from tryptophan (Sønderby et al., 2010). In the biosynthetic pathway of indolyl GS, *CYP79B2* catalyzes the conversion of tryptophan to indole-3-acetaldoxime, with *CYP83A1* and *CYP83B1* metabolizing the phenylalanine- and tyrosine-derived aldoximes (Sønderby et al., 2010). It has been reported that indolyl GS biosynthesis is modulated by the methyl jasmonate (MeJA) and the salicylic acid (SA) signal transduction pathways (Mikkelsen et al., 2003).

The biological activity of GS depends on diversity of structure of the side chain that is the last step of GS biosynthesis (Hopkins et al., 2009). For aliphatic GS, secondary modifications include oxygenations, hydroxylations, alkenylations and benzoylations. Indolic GS, in turn, can undergo hydroxylations and methoxylations (Sønderby et al., 2010). Recently, methoxylation genes involved in glucobrassicin such as *CYP81F2*, *CYP81F3, and CYP81F4* (Figure 1.3) were clearly identified by genetic engineering *Arabidopsis* indolyl GS biosynthesis into *Nicotiana benthamiana* (Pfalz et al., 2011). 4-methoxyglucobrassicin hydrolysis products have been reported to be antibiotic to fungal pathogens and to green peach aphid (*Myzus persicae*), respectively (Bednarek et al.,

2009; Kim et al., 2008) whereas neoglucobrassicin is reported as a compound that is synthesized in response to insect hervivore (Hopkins et al., 2009).

1.2.1.2. Glucosinolate Hydrolysis

Intact GS do not display bioactivity but following hydrolysis by the endogenous enzyme myrosinase, generate isothiocynates (ITCs) and other products, which have been associated with insect resistance and anti-cancer activity. In plant tissues, myrosinase is present in idioblastic myrosin cells and physically separated from GS substrates. Compartmentalization of the GS-myrosinase system is characterized in *Arabidopsis* by the presence of sulfur-rich cells (S-cells) between the phloem and the endodermis of the flower stalk, which presumably contain high concentrations of GS (Koroleva et al., 2000), and by the localization of myrosinase in neighboring cells. When the plant tissue is disrupted, myrosinase and substrate (GS) come into contact, and consequently results in GS hydrolysis. Myrosinase cleaves the glucose unit from the GS to form an unstable intermediate, which can undergo rearrangement into several different biological active compounds such as ITCs, nitriles or thiocyanates. The chemical structure of these hydrolysis products depends on the structure of the GS side chain and reaction conditions such as pH, concentration of Fe^{2+} and presence of epithiospecifier protein (ESP) (Bones and Rossiter, 1996). In the absence of ESP, the addition of Fe^{2+} ions also promotes nitrile formation, nitriles are weak anticancer compounds compared to the isothiocyanates like SF, PEITC, and AITC (Matusheski et al., 2006). The *epithiospecifier modifier 1* (*ESM1*) gene in *Arabidopsis* encodes a protein shown to inhibit function of ESP, leading to increased isothiocyanate production from GS hydrolysis (Zhang et al., 2006).

There are several types of hydrolysis products of indolyl GS (Figure 1.4). Although nitriles were dominant GS hydrolysis products from autolysis (pH 5-6) of aliphatic GS in cabbage, in the presence of ascorbic acid, ascorbigen (or neoascorbigen) and thiocyanate ion were the dominating products from glucobrassicin and neoglucobrassicin (Agerbirk et al., 1998). Depending on the pH, oligomerization patterns are different: dimerization to di(indol-3-yl)methane (DIM) happens even in neutral solutions, whereas formation of the linear and cyclic trimers requires a weakly acidic solution (Agerbirk et al., 1998).

1.2.1.3. Health Promoting Hydrolysis Products of Glucosinolates

GS have been identified as potent cancer prevention agents because some hydrolysis products induce mammalian phase II detoxification enzymes, such as quinone reductase (QR), glutathione-S-transferase (GST), and glucuronosyl transferases. Sulforaphane, the ITC derivative of 4-methylsulfinylbutyl GS or glucoraphanin (Figure 1.1), found in broccoli, has been identified as a QR inducer (Zhang et al., 1992). Previous research reported that sulforaphane putatively prevents tumor growth by blocking the cell cycle and promoting apoptosis (Gamet-Payrastre et al., 2000). It has been reported that sulforaphane also induces growth arrest and apoptosis against colon, prostate, leukemia, melanoma, and ovarian cancer cells (Chiao et al., 2002; Chuang et al., 2007; Gamet-Payrastre et al., 2000; Misiewicz et al., 2003; Wang et al., 2004). Moreover, sulforaphane displays antibiotic activity against the bacteria *Helicobacter pylori*, which promotes gastritis and stomach cancer (Fahey et al., 2002).

In addition to the sulforaphane, several indole and aromatic GS also have cancer chemopreventive activity. The best example is indole-3-carbinol (I3C), which is a hydrolysis product of glucobrassicin. I3C was reported to show dose-related decreases in tumor susceptibility as inferred by decreases in [3H] aflatoxin B1-DNA binding in trout (Dashwood et al., 1989). Kim et al. (2003) reported that I3C inhibited mouse colon carcinogenesis. According to Brew et al. (2006), treatment of the immortalized human mammary epithelial cell line MCF10A with I3C induced a G1 cell cycle arrest, elevated p53 tumor suppressor protein levels and stimulated expression of the downstream transcriptional target, p21. In both men and women, I3C significantly increased the urinary excretion of C-2 estrogens. The urinary concentrations of nearly all other estrogen metabolites, including levels of estradiol, estrone, estriol, and 16α-hydroxyestrone, were lower after 6-7 mg/kg per day of I3C treatment (Michnovicz et al., 1997). The mechanism by which I3C promotes 2-OH formation involves the selective induction of Phase I metabolizing cytochrome P450 enzymes, which facilitate the 2-hydroxylation of estrogen. Through this metabolic role, I3C promotes an increased ratio of 2-OH to l6α -OH and may improve estrogen metabolism in women with poor diets or an impaired detoxification mechanism (Michnovicz et al., 1991). I3C may also reduce the activity of the enzyme required for the 4-hydroxylation of estrogen, thereby decreasing carcinogenic 4-OH formation. Ascorbigen (ASG) is formed from its precursor, glucobrassicin. After glucobrassicin is enzymatically hydrolyzed to I3C, it reacts with L-ascorbic acid to form ASG (Wagner and Rimbach, 2009). ASG induces apoptosis, prevention of DNA damage, and the upregulation of xenobiotic metabolizing enzymes (Bonnesen et al., 2001). Moreover, 3,3'-Diindolylmethane (DIM), a major *in vivo* product

of acid-catalyzed oligomerization of I3C, is a promising anticancer agent present in vegetables of the *Brassica* genus. The effects of DIM on estrogen-regulated events in human breast cancer cells was investigated and it was observed that DIM is a promoter-specific activator of estrogen receptor (ER) function in the absence of 17β -estradiol [E(2)] (Riby et al., 2000). Incubated microsomes from rats pretreated with I3C and ASG yielded high levels of 2-hydroxyestradiol that were comparable to levels induced by β-naphthaflavone and were significantly above control group levels ($P < 0.005$) (Sepkovic et al., 1994).

Allyl isothiocyanate, derived from the aliphatic GS sinigrin, has tumor inhibition activity *in vitro* and *in vivo* (Kumar et al., 2009; Manesh and Kuttan, 2003) (Figure 1.1). Phenethyl isothiocyanate (PEITC), which is derived from the aromatic GS, gluconassturtiin, has a potential role in protection against colon cancer (Chung et al., 2000) and is also well known as a QR and GST inducer (Manson et al., 1997; Rose et al., 2000).

1.2.2. Flavonoids

Flavonoids typically have a C6-C3-C6 structure (Figure 1.5). This class of compounds, ubiquitous in the plant kingdom, is estimated to contain over 10,000 members (Dixon and Pasinetti, 2010). Flavonoids in plants have been found to be associated with a wide range of bioactive roles including beneficial microbial attractants, insect repellants, and to protect plants from Ultraviolet-B (UV-B) damage (Agati and Tattini, 2010; Dixon and Pasinetti, 2010; Tattini et al., 2005).

Dietary flavonoid consumption can delay carcinogenesis through the induction of phase II enzymes or by blocking DNA damage in the initial stage of carcinogenesis (Moon et al., 2006) (Figure 1.6). Brusselmans et al. (2005) reported that 18 naturally occurring flavonoid compounds showed a potential to induce apoptosis in cancer cells which was strongly associated with the inhibition fatty acid synthase (FAS, a key lipogenic enzyme overexpressed in many human cancers). Traditionally, flavonoids were thought to prevent cancer by their hydrogen-donating antioxidant properties but recent data suggest that their bioactivity may be associated with the inhibition of protein kinase (Hou and Kumamoto, 2010). Members of the genus *Brassica* have abundant levels of the flavonols, kaempferol and quercetin (Chen and Kong, 2004). Previous research reported that flavonol contents in kale and broccoli were positively correlated with solar or UV-B radiation (Gliszczynska-Swiglo et al., 2007; Zhang et al., 2003). These flavonols and quercetin glycosides have also been reported as QR inducers (Williamson et al., 1996; Yannai et al., 1998). Somerset and Johannot (2008) reported that broccoli is a significant source of kaempferol and quercetin in Australian diets.

1.2.3. Carotenoids

Carotenoids are tetraterpenoids whose structure is composed of a polyene hydrocarbon chain, which is sometimes terminated by rings, and may or may not have additional oxygen atoms attached. In photosynthetic organisms, carotenoids play a vital role in photosynthesis by protecting the reaction center from auto-oxidation and facilitating non-damaging energy transfer processes (Bowsher et al., 2008). For humans, some carotenoids are precursors of vitamin A, which prevents certain forms of cancers

and promotes eye health. Putative mechanisms of disease prevention by carotenoids involve reduced cell proliferation, mutagenesis and genotoxicity by their antioxidant properties, and enhanced apoptosis of cancerous cells (Krinsky et al., 2003; Palozza et al., 2003). *Brassica oleracea* vegetables like kale, cabbage, and broccoli are recognized as excellent sources of dietary carotenoids (U.S. Department of Agriculture, 2011b). Broccoli has emerged as the most commonly consumed *Brassica* vegetable in the United States and supplies an important fraction of carotenoids to the U.S. diet. Most of the variation in carotenoid and chlorophyll content in broccoli cultivars is mainly determined by genotypic differences (Farnham and Kopsell, 2009). According to a recent study, total carotenoid content among 24 broccoli cultivars ranged from 55 to 154 mg/g dry weight (Ibrahim and Juvik, 2009).

1.2.4. Vitamins, Minerals, and Other Nutrients

Brassica plants are a good source of vitamin C. Vitamin C or L-ascorbic acid is an essential nutrient for humans and certain other animal species. In living organisms ascorbate acts as an antioxidant by protecting the body against oxidative stress. Severe deficiency of vitamin C can result in scurvy. According to a recent study, commercial broccoli cultivars contain from 57 to 131 mg of vitamin C per 100 g fresh weight (Koh et al., 2009). Considering that dietry reference *intake* ranges from 13 mg to 100 mg, broccoli is excellent source of vitamin C. Kale contains 817 µg of vitamin K per 100 g fresh weights. Vitamin K is associated with blood coagulation and bone metabolism. It was reported that broccoli and cauliflower have high phytosterol content among vegetables (Piironen et al., 2003). Broccoli and cauliflower have 3408 and 4100 mg of

total phytosterols per kg of dry weight with β-sitosterol the predominant form (Gajewski et al., 2011; Piironen et al., 2003).

1.3. Jasmonic Acid

1.3.1. Synthesis, Induction and Responses to Jasmonic Acid (JA)

Jasmonates are defined as plant signal transduction compounds. JA is a defense related compound which was originally isolated from a pathogenic fungus, *Lasiodiplodia theobromae* (Aldridge et al., 1971). However, MeJA was firstly isolated from *Jasminium grandiflorum* L. flowers before JA was discovered (Demole et al., 1962). Jasmonates are synthesised via the octadecanoid pathway, beginning with linolenic acid. Synthesis is initiated with the conversion of linolenic acid to 12-oxo-phytodienoic acid (OPDA), which then undergoes a reduction and three rounds of oxidation to form (+)-7-iso-JA, jasmonic acid proper. Only the conversion of linolenic acid to OPDA occurs in the chloroplast; all subsequent reactions occur in the peroxisome (Katsir, 2008; Liechti et al., 2006). JA conjugated with the amino acid isoleucine (Ile) results in JA-Ile, which is currently the only known JA derivative effective at JA signaling (Katsir, 2008; Tamogami et al., 2008).

Plant herbivore defense related signals that induce JA biosynthesis including mechanical damage, cell wall fragmentation and the release of peptides, fatty acid-amino acid conjugates (FAC), and green leafy volatiles (GLV). Also, other factors can induce JA production such as UV-light, salt stress, ozone, and developmental cues (Howe and Jander, 2008)(Figure 1.7). The host plant defense responses can be divided into two types; direct and indirect. As an example of indirect defense, plants produce volatile compounds

that attract specific parasitoids against herbivore attack (Bruinsma et al., 2009; Paré and Tumlinson, 1999; Schnee et al., 2006). Examples of direct defense in plant include production of defensive compounds such as GS and monodesmosidic saponins (Osbourn, 1996).

1.3.2. MeJA as an Elicitor of Health Promoting Compounds

Previous studies have revealed that MeJA treatment enhances the health-promoting bioactivity of several plant species. Extracts of MeJA treated blackberry showed enhanced inhibition of lung and leukemic cancer cell proliferation and induced apoptosis of leukemic cancer cells (Wang et al., 2008). MeJA increased ascorbic acid concentrations in *Arabidopsis* and tobacco suspension cells (Wolucka et al., 2005). In radish sprouts, MeJA increased phenolics, antioxidant activity, and phenylalanine ammonia lyase (PAL) (Kim et al., 2006). However, this treatment decreased the amount of 4-methylthio-3-butenylisothiocyanate, a major isothiocyanate in radish sprouts and the activity of myrosinase. Similarly, Pérez-Balibrea et al. (2011) reported that MeJA increased GS in broccoli sprouts but higher levels of MeJA decreased glucoraphanin. Fritz et al. (2010) reported that JA increased glucoiberin, progoitrin, snigrin, and gluconapin in cabbage. Kim and Juvik (2011) reported that MeJA treatment increased gluconasturtiin and neoglucobrassicin in broccoli florets of the 'Green Magic' cultivar and gluconasturtiin, glucobrassicin and neoglucobrassicin in broccoli florets of the genotype 'VI-158' (Kim and Juvik, 2011). Kim and Juvik (2011) observed a 4.3-fold increase of neoglucobrassicin in florets of the broccoli genotype 'SU-003'. Pérez-Balibrea et al. (2011) using broccoli sprouts showed that GS increases were less than two

fold with MeJA treatment. In buckwheat, MeJA treatment increases flavonoid content (Horbowicz et al., 2011). Kim et al. (2011) reported that chlorogenic acid, catechin, isoorientin, orientin, rutin, vitexin, and quercitrin were the increased by MeJA application to buckwheat. These data suggest that MeJA can be a useful elicitor for increasing biosynthesis of health promoting compounds in the tissues of crop plants.

1.3.3. MeJA Effect on Post-harvest Vegetable Quality

MeJA applications have been reported to reduce chilling injury in mango, longquat, pineapple, and peach (Cao et al., 2009; González-Aguilar et al, 2001; González-Aguilar et al., 2000; Jin et al., 2009; Nilprapruck et al., 2008). These results suggest that antioxidant capacities and antioxidant enzyme activities enhanced by MeJA application increases chilling tolerance in fruits and vegetables. Also, MeJA was also observed to reduce microbial contamination and extended shelf life in celery and pepper (Buta and Moline, 1998). Another study reported that MeJA application suppressed green mold decay in grapefruit (Droby et al., 1999). It was suggested that MeJA reduced microbial contamination through the expression of pathogen related defense gene transcription and enzyme activity (González-Aguilar et al., 2006; Yao and Tian, 2005). However, MeJA treatment can also increase ethylene production, which initiates and stimulates senescence in broccoli (Watanabe et al., 2000). This research implies that MeJA application may reduce the shelf life and maintenance of post-harvest quality of some crops.

1.3.4. MeJA as an Elicitor of Plant Defense

Plants have lots of ways to defend themselves against herbivore or pathogen attack. Cosmetic damage by pests is a crucial concern for quality and yield of fruit and vegetable crops. Induced biochemical responses can be activated in plants by a variety of biotic and abiotic elicitors that can be induced in the absence of the pathogen or herbivore attack (Oostendorp et al., 2001). Numerous investigations have reported that induced responses by MeJA treatment affect phytophagous insect oviposition behavior and their feeding activity.

Several experiments have already shown that jasmonates can increase insect resistance in a direct or indirect manner. JA induced resistance changed oviposition preference of two specialist herbivores in cabbage (Bruinsma et al., 2007). JA induced resistance affected the development time of *Pieris rapae* and has been shown to attract parasitoids of *P. rapae* in Brussels sprouts (Bruinsma, et al., 2009). Oviposition or feeding preference of some insect pests of *Brassica* plants are influenced by GS concentrations. The intact GS, glucobrassicin and neoglucobrassicin, can serve as host recognition cues for *Plutella xylostella* although the breakdown product, indole-3-carbinol (I3C) deters the oviposition by *Plutella xylostella* in *Arabdopsis thaliana* (Sun et al., 2009). I3C derived from indolyl GS deters oviposition by *Pieris rapae* in *A. thaliana* (De Vos et al., 2008). The breakdown product from indolyl GS also increased resistance against the green peach aphid in *A. thaliana* (Kim et al., 2008; Pfalz et al., 2008). Both indolyl and aliphatic GS significantly impacted the feeding behavior of four generalist insect herbivores but had no influence on two specialist insect species (Müller et al., 2010).

There have been a few field experiments to support the potential of insect pest control by JA application. Field JA application reduced numbers of leafminers in celery and thrips and midges in winter wheat (Black et al., 2003; El-Wakeil et al., 2010). MeJA treatments have been shown to reduce lepidopteran pest populations in broccoli, Brussels sprouts, Chinese cabbage, rutabaga, and canola (McEwen, 2011). McEwen also observed that MeJA application decreased marketable yield in several of *Brassica* crops. This implies that long term MeJA application may suppress plant growth due to the programmed plant allocation of resources to defense. This phenomenon has been termed the "fitness cost of induced resistance" (Heil and Baldwin, 2002).

1.4. Broccoli, Cauliflower, and Maintenance of Post-harvest Quality

Brassica vegetables are perishable commodities compared to apples, nuts and many agronomic crops. There has been extensive research on the methods to maintain the post-harvest quality of these vegetables. Production of ethylene by harvested produce is associated with declining post-harvest quality. Inhibition of ethylene biosynthesis with the accompanying delay in product senescence to is a post-harvest protocol to maintain quality.

1.4.1. Post-harvest Senescence Physiology of Broccoli

Broccoli is harvested when flowering heads are still immature and actively growing. The head of a broccoli plant is comprised of numerous of immature florets, each made up of male and female reproductive organs surrounded by immature petals and sepals. The first sign of broccoli senescence is where chlorophyll in the sepals is

degraded (Wang, 1977). As broccoli deteriorates, the head yellows and tissues become flaccid, with cell necrosis developing during advanced stages of senescence (King and Morris, 1994b). Broccoli branchlets go through major losses of sugars, organic acids, and proteins within the first six hours after harvest, followed by increases in the free amino acid pools (especially the amides glutamine and asparagine) and ammonia accumulation (King and Morris, 1994a). Loss of membrane fatty acids is also a feature of post-harvest broccoli senescence (Page et al., 2001).

Tian et al. (1994) suggested that the reproductive structures (stamens and pistil) may have a role in determining the rate of sepal yellowing, as removing them from florets reduced the rate of sepal degreening. The pistil and stamens also had 7-fold higher levels of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) activity and more than double the ethylene production of other tissues within the floret (Tian et al., 1994). The rapid increase in *BoACO1* and *BoACO2* transcript abundance after harvest contributes to increased ethylene production by florets (Pogson et al., 1995). Transgenic broccoli lines harboring the antisense *BoACO2* gene construct displayed delayed senescence in both detached leaves and detached heads as measured by color change (Gapper et al., 2005). When harvesting broccoli, wounding to the stem increases ACO activity and its transcripts in florets (Kato et al., 2002).

1.4.2. 1-methylcyclopropene (1-MCP)

Treatment of perishable fruits and vegetables with 1-MCP is a successful protocol for maintaining post-harvest quality that is effective and safe. 1-MCP was developed by Sisler and coworkers at the University of North Carolina (Paliyath, 2008).

Carnation flowers, tomato, and banana were first tested with 1-MCP (Sisler and Serek, 1997; Sisler et al., 1996). The United States Environmental Protection Agency (EPA) approved the first commercial product, EthylBloc in 1999. This product was made available for ornamental crops. After further testing and registration of 1-MCP it was made available for application on edible crops by AgroFresh Inc., a subsidiary of Rohm and Haas (Springhouse, PA). With the approval by EPA in 2002, 1-MCP is marketed under the trade name of SmartFresh. The mechanism by which 1-MCP delays senescence is due to its ability to competitively bind to ethylene receptors in plant tissues. 1-MCP has 100-fold higher affinity for the receptor proteins than ethylene. A commercial 1-MCP application protocol was developed using a stable formulation of 1-MCP in a powder in the form of a complex with cyclodextrin. This system allows 1-MCP to be released as a gas when the powder comes in contact with water (Paliyath, 2008). Primarily, 1-MCP is used an efficient and simple technology to preserve fruit and vegetable quality after harvest. Besides that, 1-MCP has become a powerful tool to understand the fundamental mechanisms involved in ripening and senescence. While a wide range of experiments has been conducted on some climacteric, or nonclimateric fruits, and on some vegetables, response of other crops to 1-MCP is in need of further investigation.

1.4.3. Post-harvest Studies of *B. oleracea* Vegetables

An early survey observed that three cultivars of broccoli showed different patterns of ethylene production during storage at 20 °C (King and Morris, 1994b). Modified atmosphere packaging (MAP) treatments on visual quality and GS content were determined and compared with unwrapped florets. MAP treatments extended the shelf

life and reduced the post-harvest deterioration of broccoli florets stored at both 4 ° and 20 °C. All three MAP treatments showed a reduced loss of aliphatic and indolyl GS in broccoli florets when compared to those in the control (Chen-Guo et al., 2009). Long-term freezer storage did not affect total aliphatic or indole GS content in cauliflower (Volden et al., 2009). For enhanced phytochemical retention, broccoli florets should be packed in polypropylene (PP) micro-perforated film bags and stored under refrigerated conditions (Nath et al., 2011). In this experiment, samples packed in PP micro-perforated film showed significantly lower loss of moisture, ascorbic acid, chlorophyll, β-carotene and total antioxidant activity compared to controls (5.5%, 4.5%, 18.9%, 4.0% and 16.4%, respectively). Broccoli and cauliflower display distinct post-harvest physiology in a modified atmosphere packing experiment. Broccoli required 8% O_2 + 14% CO_2 while cauliflower required 1% O_2 + 21% CO_2 for the best retention of GS (Schreiner et al., 2006).

Visual color is an critical factor in retailer and consumer evaluation of product quality and subsequent purchasing decisions (Dixon, 2007). Yellowing of the foliage or discoloration of the heads and curds of broccoli and cauliflower is associated with senescence and is not acceptable to retail consumers. Consequently, color is an important factor in retailer and consumer evaluation of product quality and subsequent purchasing decisions. Thus, early research on 1-MCP applied to broccoli focused on extension of product shelf life and visual color (Ku and Wills, 1999). 1-MCP was more effective at maintaining greening in *Brassica* flowering tissue than in leafy *Brassica* vegetables (Able et al., 2002). 1-MCP treatment reduced yellowing and rate of respiration when broccoli was exposed to ethylene (Fan and Mattheis, 2000; Gaofeng et al., 2010). The effect of 1-

MCP and different packaging materials in combination treatments reduced loss of moisture, chlorophyll (Kasim et al., 2007), and chlorophyllase activity in broccoli (Gong and Mattheis, 2003). Gaofeng et al. (2010) reported that 1-MCP treatment noticeably extended broccoli shelf life, reduced post-harvest deterioration, retarded chlorophyll degradation and inhibited increases in malondialdehyde and the activities of polyphenol oxidase and lipoxygenase in florets. Previous study reported that pheophytinase (PPH) and pheophorbide a oxygenase (PaO) are key enzymes for chlorophyll breakdown in broccoli florets (Bücherta et al., 2011; Gomez-Lobato et al., 2012a). It was reported that expression of *BoPPH* and *BoPaO* were reduced by 1-MCP treatment (Gomez-Lobato et al., 2012b). Moreover, antioxidant enzymes such as superoxide dismutase, peroxidase, and catalase in florets treated with 1-MCP were higher than those in control florets. 1-MCP treatment reduced the rate of decrease of total chlorophyll, total carotenoids, ascorbic acid and GS in florets when compared to those in the control. 1-MCP treatment on broccoli significantly decreased the expression of aminocyclopropane-1-carboxylic (ACC) synthase1 *ACS1*, *ACS2*, ACC oxidase1 *ACO1*, ethylene response sensor *ERS*, and the *ethylene* receptors *ETR1* and *ETR2* (Gang et al., 2009). 1-MCP treatment down-regulated the expression of ascorbate peroxidase1 *APX1* and *APX2*, and up-regulated that of dehydroascorbate reductase *DHAR* and L-galactono-1,4-lactone dehydrogenase *GLDH* in broccoli compared with the control (Ma et al., 2010). 1-MCP treatment can contribute to the suppression of ascorbate reduction by regulation of the expression of broccoli genes.

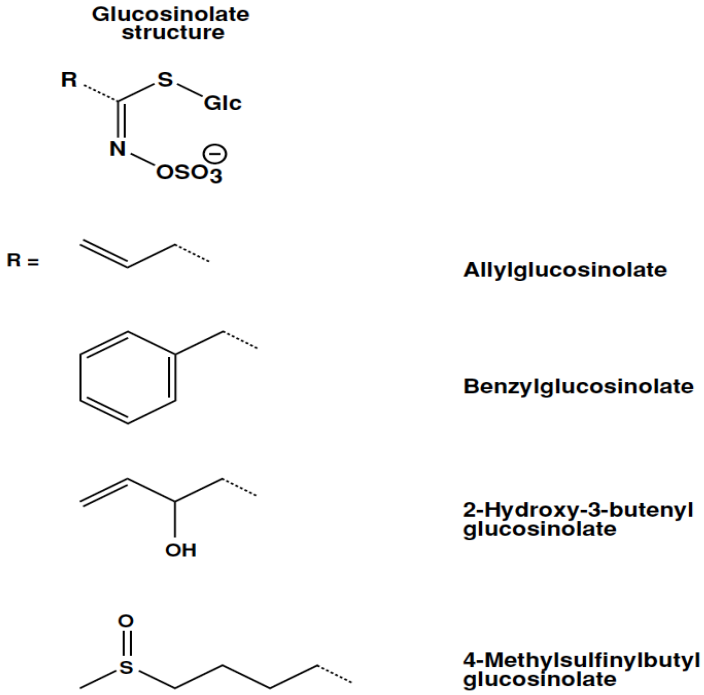


Figure 1.1. Chemical structure of GS (Halkier and Gershenzon, 2006)[1].

[1]Reproduction from Annual Review of Plant Biology, Halkier B.A., and Gershenzon, J., Biology and biochemistry of glucosinolates. 57, 303-333 Copyright (2006) with kind permission from Annual Reviews.

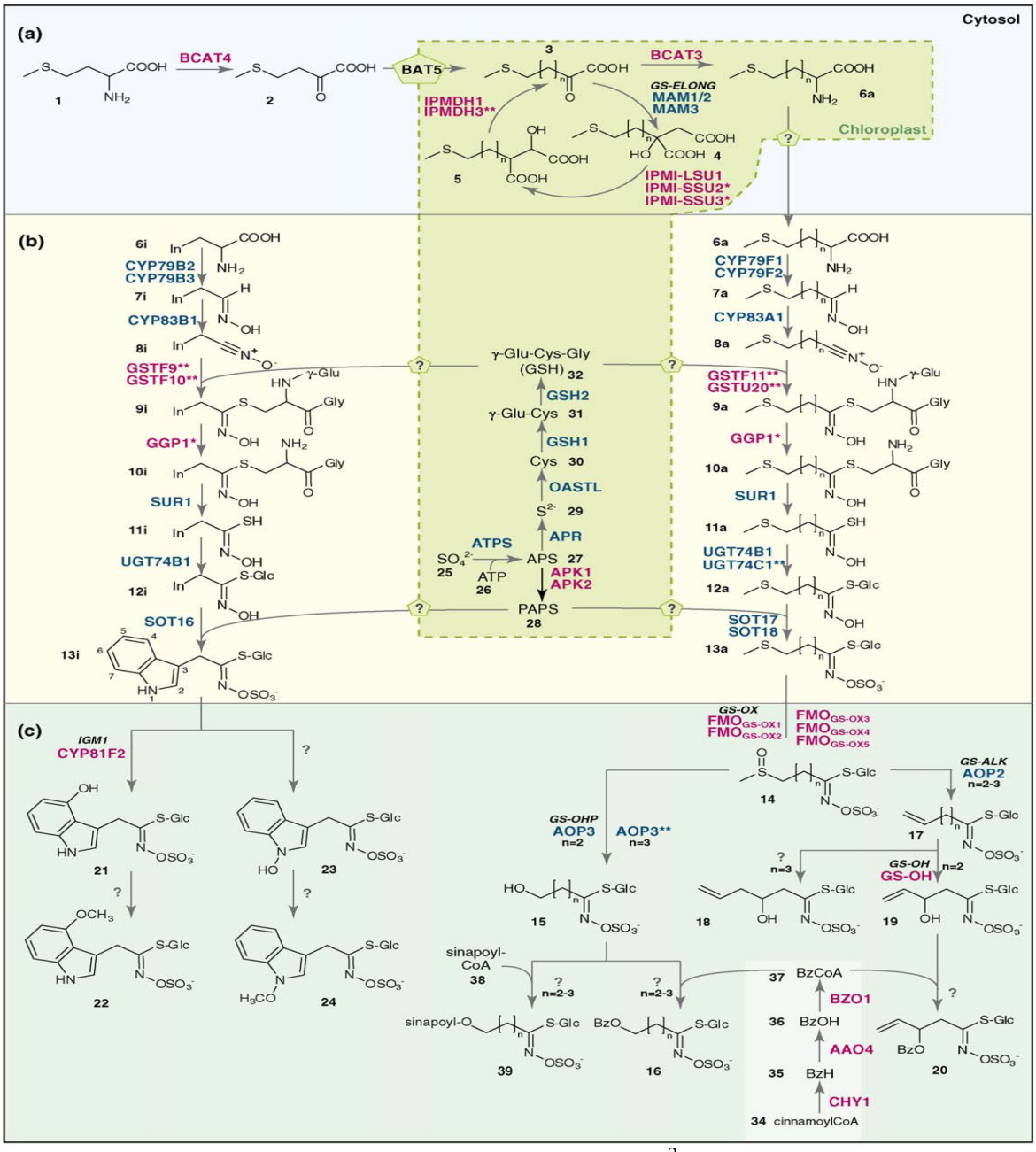


Figure 1.2. GS biosynthesis (Source: Sønderby et al., 2010)[2] (a) Chain elongation machinery. (b) Biosynthesis of core glucosinolate structure. (c) Secondary modifications.

[2]Reproduction from Trends in plant science, Sønderby, I.E., Geu-Flores, F., and Halkier, B.A., Biosynthesis of glucosinolates - gene discovery and beyond. 15, 283-290. Copyright (2010) with kind permission from Elsevier.

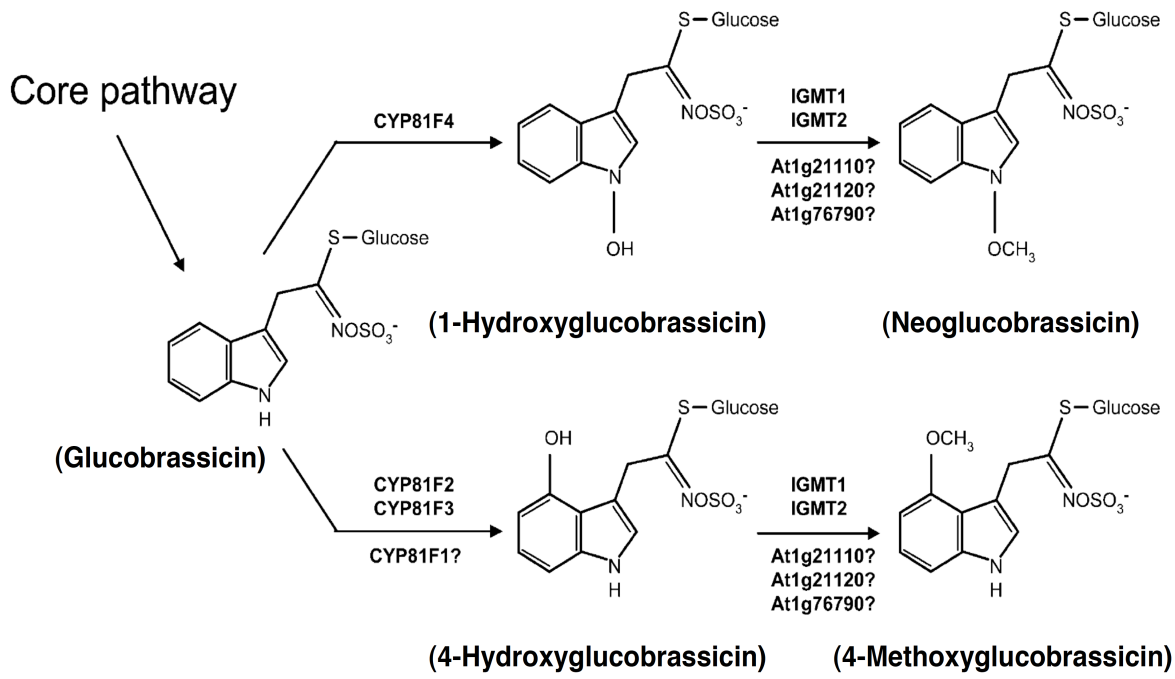


Figure 1.3. The Indolyl GS modification pathway in *Arabidopsis* (Source: Pfalz et al., 2011)[3].

[3]Reproduction from The Plant Cell, Pfalz, M., Mikkelsen, M.D., Bednarek, P., Olsen, C.E., Halkier, B.A., and Kroymann, J., Metabolic engineering in *Nicotiana benthamiana* reveals key enzyme functions in *Arabidopsis* indole glucosinolate modification. 23, 716-729. Copyright (2011) with kind permission from American Society of Plant Biologists.

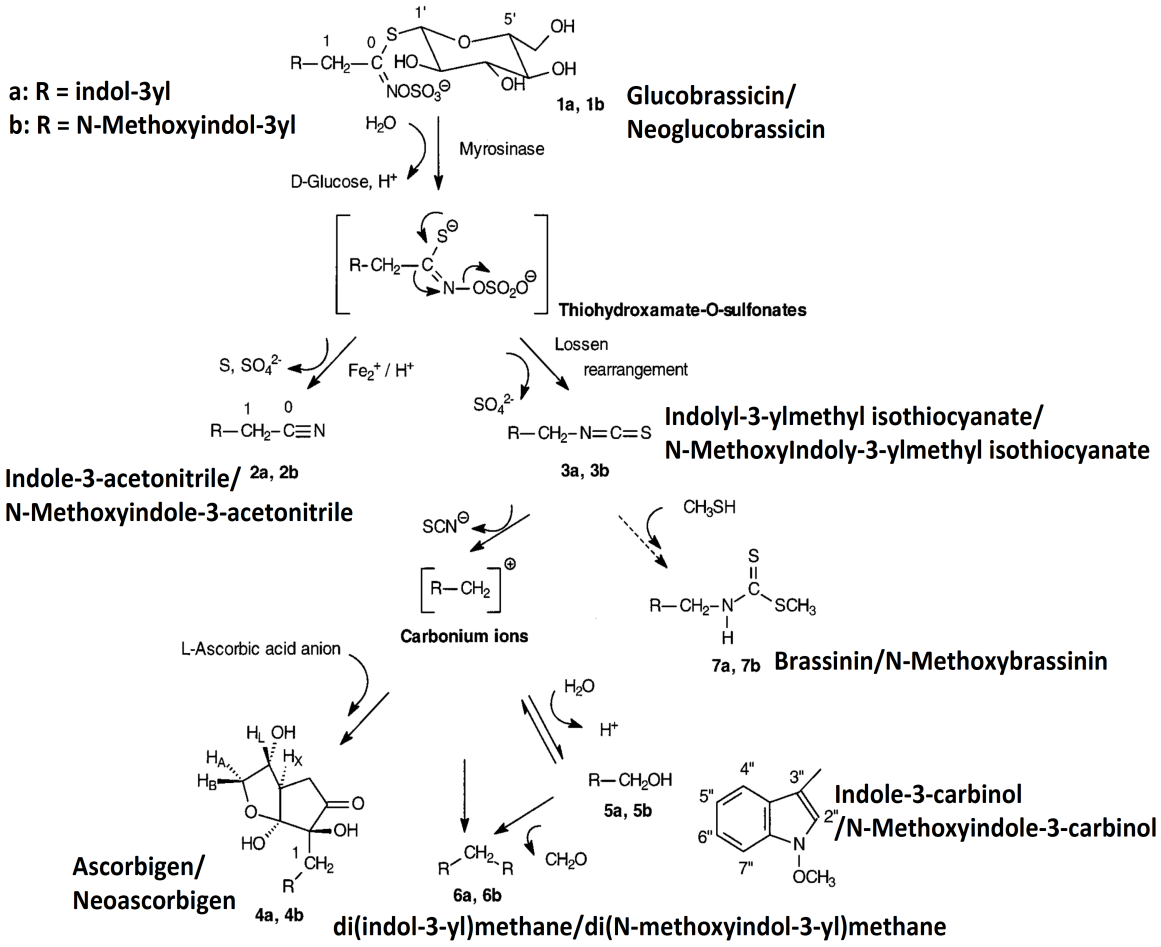
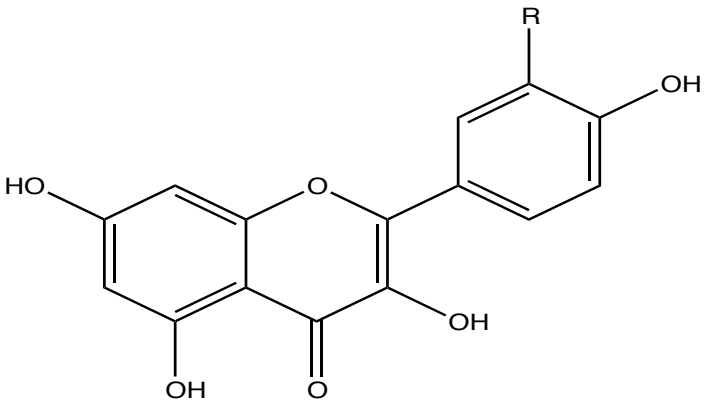


Figure 1.4. Products of the myrosinase-catalyzed hydrolysis of glucobrassicin and neoglucobrassicin (Agerbirk et al., 1998)[4].

[4]Reproduction from Journal of Agricultural and Food Chemistry, Agerbirk, N., Olsen, C.E., and Sørensen, H., Initial and final products, nitriles, and ascorbigens produced in myrosinase-catalyzed hydrolysis of indole glucosinolates. 46, 1563-1571. Copyright (1998) with permission from American Chemical Society.



Quercetin R=OH
Kaempferol R=H

Figure 1.5. Dominant flavonoids found in the *Brassica* genus.

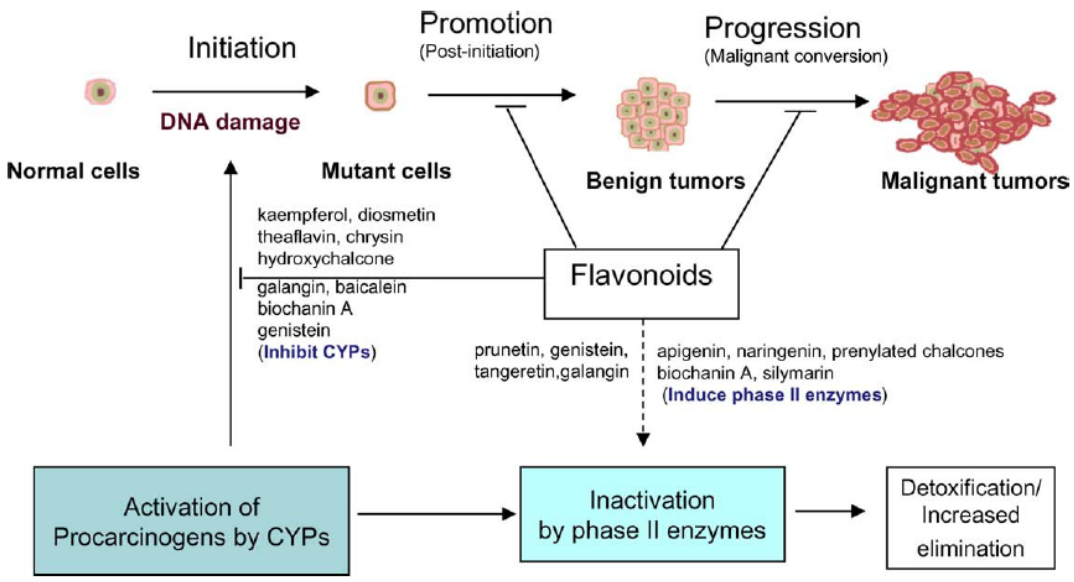


Figure 1.6. Anticarcinogenesis of flavonoids (Moon et al, 2006)[5].

[5]Reproduction from Toxicology in Vitro, Moon, Y.J., Wang, X., and Morris, M.E., Dietary flavonoids: Effects on xenobiotic and carcinogen metabolism. 20, 187-210. Copyright (2006) with kind permission from Elsevier.

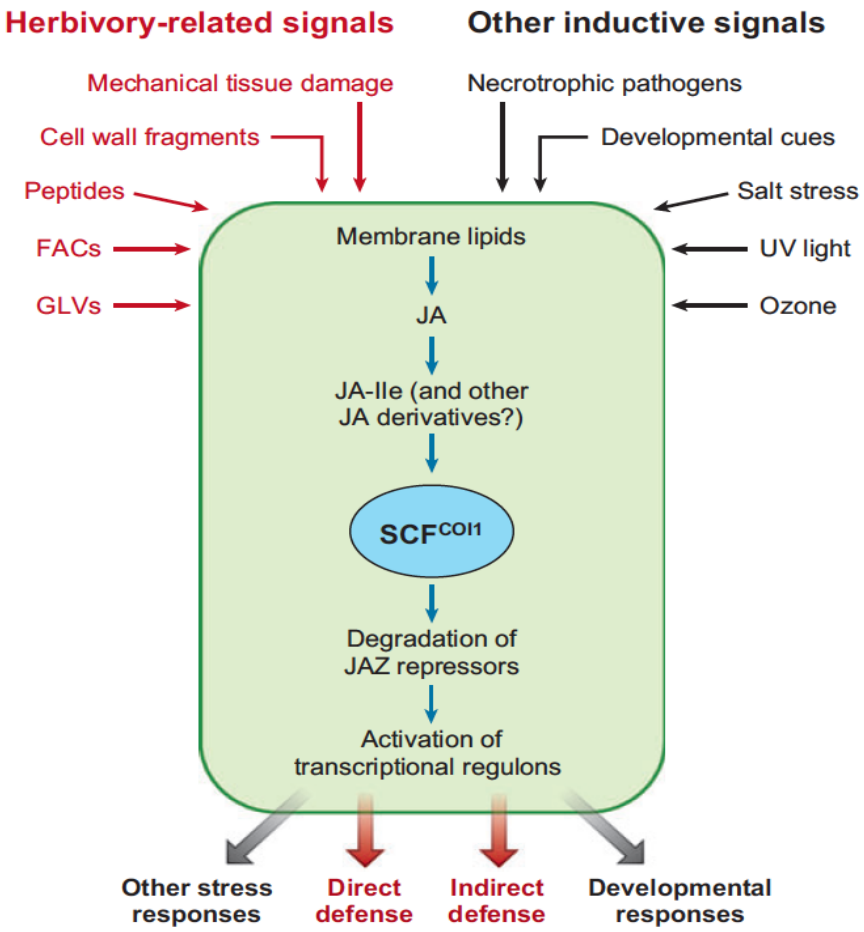


Figure 1.7. JA induction and responses (Howe and Jander, 2008)[6].

[6]Reproduction from Annual Review of Plant Biology, Howe, G.A., and Jander, G., Plant immunity to insect herbivores. 59, 41-66 Copyright (2008) with kind permission from Annual Reviews.

CHAPTER 2

Methyl Jasmonate-mediated Changes in Flavonoid Concentrations and Antioxidant Activity in Broccoli Florets and Kale Leaf Tissues[7]

2.1. Abstract

Aqueous solutions of 250 μM MeJA were sprayed on aerial plant surfaces four days prior to harvest at commercial maturity of five commercial broccoli (*Brassica oleracea* L. var. italica) hybrids, 'Pirate', 'Expo', 'Imperial', 'Gypsy', and 'Green Magic', and two kale cultivars, 'Red Winter' (*Brassica napus ssp. pabularia*) and 'Dwarf Blue Curled Vates' (*Brassica oleracea* L. var. *acephala* DC.) in replicated field trials over two years. While having no effect on broccoli florets, MeJA treatments significantly increased total phenolics by 27%, and extract antioxidant activity by 31% 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay in kale over two seasons. Partitioning experiment-wide trait variances indicated that the variability in broccoli floret concentrations of total phenolics (74%), quercetin (24%), kaempferol (34%) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) (66%) and DPPH (62%) antioxidant activity were largely influenced by year-associated environmental factors. In broccoli the differential accumulation of solar radiation among cultivars due to the variation in days to maturity was significantly correlated with total phenolics, ABTS, and DPPH antioxidant activity. Broccoli floret and kale total phenolic, quercetin, and kaempferol concentrations significantly correlated with DPPH and ABTS antioxidant

[7]Accepted in Hortscience on June 20 2013, "Environmental Stress and Methyl Jasmonate-mediated Changes in Flavonoid Concentrations and Antioxidant Activity in Broccoli Florets and Kale Leaf Tissues." Ku K.M., and Juvik J.A.

activity. To summarize, phenolic and flavonoid concentrations and their associated antioxidant activity in broccoli florets were unaffected by MeJA but varied among cultivars and over growing seasons. Apical, compared to basal leaves in kale were more responsive to MeJA mediated increases in total phenolics and ABTS and DPPH antioxidant activity.

2.2. Introduction

Numerous physiological and biochemical processes in the human body may produce oxygen-centered free radicals and other reactive oxygen species as byproducts of metabolism (Cai et al., 2004). Overproduction of free radicals can cause oxidative damage to biomolecules (e.g. lipids, proteins, DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer, aging, and other degenerative diseases in humans (Cai et al., 2004; Valko et al., 2004). Dietary antioxidants including polyphenols and flavonoids protect against free radicals such as reactive oxygen species in the human body and have been associated with the prevention of cancer, type 2 diabetes, and cardiovascular diseases (Moon et al., 2006; Poulsen et al., 1998, van Dam et al, 2013). Fruit and vegetables are good sources of natural antioxidants such as vitamins, carotenoids, flavonoids and other phenolic compounds (Dimitrios, 2006).

Broccoli (*Brassica oleracea* ssp. *italica*) and kale (*Brassica napus ssp. pabularia* and *Brassica oleracea* L. var. *acephala*) are frequently consumed vegetables in the United States and in other countries. They contain potential health promoting bioactive compounds including glucosinolates and dietary antioxidants such as carotenoids, tocopherols, and flavonoids (Eberhardt et al., 2005, Velasco et al., 2007). Both vegetables

are a good source of the dietary flavonoids, quercetin and kaempferol, which have been reported as potential anticancer agents (Koh et al., 2009; Moon et al., 2006). According to an epidemiological and animal model study, consumption of kaempferol and quercetin was inversely associated with cancer risk (Gates et al., 2007; Murakami et al., 2008; Neuhouser, 2004; Nothlings et al., 2007). Quercetin intake has also been associated with decreasing blood pressure (Larson et al., 2012). A recent study has indicated that quercetin up-regulates low density lipoprotein receptor gene expression, which can elicit hypolipidemic effects by improving the clearance of circulating LDL cholesterol levels from the blood (Moon et al., 2012). In addition to the flavonoids, both vegetables have a variety of additional polyphenol compounds such as hydroxycinnamic acid and hydroxybenzoic acid derivatives. Among them, ferulic acid and chlorogenic acid were reported to improve cardiovascular function and attenuate hypertension in hypertensive rats (Alam et al., 2013; Suzuki et al., 2006).

Biotic and abiotic factors are associated with the biosynthesis and accumulation of phenolics and flavonoids in plant tissues. Environmental factors such as temperature, solar radiation, and rainfall can influence broccoli metabolism and resulting phytochemical composition (Björkman et al., 2011; Gliszczynska-Swiglo et al., 2007). Also, herbivore or pathogen activity also can influence broccoli phytochemical composition (Hopkins et al., 2009). Recently many studies have been conducted using exogenous treatments of elicitors including jasmonic acid and salicylic acid to mimic biotic stress and increase tissue total phenolics and flavonoid concentrations. According to previous reports, methyl jasmonate (MeJA) application can enhance total phenolic concentrations in radish sprouts (Kim et al., 2006a), buckwheat sprouts (Kim et al., 2011)

and sweet basil (Kim et al., 2006b). Several reports have attempted to enhance antioxidant, antiproliferative, and anti-adipogenic activity by MeJA-mediated increases in flavonoids or phenolics in sweet basil, buckwheat, and blackberry, respectively (Kim et al., 2006b; Lee et al., 2013; Wang et al., 2008).

The objective of this research was to compare how MeJA field application affects total phenolics, flavonoid concentrations, and antioxidant activity of broccoli floret and kale leaf tissues. To our knowledge, this is the first investigation of MeJA application to broccoli and kale under field conditions. To evaluate variation in phytochemical antioxidants and antioxidant activity associated with MeJA treatments, environment effects and genotypes, we evaluated five commercial broccoli hybrids and two distinct kale cultivars in replicated field plots over two years.

2.3. Materials and Methods

2.3.1. Broccoli and Kale Cultivation. The broccoli F_1 hybrid cultivars used for this experiment were 'Pirate' (Asgrow Seed Co., Galena, MD), 'Expo', 'Imperial', 'Gypsy', and 'Green Magic' (Sakata Seed Co., Morgan Hill, CA). Kale cultivars used for this experiment were 'Red Winter' (*Brassica napus ssp. pabularia*) and 'Dwarf Blue Curled Vates' (*Brassica oleracea* L. var. *acephala* DC.). Seeds of each broccoli and kale genotype were germinated in 32 cell plant plug trays filled with sunshine® LC1 (Sun Gro Horticulture, Vancouver, British Columbia, Canada) professional soil mix. Seedlings were grown in a greenhouse at the University of Illinois at Champaign-Urbana under a 25 °C/15 °C and 14 h/10 h: day/night temperature regime with supplemental lighting. Thirty days after germination, seedling trays were placed in ground beds to harden off for

a week prior to transplanting into field plots at the University of Illinois South Farm (40˚ 04′ 38.89″ N, 88˚ 14′ 26.18″ W). Experimental design was a split plot in randomized complete block (RCB) with three replicates. The experiment plot was surrounded by one row of guard plants to avoid border effect. Ten broccoli or kale plants from each replicate block of each genotype were designated as control or MeJA treatment groups with each genotype. Transplanting of broccoli seedlings was conducted on June 24, 2009 and June 11, 2010. Harvesting broccoli occurred from August 23 to September 18 in 2009 and from August 12 to September 12 in 2010. Transplanting of kale seedlings was conducted on June 11, 2010 and June 13, 2011. Harvesting kale occurred in July 25 in 2009 and July 27 in 2010. There is considerable maturity variation among broccoli hybrids and the number of days from transplant to harvest date (DTH) was calculated for each genotype (Table 2.1). The different weather factors including growing degree days (GDD) [The formula = (min Temperature + max Temperature)/2-7.2 °C] (Dufault, 1997), solar radiation, and precipitation, which are also presented in Supplementary Table 2.S1. Weather conditions during the 2009 and 2010 growing seasons were generated from http://www.isws.illinois.edu/warm/data/cdfs/cmiday.txt and used to calculate above weather data.

2.3.2. Treatment with MeJA and Sample Preparation. An aqueous solution of 250 μM MeJA (Sigma-Aldrich, St. Louis, MO) including 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) or 0.1% Triton X-100 alone (control) were sprayed on all aerial plant tissues to the point of runoff (approximately 300 mL) four days prior to harvest at commercial maturity. This timing of harvest and treatment concentration was based on

previous studies, that generated an optimal response to MeJA application for broccoli (Ku and Juvik, 2012 and Chapter 7). Five broccoli heads and two kale leaf samples (apical: three leaves from below the meristematic growing point, at a minimum 8 cm in length; basal: three fully expanded leaves nearest the soil surface without discoloration or signs of senescence or damage) were harvested and bulked from five treated and control plants of each genotype for each replicate (five heads or leaves from five plants bulked for a replicate sample). Broccoli head tissue and kale leaf samples were frozen in liquid nitrogen, and stored at -20 °C prior to freeze-drying. Freeze-dried head and leaf tissues were ground into a fine powder using a coffee grinder and stored at -20 °C prior to chemical and bioactivity analyses.

2.3.3. Sample Extraction. Two hundred mg of fine powder of each sample was extracted with 2 mL of 70% methanol at 95 °C for 10 min. After 5 min cooling on ice, the extract was centrifuged at 3,000 g for 10 min. After a second round of extraction as described above, the supernatants were pooled. Subsequently, 1.5 mL of the pooled supernatant was transferred to a 2 mL microcentrifuge tube (Fisher Scientific, Waltham, MA) and centrifuged at 10,000 g for 2 min. This extract was used for the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant activity assays, and to quantify tissue total phenolic and flavonoid concentrations.

2.3.4. Determination of Total Phenolic Content (TPC). Analysis of TPC was conducted using a previously described protocol (Ku et al., 2010). The assay conditions

were as follows; a 10 μL sample was added to 0.2 N Folin-Ciocalteu's phenol reagent (100 μL) in 96 well plates. After 3 min, 90 μL of a saturated sodium carbonate solution was added to the mixture and subsequently incubated at room temperature for 1 h. The resulting absorbance of the mixture was measured at 630 nm using a BioTek EL 808 microplate reader (Biotek Instruments Inc., Power Wave XS, Winooski, VT). The total phenolic content was calculated on the basis of a standard curve using gallic acid (concentration range 31.25 to 500 $\mu g \cdot mL^{-1}$). Results are expressed in milligrams of gallic acid equivalents (GAE) per 100 g of dried broccoli. Three biologically replicated (block) samples were assayed with three analytical replications each.

2.3.5. Determination of Sample Flavonoid Concentrations. The sample extracts from broccoli and kale were transferred (1.2 mL) to a 2 mL microcentrifuge tube (Fisher Scientific, Waltham, MA) to which 0.24 mL of 6 M HCl was added. The tubes were then heated at 90 °C for 2 h to release the aglycone (Kurilich et al., 2002). The extract was cooled, filtered through a 0.45 μm PTFE Whatman (Clifton, NJ) membrane filter before injection onto the HPLC. Flavonoid concentrations were evaluated using an Agilent 1100 HPLC system (Agilent, Santa Clara, CA), equipped with a G1311A bin pump, a G1322A vacuum degasser, a G1316A thermostatic column compartment, a G1315B diode array detector and an HP 1100 series G1313A autosampler. Extracts were separated on a Supercosil™ LC-18 column (250 × 4 mm, particle size 5 μm) (Supelco Inc., Bellefonte, PA) with a C18 all-guard™ cartridge pre-column (Alltech, Lexington, KY). Mobile phase A was water and B was methanol with 0.1% acetic acid. Mobile phase B was 0% at injection, increasing to 60% by 15 min, 80% at 20 min, and 100% at 25 min, then held

for 5 min with a 2 mL·min^{-1} flow rate, then decreased to 0% by 35 min. Flow rate was kept at 1 mL·min^{-1} except for the 5 min where mobile phase B was held at 100%. The detector wavelength was set at 360 nm. Quercetin and kaempferol (Sigma-Aldrich, St. Louis, MO) were used as standard for determination of aglycone flavonoid concentration.

2.3.6. Determination of ABTS Radical Scavenging Activity. The ABTS assay was conducted as previously described protocol (Ku et al., 2010). Briefly, 7 mM ABTS ammonium salt was dissolved in a potassium phosphate buffer (pH 7.4) and treated with 2.45 mM potassium persulfate. The mixture was then allowed to stand at room temperature for 12-16 h for full color development (dark blue). The solution was then diluted with potassium phosphate buffer until absorbance reached 1.0 ± 0.02 at 630 nm using a BioTek EL 808 microplate reader (Biotek Instruments Inc., Power Wave XS, Winooski, VT). Subsequently, 190 µL of this solution was mixed with 10 µL of the sample. The absorbance was recorded at room temperature after 6 min. Results were expressed as a percentage of radical scavenging activity compared to controls. Three biologically replicated (block) samples were assayed in three analytical replications.

2.3.7. Determination of the Antioxidant Activity by the DPPH Free-radical Scavenging Assay. The DPPH assay was conducted as described, by Ku et al. (2010) with minor modification. Reaction mixtures containing test samples (10 µL) and 190 µL of a 200 µM DPPH ethanol solution were incubated at room temperature for 30 min in 96-well plates. The absorbance of the DPPH free radical was measured at 515 nm with a BioTek EL 808 microplate reader (Biotek Instruments Inc., Power Wave XS, Winooski,

VT). Results were expressed as percentage of scavenging activity compared to control. Three biologically replicated (block) samples were assayed in three analytical replications.

2.3.8. Statistical Analysis. JMP 10 (SAS institute Inc., Cary, NC) was used for statistical analysis. Analysis of variance (ANOVA) and partitioning of variance components were conducted using the JMP 10. Treatments, genotype, yaer effects were considered as fixed factors. Block was considered as random factors. Analysis of variance was performed using the linear model: $Y_{ijklm} = m + G_i + Y_j + T_k + GY_{ij} + GT_{ik} + YT_{jk} + GYT_{ijk} + B_{l(j)} + \varepsilon_{ijklm}$, where Y_{ijklm} is the l^{th} block of the phenotypic value of the k^{th} treatment, i^{th} genotype in year j, m is the overall mean, G, Y, T, and B indicate the effects of genotype, year (environment), treatment and blocks nested in years, and ε_{ijklm} is the experimental error associated with Y_{ijklm}, respectively. Correlation analysis and Student's t-test was conducted using the JMP 10 software (SAS institute Inc., Cary, NC). All biological sample analyses were conducted in triplicate. The results are presented as means ± SD.

2.4. Results and Discussion

2.4.1. Effect of MeJA Treatment on Total Phenolic and Flavonoid Concentrations, ABTS and DPPH Antioxidant Activities of Broccoli Floret Extracts. Treatment with 250 μM MeJA did not alter total phenolic, kaempferol, or quercetin concentrations, ABTS or DPPH antioxidant activities in broccoli florets (Table 2.2). Whereas previous research has reported that MeJA treatment increased total phenolic and flavonoid content in radish and broccoli sprouts (Kim et al., 2006a; Pérez-Balibrea et al., 2011), this was

not observed in this study with broccoli florets. The lack of response to MeJA treatment maybe due to the different tissues evaluated, plant developmental status or environmental factors. Phenolics and flavonoid biosynthesis in broccoli florets tissue is apparently not influenced by exogenous MeJA application under field conditions.

In this study, year and genotype exerted a significant effect on phytochemical content and antioxidant activity (Table 2.2). Total phenolic, quercetin and kaempferol concentrations in 2010 were 1.9, 3.0, and 1.7 fold higher than that observed in 2009 (Table 2.2). The 'Gypsy' cultivar showed the highest quercetin concentration fold change (7.5 fold) between 2009 and 2010. Different weather conditions in 2009 and 2010 are presumed to have significantly altered the ratio of quercetin/kaempferol in 'Pirate', 'Imperial', and 'Gypsy'. Antioxidant activity measured by the ABTS and DPPH assays, was 1.5 and 2.2 fold higher in 2010 than for broccoli harvested in 2009 (Table 2.2). It has been reported that solar radiation is positively correlated with flavonoid content in broccoli florets (Gliszczynska-Swiglo et al., 2007). Increased total phenolic and flavonoid content in 2010 compared to 2009 may be explained by year-associated weather factors such as solar radiation. The increased quercetin/kaempferol ratio also maybe a response to increased UV-B (Ultraviolet-B) in solar radiation (Kuhlmann and Müller, 2009). Temperature is also known to impact phytochemical content in broccoli florets (Schonhof et al., 2007). The interaction of these various environmental factors on the growth and development of broccoli cultivars very likely influenced phytochemical profiles in floret tissue.

2.4.2. Correlation between Weather Conditions and Phytochemical Change. From the correlation of extract phytochemical compound concentrations with antioxidant activity and with weather-related environmental growing conditions for each cultivar over two years, several meaningful relationships were observed (Table 2.3). Accumulated GDD, precipitation, and solar radiation to each genotype were closely related with DTH. There was a significantly positive correlation between GDD ($r = 0.703$, $P < 0.001$), and solar radiation ($r = 0.796$, $P < 0.001$) with total phenolics concentration. There were highly significant correlations between tissue ABTS antioxidant activity with total phenolics ($r = 0.927$, $P < 0.001$), quercetin ($r = 0.728$, $P < 0.001$) and kaempferol ($r = 0.785$, $P < 0.001$) (Table 2.3). There were also highly significant correlations between tissue DPPH antioxidant activity with total phenolics ($r = 0.934$, $P < 0.001$), quercetin ($r = 0.860$, $P < 0.001$), and kaempferol ($r = 0.830$, $P < 0.001$). Total phenolic content was negatively correlated with precipitation/DTH, which is precipitation per day after transplanting ($r = -0.620$, $P < 0.004$). The water deprivation condition in 2010 may have lead to increased total phenolics, which is mainly associated with antioxidant activity in broccoli. Water deprivation at flowering and during pod fill in *Brassica napus* has been associated with increased phenolic content in rapeseed (Bouchereau et al., 1996). This suggests that total phenolic and flavonoid concentrations are primarily responsible for antioxidant activity. Unlike previous reports, there was only a weak correlation between solar radiation and flavonoid (quercetin $r = 0.360$, $P = 0.120$; kaempferol $r = 0.420$, $P = 0.065$) concentrations (Gliszczynska-Swiglo et al., 2007). In addition, the ratio of quercetin to kaempferol tended to weakly correlate with solar radiation but was not significant ($r = 0.403$, $P = 0.078$).

2.4.3. Partitioning of Broccoli Phytochemical Concentrations and Antioxidant Activity Variances into MeJA Treatment, Year, and Genotype Sources of Variation.

ANOVA partitioning of the variances for phytochemical concentrations indicated that differences among broccoli genotypes described 34% and 15% of the total variation for kaempferol and quercetin, respectively while there was no significant MeJA effect (Table 2.4). Seasonal differences in environmental conditions between 2009 and 2010 were a major source of variation in total phenolic (74%), quercetin (24%), and kaempferol (34%) concentrations and in the ABTS (66%) and DPPH (62%) antioxidant activity of floret extracts (Table 2.4). There was also significant genotype by year interactions on total phenolics (12%, $P < 0.001$), flavonoids (quercetin, 37%, $P = 0.001$; kaempferol, 28%, $P < 0.001$), and antioxidant activity (ABTS, 10%, $P = 0.001$; DPPH, 12%, $P < 0.001$) (Table 2.4). Broccoli harvest maturity differed among hybrids with the days to harvest interacting with environmental factors which was associated with the accumulation of total phenolics among the different genotypes. The genotype described the largest component of variance (75%) for DTH. DTH played a major role in accumulation of phytochemicals through the interaction with environmental conditions in cultivars with longer growing seasons. A large portion of the year effects on variation in total phenolic, quercetin, and kaempferol concentrations and in the ABTS and DPPH activities are associated with genotype by environment interaction. These results suggest that appropriate cultivar selection or breeding for certain environment conditions can maximize phenolics and antioxidant bioactivity of broccoli florets. With appropriate parental material and selection schemes it may be feasible to achieve high flavonoid and

phenolic content in shorter maturing varieties as was previously reported for the development higher glucosinolate (glucoraphanin) content in early maturing broccoli germplasm (Farnham et al., 2004).

2.4.4. MeJA Effect on Kale Leaf Sample Extracts. Unlike broccoli, MeJA treatment significantly increased total phenolics and antioxidant activity in both kale species, both of two years (Table 2.5). The increase in leaf phenolic content varied between genotypes, years, and apical or basal leaf samples. MeJA treatments were observed to interact with leaf age where apical leaf tissue was more responsive to treatments in terms of increases in total phenolics, quercetin, and ABTS antioxidant activity than basal leaf tissue. MeJA response also varied between years, which may be associated with the different weather conditions observed in 2010 and 2011. Since kale leaf harvests were conducted on the same date and each genotype experienced approximately the same number of growing degree days in both years the primary weather factor that differed between years was precipitation. Our kale plots in July, 2011 received only 44% of precipitation the plots received in July of 2010. Water deprivation at flowering and during pod fill in *Brassica napus* has been associated with increased phenolic content in rapeseed (Bouchereau et al., 1996). Another study also reported that water stress increased phenolic compounds in lettuce (Oh et al., 2010). Endogenous jasmonic acid has been observed to accumulate *in planta* under drought conditions (Creelman et al., 1995). Thus, conditions in 2011 may have lead to the accumulation of endogenous JA which could have attenuated the effect of exogenous MeJA treatment.

Although the physiological relationship between antioxidant compounds and human health promoting activity has not been thoroughly established, consumers have indicated their willingness to pay a premium for produce with high nutritional value and antioxidant activity (i.e. melons with 25% more vitamin C) (Bond et al., 2008). Previous research reported that MeJA treatment increased the dietary impact of various crops on quinone reductase, antioxidant, antiproliferative, and anti-adipogenic activity as measured by the effect of extracts on cultured mammalian cells (Ku et al., 2013; Kim et al., 2006b; Lee et al., 2013; Wang et al., 2008). This study showed that MeJA could enhance levels of antioxidant phytochemicals and antioxidant activity in kale leaf tissue. It may be feasible to develop brassica vegetables with enhanced consumer health-promoting properties but the magnitude of this effect may be attenuated by interaction with biotic and abiotic stress conditions in the growing environment (Mewis et al., 2012).

2.4.5. Partitioning of Kale Phytochemical Concentrations and Antioxidant Activity Variances into MeJA Treatment, Year, and Genotype Sources of Variation.

ANOVA partitioning of the variances for phytochemical concentrations indicated that differences among genotypes described 18%, 76%, 29%, and 41% of the total variation for total phenolic and quercetin concentration and ABTS and DPPH antioxidant activity in kale apical leaf tissue, respectively (Table 2.6). In addition, MeJA treatment described 69%, 48%, and 36% of the variation in total phenolics, quercetin concentrations, and ABTS antioxidant activity in kale basal leaf tissue, respectively (Table 2.6), while no significant MeJA effect was observed on phytochemical content and antioxidant activity in broccoli floret tissue (Table 2.4). Seasonal differences in environmental conditions

between 2010 and 2011 explained a significant portion of the variance for total phenolics, quercetin, and ABTS antoxidant activity in kale apical tissue and basal tissue but the portion was smaller in basal leaf tissue.

MeJA treatment of broccoli inflorescence and kale leaf tissue appears to be a tissue-specific response. Kim and Juvik (2011) reported MeJA treatment significantly increased glucosinolate concentrations in broccoli florets but had no effect on phenolic and flavonoid concentrations. Under biotic or abiotic stress, plants tend to accumulate defense compounds in vulnerable tissues such as young leaves or florets (Zangerl and Bazzaz, 1992; van Dam et al., 1996). Accumulation of different defense compounds in plants are tissue specific and preferentially allocated to plant parts that promote plant fitness and survival that are at risk of attack from herbivores (Zangerl and Bazzaz, 1992). Since the photosynthetic capacity of leaves declines with age, young leaf tissue plays a critical role in survival and thus would show a more dramatic response to MeJA treatment. Previous studies have reported that the youngest leaves of the rosette plants of *Cynoglossum officinale*, contain 50-190 times higher concentrations of pyrrolizidine alkaloid than old leaves and that the compound acts as a defense against generalist herbivores (van Dam et al., 1996). In kale MeJA treatment increased not only flavonoids and phenolics but also certain glucosinolates including gluconasturtiin, glucobrassicin, and neoglucobrassicin (data not shown). Kale plant response to MeJA treatment may be utilized by producers to enhance potential human health promoting activity.

In conclusion, unlike previous studies on several plants, MeJA treatment did not significantly influence total phenolic and flavonoid concentrations or antioxidant bioactivity in broccoli floret extracts in five broccoli hybrids tested over two seasons

under field conditions. However, this treatment significantly increased those variables in kale leaves. MeJA treatments appear to interact with tissue type, age of leaves, and environment conditions. Selection of appropriate genotypes with manipulation of environmental conditions can increase total phenolic and flavonoid concentrations in broccoli florets resulting in elevated antioxidant activity and potential health promotion.

Table 2.1. Days to harvest, growing degree days, solar radiation, and precipitation accumulations for the five broccoli genotypes during the 2009 and 2010 growing seasons.

Year	Cultivar	Treatment	DTH	GDD (˚C)	Solar radiation (MJ·m-2)	Precipitation (mm)
2009	Expo	Control	81 ± 3	1150	1705	296
2009	Expo	MeJA	82 ± 3	1163	1729	296
2009	Green Magic	Control	58 ± 7	859	1257	223
2009	Green Magic	MeJA	57 ± 7	846	1239	222
2009	Gypsy	Control	62 ± 5	906	1342	223
2009	Gypsy	MeJA	61 ± 2	893	1318	223
2009	Imperial	Control	60 ± 3	881	1293	223
2009	Imperial	MeJA	60 ± 2	881	1293	223
2009	Pirate	Control	77 ± 5	1094	1622	296
2009	Pirate	MeJA	78 ± 5	1109	1643	296
2010	Expo	Control	88 ± 2	1531	2758	314
2010	Expo	MeJA	93 ± 3	1595	2868	318
2010	Green Magic	Control	67 ± 6	1198	2165	245
2010	Green Magic	MeJA	67 ± 6	1198	2165	245
2010	Gypsy	Control	68 ± 3	1215	2193	245
2010	Gypsy	MeJA	69 ± 2	1231	2223	245
2010	Imperial	Control	65 ± 2	1107	2100	245
2010	Imperial	MeJA	66 ± 4	1182	2136	245
2010	Pirate	Control	92 ± 6	1581	2825	314
2010	Pirate	MeJA	91 ± 4	1570	2845	314

The accumulated weather factors were calculated based on number of days from transplant to harvest (DTH). [z]Number of days from transplant to harvest. [y]Growing degree days [(Min Temperature + Max Temperature)/2 -7.2 °C]. The results are presented as means ± SD (n=3).

Table 2.2. Total phenolic and flavonoid concentrations and antioxidant activity of control and MeJA-treated broccoli florets over two seasons.

Source of variation	Treatment/Season	Total phenolic[z]	Quercetin[y]	Kaempferol[y]	Q/K ratio[x]	ABTS[w]	DPPH[w]
Treatment							
	Control	668 ± 244	112 ± 107	63 ± 26	1.55 ± 0.76	36.0 ± 9.8	30.1 ± 14.8
	MeJA	671 ± 252	117 ± 92	67 ± 23	1.59 ± 0.79	32.7 ± 9.6	25.8 ± 13.3
Year							
2009	Control	463 ± 108	57 ± 36	46 ± 14	1.17 ± 0.45	28.2 ± 5.5	18.9 ± 6.2
2010	Control	873 ± 147*	167 ± 126*	80 ± 25*	1.93 ± 0.82*	43.7 ± 6.4*	41.3 ± 12.1*
Genotype[v]							
Pirate	2009	386 ± 45	51 ± 7	36 ± 3	1.44 ± 0.18	25.4 ± 3.2	18.2 ± 5.2
	2010	942 ± 3*	139 ± 27*	62 ± 10*	2.25 ± 0.22*	40.0 ± 3.3*	39.5 ± 1.4*
Expo	2009	524 ± 28	37 ± 23	43 ± 10	0.83 ± 0.35	30.7 ± 1.4	21.1 ± 1.9
	2010	800 ± 42*	81 ± 9*	61 ± 7	1.34 ± 0.16	45.0 ± 4.0*	37.4 ± 1.2*
Green Magic	2009	631 ± 71	115 ± 34	69 ± 13	1.65 ± 0.19	34.9 ± 3.8	25.5 ± 5.5
	2010	820 ± 42*	133 ± 5	87 ± 4	1.52 ± 0.02	38.0 ± 3.3	33.5 ± 2.5
Imperial	2009	391 ± 13	25 ± 12	40 ± 2	0.62 ± 0.30	23.3 ± 5.7	11.2 ± 6.3
	2010	701 ± 40*	78 ± 3*	66 ± 10*	1.20 ± 0.13*	42.9 ± 6.5*	32.2 ± 1.3*
Gypsy	2009	385 ± 17	54 ± 10	41 ± 7	1.31 ± 0.16	26.9 ± 5.4	18.6 ± 3.4
	2010	1104 ± 55*	405 ± 8*	122 ± 2*	3.32 ± 0.12*	52.7 ± 3.1*	63.9 ± 2.6*

The results are presented as means ± SD (n=3). Three analytical replications were conducted for each biological sample. Student T-tests were conducted to determine significant at $P \leq 0.05$. MeJA treatment groups are not presented here because they were not significantly different from control groups.

[z]Total phenolic was measured by spectrophotometer using Folin-Ciocalteu reagent and expressed as mg of gallic acid equivalent concentration in 100 g of freeze-dried broccoli powder. [y]Flavonoids were measured by HPLC and expressed as mmol per 100 g of freeze-dried broccoli powder. [x]Ratio of quercetin to kaempferol concentraions. [w]Antioxidant activity is presented as percentage of free radical scavenging ability compared to negative control (solvent). [v]Values presented for individual genotype are untreated controls. Asterisks indicate means that are significantly different based on the Student t-test between treatments or different years within same genotypes ($P \leq 0.05$).

Table 2.3. Correlation between accumulated weather factors and phytochemical content and antioxidant activities.

	DTH[z]	GDD[y]	Precipitation /DTH	Solar radiation	TPC[x]	ABTS	DPPH	Quercetin	Kaempferol
DTH	1.000								
GDD	0.893***	1.000							
Precipitation/ DTH	-0.701***	-0.800***	1.000						
Solar radiation	0.773***	0.974***	-0.774***	1.000					
TPC	0.388	0.703***	-0.620**	0.796***	1.000				
ABTS	0.344	0.644***	-0.518*	0.747***	0.927***	1.000			
DPPH	0.344	0.630***	-0.539*	0.719***	0.934***	0.953***	1.000		
Quercetin	-0.019	0.260	-0.261	0.360	0.769***	0.728***	0.860***	1.000	
Kaempferol	0.002	0.304	-0.197	0.420	0.803***	0.785***	0.830***	0.868***	
Q/K ratio[w]	0.067	0.322	-0.327	0.403	0.721***	0.635***	0.803***	0.935***	0.710***

Pearson correlation coefficients were calculated based on mean values of all pair variables. Means with *, **, and *** indicate significance based on the two-tailed Pearson correlation test at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$. [z]DTH= number of days from tranplant to harvest, [y]GDD=growing degree days, [x]TPC=total phenol content. [w]Ratio of quercetin to kaempferol concentraions.

Table 2.4. Percentages of total variance described by main factors (Genotype, Treatment, Year) and factor interactions for broccoli floret phytochemical concentrations and bioactivities.

	Total phenolics[z]	Quercetin[y]	Kaempferol[y]	ABTS[x]	DPPH[x]
Genotype	6.5***	14.9***	33.6***	6.9***	14.3***
Treatment	0.0	0.9	0.1	2.9	2.0
Year	74.0***	23.9***	33.5***	64.8***	61.2***
G×T	0.4	4.8	0.3	0.6	1.2
G×Y	12.2***	36.5***	28.4***	9.5***	11.9***
T×Y	0.0	0.9	0.0	0.0	0.0
G×T×Y	2.5	4.3	1.1	4.0	2.0
Block (Year)	0.4	0.5	0.2	1.1	0.5
Residual	4.0	13.3	2.8	10.0	6.6
R Square[w]	0.96	0.97	0.86	0.90	0.93

*** indicates factor that describes a significant proportion of the total variance using ANOVA at $P \leq 0.001$ (two tailed-test). [z]Total phenolics were measured by spectrophotometer using Folin-Ciocalteu reagent and expressed as mg of gallic acid equivalent concentration in 100 g of freeze-dried broccoli powder. [y]Flavonoids were measured by HPLC and expressed as mmol per 100 g of freeze-dried broccoli powder. [x]Antioxidant activities are presented as percentage of free radical scavenging ability compared to negative control (solvent). [w]Fraction of total variance described by the regression.

Table 2.5. Total phenolic and flavonoid concentrations and antioxidant activity of untreated and MeJA-treated kale leaf tissue over two seasons.

Source of variation	Treatment/ Season	Total phenolics[z]	Quercetin[y]	Kaempferol[y]	ABTS[x]	DPPH[x]
Apical tissue Treatment						
	Control	1545 ± 219	153 ± 91	154 ± 50	4932 ± 433	3811 ± 308
	MeJA	1962 ± 282***	237 ± 139	176 ± 36	5571 ± 299***	4985 ± 299*
Year						
2010	Control	1653 ± 225	120 ± 72	177 ± 24	5130 ± 406	3476 ± 1167
	MeJA	2188 ± 132***	241 ± 147	169 ± 29	5778 ± 155**	4229 ± 711
2011	Control	1438 ± 165	186 ± 102	131 ± 61	4734 ± 393	4148 ± 1682
	MeJA	1735 ± 185*	232 ± 145	182 ± 45	5365 ± 265**	5741 ± 1232
Genotype						
	(2010)					
Dwarf Blue	Control	1846 ± 113	184 ± 34	165 ± 25	5488 ± 68	4533 ± 214
Curled Vates	MeJA	2298 ± 50**	373 ± 27**	152 ± 11	5911 ± 56**	4819 ± 359
Red Winter	Control	1461 ± 48	58.2 ± 7.7	190 ± 17	4770 ± 144	2419 ± 77
	MeJA	2079 ± 73***	109 ± 26*	186 ± 33	5645 ± 64***	3639 ±100***
	(2011)					
Dwarf Blue	Control	1579 ± 76	278 ± 19	129 ± 13	5088 ± 84	5319 ± 561
Curled Vates	MeJA	1825 ± 111*	363 ± 22**	143 ± 8	5545 ± 55**	5633 ± 592
Red Winter	Control	1296 ± 42	93.8 ± 12	133 ± 95	4380 ± 48	2662 ± 315
	MeJA	1644 ± 121*	101 ± 10	222 ± 15	5184 ± 274**	4908 ± 634*
Basal tissue Treatment						
	Control	1242 ± 133	97.8 ± 67.8	124 ± 33	4189 ± 308	2484 ± 308
	MeJA	1342 ± 172	87.7 ± 49.1	132 ± 33	4599± 353**	2707 ± 353
Year						
2010	Control	1245 ± 142	73.0 ± 36	112 ± 37	4031 ± 314	2117 ± 249
	MeJA	1370 ± 229	106 ± 55	118 ± 38	4502 ± 413	2316 ± 422
2011	Control	1240 ± 138	123 ± 86	138 ± 25	4346 ± 225	2850 ± 559
	MeJA	1314 ± 102	69.0 ± 38	146 ± 20*	4696 ± 284*	3098 ± 404
Genotype						
	(2010)					
Dwarf Blue	Control	1366 ± 11	90.0 ± 46	124 ± 33	4310 ± 87	2286 ± 223
Curled Vates	MeJA	1575 ± 52**	154 ± 19	145 ± 6.0	4836 ± 248*	2676 ± 203
Red Winter	Control	1123 ± 75	56.0 ± 16	99.5 ± 43	3753 ± 85	1949 ± 142
	MeJA	1165 ± 54	59.0 ± 22	90.7 ± 37	4169 ± 175*	1956 ± 125
	(2011)					
Dwarf Blue	Control	1348 ± 110	198 ± 37*	160 ± 4.9*	4509 ± 199	3248 ± 423
Curled Vates	MeJA	1399 ± 46	87.1 ± 51	133 ± 14.2	4826 ± 307	3067 ± 595
Red Winter	Control	1133 ± 29	47.7 ± 8.5	116 ± 3.8	4183 ± 86	2452 ± 335
	MeJA	1231 ± 55	50.9 ± 7.4	159 ± 18.5*	4566 ± 237	3129 ± 227*

The results are presented as means ± SD (n=3). Student T-tests were conducted to determine significant at $P \leq 0.05$. [z]Total phenolics were measured by spectrophotometer using Folin-Ciocalteu reagent and expressed as mg of gallic acid equivalent concentration in 100 g of freeze-dried broccoli powder. [y]Flavonoids were measured by HPLC and expressed as mmol per 100 g of freeze-dried broccoli powder. [x]Antioxidant activities are presented as percentage of free radical scavenging ability compared to negative control (solvent).

Table 2.6. Percentages of total variance described by main factors (Genotype, Treatment, Year) and factor interactions for kale leaves phytochemical concentrations and antioxidant activities.

	Total phenolics[z]	Quercetin[y]	Kaempferol[y]	ABTS[x]	DPPH[x]
Apical tissue					
Genotype	27.5***	1.4	3.7	17.8***	9.7
Treatment	17.5***	76.2***	16.8	28.7***	41.0***
Year	42.5***	12.1***	6.2	44.6***	11.7
G×T	0.3	0.3	0.5	0.0	0.0
G×Y	1.1	5.2***	5.7	4.4***	12.1
T×Y	3.5	2.4***	12.0	0.0	0.2
G×T×Y	0.1	0.4	3.8	0.1	2.8
Block (Year)	2.0	1.1	1.4	1.4	2.1
Residual	5.5	1.0	50.0	3.0	20.3
R Square[w]	0.94	0.99	0.50	0.97	0.80
Basal tissue					
Genotype	0.9	0.3	18.5	11.3	47.0***
Treatment	69.3***	47.8***	15.2	35.8***	16.4
Year	10.3***	0.8	1.3	29.6***	4.1
G×T	4.7	1.6	5.7	4.5	0.5
G×Y	0.9	1.4	2.6	0.0	1.2
T×Y	0.7	14.7	0.0	0.6	0.0
G×T×Y	3.0	14.7	15.8	0.3	7.9
Block (Year)	0.7	0.8	2.0	0.8	0.9
Residual	9.5	18.0	39.0	17.0	21.9
R Square[w]	0.90	0.82	0.61	0.83	0.78

*** indicates factor that describes a significant proportion of the total variance using ANOVA at $P \leq 0.001$ (two tailed-test). [z]Total phenolics were measured by spectrophotometer using Folin-Ciocalteu reagent and expressed as mg of gallic acid equivalent concentration in 100 g of freeze-dried broccoli powder. [y]Flavonoids were measured by HPLC and expressed as mmol per 100 g of freeze-dried broccoli powder. [x]Antioxidant activities are presented as percentage of free radical scavenging ability compared to negative control (solvent). [w]Fraction of total variance described by the regression.

Supplementary Table 2.S1. Weather information of 2009, 2010, and 2011 year in Champaign, Illinois.

Total solar radiation ($MJ \cdot m^{-2}$)					
Year	Jun	Jul	Aug	Sep	Sum
2009	681	667	617	542	2507
2010	720	730	731	510	2690
2011	667	790	726	462	2645
% of (2010/2009)	106	109	118	94	107
% of (2011/2010)	93	108	99	91	98
	Precipitation (mm)				
Year	Jun	Jul	Aug	Sep	Sum
2009	108	156	137	55	401
2010	199	91	40	16	329
2011	107	40	45	71	262
% of (2010/2009)	184	58	29	29	82
% of (2011/2010)	54	44	113	444	80
	Growing degree days (°C)				
Year	Jun	Jul	Aug	Sep	Sum
2009	360	336	342	304	1341
2010	373	408	403	293	1477
2011	362	430	381	285	1458
% of (2010/2009)	104	122	118	96	110
% of (2011/2010)	97	105	94	97	99

Growing degree days [(Min Temperature + Max Temperature)/2 -7.2 °C].

Weather data during the growing seasons was provided by the Illinois State Water Service (http://www.isws.illinois.edu/warm/data/cdfs/cmiday.txt).

CHAPTER 3

Methyl Jasmonate-mediated Induction of Glucosinolate Biosynthesis Enhances Quinone Reductase Inducing Activity of Broccoli Florets

3.1. Abstract

Methyl jasmonate (MeJA) spray treatments were utilized to alter glucosinolate composition in the florets of the commercial broccoli hybrids 'Pirate', 'Expo', 'Green Magic', 'Imperial', and 'Gypsy' grown in replicated field plantings in 2009 and 2010. Aqueous solutions of 250 μM MeJA were sprayed to drip on aerial plant tissues four days prior to harvest at commercial maturity. The MeJA treatment significantly increased glucoraphanin (11%), gluconasturtiin (59%), and neoglucobrassicin (248%) concentrations and their hydrolysis products including sulforaphane (152%), phenylethyl isothiocyanate (PEITC, 318%), N-methoxyindole-3-carbinol (NI3C, 313%), and neoascorbigen (NeoASG, 232%) in hydrolysed florets of these genotypes over two seasons. Increased QR activity was significantly correlated with increased levels of sulforaphane, NI3C, and NeoASG. Although MeJA treatments mediated two-fold higher concentrations of the hydrolysis products (NeoASG and NI3C) derived from neoglucobrassicin compared to the hydrolysis product of glucoraphnin (sulforaphane), in pure compound hydrolysis product tests sulforaphane induced much greater QR activity than NeoASG or NI3C. MeJA treatment increased the ratio of bioactive sulforaphane compared to other products generated from the hydrolysis of glucoraphanin by endogenous myrosinase. Our results suggest that sulforaphane is the major QR inducer from floret extracts of MeJA-treated broccoli. Partitioning experiment-wide trait

variances indicated that the variability in concentrations of sulforaphane (29%), NeoASG (48%) and QR activity (72%) were influenced by year-associated weather variables, whereas variation in neoglucobrassicin (63%) and NI3C (46%) concentrations were primarily attributed to MeJA treatment. Due to the different harvest maturities of broccoli cultivars and variation in growing degree days, accumulation of solar radiation and precipitation after transplanting varied among cultivars. Accumulation of these weather related variables associated with broccoli maturity, which was controlled by genetic differences among cultivars, significantly correlated with sulforaphane, NI3C, and NeoASG concentrations and QR inducing activity. These results suggest that QR inducing activity can be enhanced by MeJA treatment but the treatment effect significantly interacts with genotype and specific environmental growing conditions.

3.2. Introduction

Broccoli (*Brassica oleracea* ssp. Italica) is one of the most frequently consumed vegetables in the United States and in other countries. Broccoli is well known for its health-promoting bioactivity, with previous research reporting that regular consumption of this vegetable is associated with the prevention of prostrate, colon, breast, lung, and skin cancer (Cho et al., 2005; Cornblatt et al., 2007; Dinkova-Kostova et al., 2006; Prochaska et al., 1992; Sapone et al., 2007; Zhang et al., 1992). Moreover, epidemiological studies have reported that dietary consumption of *Brassica* vegetables is inversely correlated with cancer risk, and this association is stronger than those between cancer and fruit and vegetable consumption in general (Michaud et al., 1999).

Diet is one of the most important factors in carcinogenesis accounting for 47% of the variation in cancer risk among the non-smoking public (Doll and Peto, 1981). Certain phytochemicals have anti-carcinogenic activity and induce phase II detoxifying enzymes in mammals including glutathione S-transferase (GST) and quinone reductase (QR) that can enhance detoxification and elimination of carcinogens from the body (Prestera et al., 1993). Up-regulation of QR activity has been used as a biomarker for cancer prevention because this enzyme is a catalyst for the conversion of quinones into stable and non-toxic hydroquinones, reducing oxidative cycling (Talalay et al., 1995). Moreover, QR activity elevation in *in vitro* and *in vivo* model systems has been shown to correlate with induction of other protective phase II enzymes such as GST and provides a reasonable biomarker for the potential chemo-protective effect of test agents against cancer initiation and proliferation (Cuendet et al., 2006).

Glucosinolates (GS) are secondary metabolites existing in almost all plants of the order *Brassicales*. Although intact GS do not have strong bioactivity, products of GS generated by hydrolysis from the endogenous enzyme myrosinase in broccoli have been shown to enhance QR and other health-promoting activities. Among the GS products, sulforaphane, an isothiocyanate generated from the hydrolysis of glucoraphanin, is a potent QR inducer and is considered to be an active agent in the prevention of certain cancers (Cho et al., 2005). Phenethyl isothiocyanate (PEITC), an isothiocyanate derived from the hydrolysis of the aromatic GS, gluconasturtiin, also induces synthesis of the QR enzyme (Manson et al., 1997) and has been shown to protect against colon cancer in rats (Chung et al., 2000). N-methoxyindole-3-carbinol (NI3C), the hydrolysis product of the indolyl GS, neoglucobrassicin has been reported to induce cell cycle arrest in human

colon cancer cell lines resulting in reduced initiation and tumor growth (Neave et al., 2005).

The GS are also associated with insect defense in *Brassica* species. Jasmonic acid (JA), an endogenous plant signal transduction compound whose biosynthesis is up-regulated when *Brassica* plant species are attacked by herbivores, causes enhanced indolyl GS biosynthesis (Hopkins et al., 2009). It has been reported that the indolyl GS whose biosynthesis is up-regulated by MeJA treatment in broccoli is predominately neoglucobrassicin but it up-regulated gluconasturtiin as well (Kim and Juvik, 2011). PEITC derived from gluconasturtiin and NI3C derived from neoglucobrassicin have previously been reported as QR inducers or anticancer agents (Jump et al., 2008; Rose et al., 2000; Neave et al., 2005). In addition to NI3C, neoascorbigen (NeoASG) can be generated from neoglucobrassicin hydrolysis by condensation with ascorbic acid (Agerbirk et al., 1998). However, there is little information about the health effects of NeoASG and the variation of NeoASG concentrations associated with biotic and abiotic stresses. While there are many previous reports of MeJA mediated increases in GS concentrations nearly all of these studies do not investigate how these treatments influence abundance and activities of GS hydrolysis products which are directly associated with anti-cancer activity.

The objective of this research was to investigate which of the GS and their hydrolysis products are primarily associated with the enhanced QR induction mediated by MeJA treatments. Variance in QR activity was partitioned by ANOVA into sources of variation associated with treatment, genotype, and environment (year) main factors and their interactions. Correlation analysis was conducted to test if QR inductive activity

shows meaningful correlations with GS hydrolysis products and weather-related environmental conditions over different production seasons. This information is useful for the identification of superior broccoli germplasm and for selection strategies in *Brassica* breeding programs designed to develop cultivars with enhanced health-promoting properties.

3.3. Materials and Methods

3.3.1. Broccoli Cultivation. The five F_1 hybrid broccoli cultivars used in this experiment were 'Pirate' (Asgrow Seed Co., Galena, MD), 'Expo', 'Imperial', 'Gypsy', and 'Green Magic' (Sakata Seed Co., Morgan Hill, CA). Seeds of each broccoli genotype were germinated in 32 cell plant plug trays filled with sunshine® LC1 (Sun Gro Horticulture, Vancouver, British Columbia, Canada) professional soil mix. Seedlings were grown in a greenhouse at the University of Illinois at Champaign-Urbana under a 25 °C/15 °C and 14 h/10 h: day/night temperature regime with supplemental lighting. Thirty days after germination, seedling trays were placed in ground beds to harden off for a week prior to transplanting into field plots at the University of Illinois South Farm (40˚ 04′ 38.89″ N, 88˚ 14′ 26.18″ W). Experimental design was a split-plot arrangement in a randomized complete block (RCB) design with three replicates. The experiment plot was surrounded by a guard row to avoid border effects. Transplanting of broccoli seedlings was conducted on June 24, 2009 and June 11, 2010. Irrigation was only applied during the first week of cultivation to establish transplanted seedlings. Broccoli heads were harvested from August 23 to September 18 in 2009 and from August 12 to September 12 in 2010. Weather data during the 2009 and 2010 growing seasons was provided by the

Illinois State Water Service (http://www.isws.illinois.edu/warm/data/cdfs/cmiday.txt) and is presented in Supplementary Table 3.S1. Since accumulated solar radiation and precipitation [(PPT)/number of days from transplant to harvest (DTH)] varied between years and the number of growing degree-days (GDD) [(minT+ maxT)/2-7.2˚C] (Dufault, 1997)] varied for each genotype, these values were calculated for each year and genotype separately (Table 3.1).

3.3.2. Broccoli Treatment with MeJA and Sample Preparation. An aqueous solution of 250 µM MeJA (Sigma-Aldrich, St. Louis, MO) and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in distilled water was sprayed on all aerial plant tissues to the point of runoff (approximately 300 mL) four days prior to harvest at commercial maturity. From previous studies this application timing, concentration and surfactant maximized MeJA mediated biosynthesis of glucosinolates (Ku and Juvik, 2012 and Chapter 7). Five broccoli heads were harvested from treatments and controls of each genotype for each replicate. Broccoli heads were frozen in liquid nitrogen, and stored at -20 °C prior to freeze-drying. Freeze-dried head tissues were ground into a fine powder using a coffee grinder and stored at -20 °C prior to chemical and bioactivity analyses.

3.3.3. Isolation of Neoglucobrassicin and Generation of Hydrolysis Products. In order to measure concentrations of hydrolysis products of neoglucobrassicin from different cultivars with or without MeJA treatment and their QR inducing activity, neoglucobrassicin was isolated and purified from broccoli following the procedure described by Truscott et al. (1983) with minor modifications. Two g of MeJA-treated

‘Green Magic’ broccoli powder was extracted with 10 mL 70% methanol in a 50 mL conical centrifuge tube (BD Falcon, San Jose, CA) for 10 min. After cooling, the supernatant obtained following lead/barium acetate precipitation was loaded on to a 20 cm x 2 cm ion exchange column containing Sephadex A-25 (Sigma-Aldrich, St. Louis), indolyl GS were eluted from the column with 0.02 M pyridine acetate (20 mL) and 0.25 M pyridine acetate (20 mL). The eluent fractions from ion exchange chromatography containing neoglucobrassicin were dried using a SpeedVac® AES2010 concentrator (Thermo Savant, Waltham, MA) and quantified on a Agilent 1100 HPLC system (Agilent, Santa Clara, CA), equipped with a G1311A bin pump, a G1322A vacuum degasser, a G1316A thermostatic column compartment, a G1315B diode array detector and an HP 1100 series G1313A autosampler. Extracts were separated on a Supercosil™ LC-18 column (250 × 4 mm, particle size 5 μm) (Supelco Inc., Bellefonte, PA) with a C18 all-guard ™ cartridge pre-column (Alltech, Lexington, KY). Mobile phase A was water and B methanol. Mobile phase B was 0% at injection and held 4 min, increasing to 15% by 10 min, 35% at 20 min, and 80% at 21 min, then held 4 min, then decreased to 0% by 30 min. Flow rates were kept at 1 mL/min. The detector wavelength was set at 227 nm. The concentration of NeoGS was determined using benzylglucosinolate as the desulphoglucosinolate standard. Purity was over 98%.

Hydrolysis products of neoglucobrassicin including NI3C and NeoASG were generated by incubation of 20 g of freeze dried MeJA-treated ‘Green Magic’ broccoli in 100 mL of pH 8 distilled water without ascorbic acid or pH 5.6 distilled water with 1 mM ascorbic acid, respectively. After 4 h incubation at room temperature, 100 mL of methylene chloride were added and shaken. The emulsion was transferred to 50 mL tube

and centrifuged at 5,000 g for 10 min. The supernatant was collected and traces of water removed using sodium sulfate. After filtration with Whatman No. 1 paper filter (GE Healthcare Life Sciences, Piscataway, NJ), the methylene chloride extract was dried with nitrogen gas. The dried samples from each hydrolysis pH treatment were dissolved in 1.5 mL of 50% acetonitrile and filtered with polytetrafluoroethylene (Fisher Scientific, Waltham, MA). The solution was fractionated on the Agilent 1100 HPLC G1364C Analyst Fraction Collector (Agilent) using the same methods described above for the isolation of neoglucobrassicin.

3.3.4. Identification of Purified Compounds. Identification of desulfated GS was achieved using a Q-TOF *Ultima* electrospray ionization (ESI) mass spectrometer (MS) and MS/MS (Waters, Milford, MA). The ESI MS was operated in positive ion mode with source conditions set at: capillary voltage 3 kV; cone voltage 35 V; source temperature 120 °C; desolvation temperature 375 °C; and collision energy 12 eV. Identification of the hydrolysis products of neoglucobrassicin was achieved using electron impact (EI) direct inlet MS using a Micromass 70-VSE (Waters, Milford, MA) double-focusing magnetic sector mass spectrometer in positive ion mode at 70 eV and a source temperature of 30 °C. The instrument was scanned between *m/z* 50 and 400. High-resolution mass spectrometry was performed on same instrument above.

3.3.5. Determination of Sample GS Concentrations. Extraction and quantification of GS using high-performance liquid chromatography was performed using a protocol described by Brown et al. (2002). Freeze-dried broccoli powder (0.2 g) and 2 mL of 70%

methanol were added to 10 mL tubes (Nalgene, Rochester, NY) and heated on a heating block at 95 °C for 10 min. After cooling on ice, 0.5 mL benzylglucosinolate (1 mM) was added as internal standard (POS Pilot Plant Corp, Saskatoon, SK, Canada), mixed, and centrifuged at 3,000 × g for 15 min at 4 °C. The supernatant was saved and the pellet was re-extracted with 2 mL 70% methanol at 95 °C for 10 min and the two extracts combined. A subsample (1 mL) from each pooled extract was transferred into a 2-mL microcentrifuge tube (Fisher Scientific, Waltham, MA). Protein was precipitated with 0.15 mL of a 1:1 mixture of 1 M lead acetate and 1 M barium acetate. After centrifuging at 12,000 × g for 1 min, each sample was then loaded onto a column containing DEAE Sephadex A-25 resin (GE Healthcare, Piscataway, NJ) for desulfation with arylsulfatase (*Helix pomatia* Type-1, Sigma-Aldrich, St. Louis, MO) for 18 h and the desulfo-GS eluted. One hundred μL of each sample were injected on to a HPLC system consisting of a DIONEX GP40 gradient pump (Dionex Corporation, Sunnyvale, CA), with a AD20 variable UV detector set at 229 nm wavelength, auto-sampler, all-guard™ cartridge pre-column (Alltech, Lexington, Kentucky), and a LiChospher® 100 RP-18 column (Merck, Darmstadt, Germany). Desulfo-GS were eluted from the column over 45 min with a linear gradient of 0% to 20% acetonitrile at a flow rate of 1 mL/min. Benzylglucosinolate was used as an internal standard and UV response factors for different types of GS were used as determined by Wathelet et al. (1995). The identification of desulfo-GS profiles were validated by LC-tandom MS using a Waters 32 QT of Ultima spectrometer coupled to a Waters 1525 HPLC system and full scan LC-MS using a Finnigan LCQ Deca XP, respectively. The molecular ion and fragmentation patterns of individual desulfo-GS

were matched with the literature for GS identification (Tian et al., 2005; Velasco et al., 2011).

3.3.6. Analysis of Glucosinolate Hydrolysis Products. The extraction and analysis of isothiocyanates and other hydrolysis products was carried out according to previously published methods, with some modifications (Wilson et al., 2011). In order to determine the appropriate time for maximum GS hydrolysis by endogenous sample myrosinase, concentrations of hydrolysis products were quantified in a preliminary experiment using extracts from 'Green Magic' at various time points. Based on the preliminary results using 'Green Magic' cultivar, hydrolysis product concentrations of all samples were quantified at 2, 4, 16, 24, and 28 h using aliquots by HPLC. 75 mg of broccoli powder was suspended in 1.5 mL of water in the absence of light for 4 h (time for the maximum concentration of indolyl GS hydrolysis products) at room temperature in a sealed 2 mL microcentrifuge tube (Fisher Scientific, Waltham, MA) to facilitate GSs hydrolysis by endogenous myrosinase. Slurries were then centrifuged at 12,000 × g for 5 min and supernatants was decanted into a 2 mL microcentrifuge tube. 20 µL of butyl isothiocyanate (0.5 mg/mL) and 4-methoxyindole (1 mg/mL) were added as the internal standards for isothiocyanates and hydrolysis products of indolyl GS to quantify Indole-3-carbinol (I3C), NI3C, and neoascorbigen (NeoASG) with 0.5 mL of methylene chloride. Tubes were shaken vigorously before being centrifuged for 2 min at 9,600 g. The methylene chloride layer (200 µL) was transferred to 350 µL flat bottom insert (Fisher Scientific, Pittsburgh, PA) in a 2 mL HPLC autosampler vial (Agilent, Santa Clara, CA) for mixing with 100 µL of a reagent containing 20 mM triethylamine and 200 mM

mercaptoethanol in methylene chloride. For SF and PEITC, unlike other hydrolysis products of GS, 0.5 mL of fresh broccoli extracts were kept mixed with 0.5 mL of derivatization reagent using orbital shaker at 220 rpm for 24 h. The mixture was incubated at 30 °C for 60 min under constant stirring, and then dried under a stream of nitrogen. The residue containing isothiocyanate derivatives (isothiocyanate-mercaptoethanol derivatives) and other hydrolysis compounds was dissolved in 200 µL of acetonitrile /water (1:1) (v/v), and 10 µL of this solution injected onto a Agilent 1100 HPLC system (Agilent, Santa Clara, CA), equipped with a G1311A bin pump, a G1322A vacuum degasser, a G1316A thermostatic column compartment, a G1315B diode array detector and an HP 1100 series G1313A autosampler. Extracts were separated on a Eclipse XDB-C18 column (150 × 4 mm, particle size 5 µm, Agilent, Santa Clara, CA) with a C18 all-guard™ cartridge pre-column (Alltech, Lexington, KY). Mobile phase A was water and B was methanol. Mobile phase B was 0% at injection, increasing to 10% by 10 min, 100% at 35 min, and held 5 min, then decreased to 0% by 50 min. Flow rates were kept at 0.8 mL/min. The detector was set at wavelength 227 and 271 nm. Response factors for monomeric indolyl derivatives were used from a previous report (Agerbirk et al., 1998). Due to a lack of standards for NI3C and NeoASG the standard curve of I3C was applied for quantification of both NI3C and NeoASG. The quantities were expressed as I3C equivalent concentrations.

3.3.7. Quinone Reductase (QR) Activity. For the QR assay, broccoli extracts were collected using the same protocol for glucosinolate hydrolysis products described above with sampling at 30 min, 4 h, and 24 h of incubation. Hepa1c1c7 murine hepatoma cells

(ATCC, Manassas, VA) were grown in alpha-minimum essential medium (α-MEM), enriched with 10% heat and inactivated fetal bovine serum and maintained at 37 °C in 95% ambient air and 5% CO_2. The cells were divided every three days with a split ratio of 7. Cells with 80-90% confluence were plated into 96-well plates (Costar 3595, Corning Inc, Corning, NY), 1×10^4 cells per well, and incubated for 24 h in antibiotic-enriched media (100 units/mL penicillin, 100 μg/mL streptomycin). The QR induction activities of different samples were determined by means of the protocol described by Prochaska and Santamaria (Prochaska and Santamaria, 1988). After 24 h cells were exposed to the different sample extracts [0.5% final concentration (250 μg of freeze-dried broccoli/mL) in 200 μL of media] in new media for a further 24 h. Growth media alone and 0.2 μM SF were used as negative and positive controls, respectively. Treated cells were rinsed with phosphate buffer at pH 7.4, lysed with 50 μL 0.8% digitonin in 2 mM EDTA, incubated and agitated for 10 min. A 200-μL aliquot of reaction mix [74 mL 25 mM Tris buffer; 50 mg BSA; 0.5 mL 1.5% Tween-20 solution; 0.5 mL cofactor solution (92.7% 150 mM glucose-6-phosphate, 6.15% 4.5 mM NADP, 1.14% 0.75 mM FAD in Tris buffer)]; 150 units of glucose-6-phosphate dehydrogenase; 22.5 mg MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide]; and 75 μL 50 mM menadione in acetonitrile) was added to the lysed cells. Readings were made at five time points, 50 s apart, using a μQuant microplate reader (Bio-Tek Instruments, Winooski, VT) at 610 nm. Immediately after completion of the readings, 50 μL of 0.3 mM dicumarol in 25 mM Tris buffer was added into each well, and the plate was read again (five time points, 50 s apart) to determine non-specific MTT reduction. Total protein content was measured by the BioRad assay (Bio-Rad, Hurcules, CA, USA) using manufacture's instructions. Activity

was expressed as QR specific activity (nmol MTT reduced/mg/min) ratio of treated to negative control cells.

3.3.8. QR Activity Measurement of Hydrolysis Products of Neoglucobrassicin. QR activity of hydrolysis products of neoglucobrassicin were measured after hydrolysis with 0.5 U/mL (final concentration) commercial myrosinase (*Sinapis alba*, Sigma) and neoglucobrassicin (700 μM) for 40 mins in pH 7.2 of phosphate buffered saline (PBS) without ascorbic acid or pH 5.6 of PBS with 1 mM ascorbic acid to produce different forms of hydrolysis products, respectively and observed until completion of the hydrolysis by HPLC as described above (method for the hydrolysis products analysis). The reaction products were left on ice until adding samples for QR assay. Different concentrations of hydrolysis products were added to the media contained hepa1c1c7 cells to measured concentration required to double QR induction activity (CD) values. QR activity was measured as described above.

3.3.9. Statistical Analysis. Analysis of variance (ANOVA) and partitioning of variance components for phytochemicals and QR activity using total sums of squares were conducted using JMP 10 (SAS institute Inc., Cary, NC). Year, treatments, and genotype effects were considered as fixed factors. Year is usually considered as random effect but we considered year as fixed effect since we are interested in weather variation from year-to-year. Block was considered as random. Analysis of variance was performed using the linear model: $Y_{ijklm} = m + G_i + Y_j + T_k + GY_{ij} + GT_{ik} + YT_{jk} + GYT_{ijk} + B_{l(j)} + \varepsilon_{ijklm}$, where Y_{ijklm} is the l^{th} block of the phenotypic value of the k^{th} treatment, i^{th} genotype in

year j, m is the overall mean, G, Y, T, and B indicate the effects of genotype, year (weather), treatment and blocks nested in years, ε_{ijklm} is the experimental error associated with Y_{ijklm}, respectively. Correlation analysis and Student's t-tests were also conducted using the JMP 10 software. All sample analyses were conducted in triplicate. The results are presented as means ± SD.

3.4. Results and Discussion

3.4.1. Isolation Neoglucobrassicin and Generation of Hydrolysis Products. In order to quantify hydrolysis products, neoglucobrassicin was purified from MeJA treated 'Green Magic' floret tissue. The purified neoglucobrassicin was identified by using ESI MS on its desulfated form as previously used for GS identification. The molecular ion (399), and fragment patterns (*m/z* 237, 177, 160) were well matched with previous research (Griffiths et al., 2000) (Table 3.2). The identity of NI3C isolated from hydrolysis products of neoglucobrassicin, generated under hydrolysis at pH 8 without ascorbic acid was confirmed by EI direct inlet mass at high resolution. The positive mode provided firm evidence, showing *m/z* 177.07847 corresponded to the calculated molecular formula $C_{10}H_{11}O_2N$ (177.07898); additional evidence was obtained for the mass spectrum with *m/z* 160, 146, and 77, pointing to the presence of an hydroxyl group, methoxy group, and an aromatic ring. This result agrees with Jump et al. (2008) who reported *m/z* 177, 160 by FAB-MS for NI3C (Table 3.1). Molecular composition of the NeoASG was deduced from mass EI-MS spectra; the measured m/z 335.10053 which corresponds to the molecular composition $C_{16}H_{17}O_7N$ (calculated as 335.10050). The loss of 175 Th and 215 Th from *m/z* 335 can be rationalized as the loss of ascorbic acid ($C_6O_6H_7$, *m/z* 160) and as

the loss of ascorbic acid with methoxy group (OCH_3, *m/z* 130) (Agerbirk et al., 1998). The molecular ion (336), and fragment patterns (*m/z* 305, 160, 130) from the positive mode of ESI-MS also matched previous research (Bennett et al., 2004)(Table 3.1).

3.4.2. Time Course of GS Hydrolysis. Some GS hydrolysis products are relatively unstable in aqueous extracts including NI3 and I3C. To determine the optimal time for maximum neoglucobrassicin hydrolysis, hydrolysis product concentrations were sampled and quantified at a range of time points. The maximum concentrations of each hydrolysis product was found to be 4 h for NeoASG and I3C, 16 h for NI3C and PEITC, and, 24 h for sulforaphane (Figure 3.1). Compared to the amount of precursor GS, accumulated concentrations of PEITC, the hydrolysis product of gluconasturtiin (Figure 3.1C) and I3C, one of the hydrolysis products from GB (Figure 3.1E) were relatively low. It is reported that PEITC is relatively volatile and has very low solubility in water (0.051 mg/mL) compared to SF (8.0 mg/mL) (Wilson et al., 2012). Previous studies could not detect PEITC in hydrolyzed watercress extracts (Boyd et al., 2006; Rose et al., 2000). Thus, SF and PEITC in Table 3.3 were measured by shaking with isothiocyanate derivatization reagent for 24 h as described in the methods above.

The different peak times for hydrolysis accumulation of sulforaphane and the hydrolysis products of indolyl GS may be due to variation in isoforms of myrosinase in broccoli. James and Rossiter (1991) reported that there were two isoforms of myrosinase in *Brassica napus* and their hydrolysis efficiency varied between aliphatic and indolyl GS. In their study both myrosinase I and II were less active in cleaving glucose from the indoyl GS glucobrassicin and neoglucobrassicin, although myrosinase I was

approximately twice as active as myrosinase II in the presence of ascorbic acid. An atypical myrosinase, PEN2 has been identified which cleaves indolyl GS *in planta* preferentially as a mechanism of phytochemical defense against fungal pathogens (Bednarek et al., 2009). The observed different accumulation rates of hydrolysis products in our study may be associated with the relative activity of different isoforms of myrosinase. QR activity was tested using all samples (five genotypes with or without MeJA over two years) with sample aliquots from different time points of hydrolysis at 30 min, 4 h, and 24 h (Supplementary Figure 3.S1). Interestingly, after only 30 min of hydrolysis, aliquots of broccoli extracts showed 58% of the QR activity observed in 24 h hydrolysis aliquot extracts. The 4 h hydrolysis aliquots displayed 97% of QR activity observed after 24 h of hydrolysis. This indicates that there may have been *in situ*-hydrolysis inside cell culture media but hydrolyzing extracts for 30 min before adding to cell culturing media did not fully induce QR activity due to the lack of time for hydrolysis and induction of the QR enzyme. NI3C and NeoASG were observed to rapidly degrade after 16 h. Considering the relatively higher concentrations of NeoASG and NI3 compared to other hydrolysis products and their instability we suggest that QR inducing activity be assayed between 4-16 h after sample hydrolysis.

3.4.3. Effect of MeJA Treatment on GS Concentrations in Broccoli Florets. Over both seasons 250 μM MeJA treatments were observed to significantly increase glucoraphanin (11%), gluconasturtiin (60%), and neoglucobrassicin (248%) floret tissue concentrations across cultivars (Table 3.3). Gluconapin (data not shown) was significantly decreased (53%) by MeJA treatment across two seasons in the five broccoli

cultivars. Since glucoraphanin is upstream in the aliphatic GS biosynthesis pathway, decreased gluconapin concentration may partly be due to the increase of glucoraphnin or from a shift toward increasing neoglucobrassicin biosynthesis. Individual cultivars responded differently to MeJA treatment, with 'Pirate' showing no significant increase in any GS in 2010 while the other four hybrids displayed significantly increased concentrations of either gluconasturtiin or neoglucobrassicin. Since the 'Pirate' cultivar has late maturtity, insect activity might have up-regulated GS biosynthesis. There was significant year-to-year variation in Total GS. Total GS in control broccoli lines grown in 2010 was 43% higher than controls grown in 2009. Precipitation in August, 2010 (40 mm) was only 29% of that observed in August, 2009 (137 mm)(Supplementary Table 3.S1). This observation agrees with the finding that water stress can increase total GS, as has been previously reported for *B. napus* (Champolivier and Merrien, 1996). Average temperatures in August, 2010 were 17% higher than in August 2009 (Supplementary Table 3.S1). It has been suggested that increased temperatures can result in the accumulation of GS by up-regulating MYB transcription factors as was observed in turnips (Justen and Fritz, 2013).

3.4.4. Effect of MeJA Treatment on GS Hydrolysis Product Concentrations and QR Induction Activity. NeoASG, a hydrolysis product of neoglucobrassicin was significantly increased by MeJA treatment in four cultivars over two seasons with the exception of 'Pirate'. NI3C and NeoASG concentrations in hydrolyzed 'Pirate' floret extracts were not increased in 2009 or 2010 by MeJA treatment (Table 3.3). NeoASG concentrations in 2009 controls were significantly higher than 2010. Endogenous MeJA

is responsive to many biotic and abiotic stresses, including drought stress (Creelman and Mullet, 1995). Exogenous MeJA treatments have been observed to increase ascorbic acid concentration in broccoli florets, *Arabidopsis* and tobacco BY-2 cell suspension cultures (Kim, 2011; Wolucka et al., 2005). Drought stress conditions in 2010 and higher temperatures near harvest may have led to production of more NeoASG since there was a greater abundance of both substrates. Biotic and abiotic stress conditions can increase endogenous MeJA levels, which can stimulate GS biosynthesis and ascorbic acid biosynthesis (Wolucka et al., 2005), which suggests that stressful field conditions may be favorable for broccoli to produce more NeoASG. Sulforaphane (from 31% to 36%) and PEITC (from 28% to 51%) conversion rates from their parent GS were also significantly increased by MeJA treatment in all cultivars over two seasons (Table 3.4). MeJA treatment significantly increased gene expression of broccoli myrosinase (Chapter 5). The unbound myrosinase from ESP by increased protein amount may favors to generate isothicyanate. In addition, ESP by ESM1 interaction may be associated with the isothiocyanate formation (Baskar et al., 2012). It was reported that hydrolysis product of gluconasturtiin, phenethyl isothiocyanate (PEITC), induces synthesis of the QR enzyme (Manson et al., 1997; Rose et al., 2000). The accumulation of PEITC was relatively small compared to gluconasturtiin concentrations when we measured broccoli extracts without shaking with isothiocyanate derivatization reagents (Figure 3.1), probably associated with the low solubility and high volatility (Wilson et al., 2012). However, when the PEITC concentration was determined by shaking with isothiocyanate derivatization reagents as described in the methods, substantial concentrations of PEITC were found (PEITC conversion rates from gluconasturtiin averaged 28% for controls and 51% for MeJA

treatments). Some cultivars showed significantly increased SF and PEITC concentrations following MeJA treatment, but the effect was not consistently observed in all cultivars over both years. Although glucobrassicin was not significantly increased both years by MeJA treatment, I3C concentration was significantly increased in both 2009 and 2010. As reported previously, I3C is a relatively weak QR inducer. Based on our results, the CD value for I3C is 230 μM (Chapter 5). Based on our time course experiment, aliquots were taken after four h of broccoli extract hydrolysis for measurement of QR inducing activities. Treatment with MeJA significantly increased QR inducing activity of extracts from florets of 'Imperial', and 'Gypsy' cultivars in both years. Average QR inducing activity across all of the five broccoli genotypes was significantly increased by MeJA treatment only in 2010, which suggests an interaction between MeJA treatment and year. Several factors including pH, Fe^{2+}, and the mysosinase co-factors, ESP and ESM1 can influence glucoraphanin and gluconasturtiin hydrolysis and isothiocyanate formation (Baskar et al., 2012). Year-associated environment factors and MeJA treatment in 2010 appeared to interactively influence hydrolysis of glucoraphanin.

3.4.5. Correlation Analysis between Intact GS or Hydrolysis Products and QR activity. There was a moderate but significant correlation between gluconasturtiin with QR inducing activity where $r = 0.654$ ($P = 0.002$) (Table 3.5). Considering the precursor GS, there was only a weak and non-significant correlation between glucoraphanin ($r = 0.330$; $P = 0.155$), neoglucobrassicin $r = 0.312$ ($P = 0.181$) and QR inducing activity (Table 3.5). PEITC (Manson et al., 1997) and sulforaphane (Zhang et al., 1992) are known QR inducers, derived from gluconasturtiin and glucoraphanin, respectively. The

moderate correlation coefficient between glucoraphanin and sulforaphane concentrations ($r = 0.593$, $P = 0.006$) among the five broccoli cultivars may be associated with variation in epithiospecifier protein activity and/or epithiospecifier modifier protein activity, a cofactor in the myrosinase hydrolysis of GS, since sulforaphane formation is negatively correlated with epithiospecifier protein levels (Matusheski et al., 2006).

Although there was a significant positive correlation between gluconasturtiin and QR, there was a non-significant correlation between PEITC and QR inducing activity ($r = 0.176$, $P = 0.4594$). This lack of correlation is likely due to the high volatility and low solubility of PEITC and from variation in protein co-factors (Figure1, Table 3). However, conducting *in situ*-hydrolysis (Haack et al., 2010) with different concentrations of gluconasturtiin standards and 50 mU/mL of commercial myrosinase (*Sinapis alba* L.) we observed that to generate a two-fold increase of QR induction (CD value) required 5 μM gluconasturtiin (data not shown), comparable to what was previously reported for PEITC (Rose et al., 2000). The variation in QR inducing activity in *in situ*-hydrolysis may be associated with the accumulation of PEITC induced by enhanced myrosinase activity. Since QR induction activity is dose- and time-dependent (Hou et al., 2000), low concentrations of accumulated PEITC in broccoli extracts may reduce its contribution to enhancing QR activity. Even though the increased PEITC concentration was successively determined by using the 24 h derivitization method, it did not show a strong correlation with QR inducing activity in the *in vitro* system. The observed moderate correlation between gluconasturtiin and QR inducing activity is likely due to the correlation of gluconasturtiin with sulforaphane ($r = 0.784$, $P < 0.001$), NI3C ($r = 0.876$, $P < 0.001$), and NeoASG ($r = 0.549$, $P = 0.012$).

There were significant correlations between QR activity and the hydrolysis products of neoglucobrassicin including NI3C ($r = 0.502$, $P = 0.024$) and NeoASG ($r = 0.771$, $P < 0.001$). Recent research using an *in vitro* cancer cell line has found that NI3C can inhibit the nuclear erythroid related factor 2 (Nrf2)-dependent up-regulation of phase II detoxifying enzymes such as QR (Haack et al., 2010), interfering with its anti-cancer bioactivity. This suggests that MeJA-mediated up-regulation of neoglucobrassicin biosynthesis in broccoli florets would interfere with the QR induction associated with the isothiocyanate, SF generated from the hydrolysis of glucoraphanin. However, there was a significant positive correlation between SF and QR activity when NI3C was present at relatively high concentrations in the 4 h hydrolysis extracts, indicating it did not inhibit SF dependent increases in QR activity. In Haack et al.'s study, QR activity induced by the combination of 2.5 μM neoglucobrassicin and 5 μM glucoraphanin with myrosinase was not significantly different from the QR activity of 5 μM glucoraphanin with myrosinase alone. In addition, combination of 2.5 μM neoglucobrassicin and 10 μM glucoraphanin with myrosinase even slightly increased human *Gpx2* promoter compared to 10 μM glucoraphanin with myrosinase alone, suggesting that concentrations of NI3C in MeJA treated broccoli is not at a critical level to interfere with sulforaphane mediated QR activity induction. Haack et al. used *in situ* hydrolysis for QR activity measurement, which favors the production of NI3C. This experiment did not consider the formation of other hydrolysis products that could be generated from neoglucobrassicin. In our study neoglucobrassicin was observed to generate both NI3C and NeoASG, which is condensation product of NI3C with ascorbic acid. NeoASG maybe the primary hydrolysis product from neoglucobrassicin in the human gut where low pH and high

vitamin C concentrations (Koh et al., 2009) from broccoli consumption could favor the production of NeoASG over NI3C.

3.4.6. QR Inducers in MeJA Treated Broccoli. High correlation of QR activity with NI3C and NeoASG suggests that the hydrolysis products of neoglucobrassicin may contribute to the MeJA enhanced QR activity. CD values of each compound were were found to be 35 μM and 38 μM for NI3C and NeoASG, respectively (Figure 3.2). Previously it was reported that CD values of SF and PEITC were 0.2 μM and 5 μM, respectively (Kang and Pezzuto, 2004; Rose et al., 2000). Compared to previously reported QR inducers, NI3C and NeoASG are relatively weak QR inducing agents. Averaged CD values of hydrolysis products of neoglucobrassicin is approximately 36.5 μM. Based on these CD values the relative QR inducing ability for sulforaphane, PEITC, and the hydrolysis products of neoglucobrassicin were 182.5, 7.3, and 1, respectively. While MeJA treated broccoli was found to contain approximately twice as much hydrolysis product derived from neoglucobrassicin than sulforaphane, sulforaphane may be a more important QR inducer, having 183-fold greater potency. If the QR induction is evaluated by the relative magnitude of estimated CD values and and MeJA increased amounts of hydrolysis products, sulforaphane should possess a 38-fold greater QR inducing capacity than the hydrolysis products of neoglucobrassicin.

3.4.7. Effect of Weather Related Factors on Glucosinolates, their Hydrolysis Products, and QR Activity. Since QR activity was found to be indirectly associated with year-related weather factors, Pearson correlation coefficients were calculated

between QR activities, GS and hydrolysis product concentrations, and cultivar growing degree days (GDD), accumulated solar radiation and averaged daily precipitation (PPT/DTH). Differentially accumulated GDD among the cultivars and seasons was significantly correlated with gluconapin ($r = 0.634$, $P = 0.020$), NeoASG ($r = 0.496$, $P = 0.026$), and QR inducing activity ($r = 0.699$, $P < 0.001$) (Table 4). Differentially accumulated solar radiation was significantly correlated with gluconapin ($r = 0.634$, $P = 0.003$), NeoASG ($r = 0.586$, $P = 0.007$), and QR inducing activity ($r = 0.796$, $P < 0.001$). PPT/DTH was significant negatively correlated with SF ($r = -0.447$, $P = 0.048$), QR inducing activity ($r = -0.660$, $P = 0.002$), suggesting that drought conditions may enhance QR inducing activity by enhancing endogenous MeJA synthesis (Creelman and Mullet, 1995). It has been reported that GS concentrations in broccoli sprouts is influenced by temperature (Pereira et al., 2002) and that UV-B induces glucoraphanin biosynthesis (Mewis et al., 2012). UV-B radiation may affect phytochemical composition and QR inducing activity. DTH was significantly different among genotypes ($F_{4,19} = 18.32$, $P < 0.001$) (Table 3.1). The partitioning of total variance into variance components, indicated that the genotype accounts for 75% of DTH variation, which agres with previous research (Farnham et al., 2004). Since DTH is correlated with solar radiation and GDD, genetic variation in days to harvest plays a major role in broccoli floret phytochemical composition and QR inducing activity. QR activity is indirectly affected by weather, DTH, and GDD.

3.4.8. Partitioning of GS Concentration and Bioactivity Variances into MeJA Treatment, Year, and Genotype Sources of Variation. ANOVA partitioning of the

variances for GS concentrations indicated that differences among genotypes described 42%, 33%, 31%, and 48% of the total variation for glucoraphanin, glucobrassicin, gluconasturtiin and I3C, respectively (Table 3.6). In contrast, MeJA treatment accounted for 63%, 46%, 36%, 30% and 17%, of the total variation in floret neoglucobrassicin, NI3C, Total GS, PEITC, and sulforaphane concentrations, respectively (Table 3.6). Seasonal differences in environmental conditions between 2009 and 2010 were a major source of variation in sulforaphane (29%), NeoASG (48%), and QR inducing activity (72%). There was also a significant genotype by year interaction in concentrations of glucoraphanin (38%) and I3C (23%). MeJA treatment significantly increased QR inducing activity averaged over the two years study but this only described 5% of the variation in QR inducing activity while year associated weather effects accounted for 72%, which also agrees with previous research (Farnham et al., 2004). Environment conditions significantly correlated with GS biosynthesis and hydrolysis products of GS. These results suggest that optimal environment conditions, appropriate cultivars, and MeJA treatments can maximize phytochemical profiles and anticancer bioactivity of broccoli florets.

Table 3.1. Days from transplant to harvest (DTH) and accumulated growing degree days (GDD), solar radiation, and precipitation experienced by each of the five broccoli genotypes in 2009 and 2010.

Year	Cultivar	Treatment	DTH	GDD (˚C)	Solar radiation ($MJ \cdot m^{-2}$)	Precipitation (mm)
2009	Expo	Control	81 ± 3	1150	1705	296
2009	Expo	MeJA	82 ± 3	1163	1729	296
2009	Green Magic	Control	58 ± 7	859	1257	223
2009	Green Magic	MeJA	57 ± 7	846	1239	222
2009	Gypsy	Control	62 ± 5	906	1342	223
2009	Gypsy	MeJA	61 ± 2	893	1318	223
2009	Imperial	Control	60 ± 3	881	1293	223
2009	Imperial	MeJA	60 ± 2	881	1293	223
2009	Pirate	Control	77 ± 5	1094	1622	296
2009	Pirate	MeJA	78 ± 5	1109	1643	296
2010	Expo	Control	88 ± 2	1531	2758	314
2010	Expo	MeJA	93 ± 3	1595	2868	318
2010	Green Magic	Control	67 ± 6	1198	2165	245
2010	Green Magic	MeJA	67 ± 6	1198	2165	245
2010	Gypsy	Control	68 ± 3	1215	2193	245
2010	Gypsy	MeJA	69 ± 2	1231	2223	245
2010	Imperial	Control	65 ± 2	1107	2100	245
2010	Imperial	MeJA	66 ± 4	1182	2136	245
2010	Pirate	Control	92 ± 6	1581	2825	314
2010	Pirate	MeJA	91 ± 4	1570	2845	314

Growing degree days [(Min Temperature + Max Temperature)/2 -7.2 °C]

Table 3.2. Identification of Purified Hydrolysis Products of Neoglucobrassicin.

Compound	Fragment ions	Ref
Desulfated neoglucobrassicin	ESI (12 eV) *m/z* (%) 421 (100) [M^++ Na], 399 (83) [M^+] MS/MS fragments: 237 (100) [M^+-Glu+ H], 206 (23) [M^+-Glu-CH_3O+2H], 177 (13) [M^+-$C_7H_{11}NO_5S$], 160 (5) [M^+-$C_7H_{11}NO_5S$-OH], 130 (30) [M^+-CH_3O-$C_7H_{13}NO_6S$+H]	Griffiths et al., 2000
N-Methoxyindole-3-Carbinol	EI MS (70 eV) *m/z* (%) 177 (27) [M^+] , 160 (11)[M^+-OH], 146 (8) [M^+-OCH_3], 129 (38) [M^+-OH-OCH_3], 117 (17) [M^+-OH-$NOCH_3$+2H], 102 (18) [M^+-C_6H_5+2H], 77 (13) [M^+-$C_4H_6NO_2$], 58 (100) [M^+-C_8H_7O] Measured mass: 177.07847, calculated mass based on molecular formula $C_{10}H_{11}O_2N$ (177.07898)	Jump et al., 2008
Neoascorbigen	EI MS (70 eV) *m/z* (%) 335 (10) [M^+] , 160 (100) [M^+-$C_6O_6H_7$], 130 (42) [M $^+$-$C_6O_6H_7$-OCH_3] Measured mass: 335.10053, calculated mass based on molecular formula $C_{16}H_{17}O_7N$ (335.10050)	Agerbirk et al., 1998
	ESI MS (16eV) *m/z* (%) 374 (28) [M^+ +K], 358 (100) [M^++Na], 336 (45) [M^+ +H], 305 (5) [M^++OCH_3] 160 (38) [M $^+$-$C_6O_6H_7$+H], 130 (3) [M^+-$C_6O_6H_7$-OC H_3+H]	Benett et al., 2004

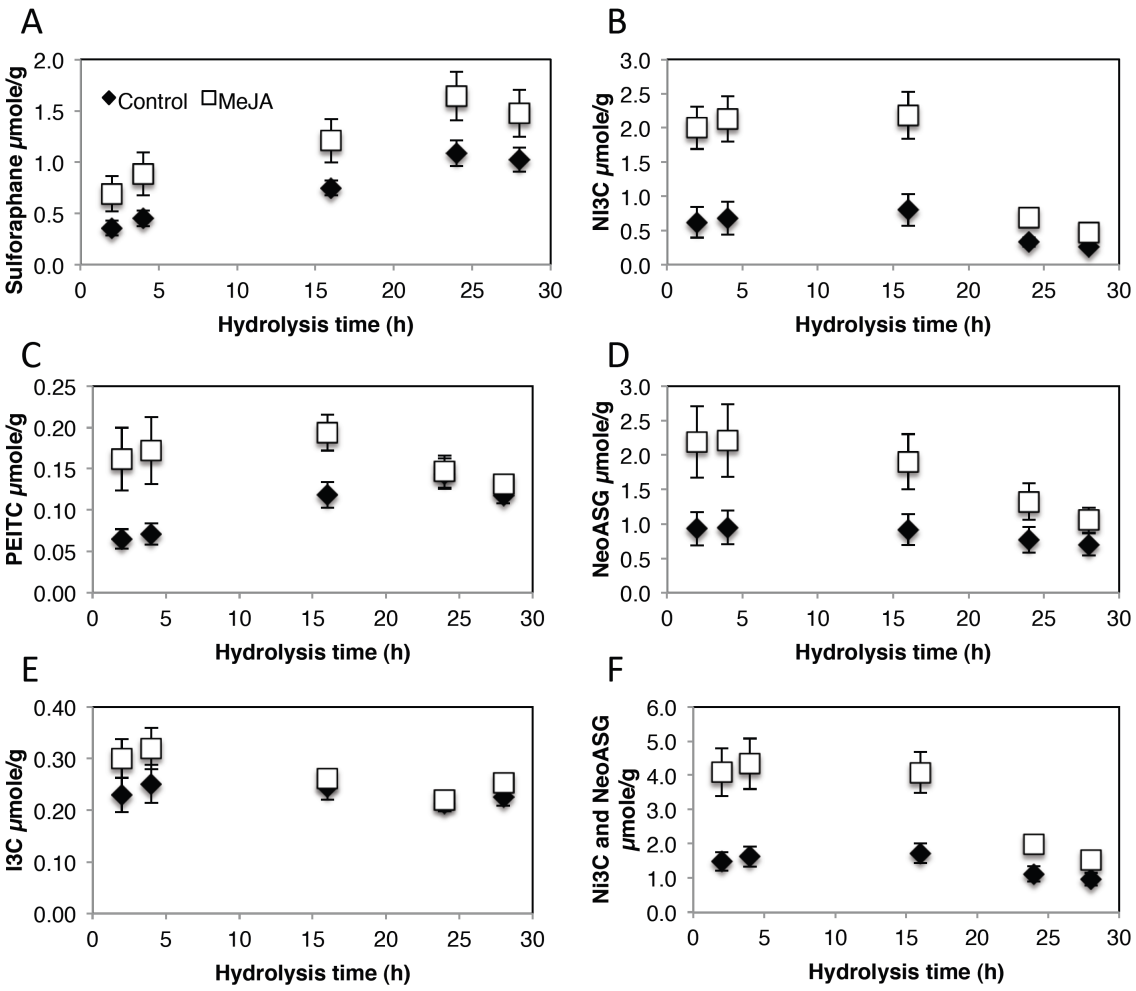


Figure 3.1. Optimization time for maximum concentration for each hydrolysis product of GS. Samples from five broccoli cultivars with or without MeJA treatment over two years were used to determine the optimum time for maximum concentration of hydrolysis products of GS. Mean ± SEM (n=30). NI3C = N-mothoxyindole-3-carbinol, PEITC = Phenethyl isothiocyanate, NeoASG = Neoascorbigen, I3C = Indole-3-carbinol.

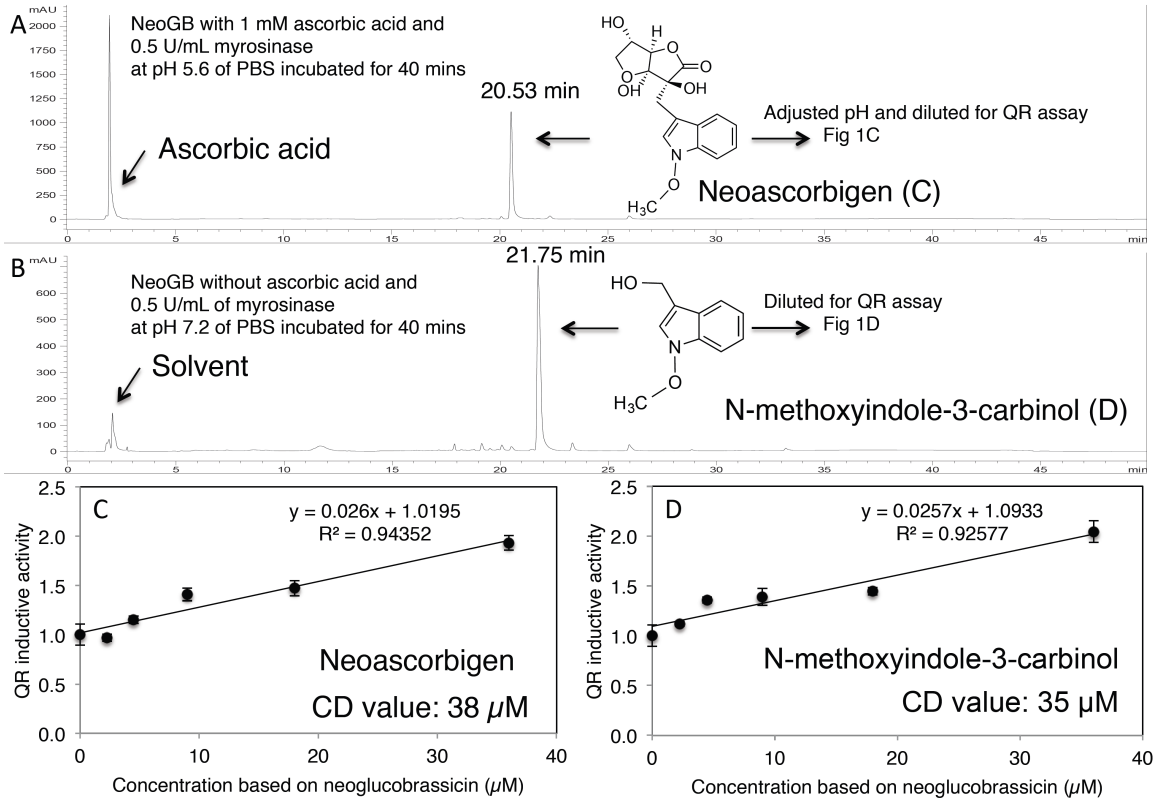


Figure 3.2. QR activity of major hydrolysis products of neoglucobrassicin. Different pH and presence of ascorbic acid produced different hydrolysis products from neoglucobrassicin. CD value = concentration required to double QR induction. 0.7 mM neoglucobrassicin was incubated at pH 5.6 with ascorbic acid (1 mM) and pH 7.2 without ascorbic acid to generate neoascorbigen and n-methoxyindole-3-carbinol, respectively. After confirming the completion of hydrolysis reactions by HPLC, the hydrolysis solution was transferred with ice and added into QR assay system with different dilutions to measure CD values. Mean ± SD (n=3).

Table 3.3. Concentrations of glucosinolates (μmol per g DW), their hydrolysis products and QR induction activity (specific activity ratio of broccoli extract treated cells to non-treated cells) in untreated (control) and MeJA-treated broccoli.

Source of variation	Treatment	GRA	(SF)	GST	(PEITC)	GB	(I3C)	NeoGB	(NI3C)	(NeoASG)	(NI3C + NeoASG)	Total GS	QR
Treatment													
	Control	4.00 ± 1.86	1.08 ± 0.31	1.68 ± 0.71	0.40 ± 0.22	2.67 ± 0.77	0.25 ± 0.12	2.88 ± 1.43	0.68 ± 0.34	0.95 ± 0.75	1.63 ± 0.95	13.6 ± 3.85	3.20 ± 0.64
	MeJA	4.42 ± 2.01*	1.64 ± 0.60*	2.68 ± 1.12*	1.27 ± 0.55*	2.64 ± 1.01	0.32 ± 0.13*	10.0 ± 3.71*	2.13 ± 1.08*	2.21 ± 1.65*	4.34 ± 2.36*	21.5 ± 6.43*	3.53 ± 0.82*
Year													
2009	Control	3.67 ± 0.64	1.08 ± 0.28	1.37 ± 0.55	0.41 ± 0.23	2.90 ± 0.66	0.26 ± 0.14	1.98 ± 1.02	0.65 ± 0.31	0.31 ± 0.13	0.96 ± 0.41	11.2 ± 2.2	2.65 ± 0.40
	MeJA	4.01 ± 1.19	1.22 ± 0.47*	2.41 ± 1.25*	1.42 ± 0.57*	3.28 ± 1.00*	0.34 ± 0.17*	10.8 ± 3.98*	1.85 ± 1.21*	0.90 ± 0.48*	2.74 ± 0.99*	21.5 ± 6.7*	2.83 ± 0.45*
2010	Control	4.32 ± 2.59	1.15 ± 0.34	1.99 ± 0.73	0.38 ± 0.22	2.43 ± 0.83	0.24 ± 0.09	3.79 ± 1.21	0.71 ± 0.40	1.59 ± 0.53	2.29 ± 1.30	16.0 ± 3.7	3.75 ± 0.31
	MeJA	4.83 ± 2.57	1.88 ± 0.66*	2.96 ± 0.93*	1.11 ± 0.50*	1.99 ± 0.48	0.30 ± 0.08*	9.30 ± 3.38*	2.41 ± 0.87*	3.53 ± 1.30*	5.95 ± 1.90*	21.4 ± 6.4*	4.23 ± 0.25*
2009 year													
Pirate	Control	2.99 ± 0.32	0.62 ± 0.02	0.75 ± 0.11	0.23 ± 0.04	3.40 ± 0.22	0.14 ± 0.02	0.74 ± 0.09	0.22 ± 0.03	0.21 ± 0.03	0.43 ± 0.06	8.72 ± 0.84	2.10 ± 0.13
	MeJA	3.32 ± 0.22	0.66 ± 0.12	1.19 ± 0.11*	0.86 ± 0.12*	3.99 ± 0.29*	0.14 ± 0.02	4.82 ± 0.87*	0.22 ± 0.03	0.22 ± 0.03	0.44 ± 0.07	13.8 ± 1.28*	2.00 ± 0.11
Expo	Control	3.44 ± 0.20	1.09 ± 0.05	2.22 ± 0.15	0.15 ± 0.01	2.81 ± 0.30	0.51 ± 0.08	1.50 ± 0.07	0.99 ± 0.15	0.52 ± 0.08	1.51 ± 0.23	11.5 ± 0.61	3.07 ± 0.21
	MeJA	5.21 ± 0.26*	2.13 ± 0.30*	4.50 ± 0.47*	1.96 ± 0.88*	4.33 ± 0.28*	0.57 ± 0.09	14.7 ± 0.60*	3.67 ± 0.55*	1.03 ± 0.15*	4.70 ± 0.71*	30.6 ± 1.49*	3.31 ± 0.13
Green Magic	Control	4.36 ± 0.16	1.07 ± 0.21	1.11 ± 0.02	0.54 ± 0.04	2.91 ± 0.53	0.21 ± 0.03	3.06 ± 0.62	0.76 ± 0.11	0.36 ± 0.05	1.11 ± 0.17	13.8 ± 1.61	2.81 ± 0.24
	MeJA	4.25 ± 1.28	1.40 ± 0.11	1.92 ± 0.11*	1.25 ± 0.18*	2.45 ± 0.17	0.26 ± 0.04	11.7 ± 0.55*	2.00 ± 0.30*	1.55 ± 0.23*	3.55 ± 0.53*	22.3 ± 1.24*	3.06 ± 0.09
Imperial	Control	3.64 ± 1.01	1.16 ± 0.13	1.60 ± 0.10	0.45 ± 0.21	3.44 ± 0.22	0.23 ± 0.03	2.02 ± 0.40	0.45 ± 0.07	0.21 ± 0.03	0.65 ± 0.10	11.5 ± 1.40	2.80 ± 0.01
	MeJA	2.67 ± 0.61	1.02 ± 0.14	2.22 ± 0.77	1.44 ± 052*	3.09 ± 1.16	0.46 ± 0.16	12.2 ± 4.56*	1.59 ± 0.56*	0.98 ± 0.34*	2.56 ± 0.90*	20.4 ± 7.09	2.95 ± 0.09*
Gypsy	Control	3.94 ± 0.26	1.16 ± 0.18	1.18 ± 0.38	0.23 ± 0.20	1.96 ± 0.62	0.21 ± 0.03	2.56 ± 1.34	0.86 ± 0.13	0.23 ± 0.04	1.09 ± 0.16	10.3 ± 2.46	2.47 ± 0.06
	MeJA	4.57 ± 1.30	1.25 ± 0.12	2.21 ± 0.93	0.86 ± 0.40*	2.51 ± 1.06	0.26 ± 0.04	10.7 ± 2.90*	1.75 ± 0.61*	0.69 ± 0.10*	2.44 ± 0.37*	20.6 ± 6.38	2.83 ± 0.08*
2010 year													
Pirate	Control	2.93 ± 1.03	1.36 ± 0.18	2.34 ± 0.94	0.50 ± 0.09	3.48 ± 1.09	0.27 ± 0.04	4.86 ± 1.83	0.81 ± 0.12	2.31 ± 0.12	3.13 ± 0.47	16.6 ± 5.76	3.86 ± 0.27
	MeJA	2.63 ± 0.59	1.77 ± 0.26	2.07 ± 1.15	0.80 ± 0.43	2.68 ± 0.38	0.39 ± 0.06*	5.12 ± 3.41	1.27 ± 0.19*	1.74 ± 0.19	3.01 ± 0.45	14.8 ± 5.92	4.21 ± 0.12
Expo	Control	3.62 ± 0.68	1.59 ± 0.36	2.98 ± 0.14	0.65 ± 0.33	2.37 ± 0.39	0.39 ± 0.06	4.83 ± 0.72	1.36 ± 0.34	1.96 ± 0.34	3.32 ± 0.50	16.5 ± 0.91	4.08 ± 0.24
	MeJA	3.73 ± 0.53	1.60 ± 0.15	3.31 ± 0.36	1.21 ± 0.20	1.74 ± 0.37	0.35 ± 0.05	7.14 ± 0.80*	1.94 ± 0.49	3.52 ± 0.49*	5.46 ± 0.82*	18.1 ± 1.84	4.41 ± 0.25
Green Magic	Control	1.82 ± 0.35	0.50 ± 0.08	1.24 ± 0.01	0.30 ± 0.02	2.41 ± 0.28	0.23 ± 0.03	3.63 ± 0.09	0.55 ± 0.08	1.31 ± 0.08	1.86 ± 0.28	11.6 ± 0.97	3.32 ± 0.10
	MeJA	2.76 ± 0.42*	1.04 ± 0.09*	2.19 ± 0.18*	0.58 ± 0.08*	1.94 ± 0.37	0.27 ± 0.04	10.7 ± 2.12*	2.33 ± 0.35*	5.17 ± 0.35*	7.50 ± 1.13*	19.6 ± 2.11*	3.86 ± 0.24*
Imperial	Control	4.54 ± 1.08	1.63 ± 0.41	1.67 ± 0.19	0.23 ± 0.06	2.54 ± 0.17	0.16 ± 0.02	3.01 ± 0.23	0.42 ± 0.06	1.19 ± 0.06	1.60 ± 0.24	15.5 ± 1.03	3.75 ± 0.13
	MeJA	6.07 ± 0.88	2.47 ± 0.32*	3.69 ± 0.52*	1.42 ± 0.19*	1.90 ± 0.42	0.30 ± 0.05*	11.7 ± 2.86*	3.29 ± 0.49*	4.29 ± 0.49*	7.59 ± 1.14*	25.4 ± 4.46*	4.31 ± 0.09*
Gypsy	Control	8.70 ± 1.03	0.66 ± 0.30	1.71 ± 0.21	0.23 ± 0.08	1.37 ± 0.19	0.17 ± 0.03	2.65 ± 0.15	0.40 ± 0.06	1.16 ± 0.06	1.56 ± 0.23	19.9 ± 1.93	3.74 ± 0.21
	MeJA	8.99 ± 0.85	3.08 ± 0.09*	3.55 ± 0.88*	1.54 ± 0.71*	1.71 ± 0.22	0.20 ± 0.03	11.6 ± 1.38*	3.23 ± 0.49*	2.93 ± 0.49*	6.16 ± 0.92*	29.4 ± 4.08*	4.36 ± 0.07*

GRA=Glucoraphanin, SF=Sulforaphane, GST=Gluconasturtiin, PEITC= Phenethyl Isothiocyanate, GB=Glucobrassicin, I3C=Indole-3-carbinol, NeoGB=Neoglucobrassicin, NI3C=N-methoxyindole-3-carbinol, NeoASG=Neoascorbigen, Total GS=Total glucosinolate, including presented glucosinolates in table and glucoiberin, progoitrin, and gluconapin. Mean ± SD (n=3).

Table 3.4. Isothiocyanate conversion rate (%) from hydrolysis of precursor GS for sulforaphane and phenethyl isothiocyanate (PEITC). Mean ± SD (n=3).

Source of variation	Treatment	sulforaphane/ glucoraphanin (%)	PEITC/ gluconasturtiin (%)
Treatment			
	Control	30.7 ± 11.5	27.8 ± 19.9
	MeJA	35.5 ± 12.4*	50.8 ± 19.2*
Year			
2009	Control	29.1 ± 6.5	36.2 ± 24.7
	MeJA	30.3 ± 10.1	64.6 ± 15.5*
2010	Control	32.2 ± 13.6	19.3 ± 7.4
	MeJA	40.7 ± 10.4*	36.9 ± 10.5*
2009 year			
Pirate	Control	20.6 ± 2.7	31.9 ± 8.9
	MeJA	20.0 ± 5.1	72.1 ± 7.7*
Expo	Control	31.7 ± 2.5	6.9 ± 0.6
	MeJA	40.1 ± 5.5*	44.7 ± 21.5*
Green Magic	Control	32.0 ± 5.1	48.6 ± 4.3
	MeJA	25.1 ± 10.3	64.9 ± 7.6*
Imperial	Control	31.9 ± 11.6	28.3 ± 13.3
	MeJA	38.0 ± 6.0	64.9 ± 4.4*
Gypsy	Control	29.4 ± 4.0	65.5 ± 32.6
	MeJA	27.4 ± 11.5	76.4 ± 14.0
2010 year			
Pirate	Control	46.5 ± 8.8	22.7 ± 6.8
	MeJA	67.2 ± 8.4*	41.0 ± 17.2
Expo	Control	44.1 ± 12.7	21.8 ± 10.9
	MeJA	43.1 ± 6.1	36.6 ± 5.4
Green Magic	Control	27.6 ± 5.7	24.3 ± 1.2
	MeJA	18.2 ± 10.6	26.3 ± 2.0
Imperial	Control	35.9 ± 6.0	14.0 ± 5.6
	MeJA	40.8 ± 8.3	38.6 ± 1.9*
Gypsy	Control	7.6 ± 3.4	13.7 ± 5.5
	MeJA	34.2 ± 3.7*	42.1 ± 14.3*

Table 3.5. Correlations among GS and hydrolysis product concentrations, QR induction activities and weather related variables.

	GRA	GNA	GB	GNS	NeoGB	SF	PEITC	I3C	NI3C	NeoASG	NI3C+ NeoASG	QR	DTH	GDD	PPT/DTH	Solar radiation
GRA	1.000															
GNA	0.513*	1.000														
GB	-0.431	-0.428	1.000													
GNS	0.390	0.187	-0.077	1.000												
NeoGB	0.247	-0.169	-0.025	0.711***	1.000											
SF	0.593**	0.021	-0.187	0.762***	0.397*	1.000										
PEITC	0.287	-0.302	0.078	0.704***	0.896***	0.784***	1.000									
I3C	0.563**	0.168	-0.224	0.489*	0.384	0.129	0.015	1.000								
NI3C	0.384	-0.039	-0.158	0.876***	0.883***	0.844***	0.817***	0.535*	1.000							
NeoASG	0.145	0.299	-0.515*	0.549*	0.492*	0.397	0.242	0.254	0.605**	1.000						
NI3C+NeoASG	0.275	0.172	-0.403	0.767***	0.733***	0.655**	0.544*	0.416*	0.862***	0.925***	1.000					
QR	0.330	0.673**	-0.550*	0.654**	0.312	0.526*	0.176	0.385	0.502*	0.771***	0.731***	1.000				
DTH	-0.206	0.228	0.181	0.358	-0.135	0.166	0.481*	0.031	0.034	0.211	0.151	0.390	1.000			
GDD	-0.057	0.514*	-0.160	0.399	-0.077	0.252	-0.097	0.128	0.101	0.496*	0.364	0.699***	0.893***	1.000		
PPT/DTH	-0.050	-0.382	0.264	-0.482*	-0.050	-0.447*	-0.108	-0.234	-0.471*	-0.471*	-0.447*	-0.660**	-0.701***	-0.800***	1.000	
Solar radiation	0.017	0.634**	-0.307	0.382	-0.058	0.280	-0.127	0.151	0.111	0.586**	0.427	0.796***	0.773***	0.974***	0.774***	1.000

Significance is indicated by asterisks: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ based on the Pearson correlation analysis.

DTH= Number of days from transplant to harvest date; GDD= Growing degree days [(Min Temperature + Max Temperature)/2 -7];

PPT = Precipitation.

Table 3.6. Percentages of total variance described by main factors (Genotype, Treatment, and Year) and factor interactions for broccoli floret phytochemical concentrations and bioactivities.

	GRA	(SF)	GST	(PEITC)	GB	(I3C)	NeoGB	(NI3C)	(NeoASG)	(NI3C + NeoASG)	Total GS	QR
Genotype	**42.9***	10.2*	**33.3***	8.1	**30.9***	**49.7***	8.2*	16.9*	6.3*	9.8*	11.4*	9.0*
Treatment	1.2	16.6*	23.0*	**52.5***	0.0	7.7*	**62.5***	**46.0***	20.1*	**37.2***	**36.3***	5.0*
Year	3.7	**29.0***	7.8	2.1	24.5*	1.1	0.0	2.1*	**48.2***	26.0*	2.5	**72.0***
G × T	0.6	7.4*	5.6	7.1	5.4	6.3	8.1*	8.8*	11.3*	11.1*	4.2	0.7
G × Y	37.5*	9.7*	6.2	2.3	4.3	24.4*	2.3	7.1*	0.8	1.7	14.8*	8.8*
T × Y	0.1	8.5*	0.0	1.4	5.2	0.2	3.5	1.4	5.8*	4.4*	5.9	1.1
G × T × Y	3.6	14.6*	7.9	5.8	5.4	2.5	5.0	11.3*	4.1*	5.3*	5.5	0.2
Block (Year)	1.6	2.9	1.4	3.7	1.2	1.3	0.9	2.3*	2.7	3.1	1.2	0.9
Residual	8.8	1.0	15.0	17.0	22.9	6.8	9.4	4.0	0.8	4.6	14.2	2.3
R Square[a]	0.91	0.96	0.85	0.83	0.77	0.87	0.91	0.94	0.97	0.95	0.84	0.95

*Indicates factor that describes a significant proportion of the total variance using partitioning sum of squares in ANOVA at $P < 0.0001$ (two tailed-test). Bold indicates which factor describes the most variance for each variable. [a]Fraction of total variance described by the regression.

Supplementary Table 3.S1. Weather information for 2009 and 2010 in Champaign, Illinois.

Total solar radiation ($MJ \cdot m^{-2}$)					
Year	Jun	Jul	Aug	Sep	Sum
2009	681	667	617	542	2507
2010	720	730	731	510	2690
% of (2010/2009)	106	109	118	94	107
	Precipitation (mm)				
Year	Jun	Jul	Aug	Sep	Sum
2009	108	156	137	55	401
2010	199	91	40	16	329
% of (2010/2009)	184	58	29	29	82
	Growing degree days (°C)				
Year	Jun	Jul	Aug	Sep	Sum
2009	360	336	342	304	1341
2010	373	408	403	293	1477
% of (2010/2009)	104	122	118	96	110

Growing degree days [(Min Temperature + Max Temperature)/2 -7]

Weather data during the growing seasons was provided by the Illinois State Water Service (http://www.isws.illinois.edu/warm/data/cdfs/cmiday.txt).

CHAPTER 4

Methyl Jasmonate-mediated Induction of Glucosinolate Biosynthesis and Inducing of Quinone Reductase Activity in Kale Leaf Tissue

4.1. Abstract

Methyl jasmonate (MeJA) spray treatments were applied to the kale varieties 'Dwarf Blue Curled Vates' and 'Red Winter' in replicated field plantings in 2010 and 2011 to investigate alteration of GS composition in the harvested leaf tissue. Aqueous solutions of 250 μM MeJA were sprayed to saturation on aerial plant tissues four days prior to harvest at commercial maturity. The MeJA treatment significantly increased gluconasturtiin (56%), glucobrassicin (98%), and neoglucobrassicin (150%) concentrations in the apical leaf tissue of these genotypes over two seasons. Induction of quinone reductase (QR) activity, a biomarker for anti-carcinogenesis, was significantly increased by MeJA in the extracts from the leaf tissue of these two cultivars. Extracts of apical leaf tissues had greater MeJA mediated increases in GS, GS hydrolysis products and QR activity than extracts from basal leaf tissue samples. The hydrolysis product of glucoraphanin, sulforphane was significantly increased in apical leaf tissue of the cultivar 'Red Winter' in both 2010 and 2011. There were significant year and year by genotype interactions in the concentrations of GS and QR activity. Drought conditions in 2011 may have reduced the effect of MeJA spray treatment by increasing endogenous jasmonate concentrations in control plants.

Correlation analysis revealed that I3C significantly correlated with QR activity ($r = 0.642$, $P = 0.007$). The concentrations required to double the specific QR inducing activity (CD values) of I3C was calculated at 230 μM, which is considerably weaker than other GS hydrolysis

products like sulforphane. To confirm relationships between GS hydrolysis products and QR inducing activity, a range of concentrations of MeJA sprays were applied to kale leaf tissues of both cultivars in 2011. Correlation analysis of these results indicated that sulforaphane, NI3C, neoascorbigen, I3C, and diindolylmethane were all significantly correlated with QR inducing activity. Thus, increased QR activity may be due to combined increases of hydrolysis product concentrations rather than individual products alone.

4.2. Introduction

Epidemiological studies have reported that the intake of *Brassica* vegetables is inversely correlated with cancer risk, and this association is stronger than those between cancer and fruit and vegetable consumption in general (Michaud et al., 1999). Kale (*Brassica oleracea* L. *acephala*) is a frequently consumed leafy vegetable. Young tender leaves are harvested for human consumption and older plant tissues for animal feed (Velasco et al., 2007). Kale is a good source of vitamins (Vitamin A, C, E and K) and of health promoting phytochemicals including glucosinolates (GS), carotenoids, phenolics, and tocopherols. In certain regions like on the Iberian Peninsula, kale (*Brassica oleracea acephala* group) leaves and flower buds are grown and harvested throughout the year.

There are several types of kale. Among them, it was previously reported that GS composition of Siberian kale (*B. napus*) was distinct from 'Vates' (*B. oleracea*) type kale (Carson et al., 1987). Red Russian and Siberian kales *(Brassica napus ssp. pabularia)* are typically more tender and have a milder flavor than the European "oleracea" kales whose young leaves are superior for use in salads. Napus kales have good cold tolerance so that they can be grown anywhere in the US over a broader range of growing seasons. Napus kales are also used

as animal forage. Forage and root vegetable cultivars of *B. napus* show high levels of progoitrin (Velasco et al., 2008), which can promote goitrogenic effects in mammals (Mithen et al., 2000). Although the species *Brassica napus* is thought to have originated from a chance hybridization between *Brassica rapa* and *Brassica oleracea,* the Red Russian type of kales were bred by artificial hybridization (http://seedambassadors.org/Mainpages/still/napuskale/napuskale.htm). The 'Red Winter' cultivar was derived from Red Russian kale types.

B. oleracea kale is a rich source of flavonoids, possessing up to 47 mg of kaempferol and 22 mg of quercetin per 100 g of fresh leaf tissue. Kale contains the highest flavonoid content among all of the *Brassica oleracea* vegetables (U.S. Department of Agriculture, 2011a). Phenolics have putative antioxidant, anticancer, and anti-cardiovascular disease activity (Dai and Mumper, 2010; Galati and O'Brien, 2004; Morton et al., 2000) . Previous research revealed that MeJA treatments enhance total polyphenolic compounds and flavonoids in kale leaf tissues (Chapter 2). The response to MeJA treatment was more dramatic in young tissue (apical leaves) compared to older leaf tissue (basal leaves) (Ku and Juvik, 2012 and Chapter 2).

Besides phenolic compounds, kale is also a good source of GS. GS are a class of secondary metabolites found in cruciferous crops. The breakdown products have been shown to affect human health, insect herbivory, and and plant resistance to pathogens (Agerbirk et al., 2009; Bednarek et al., 2009; Keum et al., 2005). Some GS breakdown products have a chemoprotective effect against certain cancers in humans (Nestle, 1998).

Up-regulation of phase II detoxification enzyme activity has been suggested as a good strategy for cancer prevention (Clapper and Szarka, 1998; Cuendet et al., 2006). Phase II detoxifying enzymes including glutathione S-transferase (GST) and quinone reductase (QR) that can enhance detoxification and elimination of carcinogens from the body (Clapper and Szarka,

1998; Cuendet et al., 2006). Hydrolysis products of GS, isothiocyanates such sulforaphane and phenethyl isothiocyanate (PEITC) have been shown to enhance quinone reductase (QR) and other chemopreventive activities (Kang and Pezzuto, 2004; Zhang et al., 1992). Previous studies have reported that the hydrolysis products of the indolyl GS including glucobrassicin and neoglucobrassicin also have cancer chemopreventive activity. Hydrolysis products of glucobrassicin including indole-3-carbinol (I3C), diindolylmethane, and ascorbigen have shown to induce QR (Kang and Pezzuto, 2004; Zhu and Loft, 2003). N-methoxyindole-3-carbinol (NI3C), the hydrolysis product of neoglucobrassicin has been reported to induce cell cycle arrest in human colon cancer cell lines (Neave et al., 2005).

The GS are also associated with insect defense in *Brassica* species. Jasmonic acid (JA), an endogenous plant signal transduction compound whose biosynthesis is up-regulated when *Brassica* plant species are attacked by herbivores, causes enhanced indolyl GSs biosynthesis (Hopkins et al., 2009). The increased GS by MeJA treatment was found to be a species-specific response (Chapter 6). MeJA treatment significantly increased gluconasturtiin and neoglucobrassicin in broccoli (Kim and Juvik, 2011) and the treatment significantly increased glucoraphanin, glucobrassicin, and neoglucobrassicin in cauliflower (Chapter 6). To date, GS compositional changes of kale leaf tissue by MeJA treatments have not been previously reported in the literature.

Compared to other *Brassica* vegetables including broccoli, watercress, and Brussels sprouts, anti-cancer bioactivity information about kale is limited (Rose et al., 2000; Zhang et al., 1992; Zhu and Loft, 2003). The objective of this research was to determine the QR inductive health promoting effect derived from elevated GS by MeJA. We also evaluated factors affecting

the GS variation of kale leaves including leaf tissue age and year associated environmental effects.

4.3. Materials and Methods

4.3.1. Kale Cultivation. The cultivars used for these experiments were 'Red Winter' (RW, *Brassica napus ssp. pabularia)* and 'Dwarf Blue Curled Vates' (DBCV, *Brassica oleracea* L. var. *acephala*) (Burpee Seed Co.). Seeds of each kale genotype were germinated in 32 cell plant plug trays filled with sunshine® LC1 (Sun Gro Horticulture, Vancouver, British Columbia, Canada) professional soil mix. Seedlings were grown in a greenhouse at the University of Illinois at Champaign-Urbana under a 25 °C/15 °C and 14 h/10 h: day/night temperature regime with supplemental lighting. Thirty days after germination, seedling trays were placed in ground beds to harden off for a week prior to transplanting into field plots at the University of Illinois South Farm (40˚ 04′ 38.89″ N, 88˚ 14′ 26.18″ W). Experimental design was a split-plot arrangement in a randomized complete block (RCB) design with three replicates. The experiment plot was surrounded by a guard row to avoid border effects. Transplanting of kale seedlings was conducted on June 11, 2010 and June 13, 2011. Harvesting kale occurred in July 25 in 2010 and July 27 in 2011. Irrigation was only applied first week of cultivation for establishing transplanted seedlings. Weather conditions during the 2010 and 2011 growing seasons collected from Illinois State Water Service (http://www.isws.illinois.edu/warm/data/cdfs/cmiday.txt) are presented in Supplementary Table 4.S1.

4.3.2. Kale Treatment with MeJA and Sample Preparation. An aqueous solution of 250 µM MeJA (Sigma-Aldrich, St. Louis, MO) and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO)

was sprayed on all aerial plant tissues to the point of runoff (approximately 300 mL) four days prior to harvest based on the result of experiments to determine when GS levels are optimized prior to harvest (Supplementary Figure 4.S1). Two different kale leaf samples (apical: three leaves from the below the meristematic growing point, at a minimum 8 cm in length; basal: three fully expanded leaves nearest the soil surface without discoloration or signs of senescence or damage) were harvested and bulked from five treated and control plants of each genotype for each replicate respectively (five heads or leaves bulked for a replicate sample). Images of apical and basal samples of each kale cultivar are shown in Figure 4.1A. In order to confirm the relationship between increased hydrolysis products of GS and QR activity, 0, 50, 250, and 500 μM MeJA were sprayed on kale leaf tissue as described above in 2011. Kale leaf tissues were frozen in liquid nitrogen, and stored at -20 °C prior to freeze-drying. Freeze-dried tissues were ground into a fine powder using a coffee grinder and stored at -20 °C prior to chemical and bioactivity analyses.

4.3.3. Determination of Sample GS Concentration. Extraction and quantification of GS using high-performance liquid chromatography was performed using a protocol described by Brown et al. (2002) (Brown et al., 2002). Freeze-dried broccoli powder (0.2 g) and 2 mL of 70% methanol were added to 10 mL tubes (Nalgene, Rochester, NY) and heated on a heating block at 95 °C for 10 min. After cooling on ice, 0.5 mL benzylglucosinolate (1 mM) was added as internal standard (POS Pilot Plant Corp, Saskatoon, SK, Canada), mixed, and centrifuged at 3,000 × g for 15 min at 4 °C. The supernatant was saved and the pellet was re-extracted with 2 mL 70% methanol at 95 °C for 10 min and the two extracts combined. A subsample (1 mL) from each pooled extract was transferred into a 2-mL microcentrifuge tube (Fisher Scientific, Waltham, MA). Protein was

precipitated with 0.15 mL of a 1:1 mixture of 1 M lead acetate and 1 M barium acetate. After centrifuging at 12,000 × g for 1 min, each sample was then loaded onto a column containing DEAE Sephadex A-25 resin (GE Healthcare, Piscataway, NJ) for desulfation with arylsulfatase (*Helix pomatia* Type-1, Sigma-Aldrich, St. Louis, MO) for 18 h and the desulfo-GS eluted. One hundred μL of each sample were injected on to a Agilent 1100 HPLC system (Agilent, Santa Clara, CA), equipped with a G1311A bin pump, a G1322A vacuum degasser, a G1316A thermostatic column compartment, a G1315B diode array detector and an HP 1100 series G1313A autosampler. UV detector set at 229 nm wavelength. All-guard™ cartridge pre-column (Alltech, Lexington, Kentucky), and a LiChospher® 100 RP-18 column (Merck, Darmstadt, Germany) were used for quantification. Desulfo-GS were eluted from the column over 45 min with a linear gradient of 0% to 20% acetonitrile at a flow rate of 1 mL/min. Benzylglucosinolate was used as an internal standard and UV response factors for different types of GS were used as determined by Wathelet et al (Wathelet et al., 1995). The identification of desulfo-GS profiles were validated by LC-tandom MS using a Waters 32 QT of Ultima spectrometer coupled to a Waters 1525 HPLC system and full scan LC-MS using a Finnigan LCQ Deca XP, respectively. The molecular ion and fragmentation patterns of individual desulfo-GS were matched with the literature for GS identification (Tian et al., 2005; Velasco et al., 2011).

4.3.4. Analysis of Glucosinolate Hydrolysis Products. The extraction and analysis of isothiocyanates and other hydrolysis products was carried out according to previously published methods, with some modifications (Wilson et al., 2011). 75 mg of kale leaf powder was suspended in 1.5 mL of water in the absence of light for 4 h (time for the maximum concentration of indolyl GS hydrolysis products) and 24 h (time for the maximum concentration

of sulforaphane) at room temperature in a sealed 2 mL microcentrifuge tube (Fisher Scientific, Waltham, MA) to facilitate GSs hydrolysis by endogenous myrosinase. Slurries were then centrifuged at 12,000 × g for 5 min and supernatants was decanted into a 2 mL microcentrifuge tube. 20 µL of butyl isothiocyanate (0.5 mg/mL) and 4-methoxyindole (1 mg/mL) were added as the internal standards for sulforphane and the hydrolysis products of indolyl GS to quantify I3C, DIM, NI3C, and NeoASG, respectively, with 0.5 mL of methylene chloride. Tubes were shaken vigorously before being centrifuged for 2 min at 9,600 g. The methylene chloride layer (200 µL) was transferred to 350 µL flat bottom insert (Fisher Scientific, Pittsburgh, PA) in a 2 mL HPLC autosampler vial (Agilent, Santa Clara, CA) for mixing with 100 µL of a reagent containing 20 mM triethylamine and 200 mM mercaptoethanol in methylene chloride. The mixture was incubated at 30 °C for 60 min under constant stirring, and then dried under a stream of nitrogen. The residue containing isothiocyanate derivatives (isothiocyanate-mercaptoethanol derivatives) and other hydrolysis compounds was dissolved in 200 µL of acetonitrile /water (1:1) (v/v), and 10 µL of this solution injected onto a Agilent 1100 HPLC system (Agilent, Santa Clara, CA), equipped with a G1311A bin pump, a G1322A vacuum degasser, a G1316A thermostatic column compartment, a G1315B diode array detector and an HP 1100 series G1313A autosampler. Extracts were separated on a Eclipse XDB-C18 column (150 × 4 mm, particle size 5 µm, Agilent, Santa Clara, CA) with a C18 all-guard™ cartridge pre-column (Alltech, Lexington, KY). Mobile phase A was water and B methanol. Mobile phase B was 0% at injection, increasing to 10% by 10 min, 100% at 35 min, and held 5 min, then decreased to 0% by 50 min. Flow rates were kept at 0.8 mL/min. The detector wavelength was set at 227 and 271 nm. Response factors for monomeric indolyl derivatives were used from a previous report (Agerbirk et al., 1998). Due to a

lack of standards for NI3C and NeoASG the standard curve of I3C was applied for quantification of both NI3C and NeoASG. The quantities were expressed as I3C equivalent concentrations.

4.3.5. Quinone Reductase (QR) Activity. For the QR assay, kale extracts were collected using the same protocol for GS hydrolysis products described above with sampling at 4 h of incubation. QR inductive activities were measured for individual apical and basal leaf tissue extracts and a pooled equal volume sample from both apical and basal leaf tissue extracts. Hepa1c1c7 murine hepatoma cells (ATCC, Manassas, VA) were grown in alpha-minimum essential medium (α-MEM), enriched with 10% heat and charcoal-inactivated fetal bovine serum and maintained at 37 °C in 95% ambient air and 5% CO_2. The cells were divided every three days with a split ratio of 7. Cells with 80-90% confluence were plated into 96-well plates (Costar 3595, Corning Inc, Corning, NY), 1×10^4 cells per well, and incubated for 24 h in antibiotic-enriched media (100 units/mL penicillin, 100 μg/mL streptomycin). The QR induction activities of different samples were determined by means of the protocol described by Prochaska & Santamaria (Prochaska and Santamaria, 1988). After 24 h cells were exposed to the different sample extracts [0.25% final concentration (125 μg of freeze-dried broccoli/mL) in 200 μL of media] in new media for a further 24 h. Growth media alone and 0.2 μM SF were used as negative and positive controls, respectively. Treated cells were rinsed with phosphate buffer at pH 7.4, lysed with 50 μL 0.8% digitonin in 2 mM EDTA, incubated and agitated for 10 min. A 200-μL aliquot of reaction mix [74 mL 25 mM Tris buffer; 50 mg BSA; 0.5 mL 1.5% Tween-20 solution; 0.5 mL cofactor solution (92.7% 150 mM glucose-6-phosphate, 6.15% 4.5 mM NADP, 1.14% 0.75 mM FAD in Tris buffer)]; 150 units of glucose-6-phosphate dehydrogenase; 22.5 mg MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide]; and 75 μL 50 mM menadione in

acetonitrile) was added to the lysed cells. Readings were made at five time points, 50 s apart, using a μQuant microplate reader (Bio-Tek Instruments, Winooski, VT) at 610 nm. Immediately after completion of the readings, 50 μL of 0.3 mM dicumarol in 25 mM Tris buffer was added into each well, and the plate was read again (five time points, 50 s apart) to determine non-specific MTT reduction. Total protein content was measured by the BioRad assay (Bio-Rad, Hurcules, CA, USA) using manufacture's instructions. Activity was expressed as QR specific activity (nmol MTT reduced/mg/min) ratio of treated to negative control cells.

4.3.6. QR Inducing Activity Measurement of I3C. QR inducing activity of hydrolysis product, I3C was measured to determine the concentrations required to double the specific activity of QR (CD value), Commercially purchased I3C (Sigma Chemical Company, St. Louis, MO) was disosolved in DMSO. Then seven concentrations (250, 125, 62.5, 31.3, 15.6, 7.8, and 3.9 μM) of I3C prepared by serial two fold dilutions were added to 96 well plates of cultured hepa1c1c7 cells. After a 24h incubation, QR activity was measured using the protocol described above.

4.3.7. Statistical Analysis. Analysis of variance (ANOVA) was conducted using JMP 10 (SAS institute Inc., Cary, NC). Year, treatments, and genotype effects were considered as fixed factors. Block was considered as random. Analysis of variance was performed using the linear model: $Y_{ijklm} = m + G_i + Y_j + T_k + GY_{ij} + GT_{ik} + YT_{jk} + GYT_{ijk} + B_{l(j)} + \varepsilon_{ijklm}$, where Y_{ijklm} is the l^{th} block of the phenotypic value of the k^{th} treatment, i^{th} genotype in year j, m is the overall mean, G, Y, T, and B indicate the effects of genotype, year (weather), treatment and blocks nested in years, ε_{ijklm} is the experimental error associated with Y_{ijklm}, respectively. Fisher's Least Significant

Difference (LSD) test, correlation analysis and Student's t-tests were also conducted using the JMP 10 software. All sample analyses were conducted in triplicate. The results are presented as means ± SD.

4.4. Results and Discusssion

4.4.1. Effect of MeJA Treatment on QR activity of Different Age of Kale Tissues. MeJA treatment significantly increased QR inducing activity in the combined apical plus basal leaf extracts of the two different kale species over two years except for the DBCV cultivar in 2011 (Figure 4.1B). There was significant year-to-year variation in QR inducing activity with 2011 samples significantly higher than those in 2010. In 2010 apical leaf tissue extracts of MeJA treated kale increased 17% and 27% of QR inducing activity for DBCV and RW, respectively, while in 2011, they increased only by 6% and 16%. QR activity of apical leaf tissue extracts were up to 2-fold greater than extracts from basal leaves (Figure 4.1C and 1D).

Effect of MeJA Treatment on GS and GS Hydrolysis Products Concentrations. Over both seasons MeJA treatments significantly increased glucobrassicin and neoglucobrassicin concentrations in both apical and basal leaves. The treatment increased apical leaf concentrations of gluconasturtiin (56%), glucobrassicin (98%), and neoglucobrassin (150%) and basal leaf concentrations of gluconasturtiin (44%), glucobrassicin (166%) and neoglucobrassin (83%) averaged across cultivars and over years (Figure 4.2A). Total GS concentration in apical leaves tissues was up to seven fold greater than basal leaves tissue.

From previous work, *B. napus* type kales (such as RW) have distinct GS composition compared with *B. oleracea* type kale (Carson et al., 1987). As Supplementary Figure 4.S1

illustrates, the major GS in both DBCV and RW are glucobrassicin and neoglucobrassicin. However, DBCV contains higher levels of glucoiberin and RW higher concentrations of progoitrin. Unlike DBCV, RW contains relatively high glucoraphanin concentrations.

MeJA mediated enhancement of GS in DBCV was greater in 2010 than in 2011 where glucobrassicin and total GS concentrations in apical leaf tissues were both 2.7 fold higher in 2010 compared to increases of 1.3 and 1.23 fold, repsectively in 2011 (Figure 4.2A). MeJA treatments may be interacting with varying weather conditions in each season of application. This variation may be related low levels of precipitation in 2011, which experienced only 51% of precipitation received in the 2010 growing season (Supplementary Table 4.S1). Endogenous jasmonic acid has been observed to accumulate *in planta* under drought conditions (Creelman et al., 1995). Conditions in 2011 may have lead to the accumulation of endogenous JA which could attenuate the effect of the exogenous MeJA treatment (Figure 4.1 and 4.2).

Only sulforaphane was significantly increased in both apical and basal leaf tissue of the RW cultivar by MeJA treatment over two years but increases of other hydrolysis products were not consistently observed in all samples (Figure 4.3). Despite significant increases in glucobrassicin, I3C and DIM concentrations in kale extracts were relatively low. I3C has been reported to be highly unstable (Bradlow, 2008) and will react with other substrates generating by-products by condensation with ascorbic acid or through oligomerization (Agerbirk et al., 2009). Following hydrolysis of the parent GS, relatively higher levels of NI3C were observed than I3C. According to previous research ascorbigen is less stable than neoascorbigen (Yudina et al., 2000). Thus, I3C may be less stable than NI3C.

Correlation Analysis between Intact GS or Hydrolysis Products and QR activity. In order to elucidate the most active QR induction hydrolysis product in MeJA treated kale leaf tissue, correlation analysis was conducted between QR inductive activity and GS and GS hydrolysis product concentrations (Table 4.1). QR inductive activity significantly correlated with glucobrassicin ($r = 0.747$, $P = 0.001$) and I3C ($r = 0.707$, $P = 0.002$).

I3C in Kale Leaf Tissue as a QR Inducer. Using different concentrations of commercial I3C, the CD value for I3C was observed to be 230 μM, which is a relatively weak QR induction agent compared to sulforaphane (0.2 μM), 7–Methylsulfinylheptyl isothiocyanate (0.2 μM), PEITC (5 μM), and brassinin (4 μM) (Kang and Pezzuto, 2004; Rose et al., 2000). In Chapter 3, we reported the that QR CD value of NI3C was 35 μM and for neoascorbigen was 38 μM, from broccoli extracts. Despite the significantly increased amount of NI3C and neoascorbigen, their contribution to enhanced QR inductive activity was relatively small. The high CD value of I3C suggests this compound does not explain the increased QR activity from kale leaf tissue extracts (Figure 4.1).

MeJA Dose Dependent Induced GS and QR Activity in Kale Leaf Tissue. To further evaluate the association between induction of QR activity and GS concentrations in kale leaf tissues, a second experiment was conducted where different MeJA concentrations (0, 50, 250, and 500 μM) were applied to two kale cultivars as described above. The MeJA treatment increased GS concentrations (glucobrassicin and neoglucobrassicin) and QR activity in a linear dose dependent fashion in apical leaf tissue of both kale cultivars (Figure 4.5A). In addition, MeJA treatment significantly increased NI3C and NeoASG in apical leaf tissue (Figure 4.5C).

Although there was a dose dependent increase in I3C by MeJA treatment in apical leaf tissue of the RW cultivar, the DBCV kale showed the inverse response to MeJA treatment (Figure 4.5C). The MeJA treatment not only changed GS biosynthesis but also hydrolysis-related gene expression (Chapter 5). Although apical leaf, indolyl GS hydrolysis product concentrations were found higher in RW compared to DBCV (Figure 4.5C), QR induction activity by RW apical leaf tissue was relatively low (Figure 4.5A). The low concentration of I3C in kale leaf tissue may be related to low stability or I3C condensation/oligomerization (Agerbirk et al., 2009; Bradlow, 2008). Other hydrolysis products of glucobrassicin such as di(indol-3-yl)methane (DIM), brassinin, or 2,3-bis(indol-3-ylmethyl)-indole (TIR) which can induce QR activity at lower CD values (Kang and Pezzuto, 2004; Zhu and Loft, 2003) may play a more important role in QR induction in kale than I3C..

Correlation Analysis of GS, GS Hydrolysis Products and QR with Varying Concentrations of MeJA. Correlations of QR inducing activity of the two kale cultivars over the two seasons (Table 4.2) were significant for gluconasturtiin ($r = 0.888$, $P < 0.0001$), glucobrassicin ($r = 0.671$, $P = 0.0005$), and neoglucobrassicin ($r = 0.980$, $P < 0.0001$). The GS hydrolysis products I3C ($r = 0.856$, <0.0001), DIM ($r = 0.788$, $P = 0.0003$), NI3C ($r = 0.974$, $P < 0.0001$), NeoASG ($r = 0.918$, $P < 0.0001$) and sulforaphane ($r = 0.770$, $P = 0.0005$) also correlated with QR activity. These data suggest that the combination of NI3C, NeoASG, and sulforaphane induction contributed to enhanced QR activity of kale leaf tissue extracts. Since correlation analysis does not necessarily imply causation, further research is needed to address which compound or compounds are dominating QR inducing activity in kale leaf tissue.

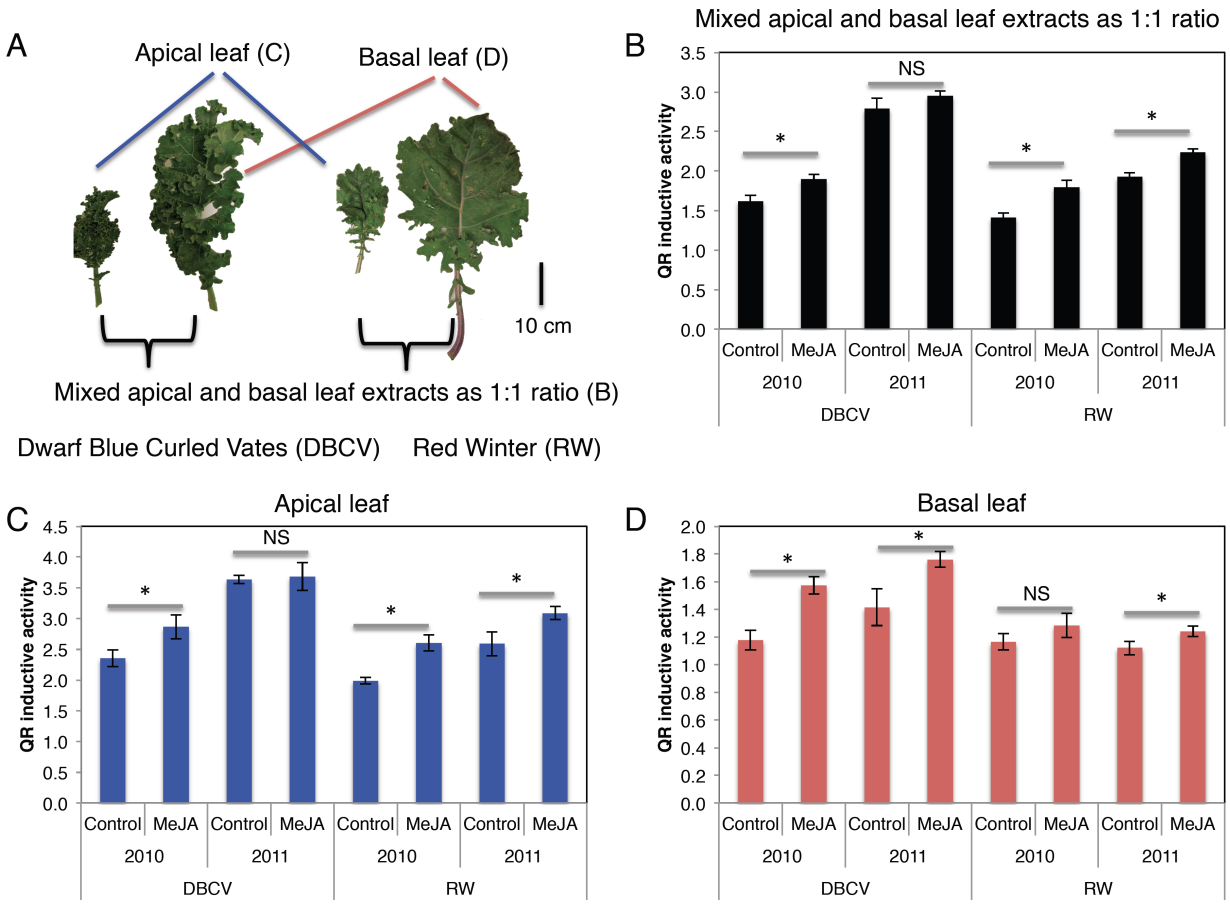


Figure 4.1. QR inducing activity of apical and basal and combined kale leaf tissue from two kale cultivars. A: Images of apical and basal leaf samples. B: QR inducing activity of mixed extract of 1:1 apical and basal leaf tissues. C: QR inducing activity of apical leaf tissue. D: QR activity of basal leaf tissue. Student T-tests were conducted to determine significance at $P \leq 0.05$. NS and * indicate non-significance and significance at $P \leq 0.05$, respectively. Mean ± SD (n=3).

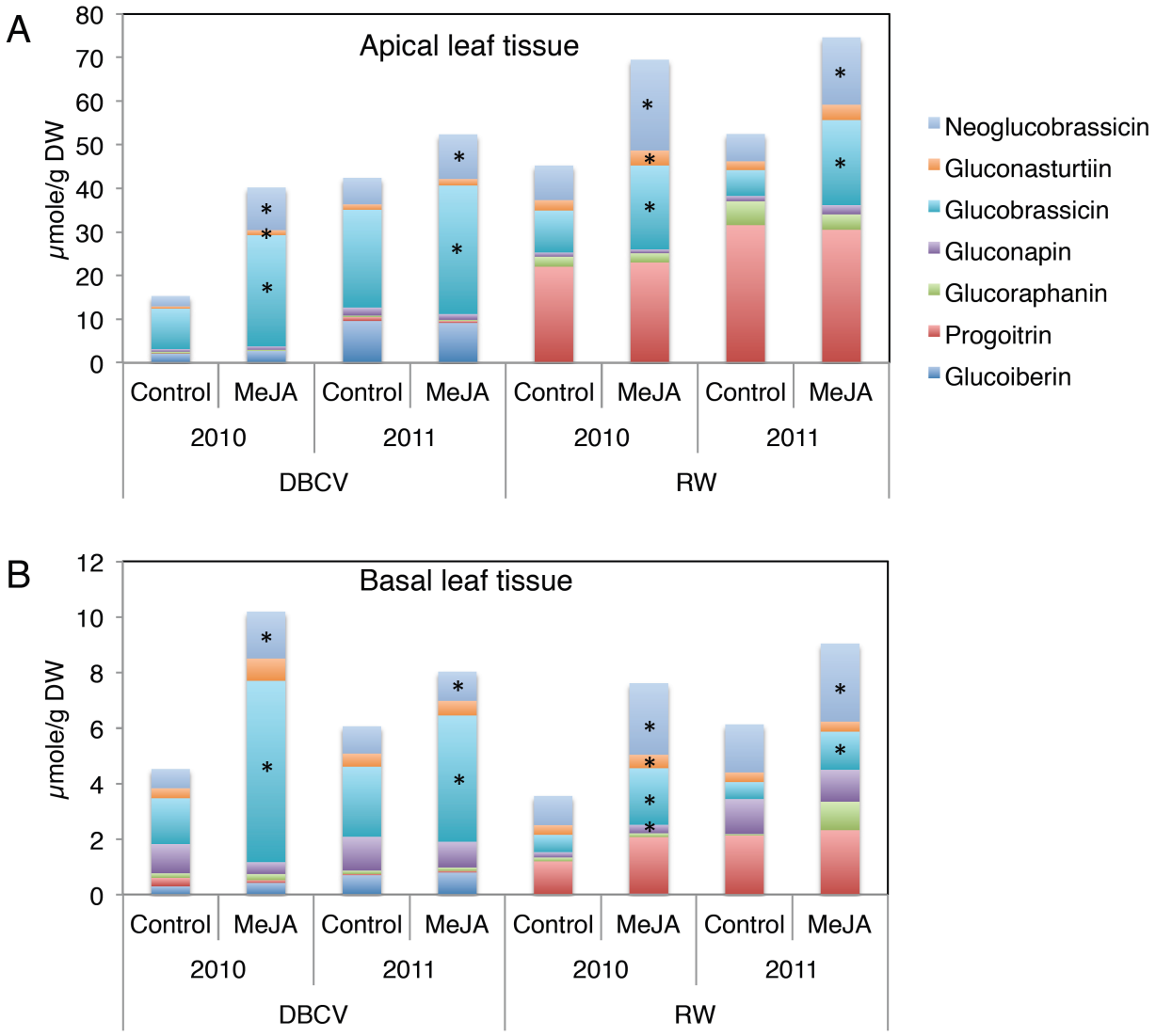


Figure 4.2. GS composition of different kale leaf tissues with or without MeJA treatment from two kale cultivars over two years. Student T-tests were conducted to determine significant at $P \leq 0.05$. * Indicates significance at $P \leq 0.05$. A: Apical leaf tissue from two kale cultivars. B: Basal leaf tissue from two kale cultivars. Mean ± SD (n=3).

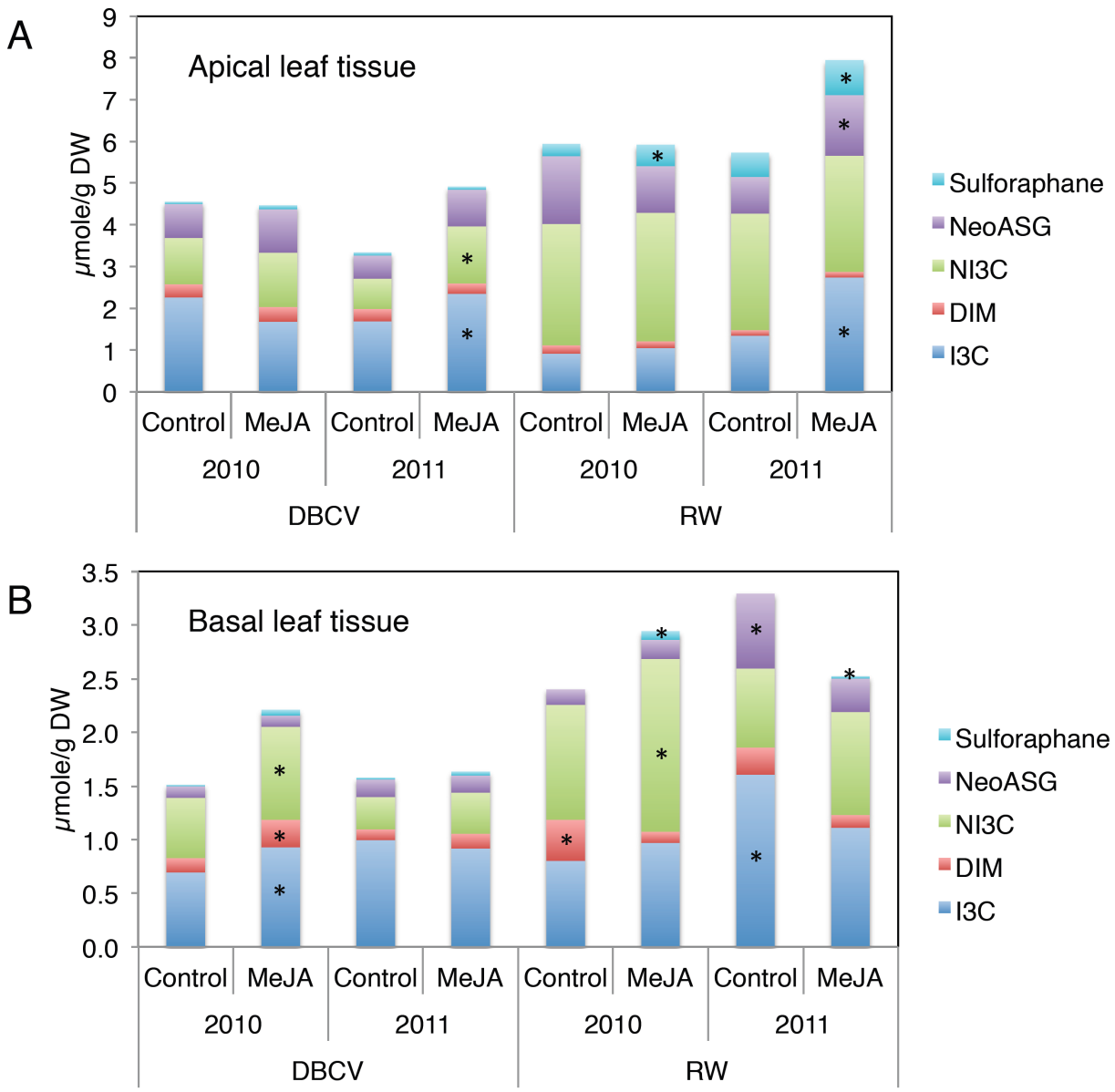


Figure 4.3. Hydrolysis product composition of apical and basal leaf tissues with or without MeJA treatment from two kale cultivars over two years. Student T-tests were conducted to determine significant at $P \leq 0.05$. * Indicates significance at $P \leq 0.05$. Mean ± SD (n=3).

Table 4.1. Correlation analysis between intact GS, GS hydrolysis products and QR activity from kale leaf tissue extracts over two years.

	Glucoraphanin	Glucobrassicin	Gluconasturtiin	Neoglucobrassicin	QR	I3C	DIM	NI3C	NeoASG
Glucoraphanin									
Glucobrassicin	0.120								
Gluconasturtiin	0.726	0.561							
Neoglucobrassicin	0.500	0.731	0.914						
QR	0.305	0.747	0.415	0.431					
I3C	0.203	0.642	0.358	0.422	0.707				
DIM	-0.374	0.308	-0.209	-0.067	0.042	0.227			
NI3C	0.810	0.330	0.880	0.767	0.176	0.209	-0.199		
NeoASG	0.584	0.590	0.802	0.754	0.381	0.548	0.075	0.788	
Sulforaphane	0.879	0.325	0.907	0.742	0.361	0.382	-0.342	0.853	0.682

Pink coloring indicates significant correlation based on the Pearson's correlation at $P \leq 0.05$.

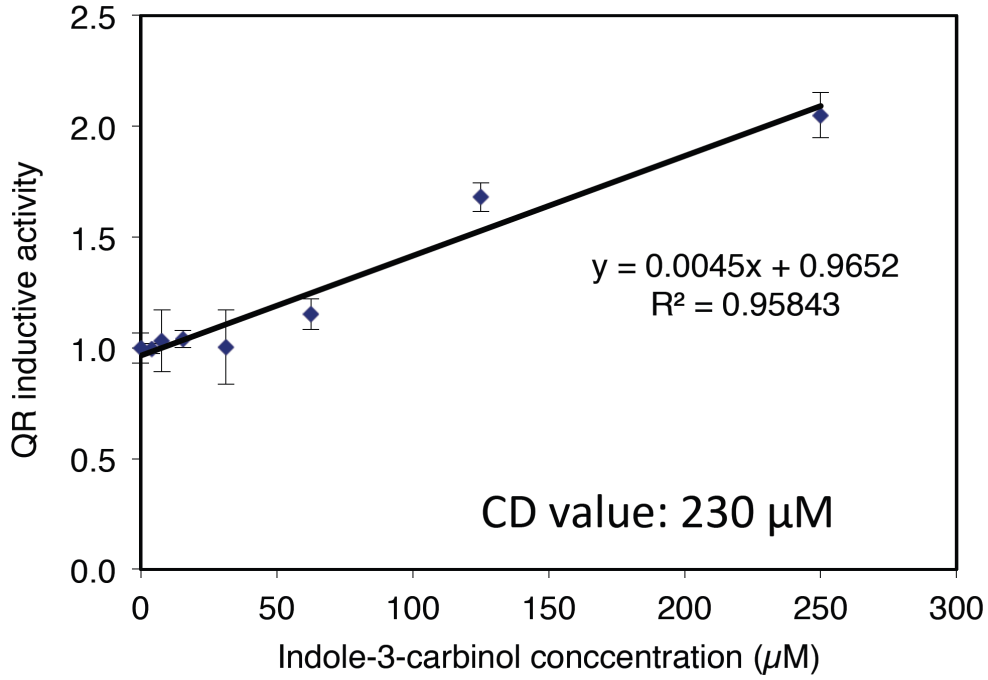


Figure 4.4. QR inductive activity of indole-3-carbinol (I3C). Seven different concentrations from 3.9 to 250 µM were tested using QR assay to determine CD value of I3C.

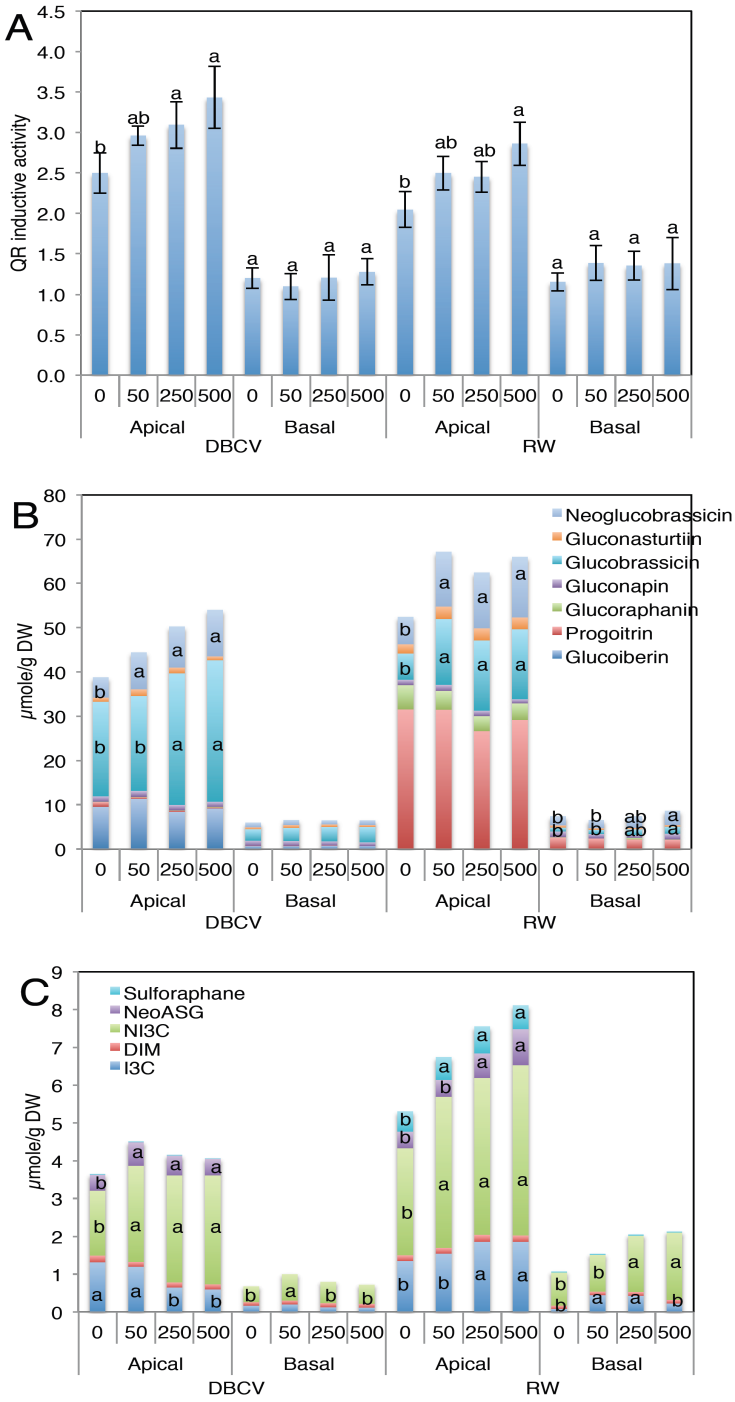


Figure 4.5. QR activity and GS and GS hydrolysis product concentrations from kale leaf tissue samples sprayed with varying concentrations of MeJA (0, 50, 250, and 500 µM). Different letters indicate significant differences among treatments based on Fisher's LSD test at $P \leq 0.05$. A: QR activity, B: GS profiles, and C: hydrolysis product profiles.

Table 4.2. Correlation analysis between intact GS, GS hydrolysis product and QR activity from kale leaf tissue across two kale cultivars over two seasons.

	Glucoraphanin	Glucobrassicin	Gluconasturtiin	Neoglucobrassicin	QR	I3C	DIM	NI3C	NeoASG
Glucoraphanin									
Glucobrassicin	0.081								
Gluconasturtiin	0.858	0.444							
Neoglucobrassicin	0.621	0.734	0.872						
QR	0.704	0.671	0.888	0.980					
I3C	0.768	0.504	0.920	0.841	0.856				
DIM	0.603	0.682	0.808	0.795	0.788	0.904			
NI3C	0.731	0.620	0.903	0.976	0.974	0.886	0.797		
NeoASG	0.581	0.742	0.845	0.914	0.918	0.890	0.867	0.897	
Sulforaphane	0.947	0.133	0.917	0.719	0.770	0.830	0.675	0.806	0.649

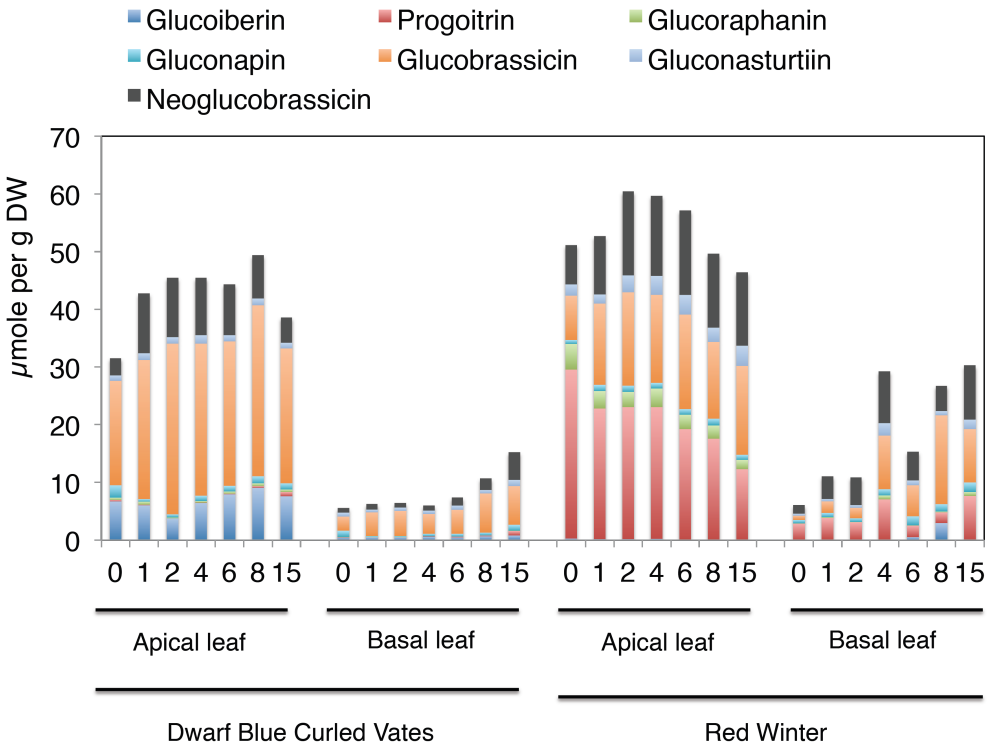
Values with a pink background indicate significant correlations based on the Pearson's correlation at $P \leq 0.05$.

Supplementary Table 4.S1. Weather information of 2009, 2010, and 2011 year in Champaign, Illinois.

	Total solar radiation ($MJ \cdot m^{-2}$)		
Year	Jun	Jul	Sum
2010	720	730	1450
2011	667	790	1457
% of (2011/2010)	93	108	100.5
	Precipitation (mm)		
Year	Jun	Jul	Sum
2010	199	91	290
2011	107	40	147
% of (2011/2010)	54	44	50.7
	Growing degree days (°C)		
Year	Jun	Jul	Sum
2010	373	408	781
2011	362	430	792
% of (2011/2010)	97	105	101.4

Growing degree days [(Min Temperature + Max Temperature)/2 -7]

Weather data during the growing seasons was provided by the Illinois State Water Service (http://www.isws.illinois.edu/warm/data/cdfs/cmiday.txt).



Supplementary Figure 4.S1. Optimum harvest date for MeJA treated kale leaf tissue based on the glucosinolate concentration.

To determine optimum harvest date, solutions of 250 µM of MeJA (Sigma-Aldrich, St. Louis, MO) containing 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) where sprayed on all aerial portions of kale plants to the point of runoff (approximately 300 mL) 1, 2, 4, 6, and 8 days prior to harvest of heads at commercial harvest maturity. Two different kale leaf samples (apical: three leaves from the top, excluding less than 8 cm length per plant; basal three leaves: three leaves from the bottom which is fully developed per plant) were harvested and bulked from five treated and control plants of each genotype for each replicate respectively (five heads or leaves bulked for a replicate sample).

CHAPTER 5

Methyl Jasmonate and 1-methylcyclopropene Treatment Effects on Quinone Reductase Induction Activity and Post-harvest Quality of Broccoli

5.1. Abstract

Pre-harvest methyl jasmonate (MeJA) and post-harvest 1-methylcyclopropene (1-MCP) treatments on broccoli floret glucosinolate (GS) concentrations and quinone reductase (QR, an anti-cancer biomarker) inducing activity were evaluated two days prior to harvest, at harvest and at 10, 20, and 30 days of post-harvest storage at 4 °C. MeJA treatments four days prior to harvest of broccoli heads was observed to significantly increase floret ethylene biosynthesis resulting in chlorophyll catabolism during post-harvest storage and reduced product quality. Post-harvest treatment with 1-MCP, which competitively binds to protein ethylene receptors, maintained post-harvest floret chlorophyll concentrations and product visual quality in both control and MeJA-treated broccoli. Transcript abundance of *BoPPH*, a gene which is responsible for the synthesis of pheophytinase, the primary enzyme associated with chlorophyll catabolism in broccoli, showed a significant, negative correlation with floret chlorophyll concentrations. The GS, glucobrassicin, neoglucobrassicin, and gluconasturtiin were significantly increased by MeJA treatments. The hydrolysis products of these GS [indole-3-carbinol (I3C), N-methoxy indole-3-carbinol (NI3C), and phenethyl isothiocyanate (PEITC)] were also quantified and found to be significantly correlated with QR. Sulforaphane, the isothiocyanate hydrolysis product of the GS glucoraphanin, was found to be the most potent QR induction agent. Increased sulforaphane formation was associated with up-regulated gene expression of myrosinase (*BoMYO*) and the myrosinase co-factor gene *epithiospecifier modifier1* (*BoESM1*). This is the first report that

MeJA treatment can increase sulforaphane formation from the hydrolysis of the parent GS, glucoraphanin. During post-harvest storage, levels of the GS, 4-methoxyglucobrassicin in MeJA-treated broccoli samples was significantly increased, whereas glucobrassicin concentrations were significantly decreased and negatively correlated (r = -0.578; P=0.019), implying GS conversion is actively occurring during post-harvest storage. This GS conversion is likely associated with modified pre- and post-harvest transcript abundance of the indolyl biosynthetic genes *BoCYP79B2* and *BoCYP83B1* and ethylene-induced 4-methoxylation enzyme activity.

5.2. Introduction

Functional plant foods that promote health have received a great deal of attention from scientists and other consumers. *Brassica oleracea* L. vegetables including broccoli, cabbage, cauliflower, kohlrabi, Brussels sprouts, and kale are commonly consumed around the world. These vegetables are recognized as functional foods that contain various phytochemicals such as glucosinolates (GS), flavonoids, carotenoids, vitamins, and minerals that have putative cancer preventive effects as shown in epidemiological and animal carcinogenesis studies (van Poppel et al., 1999). The GS (glucoraphanin, gluconasturtiin, and sinigrin) found in the tissues of accessions of *B. oleracea* have been identified as potent cancer prevention agents because products of their hydrolysis by the endogenous enzyme myrosinase generate sulforaphane (SF), phenethyl isothiocyanate (PEITC), and allyl isothiocyanate (AITC). These isothiocyanate products have been shown to induce biosynthesis and thus bioactivity of mammalian phase II detoxification enzymes such as glutathione S-transferases (GSTs), quinone reductase (QR) and UDP-glucuronosyl transferase in *in vitro* or *in vivo* systems that can enhance detoxification and elimination of carcinogens from the body (Hwang and Jeffery, 2003; Wallig et al., 1998; Zhang

et al., 1992). QR activity elevation with *in vitro* and *in vivo* systems has been shown to correlate with induction of other protective phase II enzymes such as the GSTs and provides a reasonable biomarker for the potential chemoprotective effect of phytochemical against initiation of carcinogenesis (Cuendet et al., 2006).

GS biosynthesis genes have been intensively studyied in *Arabidopsis* using biochemical assays. There is high homology of gene sequences in GS biosynthesis between *Arabidopsis* and *Brassicaceae* (Bak et al., 1998). GS biosynthesis consists of three independent steps: (i) chain elongation of selected precursor amino acids (Met and Phe), (ii) formation of the core glucosinolate structure, and (iii) secondary modifications of the amino acid side chain. A side chain amino acid precursor proceeds through a series of chain elongations prior to entering the pathway. Two genes, *methylthioalkylmalate synthase 1* (*MAM1*) and *MAM2*, have been identified in *Arabidopsis*, where *MAM1* regulates the first two methionine elongation cycles, whereas *MAM2* supoorts in only one round of elongation (Kroymann et al., 2003; Textor et al., 2004). The second step is the GS core structure formation from the precursor amino acids by reaction with various cytochrome P450 enzymes (CYP) (Sønderby et al., 2010), CYP79 catalyzes the conversion of amino acid to aldoximes. *CYP79F1* and *CYP79F2* genes are responsible for aldoxime metabolism leading to aliphatic GS derived from chain-elongated methionine derivatives, whereas *CYP79B2* and *CYP79B3* have distinct functions in indolyl GS biosynthesis, which is derived from tryptophan (Sønderby et al., 2010). In the biosynthetic pathway of indolyl GS, *CYP79B2* catalyzes the conversion of tryptophan to indole-3-acetaldoxime, with *CYP83A1* and *CYP83B1* metabolizing the phenylalanine- and tyrosine-derived aldoximes (Sønderby et al., 2010). It has been reported that indolyl GS biosynthesis is modulated by the methyl jasmonate (MeJA) and the salicylic acid (SA) signal transduction

pathways (Mikkelsen et al., 2003). The biological activity of GS depends on diversity of structure of the side chain that is the last step of GS biosynthesis (Hopkins et al., 2009). For aliphatic GS, secondary modifications include oxygenations, hydroxylations, alkenylations and benzoylations. Indolic GS, in turn, can undergo hydroxylations and methoxylations (Sønderby et al., 2010). Recently, methoxylation genes involved in glucobrassicin such as *CYP81F2*, *CYP81F3, and CYP81F4* were clearly identified by genetic engineering Arabidopsis indolyl GS biosynthesis into *Nicotiana benthamiana* (Pfalz et al., 2011).

Intact GS do not display bioactivity but following hydrolysis by the endogenous enzyme myrosinase, isothiocynates and other products are generated, which have been associated with insect resistance and anti-cancer activity. When the plant tissue is disrupted, myrosinase and substrates (GS) come into contact, resulting in GS hydrolysis. The chemical structure of hydrolysis products depends on the structure of the GS side chain and reaction conditions such as pH, concentration of Fe^{2+} and presence of epithiospecifier protein (ESP), a myrosinase co-factor that will favor formation of nitriles (Bones and Rossiter, 1996). In the absence of ESP, the addition of Fe^{2+} ions also promotes nitrile formation, which are essentially without anticancer activity compared to the isothiocyanates like sulforaphane, PEITC, and AITC (Matusheski et al., 2006). The *epithiospecifier modifier 1* (*ESM1*) gene in *Arabidopsis* encodes a protein shown to inhibit function of ESP, leading to increased isothiocyanate production from GS hydrolysis (Zhang et al., 2006).

Methyl jasmonate (MeJA), a plant signal transduction compound associated with herbivore defense, can act as an elicitor to enhance GS biosynthesis (Howe and Jander, 2008). Previous research has shown that MeJA treatments can significantly increase QR inducing activity mediated by enhancement of GS biosynthesis including glucoraphanin, glucobrassicin

and neoglucobrassicin in cauliflower (Ku et al., 2013). However, 1 mM MeJA treatment was also found to significantly promote ethylene production and increased 1-aminocyclopropane-1-carboxylate acid (ACC) concentrations and ACC oxidase activity associated with senescence and loss of product quality in broccoli (Watanabe et al., 2000). Thus, while MeJA treated broccoli can display enhanced QR activity associated with increased GS concentrations, elevated ethylene production can accelerate post-harvest senescence, phytochemical degradation and quality loss.

Visual color is critical factor in retailer and consumer evaluation of product quality and subsequent purchasing decisions (Dixon, 2007). Yellowing of the foliage or discoloration of the heads and curds of broccoli and cauliflower is associated with senescence and is not acceptable to retail consumers. Consequently, color is an important factor in retailer and consumer evaluation of product quality and subsequent purchasing decisions. Evaluation of changes in color using colorimeters provides a rapid and non-destructive means of measuring post-harvest quality of the product (Dixon, 2007). It has been reported that color change determined by hue angle measurement corresponded to increased soluble and decreased insoluble pectin, indicating senescence and loss of crispness in red radish (*Raphanus sativus*) (Schreiner et al., 2003).

Inhibition of ethylene production or blocking the ethylene receptor is an effective way to improve shelf life and quality of fruits and vegetables. 1-methylcyclopropene (1-MCP) is 100 times more effective at binding to plant ethylene receptor proteins than ethylene itself (Paliyath, 2008) and application of 1-MCP increases shelf life of broccoli (Ku and Wills, 1999). 1-MCP application has also been found to maintain the phytochemicals in broccoli such as chlorophylls, carotenoids, ascorbic acid and GS after harvest by binding to the ethylene receptors *ETR1* and *ETR2* (Gang et al., 2009; Gaofeng et al., 2010; Ma et al., 2010). As mentioned above, chlorophyll content is considered a good indicator of broccoli post-harvest quality. Previous

studies reported that pheophytinase (PPH) and pheophorbide a oxygenase (PaO) are key enzymes in post-harvest chlorophyll breakdown (Bücherta et al., 2011; Gomez-Lobato et al., 2012a). It was reported that gene expression of *BoPPH* and *BoPaO* as reduced by 1-MCP treatment (Gomez-Lobato et al., 2012b).

It was previously reported that most GS are degraded during post-harvest storage of broccoli at 0 ° and 4 °C except for gluconasturtiin, 4-methoxyglucobrassicin, and neoglucobrassicin (Rodrigues and Rosa, 1999). Chopping of harvested broccoli florets followed by storage at room temperature was observed to decrease concentrations of glucobrassicin and increase 4-methoxyglucobrassicin in broccoli (Verkerk et al., 2001). Despite detailed characterization of the pattern of indolyl GS induction and biosynthesis, relatively little is known about the mechanisms. With the high homology of gene sequences in GS biosynthesis between *Arabidopsis* and *Brassica oleracea* (Bak et al., 1998), GS biosynthesis genes from *Arabidopsis* have been transformed into *Brassica oleracea* crops such as Chinese cabbage with successfully expression and associated changes in GS profiles (Zang et al., 2009; Zang et al., 2008a; Zang et al., 2008b).

4-methoxyglucobrassicin hydrolysis products have been reported to be antibiotic to fungal pathogens and to the green peach aphid (*Myzus persicae*) (Bednarek et al., 2009; Kim et al., 2008). Many physiological responses to stress are often mediated in the plant by ethylene perception. Since the discovery of 1-MCP as an ethylene antagonist, blocking ethylene perception is a powerful tool to study not only mechanisms of action, and its effect on post-harvest quality of fruit and vegetables, but also ethylene function in plant biotic defense. Based on accumulated information on GS biosynthesis and induction from *Arabidopsis* it is the

objective of this research to investigate the interaction of MeJA and 1-MCP on anticancer inducing activity and post-harvest maintenance of quality in broccoli.

5.3. Materials and Methods

5.3.1. Plant Cultivation and Sample Preparation with Treatments. 'Green Magic' broccoli (Sakata Seed Co., Morgan Hill, CA) was used for this experiment. Broccoli seeds were germinated in 32 cell plant plug trays filled with sunshine® LC1 professional soil mix (Sun Gro Horticulture, Vancouver, British Columbia, Canada). Seedlings were grown in a greenhouse at the University of Illinois at Champaign-Urbana under a 25 °C/15 °C and 14 h/10 h: day/night temperature regime and with supplemental lighting. Forty days after seed germination, seedlings were first transferred into 1-liter pots and then after a month 150 broccoli seedlings were repotted into 3.75-L pots. These broccoli seedlings were evenly placed on three greenhouse benches and control and MeJA treatment assigned within each bench to minimize microenvironmental variation. 500 micromoles of MeJA (Sigma-Aldrich, St. Louis, MO) in solution containing 0.1% ethanol was sprayed on aerial tissues of each of the treated plants four days prior to harvest at commercial maturity. Timing of MeJA sprays and concentration of solution was previously determined to optimize up-regulation of indolyl GS (Ku and Juvik, 2012 and Chapter 7). For the control group, only a 0.1% ethanol solution was applied. At commercial market maturity 50 broccoli heads were harvested from both the control and MeJA treated plants, transported to the laboratory, and divided into branchlets of broccoli florets. King and Morris (1994) reported that branchlets are useful model systems for investigating broccoli senescence. Branchlets of treatment group were randomly divided into two groups generating four treatment groups: (1) No MeJA or 1-MCP (Control); (2) No MeJA and 500 ppb treatment with 1-MCP for

24 h (1-MCP); (3) MeJA without 1-MCP (MeJA), and (4) MeJA and 500 ppb treatment with 1-MCP for 24 hr (MeJA_1-MCP). Treatment (1) and (3) and treatment (2) and (4) were placed in airtight plastic containers at 20 °C. 1-MCP was generated in containers holding treatments (2) and (4) by adding an activator and a Smartfresh® tablet (AgroFresh, Inc. a division of Rohm and Hass, Philadelphia, PA) to the activation solution following the instructions provided by the company. After treatment, broccoli branchlets were stored in a walk-in cooler (4 °C). At each sampling date (0, 10, 20, and 30 days of post-harvest storage), three random subsamples (replications) for each treatment group were selected and assayed for ethylene and CO_2 production and visual quality. Pictures of broccoli florets and their relative visual quality from each assay date are presented in Figure 5.1. After measuring ethylene production and hue angle, a measurement of floret color change, each sample was freeze-dried. Freeze-dried broccoli floret tissue of each sample was finely ground with a commercial coffee grinder. The ground freeze-dried broccoli samples were stored at -20 °C prior to GS quantification and quinone reductase bioactivity assay. After measuring ethylene production and hue angle, a measurement of broccoli floret color change, a subsample of tissue was collected, frozen in liquid nitrogen, and stored at –80 °C until ground with a mortar and pestle in liquid nitrogen for RNA extraction.

5.3.2. Determination of Floret Ethylene Production and Respiration Rate. Respiration was measured as tissue CO_2 production. Three subsamples (300 g each) of broccoli branchlets from each treatment were placed into 3 L jars and enclosed with a silicon rubber cap for 1 h at 20 °C. Sample CO_2 was estimated using 2% CO_2 in nitrogen gas (v/v) standard for each experiment. The headspace gas in the jar was sampled with a 0.2 mL plastic hypodermic syringe and injected into a GC (model Perkin Elmer AutoSystem Gas Chromatograph) equipped with a Propak®

(Waters Co., Milford, Ma) column and thermal conductivity detector (TCD). Temperature of the injector, detector and column was 100, 150 and 30 °C, respectively. The results were expressed as mL of CO_2/kg/h. Ethylene measurement was measured as previously reported (Ku et al., 2013) using pure ethylene gas as a standard for estimating sample concentrations. Headspace gas in the same jar that was used for CO_2 evaluation, were sampled with a 1 mL plastic hypodermic syringe and injected into a GC (model Perkin Elmer AutoSystem Gas Chromatograph) equipped with a activated aluminum packed column and flame ionization detector (FID). The temperature of the injector, detector and column was 100, 200 and 80 °C, respectively. The flow rate of hydrogen was 40 mL/min. The rate of ethylene production was expressed as µL of ethylene/kg/h.

5.3.3. Determination of Total Chlorophyll Content. Floret tissue samples (75 mg) were ground and extracted in 1.5 ml of 80% acetone in a 2 mL tube using vigorous vortexing for 1 h. Total chlorophyll content was determined by reading the absorbance at 645 and 663 nm with a µQuant microplate reader (Bio-Tek Instruments, Winooski, VT). Total chlorophyll content was calculated by summation of estimated chlorophyll a and chlorophyll b concentrations. The total chlorophyll was estimated as mg/g fresh weight using the equation listed below (Arnon, 1949).

Total chlorophyll (µg/mg) = 20.2 (A_{645}) + 8.02 (A_{663})

Chlorophyll a (µg/mg) = 12.7 (A_{663}) - 2.69 (A_{645})

Chlorophyll b (µg/mg) = 22.9 (A_{645}) - 4.68 (A_{663})

5.3.4. Hue Angle Measurement. For purposes of data analysis and data interpretation, it is important to have a reliable measure of color change. Hue is one property of color. Hue is how we perceive an object's color (red, orange, green, blue, etc.)

(http://personal.uncc.edu/lagaro/cwg/color/color_percept.html). Hue degree allows us to get digitalized data by using quadrant space for color (0° = red, 90° yellow, 180° = green, 270° = blue). Sample post-harvest visual quality was measured by using a LabScan XE colorimeter (Hunter Associates Laboratory, Reston, VA, USA) generating values for a* (redness and greenness), and b* (yellowness and blueness). The instrument was calibrated with a standard white and black tile. The average of four different broccoli branchlets were recorded in each replication. Hue degree (h°) was calculated as $h° = \tan^{-1} (b*/a*)$ when $a*>0$ and $b*>0$, or as $h° = 180° - \tan^{-1} (b*/a*)$ when $a<0$ and $b>0$.

5.3.5. Determination of Sample GS Concentrations. Freeze-dried broccoli powder (0.2 g) and 2 mL of 70% methanol were added to 10 mL tubes (Nalgene, Rochester, NY) and heated on a heating block at 95 °C for 10 min. After cooling on ice, 0.5 mL benzylglucosinolate (1 mM) was added as an internal standard (POS Pilot Plant Corp, Saskatoon, SK, Canada), mixed, and centrifuged at 3,000 × g for 15 min at 4 °C. The supernatant was saved and the pellet was re-extracted with 2 mL 70% methanol at 95 °C for 10 min and the two extracts combined. A subsample (1 mL) from each pooled extract was transferred into a 2-mL microcentrifuge tube. Protein was precipitated with 0.15 mL of a 1:1 mixture of 1 M lead acetate and 1 M barium acetate. After centrifuging at 12,000 × g for 1 min, each sample was then loaded onto a column containing DEAE Sephadex A-25 resin (Sigma-Aldrich, St. Louis, MO) for desulfation with arylsulfatase (*Helix pomatia* Type-1, Sigma-Aldrich, St. Louis, MO) for 18 h and the desulfo-GS eluted. One hundred µL of each sample were injected on to a HPLC. Quantification of GS using high-performance liquid chromatography was performed using a previously described protocol (Brown et al., 2002).

5.3.6. Quinone Reductase (QR) Inductive Activity. For the QR assay, 75 mg of broccoli floret powder from each sample were suspended in 1.5 mL of water in the absence of light for 4 h at room temperature in a sealed 2 mL microcentrifuge tube (Fisher Scientific, Waltham, MA) to facilitate GS hydrolysis by endogenous myrosinase. Slurries were then centrifuged at 12,000 × g for 10 min and supernatants were diluted to 0.25% final concentration in the QR activity assays. The QR induction activities of different samples were determined by means of the protocol described by Prochaska and Santamaria (Prochaska and Santamaria, 1988). Hepa1c1c7 murine hepatoma cells (ATCC, Manassas, VA) were grown in alpha-minimum essential medium (MEM), enriched with 10% heat and charcoal-inactivated fetal bovine serum and maintained at 37 °C in 95% ambient air and 5% CO_2. After 24 h cells were exposed to the different sample extracts (0.25% final concentration in 200 μL of media) in new media for a further 24 h. Treated cells were rinsed with phosphate buffer at pH 7.4, lysed with 50 μL 0.8% digitonin in 2 mM EDTA, incubated and agitated for 10 min. A 200-μL aliquot of reaction mix (Prochaska and Santamaria, 1988) was added to the lysed cells. Readings were made at five time points, 50 s apart, using a μQuant microplate reader (Bio-Tek Instruments, Winooski, VT) at 610 nm. Immediately after completion of the readings, 50 μL of 0.3 mM dicumarol in 25 mM Tris buffer was added into each well, and the plate was read again (five time points, 50 s apart) to determine non-specific MTT (methylthiazolyldiphenyl-tetrazolium bromide) reduction. Total protein content was measured by the BioRad assay (Bio-Rad, Hurcules, CA, USA) following manufacturer's instructions. Activity was expressed as QR specific activity (nmol MTT reduced/mg/min) ratio of treated to control cells.

5.3.7. Analysis of Glucosinolate Hydrolysis Products. The extraction and analysis of isothiocyanates and other hydrolysis products was carried out according to previously published methods with some modifications (Wilson et al., 2011). Broccoli powder (75 mg) was suspended in 1.5 mL of water in the absence of light for 4 h (optimal time for hydrolysis products of indolyl GS) and 24 h (optimal time for sulforaphane and PEITC) at room temperature in a sealed 2 mL microcentrifuge tube (Fisher Scientific, Waltham, MA) to facilitate GS hydrolysis by endogenous myrosinase. Slurries were then centrifuged at 12,000 × g for 5 min and supernatants was decanted into a 2 mL microcentrifuge tube. 20 µL of butyl isothiocyanate (0.5 mg/mL) and 4-methoxyindole (1 mg/mL) were added as the internal standards for isothiocyanates and hydrolysis products of indolyl GS to quantify indole-3-carbinol (I3C), NI3C, and neoascorbigen (NeoASG), respectively, with 0.5 mL of methylene chloride. Tubes were shaken vigorously before being centrifuged for 2 min at 9,600 g. The methylene chloride layer (200 µL) was transferred to 350 µL flat bottom insert (Fisher Scientific, Pittsburgh, PA) in a 2 mL HPLC autosampler vial (Agilent, Santa Clara, CA) for mixing with 100 µL of a reagent containing 20 mM triethylamine and 200 mM mercaptoethanol (derivatization reagent) in methylene chloride. For SF and PEITC, unlike other hydrolysis products of GS measurement, 0.5 mL of fresh broccoli extracts were mixed with 0.5 mL of derivatization reagent using orbital shaker at 220 rpm for 24 hours. Then, internal standards were added as described above. The mixture was incubated at 30 °C for 60 min under constant stirring, and then dried under a stream of nitrogen. The residue containing isothiocyanate derivatives (isothiocyanate-mercaptoethanol derivatives) and other hydrolysis compounds was dissolved in 200 µL of acetonitrile/water (1:1) (v/v), and 10 µL of this solution injected onto a Agilent 1100 HPLC system (Agilent, Santa Clara, CA), equipped with a G1311A bin pump, a G1322A vacuum degasser, a G1316A thermostatic column

compartment, a G1315B diode array detector and an HP 1100 series G1313A autosampler. Extracts were separated on a Eclipse XDB-C18 column (150 × 4 mm, particle size 5 μm, Agilent, Santa Clara, CA) with a C18 all-guard™ cartridge pre-column (Alltech, Lexington, KY). Mobile phase A was water and B methanol. Mobile phase B was 0% at injection, increasing to 10% by 10 min, 100% at 35 min, and held 5 min, then decreased to 0% by 50 min. Flow rates were kept at 0.8 mL/min. The detector wavelength was set at 227 and 271 nm. Response factors for monomeric indolyl derivatives were used from a previous report (Agerbirk et al., 1998). Due to a lack of standards for NI3C and NeoASG the standard curve of I3C was applied for quantification of both NI3C and NeoASG. The quantities were expressed as I3C equivalent concentrations.

5.3.8. Cloning of broccoli *epithiospecifier modifier* 1 (*BoESM1*). Using known *Arabidopsis* (NM_112278.2)*, Brassica rapa* (FJ830451.1) and *Brassica napus* (FJ830448.1) gene sequence information, PCR primers were designed with the Primer3 software (http://frodo.wi.mit.edu/primer3) to isolate the broccoli, cabbage, and cauliflower homologous *ESM1* gene*,* known to be associated with GS hydrolysis. PCR amplification was performed using the GoTaq® PCR Core System (Promega, Madison, WI) following the protocol described by the manufacturer. The PCR product was separated on 1% TAE gels and purified by using a Qiagen gel extraction kit (QIAGEN, Valencia, CA) according to the manufacturer's protocols. The amplified PCR products were cloned with pGEM®-T Easy Vector System (Promega), and the clones were sequenced in the W. Carver Biotechnology Center, University of Illinois at Urbana-Champaign using the ABI 3730XL Capillary Sequencer (Applied Biosystems, Foster City, CA). The amino acid sequences deduced from the isolated cDNA sequences were subjected to phylogenetic tree analysis using Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/).

(Supplementary Figure 5.S1). Quantitative RT-PCR (qRT-PCR) primers for *BoESM1* were designed based on the consensus sequences of *B. oleracea* (broccoli, cauliflower, and cabbage), *B. napus*, and *B. rapa* cDNA (Supplementary Table 5.S1).

5.3.9. RNA Extraction and Quantitative Real Time-PCR. Total RNA was isolated from control and MeJA treated floret tissue samples using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The general quality of RNA was determined by agarose gel electrophoresis. The quantity of RNA was measured by the NanoDrop 3300 spectrophotometer (Thermo Scientific, Waltham, MA). 1 μg of the total RNA was reverse-transcribed with Superscript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The resulting cDNA samples were diluted to 1 tenth their concentrations (v/v) for qRT-PCR. Previously reported primer sets of GS biosynthesis (*BoCYP79B2*, *BoCYP83B1*, *BoCYP79F1*, *BoCYP83A1*) genes, hydrolysis (*BoMYO*, *BoESP, BoESM1*) genes, a pathogenesis-related (PR) protein (*BoPR*) gene known to be responsive to MeJA, chlorophyll catabolism (*BoPPH*, *BoPaO*) genes, and the actin gene (*BoAct1*) as a normalization standard were used for qRT-PCR (Hasperué et al., 2013; Kim, 2011) (Supplementary Table S1). The primer sequence sets were synthesized by Integrated DNA Technologies (Coralville, IA). Real-time PCR was carried out with the real-time Power SYBR® Green PCR Master Mix (QIAGEN, Valencia, CA) using Taqman ABI 7900 (Applied Biosystems, Foster city, CA) according to the manufacturer's instructions. Each reaction contained 0.375 μL of 10 μM of forward and reverse primers, respectively and 6.75 μL of template cDNA RT-PCR and 7.5 μL of Power SYBR® Green PCR Master Mix. The thermal cycling program was as follows: 50 °C for 2 min, then 95 °C for 10 min followed by 40 cycles of

95 °C for 15 s and 60 °C for 60 s. Quantitative RT-PCR was performed in two technical replicates for each biological sample. The relative expression ratio was determined with the equation $2^{-\Delta\Delta Ct}$ by normalizing with *BoAct1*, using the Ct values generated by the Taqman ABI 7900 Sequence Detection System Software 2.4 (Applied Biosystems).

5.3.10. Measurement of Myrosinase Activity. Myrosinase activity was optimized according to previous studies (Li and Kushad, 2005; Pang et al., 2009; Penas et al., 2011). Crude extracts were prepared by adding 0.3 g of a finely ground freeze-dried sample in 4 mL of an extraction buffer consisting of 10 mM potassium phosphate, 1 mM EDTA, 3 mM DTT and 5% glycerol (pH 7.0) for 20 min in an ice bath (Pang et al., 2009). The crude extracts were centrifuged at 15,000 g for 30 min at 4 °C. To remove endogenous GS and glucose, the crude extract was filtered through an Amicon ultrafiltration cell (Millipore, Billerica, MA) with a 10 KDa molecular weight cutoff (Penas et al., 2011) and washed several times at 4 °C using the same extraction buffer at pH 7.0 (Pang et al., 2009). 50 µL of purified extracts and 450 µL of 0.2 mM sinigrin in 33.3 M phosphate buffer, pH 6.5 were mixed and incubated for 40 min (Li and Kushad, 2005). To stop the enzyme reaction extracts were heated at 95 °C for 10 min. The release of glucose was determined by the glucose oxidase/peroxidase/ABTS method (Bergmeyer, 1974) using a microplate reader (Biotek Instruments, Winooski, VT). Glucose concentrations were calculated using a linear standard curve. By calculating the glucose amount in aliquots of purified extracts without sinigrin, endogenous glucose levels were subtracted in purified extracts for myrosinase activity measurement.

5.3.11. Statistical Analysis. Statistical analyses were conducted using the JMP 10 software (SAS institute Inc., Cary, NC). Student's T-tests was used for comparing two treatment groups. Fisher's Least Significant Difference (LSD) test was conducted for comparing treatment group means at $P \leq 0.05$. Pearson correlation was conducted on all pairs of a GS, hydrolysis product and chlorophyll concentrations, gene expression, and QR inductive activity based on the mean values of each treatment across post-harvest storage dates. The results are presented as means ± SD.

5.4. Results and Discusssion

5.4.1. Ethylene Production and Respiration Rate for Broccoli Floret Subjected to MeJA Treatments. Treatment with 500 μM MeJA significantly increased ethylene production (1.9 fold) in broccoli floret tissues four days after treatment (Supplementary Figure 5.S2A). Ethylene production dropped significantly during post-harvest storage at 4 °C. Ethylene production between control and MeJA treated groups were not significantly different at 10 and 20 days of storage regardless of 1-MCP treatment. There was also no consistent difference in respiration rates among the different treatments at harvest or during post-harvest storage. Respiration rates in samples peaked at harvest and gradually decreased during post-harvest storage at 4 °C (Supplementary Figure 5.S2B).

5.4.2. Product Color Measurement for Visual Quality Change and Chlorophyll Concentrations. Visual color is an important factor in retailer and consumer evaluation of broccoli product quality and subsequent purchasing decisions (Dixon, 2007). Loss of visual quality associated with degradation of floret chlorophyll concentrations enhanced by higher

ethylene production impacts on broccoli shelf life. Images of broccoli florets from the four different treatments from each assay date are presented in Figure 5.1. There were significant differences in visual quality between control and 1-MCP treatment groups over the period of post-harvest storage, regardless of MeJA treatment (Figure 5.1). In order to determine this objectively, tissue chlorophyll concentrations and floret hue angle were measured to quantify visual quality. There was a significant reduction in total chlorophyll content in broccoli florets two and four days after MeJA treatment compared to controls (Figure 5.2A). It was reported that MeJA treatment reduced total chlorophyll content previously in *Arabidopsis thaliana* (Jung, 2004). Chlorophyll b concentrations are much higher than chlorophyll a in broccoli and showed more dramatic losses during post-harvest storage (data not shown). Hue angle measurements indicated that 1-MCP treatments were associated with superior visual quality throughout the period of post-harvest storage compared to controls and the MeJA treatment (Figure 5.2B).

5.4.3. Chlorophyll Catabolism Gene Expression by Preharvest MeJA and Post-harvest 1-MCP Treatments. To investigate the mechanism of chlorophyll and visual quality loss during the post-harvest period, relative transcript abundance of two genes associated with chlorophyll catabolism *BoPaO* and *BoPPH* were assayed by qRT-PCR. Transcript abundance of these genes was significantly greater than in the control or MeJA treated broccoli for *BoPPH* at 20 and 30 days of post-harvest storage and for *BoPaO* at 30 days post-harvest storage (Figure 5.2C and 5.D). There was a significant negative correlation between *BoPPH* gene expression (Figure 5.2C) and total chlorophyll concentrations (r = -0.642, P = 0.007) (Figure 5.2A). Hue angle measurements of visual quality were negatively correlated with both *BoPPH* (r = -0.868, $P <$ 0.001) and *BoPaO* gene expression (r = -0.641, P = 0.014). Down-regulation of expression of

these genes to maintain visual quality has been previously reported in broccoli (Bücherta et al., 2011). 1-MCP mediated reduction of ethylene binding to receptor proteins was responsible for reduced expression of *BoPaO* and *BoPPH*, chlorophyll degradation and associated visual quality loss during post-harvest storage. A previous study has shown that chlorophyll degradation in broccoli florets during post-harvest senescence is positively associated with ethylene and negatively associated with cytokinin (Costa et al., 2005; King and Morris, 1994; Pogson et al., 1995). These hormones also regulate the activity of catabolism-related enzymes. For example, 1-MCP treatment delayed broccoli yellowing and decreased chlorophyll-degrading peroxidase and chlorophyllase activities, resulting in maintaining visual quality (Gong and Mattheis, 2003).

5.4.4. Pre-harvest MeJA and Post-harvest 1-MCP Treatments Influence GS and GS Hydrolysis Product Concentrations. Treatment with MeJA significantly increased glucobrassicin, gluconasturtiin, 4-methoxyglucobrassicin and neoglucobrassicin concentrations in broccoli floret samples. At harvest, the relative increase of these GS was 1.58, 2.28, 4.75, and 4.71 fold over controls, respectively (Figure 5.3). Neoglucobrassicin and 4-methoxyglucobrassicin, which are products of the glucobrassicin biosynthesis pathway following hydrolxylation then methoxylation, respectively increased by similar fold values. Previous reports indicate MeJA treatments increased glucoraphanin, glucobrassicin, and neoglucobrassicin concentrations in cauliflower curds (Ku et al., 2013) and glucoiberin, progoitrin, sinigrin, and gluconapin concentrations in cabbage (Fritz et al., 2010). Both jasmonate (JA) and MeJA induced significant increases (up to 20-fold) in the concentration of specific indolyl GS in *Brassica napus* (primarily the GS, glucobrassicin) and *B. rapa*, (primarily the GS, 4-hydroxy glucobrassicin) and in mustard (*B. juncea*, where both where increased) (Bodnaryk, 1994). The

different responses to MeJA treatment observed in this study suggest that variation in GS response to MeJA is species specific.

The enhanced glucobrassicin concentrations of MeJA treated broccoli observed at harvest decreased over the duration of post-harvest storage whereas neoglucobrassicin concentrations remained relatively stable. MeJA treated broccoli showed significant degradation in glucobrassicin concentrations during the first 10 days of post-harvest storage (Figure 5.3B and 5.D). MeJA treatments were found to significantly increase gluconasturtiin concentrations at harvest (Figure 5.3E). Concentrations of gluconasturtiin were observed to increase over the period of post-harvest storage in all the treatments except for the MeJA treated group. The concentration of 4-methoxyglucobrassicin was increased in all four treatment groups, with the MeJA treatment showing the largest increment. There was a significant correlation between the loss of glucobrassicin and increases of 4-methoxyglucobrassicin (r = -0.578, P = 0.019), implying active GS conversion.

Interestingly, SF formation was significantly increased by MeJA treatment even though there were no significant increases in glucoraphanin concentrations (Figure 5.4 and Figure 5.6). This can be explained by the significantly increased levels in gene expression of myrosinase (*BoMYO*) and *BoESM1* compared to the *BoESP* gene (Figure 5.5). Myrosinase activity was significantly (58%) increased by MeJA treatment at four days after treatment (Supplementary Figure 5.S3). MeJA treatment not only increased gluconasturtiin biosynthesis but also PEITC formation (Figure 5.4 and Figure 5.6). NeoASG concentrations were significantly increased by MeJA treatment and maintained elevated concentrations during post-harvest storage (Figure 5.4). The major hydrolysis product of neoglucobrassicin was NeoASG (Figure 5.4C and D). I3C concentrations were only observed to be significantly increased in the MeJA treatment at harvest.

5.4.5. GS Biosynthetic, Hydrolytic, and Pathogenesis Related-Protein (PR) Gene Expression Changes Mediated by MeJA and/or 1-MCP treatments. The amino acid sequences deduced from the isolated *B. oleracea ESM1* gene sequences corresponded to the *B. napus* (96%), B. *rapa* (95%) and *Arabidopsis thaliana* protein, ESM1 (79%), respectively, which suggests similar gene product function in broccoli by sharing homologous protein motifs (Supplementary Figure 5.S1). In order to evaluate the effects of MeJA and 1-MCP treatments at harvest and during post-harvest storage, gene expression of GS biosynthetic (*BoCYP79B2*, *BoCYP83B1*, and *BoCYP83A1*), hydrolytic (*BoMYO, BoESP*, and *BoESM1*), and pathogenesis related (*BoPR*) genes were measured by qRT-PCR. Gene expression of *BoCYP79B2* (10.0 fold), *BoCYP83B1* (2.7 fold), *BoCYP83A1* (1.5 fold), *BoMYO* (3.0 fold), *BoESP* (1.3 fold), *BoESM1* (1.9 fold) and *BoPR* (1.5 fold) were significantly increased by MeJA treatment compared to control at two days after the treatment (Figure 5.5). Jun et al. (2011) also reported that MeJA treatment increased transcript level of broccoli sprouts. In contrast, gene expression of *BoCYP79F1* (0.7 fold) was significantly decreased by MeJA treatment compared to control at two days after the treatment. The elevated transcript abundance observed at 2 days after MeJA treatment was dramatically reduced with post-harvest storage at 4 °C (Figure 5.5). While gene expression levels during post-harvest storage were low and not dramatically different among treatments for many of the genes, transcript abundance for *BoCYP79B2*, *BoCYP83B1,* and *BoPR* gradually increased over the duration of storage. The mRNA expression levels of these genes may explain the increase in indolyl GS during post-harvest storage. Decreased gene expression of *BoCYP79B2* and *BoCYP83B1* associated with 1-MCP treatments is in agreement with previous research in *Arabidopsis* showing that that these GS biosynthetic genes are stimulated by

elevated MeJA-mediated ethylene production (Chang and Shockey, 1999; Mikkelsen et al., 2003; Stotz et al., 2000).

5.4.6. Correlation between Gene Expression and Glucosinolate Concentrations. There were significant correlations between glucobrassicin concentrations and *BoCYP79B2* ($r = 0.625$, $P = 0.017$) and *BoCYP83B1* ($r = 0.573$, $P = 0.032$) expression, genes which are involved in up-stream biosynthesis of indolyl and aromatic GS. *BoCYP79B2* ($r = 0.625$, $P = 0.017$) and *BoCYP83B1* ($r = 0.573$, $P = 0.032$) gene expression also correlated with ethylene production indicating that GS biosynthesis is ethylene-mediated response as described above (Chang and Shockey, 1999; Mikkelsen et al., 2003; Stotz et al., 2000) (Supplementary Table 5.S2). In addition, *BoPR* gene expression was significantly correlated with ethylene production in our samples ($r = 0.694$, $P = 0.006$). *BoPR* expression is responsive to the salicylic acid (SA), MeJA, and the ethylene signaling pathway (Mikkelsen et al., 2003).

The observed reduction in glucobrassicin with complimentary increases in 4-methoxyglucobrassicin concentrations are a likely result of the 4-methoxylation of glucobrassicin. Recently the indolyl GS modification from glucobrassicin to 4-methoxyglucobrassicin was characterized by the transformation of *Nicotiana benthamiana* with known GS biosynthetic genes from *Arabidopsis* (Pfalz et al., 2011). The *Arabidopsis CYP81F2* gene is involved in accumulation of 4-methoxyglucobrassicin in response to pathogen infection (Bednarek et al., 2009). The *CYP81F* subfamily of cytochrome P450s are responsible for hydroxylation of glucobrassicin. Hydroxy intermediates are then converted to neoglucobrassicin and 4-methoxyglucobrassicin, respectively, by either of two families of 2 O-methyltransferases, named *indolyl glucosinolate methyltransferase 1* (*IGMT1*) and *IGMT2*. The loss of

glucobrassicin in MeJA treated broccoli during the post-harvest storage is possibly due to the higher levels of *BoCYP81F* subfamily transcripts compared to *BoCYP79B2* and *BoCYP83B1* gene expression. Mikkelsen et al. (2003) also reported that enzyme activities responsible for the N-methoxylation of glucobrassicin are strongly induced by MeJA treatment and this induction is suppressed by ACC. Recently, it has been identified that *CYP81F4* is involved in N-hydroxylation of glucobrassicin to synthesize neoglucobrassicin (Pfalz et al., 2011). As Mikkelsen et al. (2003) observed, it has been reported that *CYP81F4* was up-regulated by MeJA and down-regulated by ethylene in *Arabidopsis* (5.81 fold) (Hall et al., 2012; Kai et al., 2011). It was reported that 1-MCP treatments did not completely inhibit the accumulation of ACC in broccoli florets (Ma et al., 2009). In addition, 1 mM of MeJA post-harvest treatment also significantly increased ACC concentrations compared with control broccoli (Watanabe et al., 2000). Thus, gene expression of *CYP81F2* in broccoli may be relatively greater than *CYP81F4* because accumulation of ACC or ethylene during the post-harvest storage may down regulate gene expression of *CYP81F4* (Hall et al., 2012). Consequently, ethylene accumulation favors the pathway of methoxylation from glucobrassicin to 4-methoxyglucobrassicin rather than formation of neoglucobrassicin during post-harvest storage. Favoring the methoxylation pathway by accumulation of ethylene or the ethylene precursor, ACC would facilitate to defense against post-harvest pathogens since 4-methoxyglucobrassicin has been shown to be antibiotic to fungi (Bednarek et al., 2009; Clay et al., 2009). PEITC, the hydrolysis product of gluconasturtiin has also been reported to possess antifungal activity (Drobnica et al., 1967). The increasing levels of gluconasturtiin may be associated with upregulation of *BoCYP83B1,* which is involved in both indolyl and aromatic GS biosynthesis. Enhanced levels of PEITC during the post-harvest storage could also be associated with antifungal defense.

5.4.7. Correlations between QR Induction Activity of Broccoli and Enhanced GS Hydrolysis Products. QR induction activity of broccoli floret extracts treated with MeJA was significantly increased compared to controls at 2 and 4 days after MeJA treatment and throughout the course of post-harvest storage (Figure 5.4F). The increased QR activity may be due to the increased concentrations of sulforaphane, PEITC, and/or hydrolysis products of neoglucobrassicin including NI3C and NeoASG. Significant positive correlations were observed between QR activity and the GS concentrations of gluconasturtiin ($r = 0.673$, $P = 0.008$), 4-methoxyglucobrassicin ($r = 0.716$, $P = 0.002$), and neoglucobrassicin ($r = 0.826$, $P < 0.001$) in broccoli floret sample extracts during post-harvest storage at 4 °C (Supplementary Table 5.S2, Figure 5.7). Hydrolysis products of GS, sulforaphane ($r = 0.865$, $P < 0.001$), PEITC ($r = 0.857$, $P < 0.001$), NI3C ($r = 0.899$, $P < 0.001$), and NeoASG ($r = 0.874$, $P < 0.001$) were also correlated with QR activity (Supplementary Table 5.S2, Figure 5.7). Previously, 4-methoxyindole 3-carbinol has been reported to provide antiproliferation activity in two different human colon cancer cells lines *in vitro* (Kronbak et al., 2010). Interestingly, there was significant correlation between total chlorophyll concentrations and glucoraphanin concentration, suggesting greenness may be good indicator of glucoraphanin concentration during post-harvest storage ($r = 0.590$, $P = 0.016$). Since only one cultivar was used for post-harvest experiment, more study is needeeds.

Sulforaphane formation was significantly increased by MeJA treatment (Figure 5.6). In most previous studies typically only MeJA mediated GS concentration changes were reported without information about the hydrolysis products. This present study suggests that MeJA treatment not only increases concentrations of certain GS, but can also increase SF and PEITC conversion rates from precursor GS, which are the hydrolysis products with the highest activity

for QR induction and putative anticancer activity. Concentrations required for a two-fold increase in QR activity (the CD value) of NI3C, NeoASG, PEITC and SF were 35, 38.5, 5.0 and 0.2 μM, respectively (Zhang et al., 1992; Kang and Pezzuto, 2004; Chapter 3). Even though the increased SF concentrations observed in our study is smaller than that of NeoASG, the relative SF bioactivity suggest it is the major contributor toward enhanced QR induction activity in our extracts, although MeJA-mediated increases in other GS and their hydrolysis products are also likely contributors. 1-MCP treatment was observed to reduce post-harvest degradation of GS to improve the delivery of bioactive compounds to consumers.

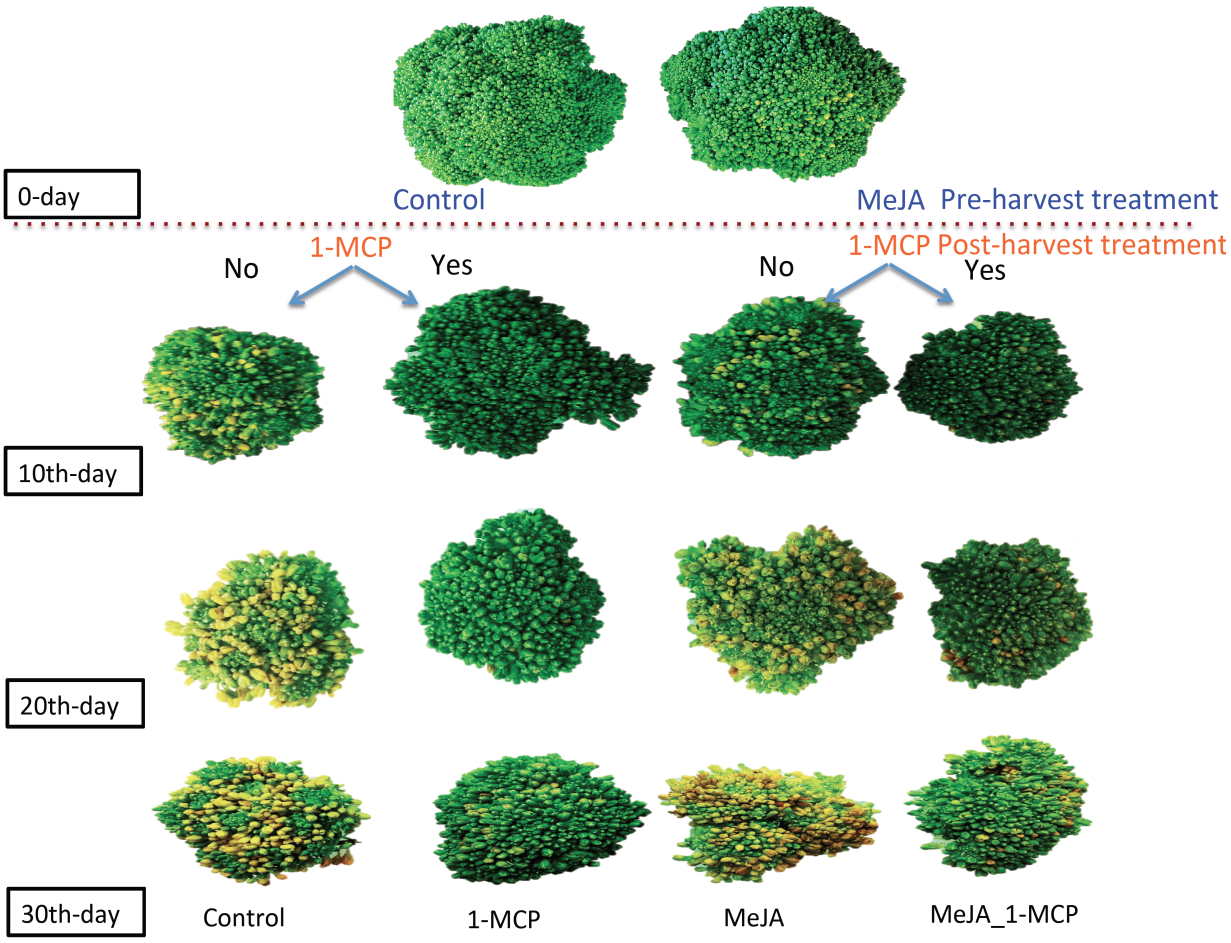


Figure 5.1. Representative samples of broccoli branchlets with or without pre-harvest MeJA and 1-MCP treatments for 0, 10, 20, and 30 days post-harvest storage at 4 °C.

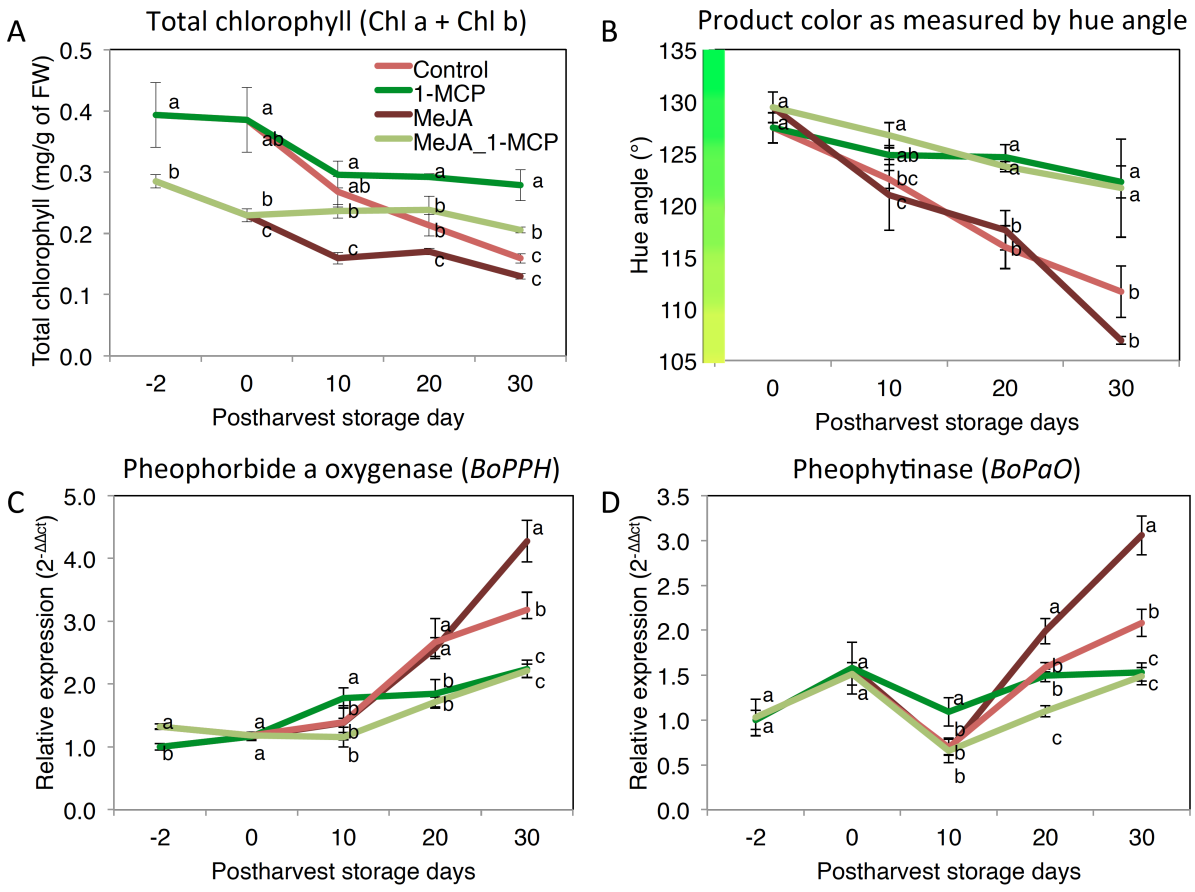


Figure. 5.2. Changes in total chlorophyll content, hue angle, and gene expression of chlorophyll catabolism genes from pre-harvest MeJA and post-harvest 1-MCP treatments two days prior to harvest, at harvest and during post-harvest storage at 4 °C. C: broccoli pheophytinase (*BoPPH*) transcript abundance D: broccoli pheophorbide a oxygenase, (*BoPaO*) transcript abundance. Different letters indicate significant differences among treatments based on Fisher's LSD test at $P \leq 0.05$. Mean ± SD (n=3).

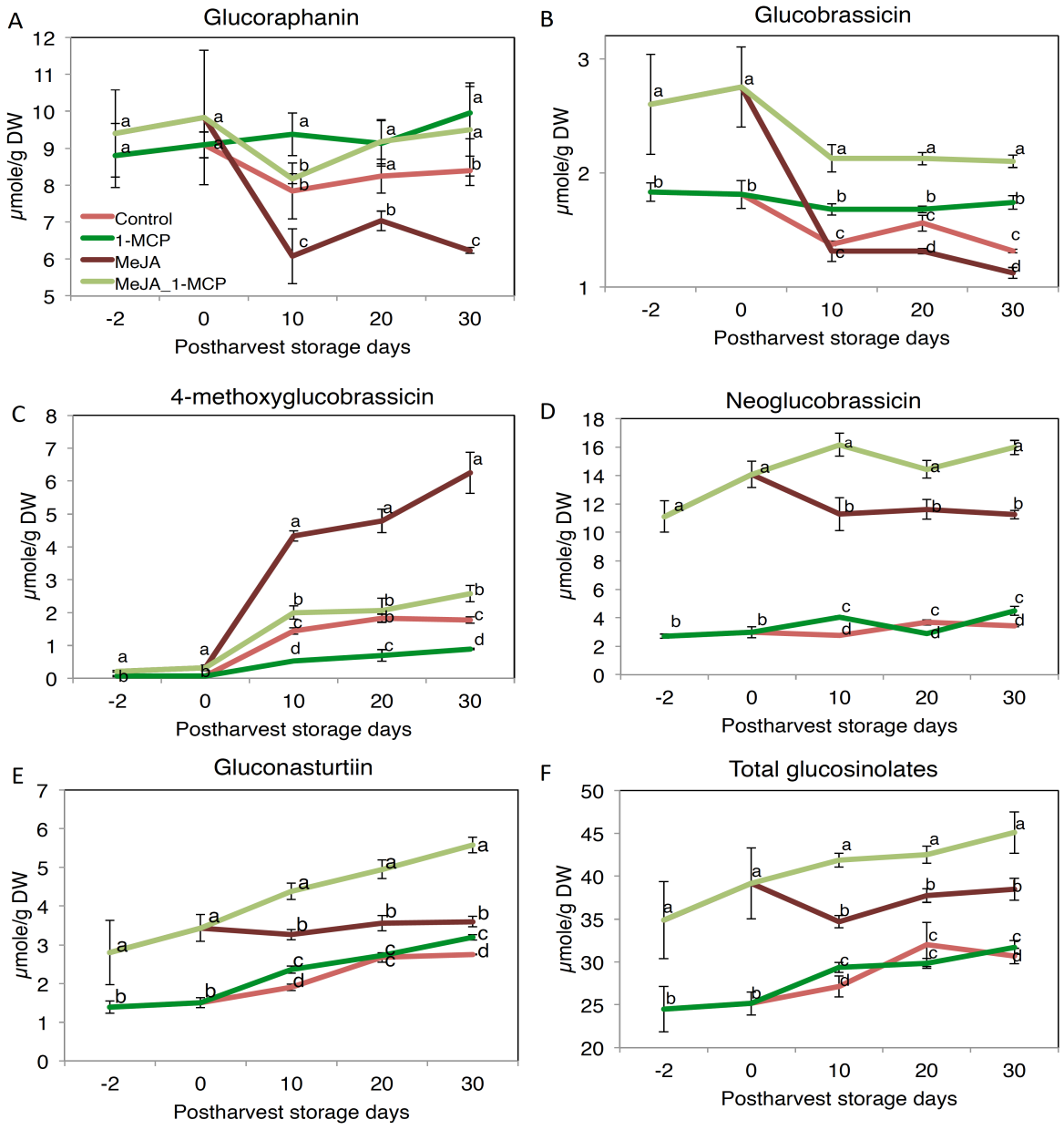


Figure 5.3. Effect of pre-harvest MeJA and post-harvest 1-MCP treatments on glucosinolates concentrations of florets at 2 days prior to harvest, at harvest (0 days) and during post-harvest storage at 4 °C. A: glucoraphanin B: glucobrassicin, C: 4-methoxyglucobrassicin, D: neoglucobrassicin E: gluconasturtiin, and F: total glucosinolates. Data are means ± SD (n=3). Different letters indicate significant differences among treatments based on Fisher's LSD test at $P \leq 0.05$.

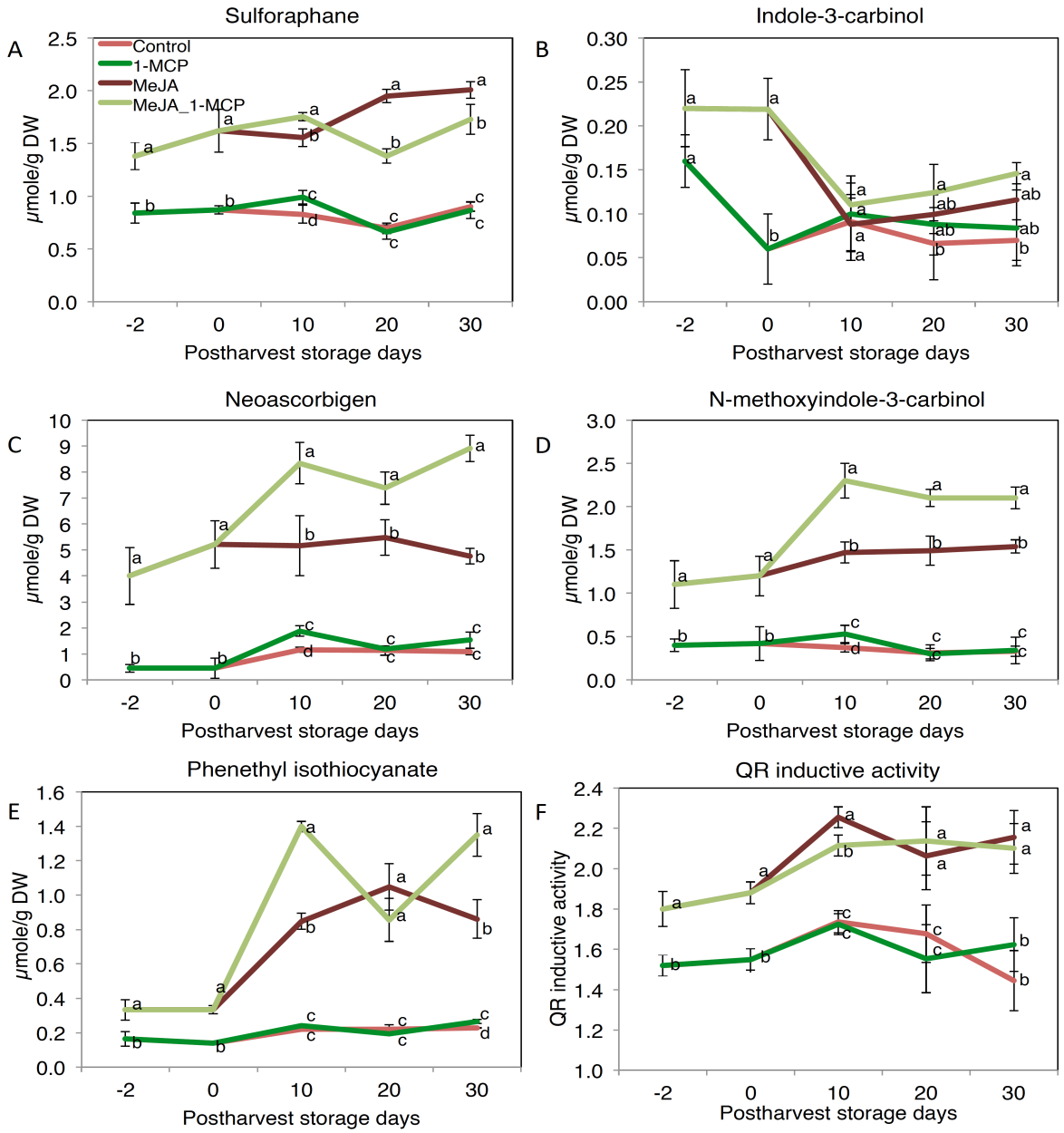


Figure 5.4. Effect of pre-harvest MeJA and post-harvest 1-MCP treatments on GS hydrolysis product concentrations and QR activity of floret extracts at two days before harvest, at harvest and during post-harvest storage at 4 °C. A: sulforaphane; B: indole-3-carbinol; C: neoascorbigen; D: N-methoxyindole-3-carbinol; E: phenethyl isothiocyanate; and F: QR inductive activity. Data are means ± SD (n=3). Different letters indicate significant differences among treatments based on Fisher's LSD test at $P \leq 0.05$. [Z]I3C equivalent concentration (µmole/g DW).

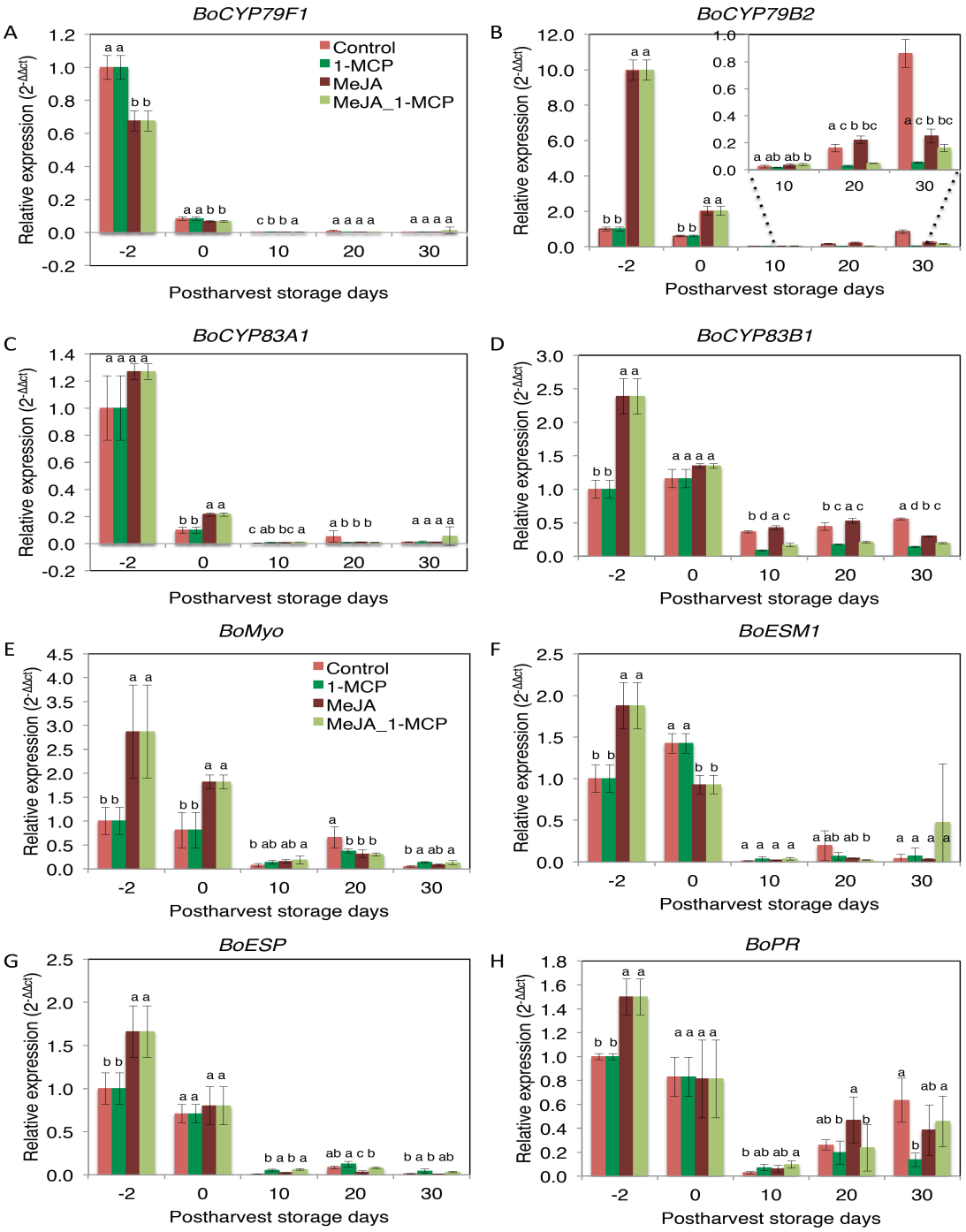


Figure 5.5. Effect of pre-harvest MeJA and post-harvest 1-MCP treatment on gene expression of GS biosynthetic, hydrolytic, and *PR* genes in broccoli floret two days after MeJA treatment, at harvest and during post-harvest storage at 4 °C. Different letters indicate significant differences among treatments based on Fisher's LSD test at $P \leq 0.05$. Mean ± SD (n=3).

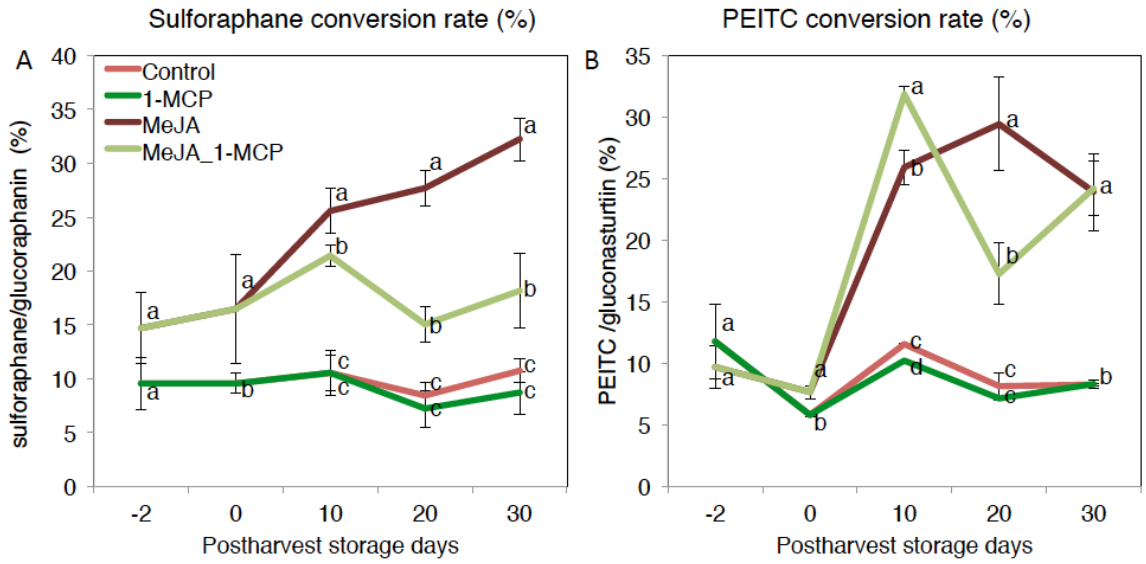


Figure 5.6. Sulforaphane and phenethyl isothiocyanate (PEITC) conversion from glucoraphanin and gluconasturtiin at two days before harvest, at harvest, and during post-harvest storage at 4 °C. Different letters indicate significant differences among treatments based on Fisher's LSD test at $P \leq 0.05$. Mean ± SD (n=3).

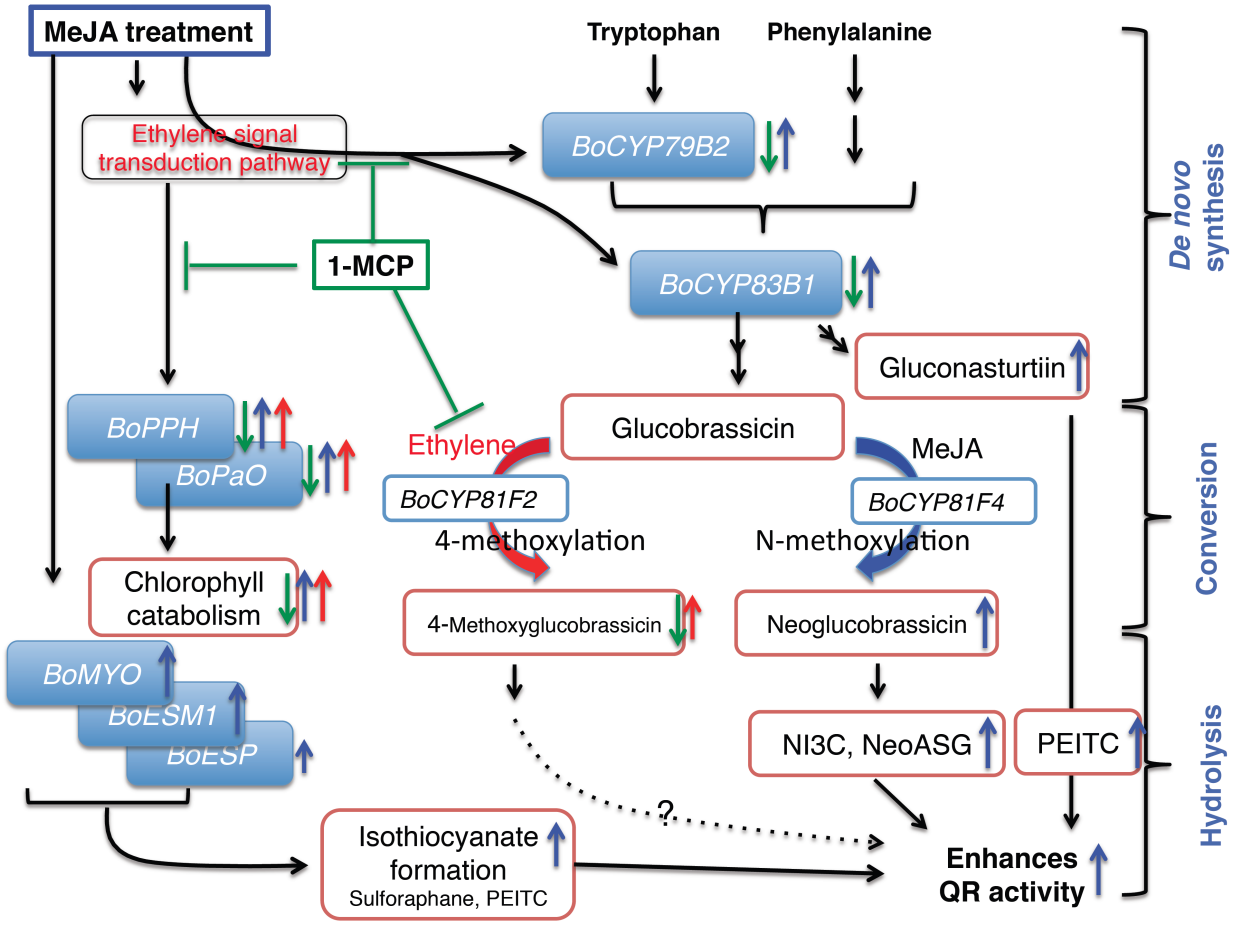
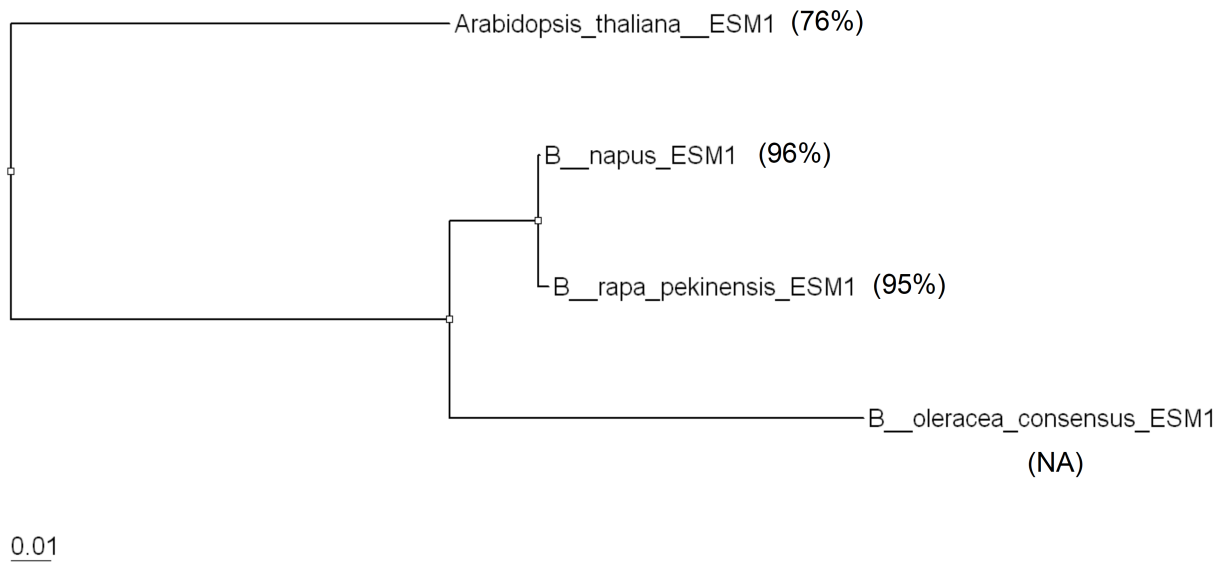


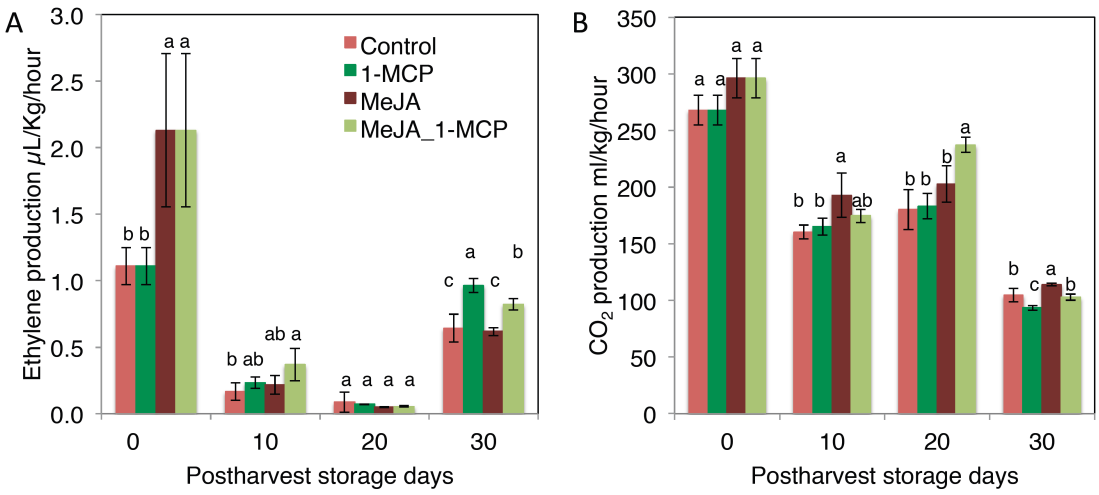
Figure 5.7. Proposed model of pre-harvest MeJA and post-harvest 1-MCP treatment effects on GS biosynthesis, hydrolysis, QR bioactivity, and visual quality of broccoli florets during post-harvest storage at 4 °C. Pre-harvest MeJA increases indolyl and aromatic GS biosynthesis (*de novo* GS biosynthesis). Ethylene accumulation induces 4-methoxylation of glucobrassicin rather than N-methoxylation of glucobrassicin during post-harvest but 1-MCP maintains glucobrassicin concentrations and reduces indolyl GS biosynthesis during post-harvest by inhibiting ethylene mediated GS biosynthesis. MeJA enhances synthesis of myrosinase and the hydrolysis of GS to favor isothiocyanate formation in the case of glucoraphanin and gluconasturtiin. Blue arrows describe MeJA regulated gene expression; green arrows 1-MCP regulated gene expression; and red arrows ethylene regulated gene expression.

Supplementary Table 5.S1. List of primers used for qRT-PCR in broccoli.

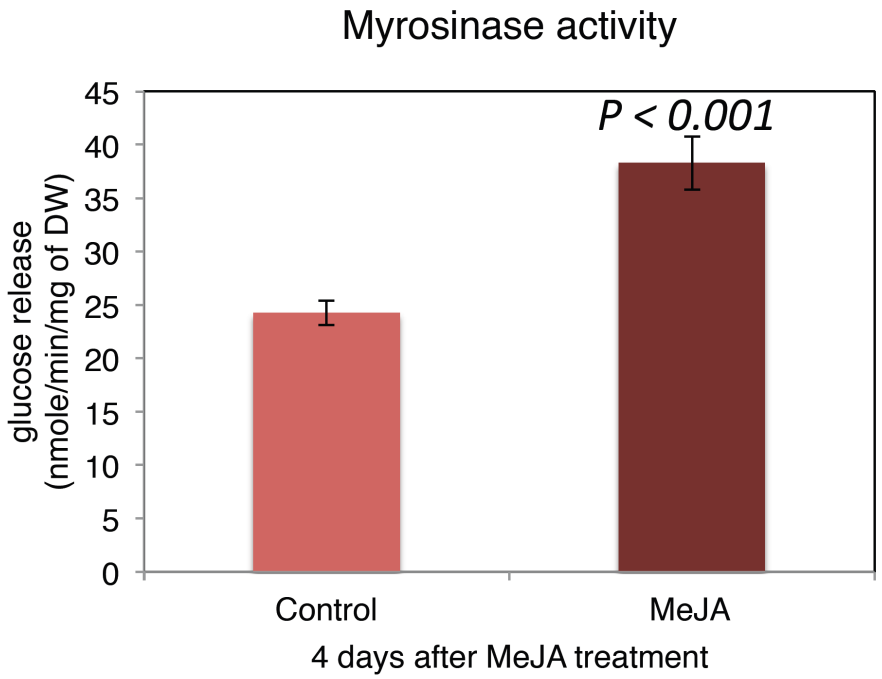
Target gene (Accession number)	Description	Forward Primer (5'-3')	Reverse Primer (5'-3')	Ref
Glucosinolate biosynthesis				
BoCYP79B2	*Brassica oleracea* var. *italica* cytochrome P450 (CYP79B2)	AGCCAAGTCCTTCTCAGTCG	ACGAGATAAACCGGAGATCG	(Kim, 2011)
BoCYP83B1	*Brassica oleracea* var. *italica* cytochrome P450 (CYP83B1)	ACGGAACCGAGATGAAGAGA	CTCTCTTGAGACGCGCACTA	(Kim, 2011)
BoCYP79F1	*Brassica oleracea* var. *italica* cytochrome P450 (CYP79F1)	TCCGATGGTTCTCATGTTGA	AACCGGATATCGCATGTTTC	(Kim, 2011)
BoCYP83A1	*Brassica oleracea* var. *italica* cytochrome P450 (CYP83A1)	TCAAGACGCAAGACGTCAAC	CAAGTGGTTCATCCCCATCT	(Kim, 2011)
Glucosinolate hydrolysis				
BoMYO (EU004075)	*Brassica oleracea* myrosinase (MYO)	AACGCCTTTCGTTACCCTCT	TCACCTTTCCACCAAATTCC	(Kim, 2011)
BoESP (DQ059298)	*Brassica oleracea* var. *italica* epithiospecifier (ESP) protein	CGAGAAGCTCACATGGCATA	CTTGGACGGAGAGATTGACC	(Kim, 2011)
BoESM1 (FJ830448.1)	*Brassica oleracea* epithiospecifier modifier 1 (ESM1)	ATTCCAAACGGAATCCCGCC	CCGGAGCCCCAAGAATAGAA	
Plant defense				
BoPR (EF423806)	*Brassica oleracea* var. *gemmifera* pathogenesis-related (PR) protein	CCACCATTGTTACACCTTGCT	AACCTTTGGGTCAACGAGAA	(Kim, 2011)
Chlorophyll catabolism				
BoPPH (OL386R)	*Brassica oleracea pheophytinase*	AGAGGTTATCGGTGAGCCA	GACGAGATGAGGATGGG	(Hasperué et al., 2013)
BoPaO (*AM388844.1)*	*Brassica oleracea pheophorbide a oxygenase*	GCGAAATTCCCGTCCAGAGTCTC	TTATCTCCGCCGTGCTCTTCTTC	(Hasperué et al., 2013)
qRT-PCR controls				
BoACT1 (AF044573)	*Brassica oleracea* actin (ACT1)	TCTCGATGGAAGAGCTGGTT	GATCCTTACCGAGGGAGGTT	(Kim, 2011)



Supplementary Figure 5.S1. Phylogenetic tree of epithiospecifier modifier 1 (ESM1) putatively associated with glucosinolate hydrolysis process based on the amino acids sequences deduced from the isolated cDNA sequence. *Brassica oleracea* consensus (cabbage, broccoli, and cauliflower), *Brassica rapa ssp. perkinesis* (ACO57702.1), *Brassica napus* (ACO57703.1), and *Arabidopsis thaliana ESM1* (XP_002882872.1) used to construct phylogenetic tree. The values in parenthesis are amino acid sequence similarity with *B. oleracea* consensus by using NCBI BLAST search. The tree was constructed using Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/).



Supplementary Figure 5.S2. Effects of pre-harvest MeJA and post-harvest 1-MCP treatments on ethylene production and respiration rate of broccoli florets at harvest and at 10, 20, and 30 days post-harvest storage at 4 °C. Different letters indicate significant differences among treatments based on Fisher's LSD test at $P \leq 0.05$. Mean ± SD (n=3).



Supplementary Figure 5.S3. Effect of MeJA treatment on broccoli floret myrosinase activity at harvest. Student's T-test was conducted to determine significance. Mean ± SD (n=3).

Supplementary Table 5.S2. Correlations among phytochemical, QR inducing activity and gene expression of broccoli florets during post-harvest storage at 4 °C. Pearson's correlation coefficients and *P*-values were calculated based on the means of each treatment over the duration of post-harvest storage sampling (n=16, except for ethylene production and hue angle: n=14). Significant positive and negative correlations were filled with pink and light green in the cell of the table, respectively based on $P \leq 0.05$.

No	Variable	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	Glucoraphanin																			
2	Glucobrassicin	0.678																		
3	Gluconasturtiin	0.115	0.392																	
4	4-methoxy glucobrassicin	-0.836	-0.578	0.333																
5	Neoglucobrassicin	-0.048	0.494	0.879	0.349															
6	Sulforaphane	-0.416	0.091	0.705	0.697	0.860														
7	PEITC	-0.356	-0.051	0.768	0.636	0.760	0.827													
8	I3C	0.338	0.703	0.349	-0.211	0.604	0.391	0.091												
9	NeoASG	-0.127	0.266	0.914	0.461	0.930	0.842	0.920	0.373											
10	NI3C	-0.225	0.214	0.854	0.513	0.909	0.862	0.929	0.333	0.980										
11	QR	-0.490	-0.009	0.710	0.716	0.804	0.865	0.857	0.208	0.874	0.899									
12	*BoCYP79B2*	0.254	0.512	0.029	-0.294	0.278	0.081	-0.192	0.676	0.008	-0.003	-0.091								
13	*BoCYP83B1*	0.234	0.622	-0.105	-0.366	0.204	0.042	-0.319	0.667	-0.121	-0.107	-0.197	0.882							
14	*BoCYP83A1*	0.255	0.427	-0.228	-0.404	0.067	-0.075	-0.275	0.687	-0.150	-0.120	-0.237	0.815	0.814						
15	*BoCYP79F1*	0.197	0.285	-0.365	-0.396	-0.089	-0.162	-0.293	0.558	-0.241	-0.186	-0.304	0.575	0.649	0.941					
16	Ethylene	0.440	0.714	0.248	-0.312	0.287	0.147	-0.168	0.609	0.038	-0.023	-0.140	0.843	0.732	0.856	0.760				
17	Hue Angle	0.602	0.752	0.119	-0.703	0.164	-0.178	-0.082	0.317	0.105	0.053	-0.073	0.176	0.275	0.427	0.488	0.296			
18	*BoPAO*	-0.005	-0.250	-0.017	0.169	-0.242	-0.108	-0.215	-0.269	-0.268	-0.296	-0.363	-0.098	-0.061	-0.228	-0.240	0.218	-0.641		
19	*BoPPH*	-0.269	-0.581	0.057	0.469	-0.180	0.057	0.045	-0.393	-0.090	-0.114	-0.099	-0.200	-0.325	-0.371	-0.382	-0.152	-0.868	0.867	
20	Total chlorophyll	0.590	0.396	-0.493	-0.811	-0.433	-0.602	-0.541	0.098	-0.497	-0.463	-0.603	0.161	0.317	0.460	0.557	0.150	0.719	-0.372	-0.642

CHAPTER 6

Pre-harvest Methyl Jasmonate Treatment Enhances Cauliflower Chemoprotective Attributes without a Loss in Post-harvest Quality[8]

6.1. Abstract

Methyl jasmonate (MeJA) treatment can significantly increase glucosinolate (GS) concentrations in *Brassica* vegetables and potentially enhance anticancer bioactivity. Although MeJA treatment may promote ethylene biosynthesis, which can be detrimental to post-harvest quality, there are no previous reports of its effect on cauliflower post-harvest quality. To address this, cauliflower curds in field plots were sprayed with either 0.1% Triton X-100 (control) or 500 μM MeJA solutions four days prior to harvest, then stored at 4 °C. Tissue subsamples were collected after 0, 10, 20, and 30 days of post-harvest storage and assayed for visual color change, ethylene production, GS concentrations, and extract quinone reductase inductive activity. MeJA treatment increased curd GS concentrations of glucoraphanin, glucobrassicin, and neoglucobrassicin by 1.5, 2.4, and 4.6 fold over controls, respectively. MeJA treated cauliflower showed significantly higher quinone reductase activity, a biomarker for anticancer bioactivity, without reducing visual color and post-harvest quality for 10 days at 4 °C storage.

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[8] Reprinted from Plant Foods for Human Nutrition, Ku, K.M., Choi J.-H., Kushad, M.M., Jeffery, E.H, and Juvik J.A., Pre-harvest methyl jasmonate treatment enhances cauliflower chemoprotective attributes without a loss in post-harvest quality 68 (2), 113-117. Copyright (2013) with kind permission from Springer Science and Business Media. The designated DOI is 10.1007/s11130-013-0356-y.

6.2. Introduction

Consumption of cruciferous vegetables, in particular subspecies of *Brassica oleracea* including broccoli, cauliflower, kale, and Brussels sprouts, provide putative cancer preventive effects as shown in epidemiological and animal carcinogenesis studies (van Poppel et al., 1999). The glucosinolates (GS) found in accessions of *B. oleracea* have been identified as potent cancer prevention agents because certain GS hydrolysis products induce biosynthesis and bioactivity of mammalian phase II detoxification enzymes such as glutathione S-transferases (GSTs), quinone reductase (QR) and UDP-glucuronosyl transferase that can enhance detoxification and elimination of carcinogens from the body (Cuendet et al., 2006). Up-regulation of QR activity has been used as a biomarker for cancer prevention because this enzyme is a catalyst for the conversion of quinones into stable and non-toxic hydroquinones, reducing oxidative cycling (Cuendet et al., 2006). Moreover, QR activity elevation with *in vitro* and *in vivo* systems has been shown to correlate with induction of other protective phase II enzymes such as the GSTs and provides a reasonable biomarker for the potential chemoprotective effect of phytochemical test agents active against cancer initiation (Cuendet et al., 2006).

Methyl jasmonate (MeJA), a plant signal transduction compound, can act as an elicitor to enhance GS biosynthesis. Previous research has shown that MeJA can significantly increase neoglucobrassicin and gluconasturtiin in broccoli (Kim and Juvik, 2011) but the magnitude of this effect is cultivar dependent. This suggests that among subspecies of *B. oleracea* biosynthesis of specific GS may show a differential response to MeJA treatment.

Cauliflower (*Brassica oleracea* L. var. *botrytis*) is known as a good source of glucobrassicin and minerals including iron, copper, and zinc (Singh et al., 2001). It is closely related to broccoli and is also a perishable horticultural crop. Consequently, visual color is an important factor in retailer and consumer evaluation of product quality and subsequent purchasing decisions (Dixon, 2007). Previous research reported that 1 mM MeJA post-harvest treatment significantly enhanced ethylene biosynthesis and promoted senescence in broccoli (Watababe et al., 2007), adversely affecting post-harvest product quality. There are no reports that evaluate pre-harvest MeJA treatment effects on cauliflower post-harvest quality and physiology. Thus, the objectives of this research are to evaluate the MeJA effect on glucosinolate composition and quinone reductase bioactivity of cauliflower curd extracts and post-harvest quality.

6.3. Materials and Methods

6.3.1. Plant Cultivation. 'Candid Charm' cauliflower (Territorial Seed Company, Cottage Grove, OR) seeds were germinated in 32 cell plant plug trays filled with sunshine® LC1 professional soil mix (Sun Gro Horticulture, Vancouver, British Columbia, Canada). Seedlings were grown in a greenhouse at the University of Illinois at Champaign-Urbana under a 25 °C/15 °C and 14 h/10 h: day/night temperature regime and with supplemental lighting. Three weeks after germination, seedlings were placed in ground beds to harden off for a week prior to transplanting into field plots at the University of Illinois South Farm (40˚ 04′ 38.89″ N, 88˚ 14′ 26.18″ E). Transplanting of cauliflower seedlings was done on 24 June 2011 and harvesting was conducted on 8 October 2011.

6.3.2. MeJA Treatments and Sample Preparation. An aqueous solution of 500 μM MeJA (Sigma-Aldrich, St. Louis, MO) and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) was sprayed on plant leaf tissues to the point of runoff four days prior to harvest at commercial maturity. For the control group, an aqueous solution of 0.1% Triton X-100 solution was applied to plants. Harvested cauliflower curds from both control and MeJA treatments were immediately transported to the laboratory, divided into branchlets and stored at 4 °C. Three random subsamples of cauliflower branchlets in each treatment were collected for each of the following assay dates: 0, 10, 20, and 30 days. Pictures of cauliflower curds showing visual quality from each assay date are presented in Figure 6.1. After measuring ethylene production and hue degree, a measure of visual color quality, each sample was freeze-dried. Freeze-dried cauliflower curd tissue of each sample was finely ground with a commercial coffee grinder. The ground freeze-dried cauliflower samples were stored at -20 °C prior to GS quantification and quinone reductase bioactivity assay.

6.3.3. Hue Degree Measurement. Sample post-harvest visual quality was measured by using a LabScan XE colorimeter (Hunter Associates Laboratory, Reston, VA, USA) generating values for a* (redness and greenness), and b* (yellowness and blueness). The instrument was calibrated with a standard white and black tile. Average of four different cauliflower branchlets were recorded in each replication. Hue degree (h°) was calculated as $h° = \tan^{-1} (b*/a*)$ when $a*>0$ and $b*>0$, or as $h° = 180° - \tan^{-1} (b*/a*)$ when $a<0$ and $b>0$.

6.3.4. Determination of Ethylene Production and Respiration Rate. Three random subsamples (300 g each) of cauliflower curd branchlets from each treatment and post-harvest storage date were placed into 3 L jars and sealed with a silicon rubber cap for 1 h at 20 °C. 1mL of headspace gas in the jar was injected into a GC (Perkin Elmer AutoSystem Gas Chromatograph, Waltham, MA, USA) equipped with an activated alumina column and flame ionization detector. The temperature of the injector, detector and column was 100 °C, 200 °C and 80 °C, respectively. The flow rate of carrier gas helium, hydrogen, and air were 70, 30, and 300 mL/min, respectively. Samples were calibrated a using 10 µL/L ethylene standard. The rate of ethylene production was expressed as µL of ethylene/kg of sample/h.

6.3.5. Determination of Sample GS Concentrations. Freeze-dried broccoli powder (0.2 g) and 2 mL of 70% methanol were added to 10 mL tubes (Nalgene, Rochester, NY) and heated on a heating block at 95 °C for 10 min. After cooling on ice, 0.5 mL benzylglucosinolate (1 mM) was added as internal standard (POS Pilot Plant Corp, Saskatoon, SK, Canada), mixed, and centrifuged at 3,000 × g for 15 min at 4 °C. The supernatant was saved and the pellet was re-extracted with 2 mL 70% methanol at 95 °C for 10 min and the two extracts combined. A subsample (1 mL) from each pooled extract was transferred into a 2-mL microcentrifuge tube. Protein was precipitated with 0.15 mL of a 1:1 mixture of 1 M lead acetate and 1 M barium acetate. After centrifuging at 12,000 × g for 1 min, each sample was then loaded onto a column containing DEAE Sephadex A-25 resin (Sigma-Aldrich, St. Louis, MO) for desulfation with arylsulfatase (*Helix pomatia* Type-1, Sigma-Aldrich, St. Louis, MO) for 18 h and the desulfo-GS eluted. One

hundred μL of each sample were injected on to a HPLC. Quantification of GS using high-performance liquid chromatography was performed using a protocol described by Brown et al. (2002).

6.3.6. Quinone Reductase (QR) Inductive Activity. For the QR assay, 50 mg of cauliflower curd powder from each sample were suspended in 1 mL of water in the absence of light for 24 h at room temperature in a sealed 2 mL microcentrifuge tube (Fisher Scientific, Waltham, MA) to facilitate GS hydrolysis by endogenous myrosinase. Slurries were then centrifuged at 12,000 × g for 10 min and supernatants filtered through a 0.45 μm nylon membrane and diluted to 1% final concentration in the QR activity assays. The QR induction activities of different samples were determined by means of the protocol described by Prochaska and Santamaria (1988). Hepa1c1c7 murine hepatoma cells (ATCC, Manassas, VA) were grown in alpha-minimum essential medium (MEM), enriched with 10% heat and charcoal-inactivated fetal bovine serum and maintained at 37 °C in 95% ambient air and 5% CO_2. After 24 h cells were exposed to the different sample extracts (0.25% final concentration in 200 μL of media) in new media for a further 24 h. Treated cells were rinsed with phosphate buffer at pH 7.4, lysed with 50 μL 0.8% digitonin in 2 mM EDTA, incubated and agitated for 10 min. A 200-μL aliquot of reaction mix [8] was added to the lysed cells. Readings were made at five time points, 50 s apart, using a μQuant microplate reader (Bio-Tek Instruments, Winooski, VT) at 610 nm. Immediately after completion of the readings, 50 μL of 0.3 mM dicumarol in 25 mM Tris buffer was added into each well, and the plate was read again (five time points, 50 s apart) to determine non-specific MTT (methylthiazolyldiphenyl-tetrazolium bromide)

reduction. Total protein content was measured by the BioRad assay (Bio-Rad, Hurcules, CA, USA) following the instruction. Activity was expressed as QR specific activity (nmol MTT reduced/mg/min) ratio of treated to control cells.

6.3.7. Statistical Analysis. Statistical analyses were conducted using the JMP 9 software (SAS institute Inc., Cary, NC). Student's T-tests was conducted for significance at $P = 0.05$. Pearson correlation was conducted between QR inductive activity and GS concentrations based on the mean values of each treatment across post-harvest storage dates. All sample analyses were conducted in triplicate. The results are presented as Means ± SD.

6.4. Results and Discusssion

6.4.1. Change in Hue and Ethylene Production for Cauliflower Curds Subjected to MeJA Treatments. Treatment with 500 μM MeJA did not significantly affect either sample hue degree (90° = yellow and 0° = red) or ethylene production of cauliflower curds at 4 °C post-harvest storage temperature (Table 6.1). Visual color of cauliflower is an important factor in consumers' purchasing decisions (Dixon, 2007). MeJA treatment did not alter sample hue degree, indicating that these treatments did not negatively impact cauliflower visual quality over the course of post-harvest storage (Figure 6.1).

6.4.2. Response in GS Levels to MeJA Treatments. Treatment with MeJA significantly increased glucoraphanin, glucobrassicin, and neoglucobrassicin concentrations in cauliflower curd samples. At harvest, the relative increase of these GS was 1.53, 2.38,

and 4.61 fold over controls, respectively (Figure 6.2). The enhanced GS concentrations observed in the MeJA treatments compared to controls were maintained over the duration of post-harvest storage. Previous reports indicate jasmonate treatments increased gluconasturtiin and neoglucobrassicin concentrations in broccoli florets (Kim and Juvik, 2011) and glucoiberin, progoitrin, sinigrin, and gluconapin concentrations in cabbage (Fritz et al., 2010). Both jasmonate (JA) and MeJA induced significant increases (up to 20-fold) in the concentration of specific indolyl GS in cotyledons and leaves of oilseed rape, *Brassica napus* (the GS, glucobrassicin) and *B. rapa*, (the GS, 4-hydroxy glucobrassicin) and the mustard *B. juncea* (both) (Bodnaryk, 1994). The different responses to MeJA treatment observed in this study suggest that variation in GS response to MeJA is species specific. Treatment with MeJA increased glucoraphanin in our study, implying aliphatic GS can be increased by MeJA treatment, as was observed for cabbage (Fritz et al., 2010).

6.4.3. QR Induction Activity of Cauliflower Treated with MeJA. QR induction activity of cauliflower curd extracts treated with MeJA was significantly increased compared to controls at 4 °C postharvest storage (Figure 6.3). The increased QR activity may be due to the higher concentrations of glucoraphanin and/or glucobrassicin for which hydrolysis products have been reported to induce QR (Zhang et al., 1992; Zhu and Loft, 2003). The enhanced QR activity in the MeJA treated group was maintained through thirty days at 4 °C but only significantly higher than controls at 0 and 10 days postharvest storage (Figure 6.3).

6.4.4. Correlations between QR Induction Activity and Enhanced GS. Significant correlations were observed between extract induced QR activity and the GS concentrations of glucoraphanin ($r = 0.831$, $P = 0.011$), glucobrassicin ($r = 0.893$, $P = 0.003$), and neoglucobrassicin ($r = 0.807$, $P = 0.016$) in cauliflower curd samples during postharvest storage at 4 °C (Figure 6.4). Hydrolysis products of glucoraphanin (sulforaphane) and glucobrassicin (indole-3-carbinol, I3C) are known as relatively strong and moderate QR inducers, respectively (Zhang et al., 1992; Zhu and Loft, 2003). This implies that GS concentrations in cauliflower increased by MeJA treatments may enhance the induction of QR activity in Hepa1c1c7 murine hepatoma cells.

N-methoxy-indole-3-carbinol (NI3C) derived from the hydrolysis of neoglucobrassicin has been shown to exert an antiproliferative effect on a human colon cancer cell line (Neave et al., 2005). It has been reported that a combination of I3C and NI3C in a 1:1 ratio has stronger antiproliferative activity on human colon cancer cells than either compound alone (Bitir et al., 2011). The MeJA treatment changed the ratio of I3C and NI3C from approximately 6:1 in control samples to 3:1 in MeJA treated samples, which being closer to the 1:1 ratio should favor increased inhibition of colon cancer cell proliferation. Recent research has also reported that NI3C may inhibit QR activity induced by sulforaphane in HepG2 cells (Haack et al., 2010). In contrast, in our study we observed that increased neoglucobrassicin concentrations did not block increases in QR and were positively associated with enhanced QR activity. This contrasting observation may be due to the different cell line used for our QR assays. It is also possible that the increased neoglucobrassicin observed in our experiments was at too low a concentration

to interfere with QR activity induced by sulforaphane. These contradictory results suggest a need for additional research on the role of NI3C in cancer prevention.

To our knowledge, this is the first study investigating the influence of pre-harvest MeJA treatments on the postharvest quality and anticancer bioactivity of cauliflower. Pre-harvest treatments of 500 μM MeJA were observed to enhance QR, likely due to increasing glucoraphanin, glucobrassicin, and/or neoglucobrassicin cauliflower curd concentrations, without a detrimental effect on the maintenance of postharvest visual quality. Therefore, MeJA treatment can be a useful elicitor to enhance the potential health-promoting properties of cauliflower, without, a loss of postharvest quality.

Table 6.1. Effect of pre-harvest MeJA treatment on visual color change (hue degree) and ethylene production of cauliflower curds during postharvest storage at 4 °C.

Hue degree				
Storage days at 4 °C	0	10	20	30
Control	91.9 ± 0.8	89.9 ± 0.8	89.6 ± 1.2	87.9 ± 0.8
MeJA	91.0 ± 0.6	89.9 ± 0.1	89.9 ± 0.3	88.1 ± 0.8
Ethylene production ($\mu L\ Kg^{-1}\ hr^{-1}$)				
Storage days at 4 °C	0	10	20	30
Control	0.42 ± 0.11	1.10 ± 0.10	0.74 ± 0.24	0.67 ± 0.10
MeJA	0.32 ± 0.09	0.90 ± 0.10	0.44 ± 0.25	0.82 ± 0.43

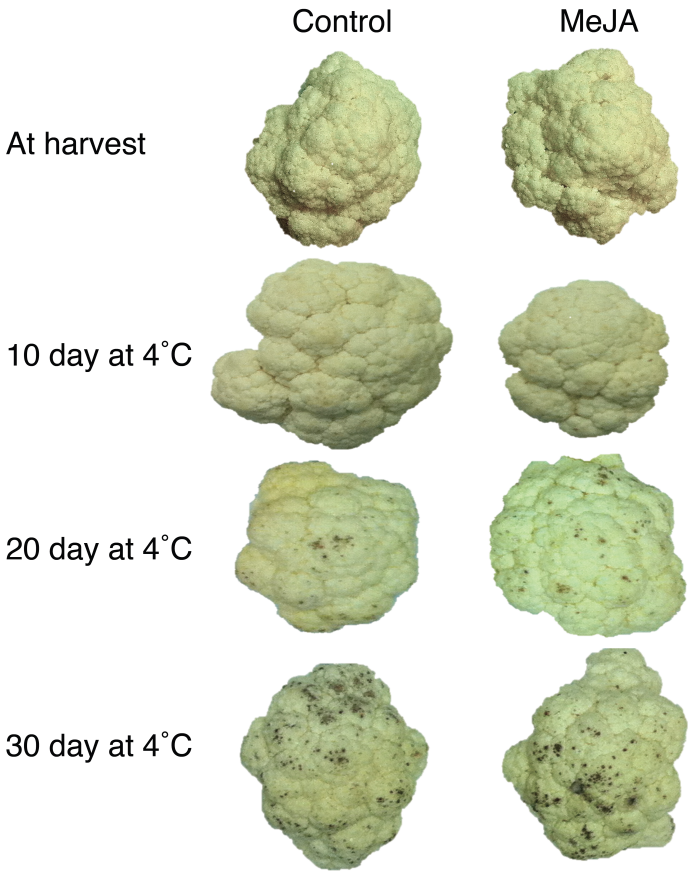

Figure. 6.1. Representative samples of cauliflower curds with or without preharvest MeJA (500 µM) treatment during postharvest storage at 4 °C at each of the assay dates: 0, 10, 20 and 30 days.

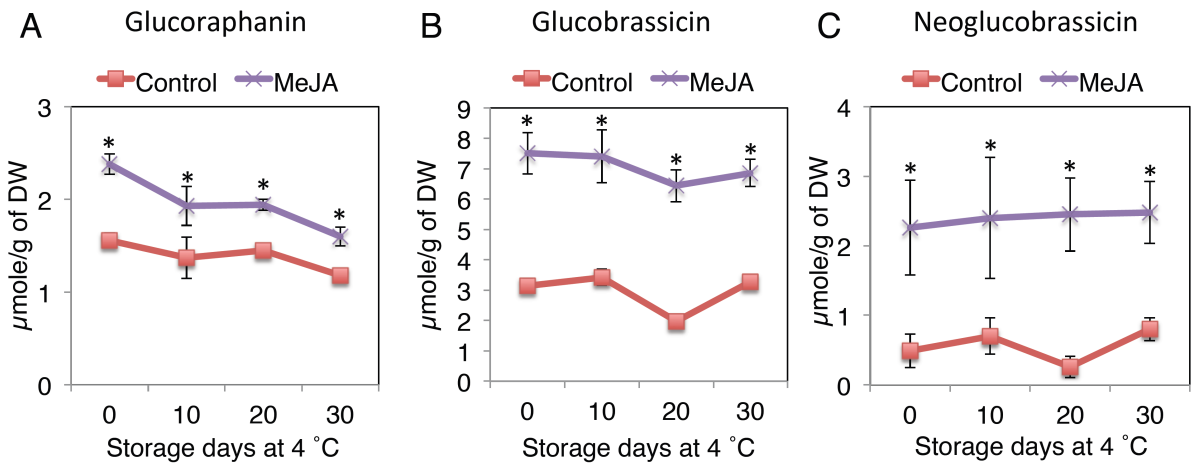

Figure 6.2. Effect of pre-harvest MeJA treatment on glucosinolates concentrations of cauliflower curds and glucosinolate concentration change during postharvest storage at 4 °C. A: glucoraphanin B: glucobrassicin, and C: neoglucobrassicin.

Data are means ± SD (n=3). Asterisks indicate significant differences between treatments at $P \leq 0.05$.

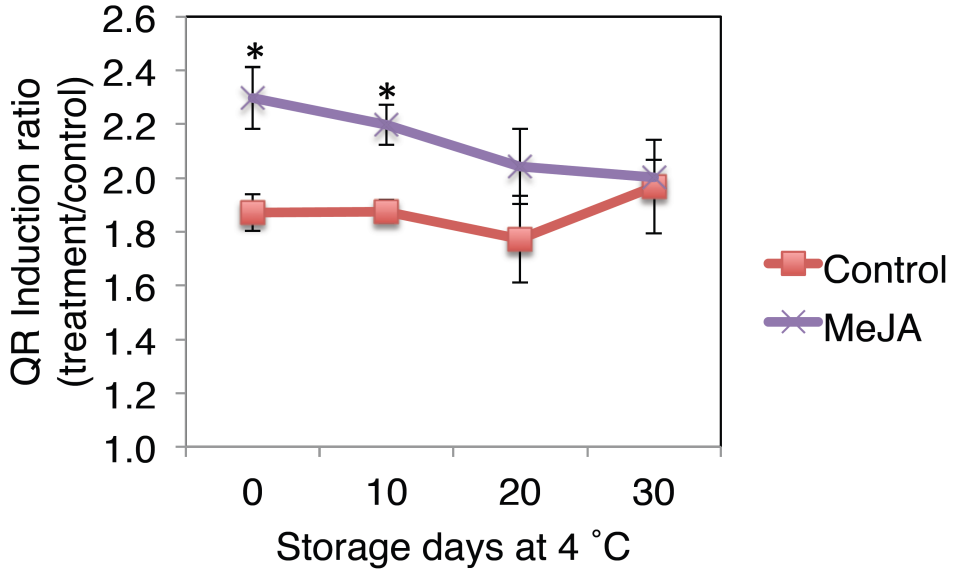

Figure 6.3. Effect of pre-harvest MeJA (500 µM) treatment on QR activity of cauliflower curd extracts during postharvest storage at 4 °C.

Data are means ± SD (n=3). Asterisks indicate significant differences between treatments at $P \leq 0.05$.

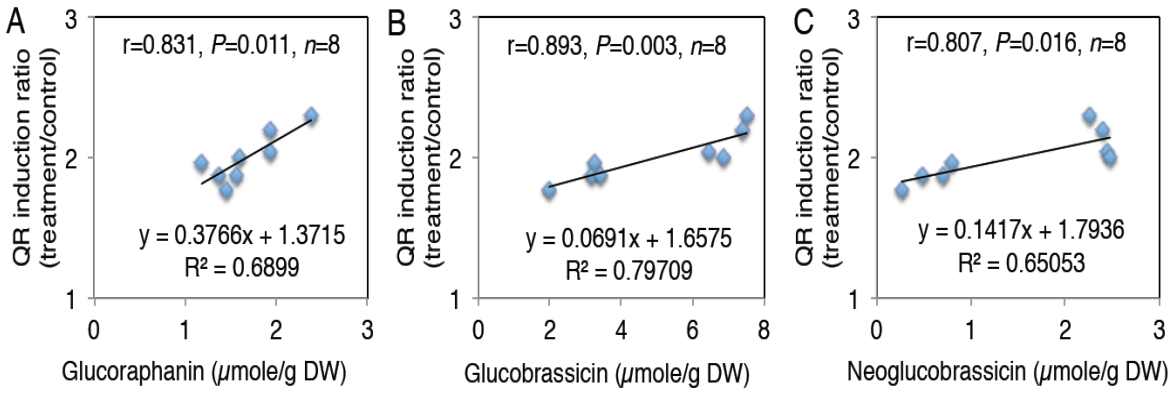

Figure 6.4. Correlations between QR inductive activity and enhanced glucosinolates during postharvest storage at 4 °C.

Pearson's correlation coefficients and p-values were calculated based on the means of each treatment over the duration of postharvest storage sampling (n=8).

CHAPTER 7

Optimization of MeJA Application to Broccoli Florets for Enhancing Potential Human Health Promoting Compounds

7.1. Abstract

Isothiocynates derived from glucosinolates (GS) in brassica vegetables have been reported to induce quinone reductase activity, an important cancer chemopreventative biomarker. Methyl jasmonate (MeJA) has been shown to increase GS and reduce insect damage in broccoli. For these reasons we have conducted several studies to determine application protocols that maximize accumulation of GS and other phytochemicals in broccoli florets. We investigated the effect of Triton X-100 surfactant and varying MeJA application concentrations (0, 62.5, 125, 250, and 500 μM), number, and application date in days prior to harvest of broccoli florets of the cultivar 'Green Magic'.

MeJA application four days prior to harvest generated broccoli florets with the highest concentrations of GS. Although a single application of 250 μM MeJA significantly increased GS concentrations in broccoli florets, two consecutive days of treatment (four and three days prior to harvest) of 250 μM MeJA further increased total GS concentrations (primarily neoglucobrassicin) and QR activity four days prior to harvest. In addition to GS levels the flavonoids, quercetin and kaempherol, were observed to show minor but significant increases when 62.5 μM MeJA was applied four days prior to harvest, with higher treatment concentrations decreasing flavonoids and total phenolics. With increasing treatment concentrations of MeJA to broccoli florets gluconasturtiin, neoglucobrassicin, and sulforaphane floret concentrations were gradually

increased. Concentration of these compounds also showed positive correlations with QR inductive and nitric oxide production inhibitory activity. These application protocols were found to maximize GS concentrations and putatively enhance the health promoting properties of broccoli florets.

7.2. Introduction

Jasmonic acid (JA) and its derivatives are signal transduction compounds, associated with the regulation of plant defense against herbivores. JA was originally isolated from a pathogenic fungus, *Lasiodiplodia theobromae* (Aldridge et al., 1971). However, methyl jasmonic acid (MeJA) was firstly isolated from *Jasminium grandiflorum* L. flower before JA was discovered (Demole et al., 1962). MeJA is used as a fragrance in many products (Scognamiglio et al., 2012). The LD_{50} of MeJA was found to be greater than 5 g/kg administered by to Sherman Wistar rats (Scognamiglio et al., 2012) making it safer to consume than table salt (LD_{50} = 3 g/kg).

Research has shown the potential of JA application to *Brassica* vegetables for insect management (Brader et al., 2006; Bruinsma et al., 2007; McEwen, 2011) and health promotion. According to McEwen (2011), MeJA application deterred feeding by flea beetles (*Phyllotreta* spp.) and lepidopteran species (*Pieris rapae* and *Trichoplusia ni*). MeJA application can enhance total phenolic concentrations in radish (Kim et al., 2006a) and buckwheat sprouts (Kim et al., 2011) and sweet basil (Kim et al., 2006b). Several studies have shown that MeJA mediated increases in flavonoids or phenolics can enhance antioxidant, antiproliferative, and anti-adipogenic activity of sweet basil, buckwheat, and blackberry (Kim et al., 2006b; Lee et al., 2013; Wang et al., 2008).

Pérez-Balibrea et al. (2011) reported that MeJA increased glucosinolate concentrations (GS) in broccoli sprouts. Fritz et al. (2010) also reported that JA increased glucoiberin, progoitrin, sinigrin, and gluconapin in cabbage. For these reasons, JAs could be used in vegetable production fields.

GS are secondary metabolites mainly found in almost all plants of the order *Brassicales*. The myrosinase-derived hydrolysis products of GS in broccoli have been shown to enhance phase II detoxifying enzymes and other health-promoting activities such as anti-inflammation (Bonnesen et al., 2001; Lin et al., 2008; Zhang et al., 1994). Among various hydrolysis products of GS, isothiocyanates including sulforaphane, allyl isothiocyanate, and phenethyl isothiocyanate have anti-carcinogenic activity and induce phase II detoxifying enzymes including glutathione S-transferase (GST) and quinone reductase (QR) (Kang and Pezzuto, 2004; Rose et al., 2000; Zhang et al., 1994). Up-regulation of QR activity has been used as a biomarker for cancer prevention because this enzyme is a catalyst for the conversion of quinones into stable and non-toxic hydroquinones, reducing oxidative cycling (Talalay et al., 1995).

Nitric oxide (NO) is an essential mammalian signaling molecule that mediates many physiological processes, including vasodilatation, host-defense, platelet aggregation, and iron metabolism. However, the body of evidence suggests that elevated levels of NO produced during chronic inflammation can attribute to a variety of pathological disorders, including cancer (Cheng et al., 2010; Hofseth et al., 2003). NO and NO-derived reactive nitrogen species induce oxidative and nitrosative stress which results in DNA damage and inhibition of DNA repair enzymes (Hofseth et al., 2003; Muntane and la Mata, 2010). NO is synthesized by three differentially gene-encoded NO

synthase genes (NOS) in mammals: neuronal NOS (*nNOS* or *NOS-1*), inducible NOS (*iNOS* or *NOS-2*) and endothelial NOS (*eNOS* or *NOS-3*). The expression of *NOS-2* and *NOS-3* has been found to be increased in a variety of human cancers (Muntane and la Mata, 2010). Thus, inhibition of excessive NO production can be a good strategy for anti-inflammation and its association with carcinogenesis.

Previous research in our lab observed a broccoli genotype by MeJA treatment interaction in greenhouse studies (Kim and Juvik, 2011). However, there exists no standardized protocol for MeJA application on *Brassica* species that optimize beneficial phytochemical concentrations. The objectives of this study were to (1) examine application concentration and harvest time of broccoli in order to optimize efficacy, (2) compare the effect of different plant target tissues for application and (3) examine the effect of multiple applications of MeJA and the interaction with different surfactants.

7.3. Materials and Methods

7.3.1. Broccoli Cultivation. The cultivar used for these experiments was the F_1 hybrid broccoli cultivar 'Green Magic' (Sakata Seed Co., Morgan Hill, CA). Seeds were germinated in small pots filled with sunshine® LC1 professional soil mix. Seedlings were grown in a greenhouse at the University of Illinois at Champaign-Urbana under a 25 °C/15 °C and 14 h/10 h : day/night temperature regime and with supplemental lighting. Thirty days after germination, seedling trays were placed in ground beds to harden off for a week prior to transplanting into field plots at the University of Illinois South Farm (40˚ 04′ 38.89″ N, 88˚ 14′ 26.18″ E). Experimental design was a randomized complete block with three replicates. The experiment plot was surrounded by a guard row to avoid border

effects. Transplanting of broccoli seedlings was conducted on June 11, 2010 and June 13, 2011. Harvesting broccoli occurred in mid August to early September in both 2010 and 2011.

7.3.2. MeJA Spray Treatments.

7.3.2.1. *Experiment 1.* Determination of the appropriate Harvest Date after MeJA Treatment. To determine optimum harvest date, solutions of 250 μM of MeJA (Sigma-Aldrich, St. Louis, MO) containing 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) where sprayed on all aerial portions of 'Green Magic' broccoli plants 1, 2, 4, 6, and 8 days prior to harvest of heads at commercial harvest maturity. Each plant received approximately 300 ml of solution over all aerial portions until leaves and heads were completely saturated. Using these broccoli samples, GS concentration was measured as described below.

7.3.2.2. *Experiment* 2. Multiple MeJA Spray Treatments, GS concentrations, and QR Activity. The effect of multiple spray treatments were tested where treatments included, spraying once (4 days before harvest) or twice (4 and 3 days before harvest) or three times (4, 3, and 2 days before harvest) over all aerial portions of broccoli before harvesting heads at commercial maturity. This experiment was done in 2010 and 2011. Using these broccoli samples, GS concentration and QR activity were measured as described below.

7.3.2.3. *Experiment 3.* Optimal Concentrations of MeJA in applications. To determine optimum concentrations for single application, 62.5, 125, 250, and 500 µM of MeJA solutions containing 0.1% Triton X-100 were sprayed on broccoli aerial plant tissues four days prior to harvest at commercial maturity. Using these broccoli samples, GS, flavonoid, total phenolics, and sulforaphane concentration as well as QR activity and NO production inhibitory activities were measured as described below.

7.3.2.4. *Experiment 4.* MeJA Applications to Different Plant Tissues. In order to determine the effect of MeJA on specific plant tissues, 250 µM MeJA containing 0.1% Triton X-100 was applied exclusively to leaf tissue only, to both leaf and head tissues, and poured onto the soil surrounding the crown of the tap root using same volume of MeJA (300 mL) four days prior to harvest at commercial maturity. 0.1% Triton X-100 applied to both leaf and head tissues was used as a control. Each of these treatments were applied to five different plants in each of three replicates.

7.3.2.5. *Experiment 5.* Influence of Triton X-100 on Broccoli Head Color. In order to determine if the surfactant or solvent effect had any effect in the control treatments, solutions of 0.1% of Triton X-100 were tested on 'Green Magic' broccoli multiple times as described above in experiment 2. 0.1% of ethanol with MeJA also was applied to 'Green Magic' as a control. After harvest product color of broccoli florets was measured by colorimeter as described below.

At the commercial maturity, five broccoli heads were harvested for each replicate in each of the above experiments, in a randomized complete block design. Broccoli heads

were frozen in liquid nitrogen, and stored at -20 °C prior to freeze-drying. After lyophilization, samples were ground into fine powder and stored at -20°C until chemical analysis and bioactivity measurement.

7.3.3. Determination of GS Content. GS in lyophilized tissues were extracted and analyzed by high-performance liquid chromatography using a reverse phase C18 column as described by Kim and Juvik (2011). GS were desulfated with sulfatase solution (Sigma-Aldrich, St. Louis, MO) in columns containing DEAE Sephadex A-25 resin (Sigma-Aldrich, St. Louis, MO), and eluted desulfo-GS were separated on a HPLC system consisting of a DIONEX GP40 gradient pump, with a AD20 variable UV detector set at λ229 nm wavelength, auto-sampler, all-guard ™ cartridge precolumn (Alltech, Lexington, Kentucky), and a LiChosphere® 100 RP-18 column (Merck, Darmstadt, Germany). The type and amount of GS in each sample were calculated in comparison to certified GS levels in a standard rapeseed reference material (BCR 367, Commission of the European Community Bureau of References, Brussels, Belgium). GS were quantified with benzylglucosinolate (POS Pilot Plant Corp, Saskatoon, SK, Canada) as an internal standard using UV response factors for different types of GS determined by Wathelet et al. (1995). The identification of intact and desulfo-GS profiles were validated by LC-tandom MS using a Waters 32 QT of Ultima spectrometer coupled to a Waters 1525 HPLC system and full scan LC-MS using a Finnigan LCQ Deca XP, respectively. The molecular ion and fragmentation patterns of individual intact and desulfo GS were matched with the literature for GSs identification (Tian et al., 2005; Velasco et al., 2011).

7.3.4. Quinone Reductase (QR) Activity. Hepa1c1c7 murine hepatoma cells were grown in alpha-minimum essential medium (MEM), enriched with 10% heat and charcoal-inactivated fetal bovine serum and maintained at 37 °C in 95% ambient air and 5% CO_2. The cells were split every 4 days with a split ratio of 7. Cells with 80-90% confluence were plated into 96-well plates (Costar 3595, Corning Inc, Corning, NY), 1×10^4 cells per well, and incubated for 24 h in antibiotic-enriched media (100 units/mL penicillin, 100 μg/mL streptomycin). The QR induction activities of different samples were determined by means of QR assay (Prochaska & Santamaria, 1988). The cells were grown in 96-well plates (Costar 3595, Corning Inc, Corning, NY) for 24 h and then exposed to the different samples for 24 hr. Growth media and 1 μM b-naphthoflavone were used as negative and positive controls, respectively. Treated cells were rinsed with phosphate buffer at pH 7.4, lysed with 50 μL 0.8% digitonin in 2 mM EDTA, incubated and agitated for 10 min. A 200-μL aliquot of mixed solution [74 mL of 25 mM Tris buffer; 50 mg of BSA; 0.5 mL of 1.5% Tween-20 solution; 0.5 mL of thawed cofactor solution (92.7%, 150 mM glucose-6-phosphate; 6.15%, 4.5 mM NADP; 1.14%, 0.75 mM FAD in Tris buffer]; 150 units of glucose-6-phosphate dehydrogenase; 22.5 mg of MTT (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide); and 75 μL of 50 mM menadione in acetonitrile) was added into lysed cells. Readings were made at five time points, 50 s apart, using a μQuant microplate reader (Bio-Tek Instruments, Winooski, VT) at 610 nm. Immediately after completion of the readings, 50 μL of 0.3 mM dicumarol in 25 mM Tris buffer was added into each well, and the plate was read again (five time points, 50 s apart). Total protein content was measured by BioRad assay (Bradford, 1976).

QR induction activity was expressed as the specific activity (nmol MTT reduced/mg/mins) ratio of treated to control cells.

7.3.5. Analysis of Sulforphane Production. The extraction and analysis of sulforphane, the bioactive hydrolysis product of glucoraphanin was carried out according to previously published methods, with some modifications (Wilson et al., 2011). 75 mg of broccoli powder was suspended in 1.5 mL of water in the absence of light for 24 h at room temperature in a sealed 2 mL microcentrifuge tube (Fisher Scientific, Waltham, MA) to facilitate GS hydrolysis by endogenous myrosinase. Slurries were then centrifuged at 12,000 × g for 5 min and supernatants was decanted into a 2 mL microcentrifuge tube. 20 µL of benzyl isothiocyanate (0.5 mg/mL) was added as the internal standard to quantify SF with 0.5 mL of methylene chloride. Tubes were shaken vigorously before being centrifuged for 2 min at 9,600 g. The methylene chloride layer (200 µL) was transferred to 350 µL flat bottom insert (Fisher Scientific, Pittsburgh, PA) in a 2 mL HPLC autosampler vial (Agilent, Santa Clara, CA) for mixing with 100 µL of a reagent containing 20 mM triethylamine and 200 mM mercaptoethanol in methylene chloride. The mixture was incubated at 30 °C for 60 min under constant stirring, and then dried under a stream of nitrogen. The residue containing the sulforphane derivative (sulforphane-mercaptoethanol derivative) was dissolved in 200 µL of acetonitrile /water (1:1) (v/v), and 10 µL of this solution injected onto a Agilent 1100 HPLC system (Agilent, Santa Clara, CA), equipped with a G1311A bin pump, a G1322A vacuum degasser, a G1316A thermostatic column compartment, a G1315B diode array detector and an HP 1100 series G1313A autosampler. Extracts were separated on a Eclipse XDB-

C18 column (150 × 4 mm, particle size 5 μm, Agilent, Santa Clara, CA) with a C18 all-guard™ cartridge pre-column (Alltech, Lexington, KY). Mobile phase A was water and B methanol. Mobile phase B was 0% at injection, increasing to 10% by 10 min, 100% at 35 min, and held 5 min, then decreased to 0% by 50 min. Flow rates were kept at 0.8 mL/min. The detector wavelength was set at 227 and 271 nm.

7.3.6. Determination of Flavonoid Concentrations. 200 mg samples of broccoli powder were weighed and added with 4 mL of 60% methanol to a 15 mL conical centrifuge tube (BD Falcon, San Jose, CA). After a 20 min extraction at 90 °C, the tubes were centrifuged and 1.2 mL of extract supernatant transferred to 2 mL microcentrifuge tube (Fisher Scientific, Waltham, MA) to which 0.24 mL of 6N HCl was added. The tubes were then heated at 90°C for 2 hr. The extract was cooled, filtered through a 0.45 μm Whatman (Clifton, NJ) membrane filter before injection into the HPLC (Kurilich et al. 2002). Hydrolyzed flavonoid concentrations were evaluated using an Agilent 1100 HPLC system (Agilent, Santa Clara, CA), equipped with a G1311A bin pump, a G1322A vacuum degasser, a G1316A thermostated column compartment, a G1315B diode array detector and an HP 1100 series G1313A autosampler. Samples were analyzed on a Supercosil™ LC-18 (250 × 4 mm, particle size 5 μm) (Supelco Inc., Bellefonte, PA) with a C18 all-guard ™ cartridge precolumn (Alltech, Lexington, KY). The mobile phase A was water and mobile phase B was methanol with 0.1% acetic acid. The mobile phase B started at 0% in the initial stage, increasing to 60% by 15 mins, 80% at 20 mins, and 100% at 25 mins, and held 5 mins with 2 mL/min of flow rate, then decreased to 0% at 35 mins. Prior flow rates were kept at 1 mL/min. The detector wavelength set at was λ360 nm.

7.3.7. Determination of Total Polyphenol Content (TPC). 200 mg samples of fine powder were extracted with 4 mL of 70% methanol solvent at 95 °C for 10 min. After 5 min cooling on ice, the extract was centrifuged at 1500 g for 10 min. Subsequently, 1 mL of the supernatant was transferred to a 2 mL microcentrifuge tube (Fisher Scientific, Waltham, MA). After centrifuging, the supernatant was used to quantify TPC. TPC was analyzed using the method of Ku et al. (2010). The assay conditions were as follows: a 10 μL sample was added to 0.2 N Folin-Ciocalteu's phenol reagent (100 μL) in 96 well plates. After 3 min, 90 μL of a saturated sodium carbonate solution was added to the mixture and subsequently incubated at room temperature for 1 hr. The resulting absorbance of the mixture was measured at 630 nm using a BioTek EL 808 microplate reader (Biotek Instruments Inc., Power Wave XS, Winooski, VT). The total polyphenol content was calculated on the basis of a standard curve with gallic acid. The standard solution concentrations ranged from 31.25 to 500 μg/mL. Results were expressed in milligrams of gallic acid equivalent (GAE) per 100 g of dried broccoli. Samples were assayed in triplicate.

7.3.8. Inhibitory Effect on Nitric Oxide (NO) Production Activated by Lipopolysaccharide (LPS). The mouse macrophage cell line, Raw 264.7, was grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Hyclone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone), 4 mM glutamine and penicillin (100 U/ml) / streptomycin (100 μg/ml) (Gibco BRL, Grand Island, NY). Cell suspensions of 2×10^4 cells/well were cultured in a flat-bottom 96-well plate for 2 days. Thereafter, 200 μL of medium was replaced with fresh high-glucose, phenol red free

DMEM (Gibco) containing either LPS (1 µg/ml, E. coli, serotype 0.55:B5) alone or LPS with various concentrations of broccoli MeJA treated samples. Cultured cells with or without LPS served as a positive or negative control, respectively. The production of NO was determined by measuring nitrite in the culture medium using the Griess reaction. Briefly, 100 µL aliquots of medium were incubated with an equal volume of modified Griess reagent (50 µL of 1% sulfanilamide in 5% H_3PO_4 and 50 µL of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in water). After 10 min, the absorbance was measured at 570 nm using a microplate reader.

7.3.9. Browning Discoloration Measurements by Hunter Colorimeter. For purposes of data analysis and data interpretation, it is important to have a reliable measure of color change. Hue is one property of color. Hue is how we perceive an object's color (red, orange, green, blue, etc.) (http://personal.uncc.edu/lagaro/cwg/color/color_percept.html). Hue degree allows us to get digitalized data by using quadrant space for color (0° = red, 90° yellow, 180° = green, 270° = blue). Superficial color was measured by using a LabScan XE colorimeter (Hunter Associates Laboratory, Reston, VA, USA) generating values for a* (redness and greenness). The instrument was calibrated with a standard white and black tile. An average from four different broccoli (branchlets) florets were recorded in each replication.

7.3.10. Statistical Analysis. Statistical analyses were conducted using the JMP 10 software (SAS institute Inc., Cary, NC). Data was subjected to analysis of variance (ANOVA model one-way). A source of variation was MeJA treatment. The means were

compared by the least significant differences (LSD) test at a significance level of $P = 0.05$. Pearson correlation was conducted between bioactivities and phytochemicals.

7.4. Results and Discussion

7.4.1. *Experiment 1.* Determination of the appropriate Harvest Date after MeJA Treatment. In order to determine the harvest date after MeJA treatment where GS levels were maximized in broccoli floret tissues, broccoli heads were harvested at different days after MeJA treatment. While neoglucobrassicin concentrations in broccoli florets were significantly greater from plants harvested one or two days after MeJA treatment significant concentrations peaked in heads harvested four days after treatment (Figure 7.1), consistent with a previous report (Kim and Juvik, 2011). Kim and Juvik reported that MeJA treatment four days prior to harvest significantly increased gluconasturtiin and neoglucobrassicin. In *Arabidopsis thaliana* MeJA treatment significantly increased transcript abundance of the indolyl GS biosynthesis gene *AtCYP79B3* as early as two hr after treatment but significant increases in glucobrassicin concentrations were not observed until two days after treatment (Brader et al., 2001). Our results indicate that the elevated GS concentrations of neoglucobrassicin were maintained for at least 8 days after MeJA treatment.

7.4.2. *Experiment* 2. Multiple MeJA Spray Treatments, GS Concentrations, and QR Activity. 250 μM MeJA treatments at both four and three days prior to harvest increased floret total GS and neoglucobrassin concentrations above those observed in a single treatment at four days prior to harvest but not significantly higher (Figure 7.2A). Multiple

MeJA treatments showed consistent increases in neoglucobrassicin in both 2010 and 2011 (data not shown). Averaged QR inductive activity was gradually increased as additional MeJA spray treatments were applied (Figure 7.2B). Extracts of broccoli florets showed a similar pattern of QR inductive activity with increasing numbers of applications in both 2010 and 2011 (data not shown).

7.4.3. *Experiment 3.* Optimal Concentrations of MeJA in Applications. Various MeJA concentration treatments were applied to broccoli plants to determine which concentration will maximize induction of GS biosynthesis with a single application. There were significant positive correlations between MeJA application concentrations and enhanced levels of glucoraphanin, gluconasturtiin and neoglucobrassicin (Table 7.1). Increased glucoraphanin, neoglucobrassicin, gluconasturtiin, and sulforaphane concentrations correlated with both QR inductive activity and NO inhibitory activity. Sulforaphane has been reported to suppress LPS-induced inflammation (Lin et al., 2008) and also identified as a potent QR inducer in broccoli (Zhang et al., 1992). In addition, phenethyl isothiocyanate (PEITC), which is hydrolysis product of gluconasturtiin was also reported to suppress nitric oxide production induced by LPS (Okubo et al., 2010). The increased QR inductive and NO inhibitory activity observed in the floret extracts may be associated with these isothiocyanate hydrolysis products. Haack et al., (2010) reported that N-methoxyindole-3-carbinol the hydrolysis product of neoglucobrassicin suppresses sulforaphane induced QR enzyme activity. Although we observed significant increases in neoglucobrassicin concentrations, we did not observe suppression of QR inductive activity. In contrast to previous studies, MeJA treatments were only observed to

increase total phenolic and flavonoid concentrations at 62.5 μM (Table 7.1). Application of higher concentrations of MeJA treatment had no effect on these compounds compared to controls except at 500 μM where total phenolics were significantly reduced. Considering the saturation trends associated with application of different concentrations of MeJA to increase QR inductive and NO production inhibitory activity the cost effective concentration should be 250 μM to enhance health promotion of broccoli in a commercial production system.

7.4.4. *Experiment 4.* MeJA applications to Different Plant Tissues. Application of 300 ml of 250 μM MeJA to the soil surrounding the root crown of broccoli plants had no effect on floret GS concentration four days after application (data not shown). Root applications of MeJA increased aliphatic GS concentrations in the shoot of *B. oleracea* (Van Dam et al., 2004). MeJA sprays on broccoli leaf tissue increased GS concentrations in floret tissues comparable to spray applied to both broccoli head and leaf tissues (Figure 7.3).

7.4.5. *Experiment 5.* Influence of Triton X-100 on Broccoli Head Color. It was observed on some occasions that MeJA treatments using the surfactant Triton X-100 resulted in some browning discoloration of broccoli florets. This discoloration was more dramatic with additional spray applications. In *Brassica* vegetables, visual color is an important factor in retailer and consumer evaluation of product quality and subsequent purchasing decisions (Dixon, 2007). To quantify the observed browning we also measured floret color of broccoli samples using a Hunter colorimeter. Although MeJA

dissolved in 0.1% ethanol did not affect broccoli color, a single spray of 0.1% Triton X-100 significantly increased the hunter's a value (Figure 7.4A and 7.4B). MeJA solution with 0.1% Triton X-100 as a surfactant further increased hunter's a value. There was a significant linear relationship between broccoli discoloration and number of Triton X-100 applications with or without MeJA. It was previously reported that Triton X-100 is responsible for phytotoxicity on leaves of plants using three different plant species (Falk et al., 1994). MeJA dissolved in 0.1% ethanol increased GS concentration levels comparable to those observed in MeJA treatments with 0.1% Triton X-100 (data not shown). After this observation we eliminated the use of Triton X-100 in our applications.

In conclusion, various factors for practical field application of MeJA in field production environments were tested in this present study. The results suggest that MeJA application is recommened to spray on leaf tissue of broccoli using 0.1% ethanol containing 250 μM MeJA four days prior to harvest. This information should be helpful for commercial production of vegetables with enhanced health promoting activity.

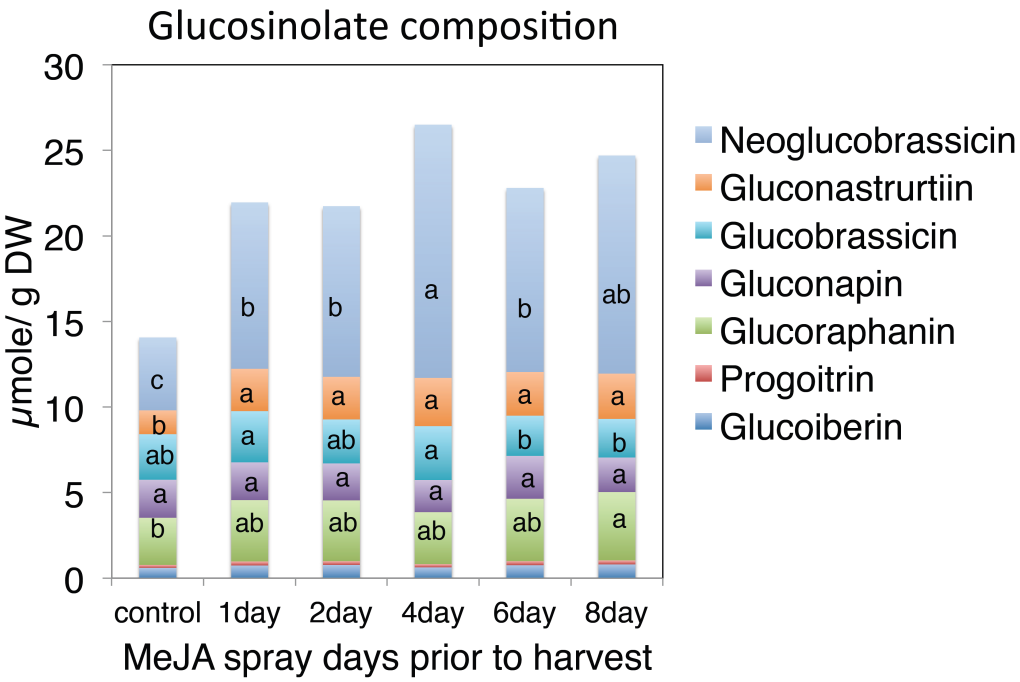

Figure 7.1. *Experiment 1.* Days before harvest of broccoli treated with 250 µM MeJA and comparative floret GS concentrations. Data are means ± SD (n=3).

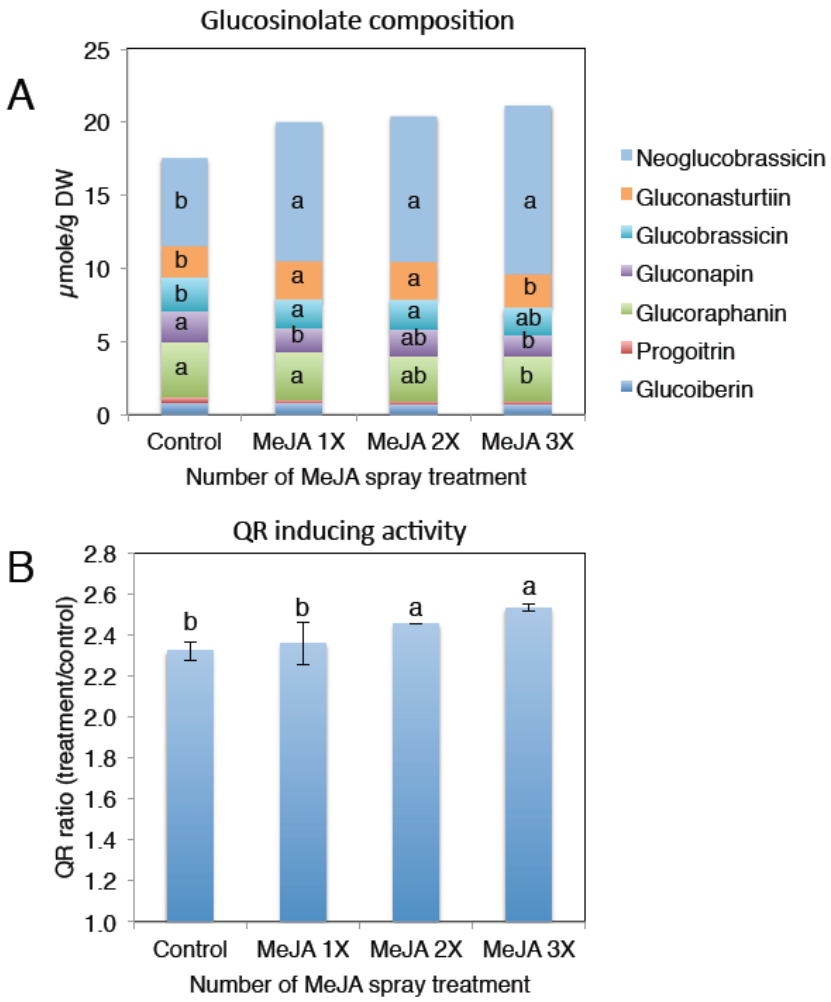

Figure 7.2. *Experiment 2.* Multiple MeJA treatment significantly increased neoglucobrassicin and QR inducing activity in averaged values of both 2010 and 2011 year. Multiple MeJA treatments were applied with 0 (control), MeJA 1X (once, 4 days prior to harvest), MeJA 2X (twice, at four and three days prior to harvest), and MeJA 3 X (three times at four, three, and two days prior to harvest). A: averaged glucosinolate composition for multiple MeJA applications over two years; B: averaged QR inductive activity for multiple MeJA applications over two years. Data are means ± SD (n=3).

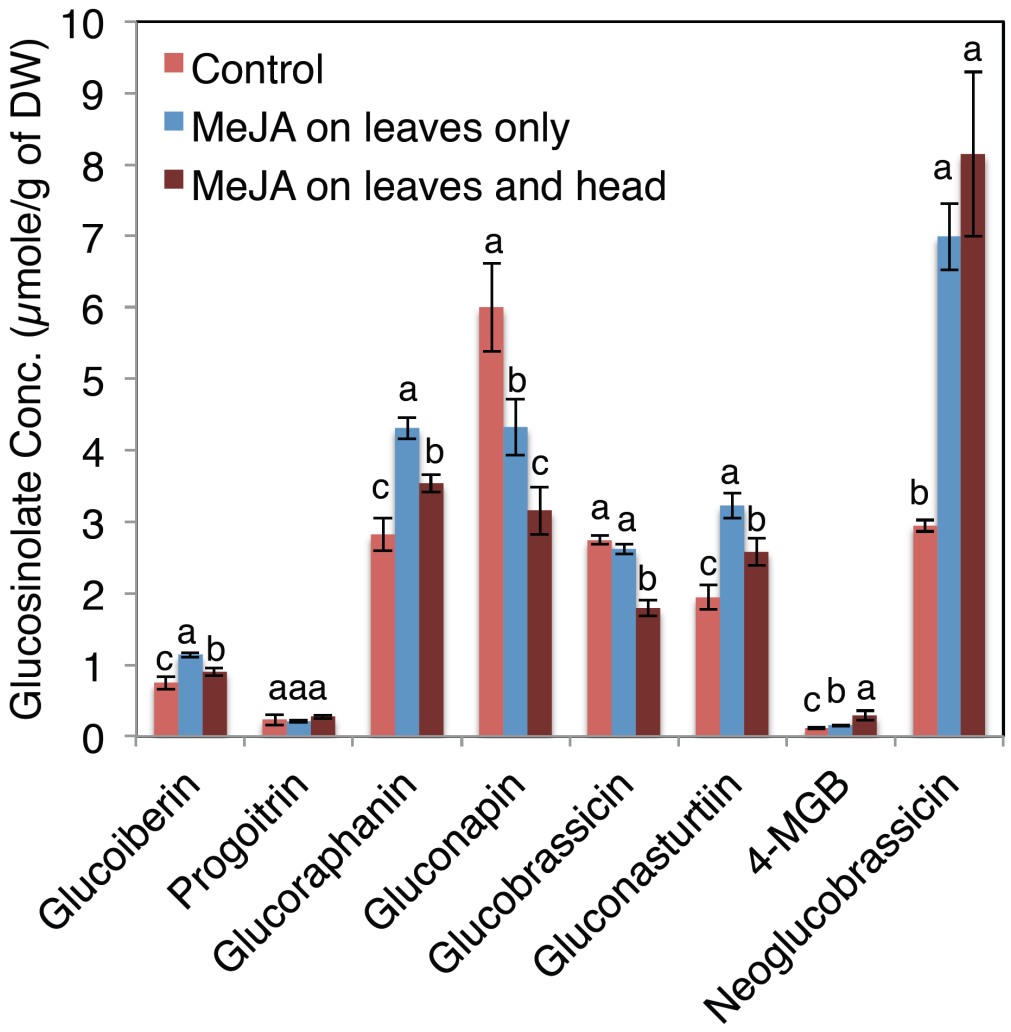

Figure 7.3. *Experiment 4.* Glucosinolate composition of control florets, florets where MeJA sprays was applied to leaf tissue, and florets where MeJA sprays were applied to both leaf and head tissue. *4-MGB = 4-methoxyglucobrassicin. Data are means ± SD (n=3).

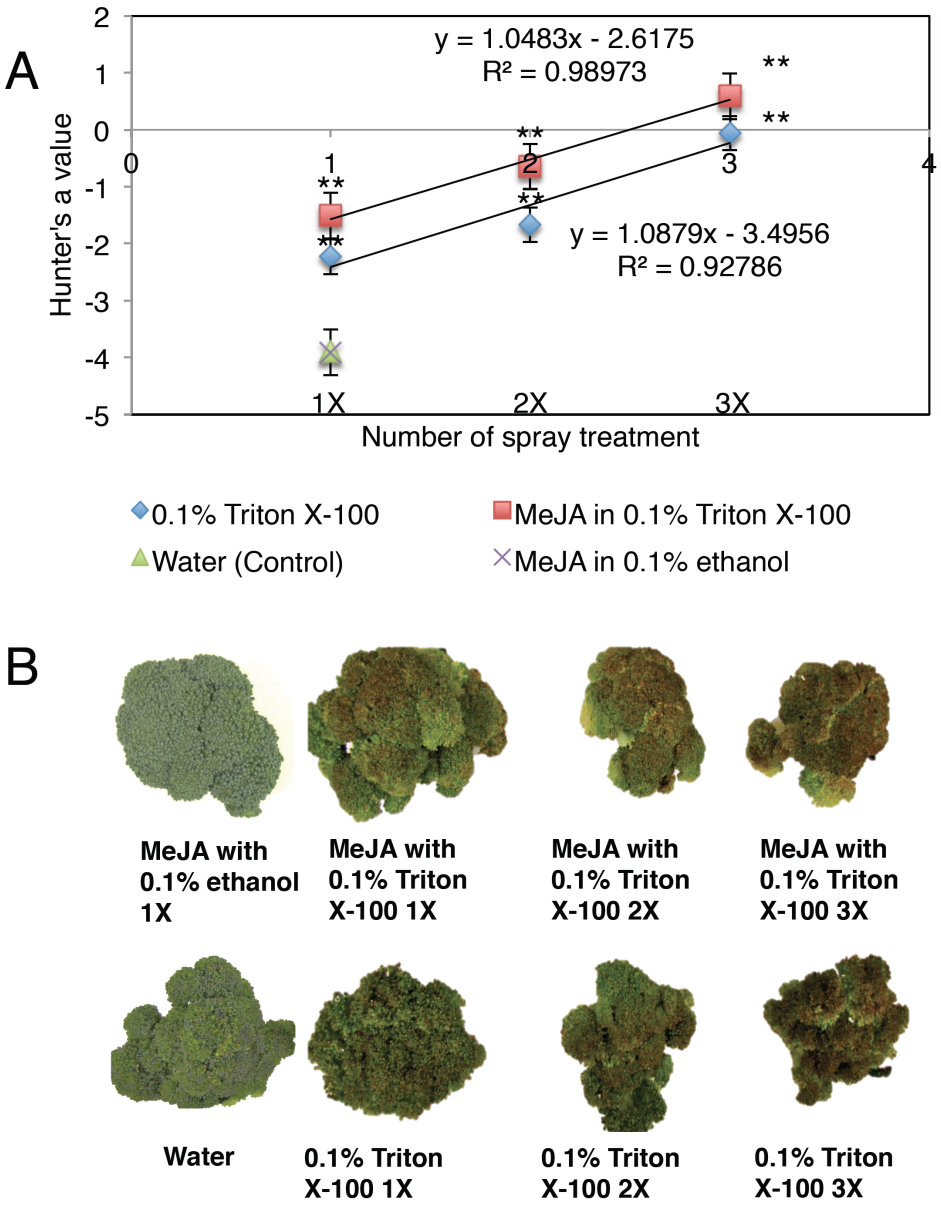

Figure 7.4. *Experiment 5.* Effect of multiple MeJA applications with Triton X-100 on browning discoloration of broccoli heads that negatively influence product visual quality. A: hunter's a value as a browning indicator. B: representative sample image of each treatment. Data are means ± SD (n=3).

Table 7.1. *Experiment 3.* Phytochemicals and inducing QR activity following application of varying concentrations of MeJA on 'Green Magic' broccoli florets.

Treatment MeJA (µM)	Glucoraphanin[a]	Neoglucobrassicin[z]	Gluconasturtiin[z]	Sulforaphane[z]	Quercetin[y]	Kaempferol[y]	Total phenolics[x]	QR[w]	NO production inhibitory assay[v]
0	2.05 ± 0.23 b	4.53 ± 1.03 d	1.28 ± 0.26 d	0.75 ± 0.03 b	139 ± 17 bc	79 ± 8 bc	645 ± 20 ab	2.21 ± 0.06 b	6.08 ± 0.58 b
62.5	2.23 ± 0.14ab	10.85 ± 0.97 c	2.48 ± 0.14 c	1.12 ± 0.19 a	175 ± 18 a	101 ± 9 a	715 ± 88 a	2.45 ± 0.37 ab	5.14 ± 0.43 ab
125	2.26 ± 0.07 a	14.25 ± 1.78 b	2.94 ± 0.18 b	1.20 ± 0.22 a	155 ± 24 ab	96 ± 13 ab	670 ± 88 ab	2.59 ± 0.37 ab	3.99 ± 0.77 a
250	2.41 ± 0.04 a	14.77 ± 2.08 b	2.97 ± 0.19 b	1.27 ± 0.25 a	121 ± 12 c	72 ± 7 c	625 ± 62 ab	2.73 ± 0.62 a	3.91 ± 0.45 a
500	2.42 ± 0.03a	20.18 ± 0.20 a	3.43 ± 0.12 a	1.36 ± 0.31 a	117 ± 18 c	75 ± 10 c	580 ± 18 b	2.81 ± 0.30 a	3.89 ± 1.25 a
Correlation with QR (*r*)	0.985 (*P*=0.002)	0.979 (*P*=0.004)	0.976 (*P*=0.005)	0.922 (*P*=0.026)	-0.523 (*P*=0.367)	-0.325 (*P*=0.593)	-0.554 (*P*=0.332)	-	-0.955 (*P*=0.012)
Correlation with MeJA concentrations (*r*)	0.854 (*P*=0.065)	0.908 (*P*=0.033)	0.808 (*P*=0.098)	0.782 (*P*=0.118)	-0.707 (*P*=0.026)	-0.533 (*P*=0.026)	-0.801 (*P*=0.026)	0.880 (*P*=0.049)	-0.740 (*P*=0.260)
Correlation with NO (*r*)	-0.916 (*P*=0.029)	-0.922 (*P*=0.026)	-0.955 (*P*=0.011)	-0.945 (*P*=0.015)	0.398 (*P*=0.506)	0.178 (*P*=0.775)	0.419 (*P*=0.483)	-0.955 (*P*=0.012)	-
Negative control	-	-	-	-	-	-	-	-	0.00 ± 0.18 a
LPS induced cell	-	-	-	-	-	-	-	-	10.92 ± 0.89 a

Values are means ± SD (n=3). Different letters are significantly different within column based on the Fisher's LSD multiple comparison test ($P < 0.05$). [z]µmole per g dry weight. [y]mmole/100 g of DW. [x]mg of gallic acid equivalents in 100 g of DW. [w]specific activity ratio of broccoli extract treated cells to untreated cells. [v]Nitrite concentration (µmole/mL). NO assay was conducted using five analytical replicates for each biological replicate sample. Data are means ± SD (n=3).

SUMMARY AND FUTURE PERSPECTIVES

Specific phytochemicals (glucosinolates, flavonoids, tocophenols, carotenoids and vitamins) from brassica vegetables have been shown to reduce incidence of cardiovascular disease and various cancers. Brassica vegetables are more effective in reducing cancer risk than other vegetables. Increasing the concentration of health-promoting compounds in *brassica* vegetables could effectively to contribute public health. Previous research suggests MeJA treatment increase GS and phenolic compounds. Hydrolysis products of GS have been reported to enhance activity of the cancer chemopreventive biomarker, QR. The objectives of this research was to exam whether MeJA treatment enhances human health promoting bioactivity (QR induction, antioxidation, and nitric oxide production inhibitory activity) by increasing GS and phenolic compound concentrations.

The objective of study 1 (Chapter 2) was to test MeJA treatment could increase total phenolic content in different edible tissue of brassica plants including broccoli florets and kale leaf tissues. The MeJA treatment significantly increased phenolic content and ABTS antioxidant activity in apical leaf tissue of kale but it did not significantly increased in broccoli florets. This indicates that MeJA treatment application only can increase total phenolic content in specific edible tissue.

The objective of study 2 (Chapter 3) was to test MeJA treatment could enhance QR inducing activity by increasing GS concentration. The MeJA treatment significantly increased not only gluconasturtiin and neoglucobrassicin concentration but also isothicyanate formation of GS including sulforaphane and PEITC over two seasons. To date, there were many experiments using MeJA to change GS concentration but hydrolysis products were not intensively measured. To our knowledge, this is the first report that MeJA treatment increases isothiocyanate formation,

which is meaningful for human health promoting. With increased sulforaphane and hydrolysis products of neoglucbrassicin, QR inducing activity of MeJA-treated broccoli was significantly increased. Since there was interaction between MeJA treatment and growing environment conditions, more studies on growing environment condition for MeJA treatment are needed to maximize the human health promoing activity. In addition, MeJA treatment may interact with weather conditions of the spraying time. The sunlight, humidity and wind of the spraying day may interact with MeJA treatment. The more research is needed on this.

The objective of study 3 (Chapter 4) was to test MeJA treatment could enhance QR inducing activity by increasing indolyl GS concentration in kale leaf tissues. In order to test this, we choose two different kale species [Dwarf Blue Curled Vates (*B. oleracea*) and Red Winter (*B. napus*)]. Increased hyrolysis proeducts of glucobrassicin and neoglucobrassicin including I3C, DIM, NI3C, and NeoASG were significantly correlated with QR inducing activity. Even though Dwarf Blue Curled Vates cultivar does not have high concentration of sulforaphane, QR inducing activity were significantly increased. This indicates that the MeJA treatment significantly enhanced QR inducing activity by increasing glucobrassicin and/or neoglucosinolate in kale leaf tissues.

The objective of study 4 (Chapter 5) was to test enhanced QR inducing activity and reduced postharvest quality of broccoli by MeJA treatment could be compensated by 1-MCP treatment. The combination treatment of MeJA and 1-MCP showed highest GS concentration and significantly higher QR inducing activity than control broccoli. In addition, GS biosynthesis and GS conversion during postharvest storage were observed. Ethylene accumulation during the postharvest storage may be the factor inducing conversion from glucobrassicin to 4-methoxyglucobrassicin. There were limited reports about bioactivity of 4-methoxyglucobrassicin.

In addition to 4-methoxyglucobrassicin, gluconasturtiin concentration was also gradually increased during the postharvest storage. To date, maintaining product quality and phytochemical concentration were the main research focus of numerous studies. However, my results suggest that modifying conditions during postharvest storage (1-MCP treatment) can enhance phytochemical and bioactivity.

The objective of study 5 (Chapter 6) was to exam whether MeJA treatment increases QR activity in cauliflower without a loss of postharvest quality. In this experiment we only sprayed MeJA solutions on cauliflower leaf tissue. MeJA treatment did not significantly increase ethylene production of cauliflower (Chapter 6) and broccoli (data not shown) when MeJA was applied only to leaf tissues.

In Chapter 7 (study 6), we conducted several experiments to establish optimizing MeJA application method for future MeJA application for vegetable production.

Overally, my dissertation research revealed that MeJA treatment can enhance QR inducing activity by increasing isothicyanate formation and GS biosynthesis mainly in the form of indolyl GS and enhanced biosynthesis of myrosinase. However, increased isothicyanate cannot be fully explained with gene expression of *BoMYO*, *BoESP*, and *BoESM1*. Further elucidation of this hydrolysis machanism for isothicyanate formation may facilitate our understanding plant herbivore defense and to improve the nutritional quality of brassica vegetables. This experiment results also suggest that application of MeJA or other elicitors for improving health promoting compounds of vegetables may require for an additional treatments for maintenance of quality during postharverst storage. To elucidate unknown or unwanted side effects from elicitor treatments, an omics based-approach may be useful including RNA-seq (transcriptome), iTRAQ (proteome), and untargeted metabolomics.

LITERATURE CITED

Abdulla, M. and Gruber, P., 2000. Role of diet modification in cancer prevention. *Biofactors* 12:45-51.

Able, A.J., Lung Sing, W., Amikha, P., and O'Hare, T.J., 2002. 1-MCP is more effective on a floral brassica (*Brassica oleracea var italica* L) than a leafy brassica (*Brassica rapa var chinensis*). *Postharvest Biology and Technology* 26:147--155.

Agati, G. and Tattini, M., 2010. Multiple functional roles of flavonoids in photoprotection. *New Phytologist* 186:786-793.

Agerbirk, N., De Vos, M., Kim, J.H., and Jander, G., 2009. Indole glucosinolate breakdown and its biological effects. *Phytochemistry Reviews* 8:101-120.

Agerbirk, N., Olsen, C.E., and Sørensen, H., 1998. Initial and final products, nitriles, and ascorbigens produced in myrosinase-catalyzed hydrolysis of indole glucosinolates. *Journal of Agricultural and Food Chemistry* 46:1563-1571.

Alam, M.A., Sernia, C., and Brown, L., 2013. Ferulic Acid improves cardiovascular and kidney structure and function in hypertensive rats. *J Cardiovasc Pharmacol* 61:240-249.

Aldridge, D.C., Galt, S., Giles, D., and Turner, W.B., 1971. Metabolites of lasiodiplodia theobromae. *Journal of the Chemical Society C: Organic*:1623-1627.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. polyphenoloxidase in *Beta Vulgaris*. *Plant Physiol* 24:1-15.

Bak, S.r., Nielsen, H., and Halkier, B., 1998. The presence of CYP79 homologues in glucosinolate-producing plants shows evolutionary conservation of the enzymes in the conversion of amino acid to aldoxime in the biosynthesis of cyanogenic glucosides and glucosinolates. *Plant Molecular Biology* 38:725-734.

Baskar, V., Gururani, M., Yu, J., and Park, S., 2012. Engineering glucosinolates in plants: current knowledge and potential uses. *Applied Biochemistry and Biotechnology* 168:1694-1717.

Bednarek, P., Piślewska-Bednarek, M., Svatoš, A., Schneider, B., Doubský, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., Sanchez-Vallet, A., Molina, A., and Schulze-Lefert, P., 2009. A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* 323:101-106.

Bennett, R.N., Mellon, F.A., Rosa, E.A.S., Perkins, L., and Kroon, P.A., 2004. Profiling glucosinolates, flavonoids, alkaloids, and other secondary metabolites in tissues of *Azima tetracantha* L. (*Salvadoraceae*). *Journal of Agricultural and Food Chemistry* 52:5856-5862.

Bergmeyer, H.U., 1974. *Methods of Enzymatic Analysis*.

Bitir, B., Erginer, M., Engin, E., and Vang, O., 2011. Determination combinatory effect of indole 3 carbinol and N-methoxy indole-3-carbinol on cell proliferation in HCT 116 cell line.

Björkman, M., Klingen, I., Birch, A.N.E., Bones, A.M., Bruce, T.J.A., Johansen, T.J., Meadow, R., Mølmanne, J., Seljåsenf, R., Smart, L.E., and Stewart, D., 2011. Phytochemicals of *Brassicaceae* in plant protection and human health, influences of climate, environment and agronomic practice. *Phytochem.* 72:538-556.

Black, C.A., Karban, R., Godfrey, L.D., Granett, J., and Chaney, W.E., 2003. Jasmonic acid: a vaccine against leafminers (*Diptera*: *Agromyzidae*) in celery. *Environmental Entomology* 32:1196-1202.

Bodnaryk, R.P., 1994. Potent effect of jasmonates on indole glucosinolates in oilseed rape and mustard. *Phytochemistry (Oxford)* 35:301-305.

Bones, A.M. and Rossiter, J.T., 1996. The myrosinase-glucosinolate system, its organisation and biochemistry. *Physiologia Plantarum* 97:194-208.

Bonnesen, C., Eggleston, I.M., and Hayes, J.D., 2001. Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. *Cancer Res* 61:6120-6130.

Bouchereau, A., Clossais-Besnard, N., Bensaoud, A., Leport, L., and Renard, M., 1996. Water stress effects on rapeseed quality. *European J. of Agron.* 5:19-30.

Bowsher, C., Steer, M.W., and Tobin, A.K., 2008. *Plant biochemistry*. Garland Science, New York.

Boyd, L.A., McCann, M.J., Hashim, Y., Bennett, R.N., Gill, C.I.R., and Rowland, I.R., 2006. Assessment of the anti-genotoxic, anti-proliferative, and anti-metastatic potential of crude watercress extract in human colon cancer cells. *Nutrition and Cancer* 55:232-241.

Brader, G., Mikkelsen, M.D., Halkier, B.A., and Palva, E.T., 2006. Altering glucosinolate profiles modulates disease resistance in plants. *Plant Journal* 46:758-767.

Brader, G., Tas, E., and Palva, E.T., 2001. Jasmonate-dependent induction of indole glucosinolates in Arabidopsis by culture filtrates of the nonspecific pathogen Erwinia carotovora. *Plant Physiol* 126:849-860.

Bradlow, H.L., 2008. Indole-3-carbinol as a chemoprotective agent in breast and prostate cancer. *In Vivo* 22:441-445.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254.

Brew, C.T., Aronchik, I., Hsu, J.C., Sheen, J.H., Dickson, R.B., Bjeldanes, L.F., and Firestone, G.L., 2006. Indole-3-carbinol activates the ATM signaling pathway independent of DNA damage to stabilize p53 and induce G1 arrest of human mammary epithelial cells. *Int J Cancer* 118:857-868.

Brown, A.F., Yousef, G.G., Jeffery, E.H., Klein, B.P., Wallig, M.A., Kushad, M.M., and Juvik, J.A., 2002. Glucosinolate profiles in broccoli: variation in levels and implications in breeding for cancer chemoprotection. *Journal of the American Society for Horticultural Science* 127:807-813.

Bruinsma, M., Posthumus, M.A., Mumm, R., Mueller, M.J., van Loon, J.J.A., and Dicke, M., 2009. Jasmonic acid-induced volatiles of *Brassica oleracea* attract parasitoids: effects of time and dose, and comparison with induction by herbivores. *Journal of Experimental Botany* 60:2575-2587.

Bruinsma, M., Van Dam, N., Van Loon, J., and Dicke, M., 2007. Jasmonic acid-induced changes in *Brassica oleracea* affect oviposition preference of two specialist herbivores. *Journal of Chemical Ecology* 33:655-668.

Brusselmans, K., Vrolix, R., Verhoeven, G., and Swinnen, J.V., 2005. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. *Journal of Biological Chemistry* 280:5636-5645.

Bücherta, A.M., Civello, P.M., and Martínez, G.A., 2011. Chlorophyllase versus pheophytinase as candidates for chlorophyll dephytilation during senescence of broccoli. *Journal of Plant Physiology* 168:337-343.

Buta, J.G. and Moline, H.E., 1998. Methyl jasmonate extends shelf life and reduces microbial contamination of fresh-cut celery and peppers. *Journal of Agricultural and Food Chemistry* 46:1253-1256.

Cai, Y., Luo, Q., Sun, M., and Corke, H., 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci.* 74:2157-2184.

Cao, S., Zheng, Y., Wang, K., Jin, P., and Rui, H., 2009. Methyl jasmonate reduces chilling injury and enhances antioxidant enzyme activity in postharvest loquat fruit. *Food Chemistry* 115:1458-1463.

Carson, D.G., Daxenbichler, M.E., and VanEtten, C.H., 1987. Glucosinolates in crucifer vegetables: broccoli, Brussels sprouts, cauliflower, collards, kale, mustard greens, and kohlrabi. *J. Amer. Soc. Hort. Sci.* 112:173-178.

Champolivier, L. and Merrien, A., 1996. Effects of water stress applied at different growth stages to *Brassica napus* L. var. oleifera on yield, yield components and seed quality. *European Journal of Agronomy* 5:153-160.

Chang, C. and Shockey, J.A., 1999. The ethylene-response pathway: signal perception to gene regulation. *Current opinion in plant biology* 2:352-358.

Chen, C. and Kong, A.N.T., 2004. Dietary chemopreventive compounds and ARE/EpRE signaling. *Free Radical Biology and Medicine* 36:1505-1516.

Cheng, R., Ridnour, L., Glynn, S., Switzer, C., Flores-Santana, W., Hussain, P., Thomas, D., Ambs, S., Harris, C., and Wink, D., 2010. Nitric oxide and cancer: an overview, p. 3-20. In: Bonavida, B. (ed.), Nitric Oxide (NO) and Cancer. Springer New York.

Cheng-Guo, J., Chao-Jiong, X., Jia, W., Jing, Y., Gao-Feng, Y., Bing-Liang, W., and Qiao-Mei, W., 2009. Effect of modified atmosphere packaging on visual quality and glucosinolates of broccoli florets. *Food Chemistry* 114:28--37.

Chiao, J.W., Chung, F.L., Kancherla, R., Ahmed, T., Mittelman, A., and Conaway, C.C., 2002. Sulforaphane and its metabolite mediate growth arrest and apoptosis in human prostate cancer cells. *Int J Oncol* 20:631-636.

Cho, S.D., Li, G., Hu, H., Jiang, C., Kang, K.S., Lee, Y.S., Kim, S.H., and Lu, J., 2005. Involvement of c-Jun N-terminal kinase in G2/M arrest and caspase-mediated apoptosis induced by sulforaphane in DU145 prostate cancer cells. *Nutr Cancer* 52:213-224.

Chuang, L.T., Moqattash, S.T., Gretz, H.F., Nezhat, F., Rahaman, J., and Chiao, J.W., 2007. Sulforaphane induces growth arrest and apoptosis in human ovarian cancer cells. *Acta Obstet Gynecol Scand*:1-6.

Chung, F.L., Conaway, C.C., Rao, C.V., and Reddy, B.S., 2000. Chemoprevention of colonic aberrant crypt foci in Fischer rats by sulforaphane and phenethyl isothiocyanate. *Carcinogenesis* 21:2287-2291.

Clapper, M.L. and Szarka, C.E., 1998. Glutathione S-transferases--biomarkers of cancer risk and chemopreventive response. *Chem Biol Interact* 111-112:377-388.

Clay, N.K., Adio, A.M., Denoux, C., Jander, G., and Ausubel, F.M., 2009. Glucosinolate metabolites required for an Arabidopsis innate immune response. *Science* 323:95-101.

Cornblatt, B.S., Ye, L., Dinkova-Kostova, A.T., Erb, M., Fahey, J.W., Singh, N.K., Chen, M.S., Stierer, T., Garrett-Mayer, E., Argani, P., Davidson, N.E., Talalay, P., Kensler, T.W., and Visvanathan, K., 2007. Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast. *Carcinogenesis* 28:1485-1490.

Costa, M.L., Civello, P.M., Chaves, A.R., and Martínez, G.A., 2005. Effect of ethephon and 6-benzylaminopurine on chlorophyll degrading enzymes and a peroxidase-linked chlorophyll bleaching during post-harvest senescence of broccoli (*Brassica oleracea* L.) at 20 °C. *Postharvest Biology and Technology* 35:191-199.

Creelman, R.A. and Mullet, J.E., 1995. Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. *Proc Natl Acad Sci U S A* 92:4114-4119.

Cuendet, M., Oteham, C.P., Moon, R.C., and Pezzuto, J.M., 2006. Quinone reductase induction as a biomarker for cancer chemoprevention. *Journal of Natural Products* 69:460-463.

Dai, J. and Mumper, R.J., 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules* 15:7313-7352.

Dam, N.M.V., Jong, T.J.D., Iwasa, Y., and Kubo, T., 1996. Optimal distribution of defences: are plants smart investors? *Functional Ecology* 10:128-136.

Dashwood, R.H., Arbogast, D.N., Fong, A.T., Pereira, C., Hendricks, J.D., and Bailey, G.S., 1989. Quantitative inter-relationships between aflatoxin B1 carcinogen dose, indole-3-carbinol anti-carcinogen dose, target organ DNA adduction and final tumor response. *Carcinogenesis* 10:175-181.

De Vos, M., Kriksunov, K.L., and Jander, G., 2008. Indole-3-acetonitrile production from indole glucosinolates deters oviposition by *Pieris rapae*. *Plant Physiology* 146:916-926.

Demole, E., Lederer, E., and Mercier, D.E., 1962. Isolement et détermination de la structure du jasmonate de méthyle, constituant odorant charactéristique de l'essence de jasmin. *Helvetica Chimica Acta,* 45:675-685.

Dimitrios, B., 2006. Sources of natural phenolic antioxidants. *Trends in Food Sci. & Tech.* 17:505-512.

Dinkova-Kostova, A.T., Jenkins, S.N., Fahey, J.W., Ye, L., Wehage, S.L., Liby, K.T., Stephenson, K.K., Wade, K.L., and Talalay, P., 2006. Protection against UV-light-induced skin carcinogenesis in SKH-1 high-risk mice by sulforaphane-containing broccoli sprout extracts. *Cancer Lett* 240:243-252.

Dixon, G.R., 2007. *Vegetable Brassicas and Related Crucifers*. CABI Pub.

Dixon, R.A. and Pasinetti, G.M., 2010. Flavonoids and isoflavonoids: from plant biology to agriculture and neuroscience. *Plant Physiol.* 154:453-457.

Doll, R. and Peto, R., 1981. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst* 66:1191-1308.

Drobnica, L., Zemanova, M., Nemec, P., Antos, K., Kristian, P., Stullerova, A., Knoppova, V., and Nemec, P., Jr., 1967. Antifungal activity of isothiocyanates and related compounds. I. naturally occurring isothiocyanates and their analogues. *Appl Microbiol* 15:701-709.

Dufault, R.J., 1997. Determining heat unit requirements for broccoli harvest in coastal south carolina. *J. Amer. Soc. Hort. Sci.* 122:169-174.

Droby, S., Porat, R., Cohen, L., Weiss, B., Shapiro, B., Philosoph-Hadas, S., and Meir, S., 1999. Suppressing green mold decay in grapefruit with postharvest jasmonate application. *Journal of the American Society for Horticultural Science* 124:184-188.

Eberhardt, M.V., Kobira, K., Keck, A.-S., Juvik, J.A., and Jeffery, E.H., 2005. Correlation analyses of phytochemical composition, chemical, and cellular measures of antioxidant activity of broccoli (*Brassica oleracea* L. var. *italica*). *J. of Agricultural and Food Chem.* 53:7421-7431.

El-Wakeil, N.E., Volkmar, C., and Sallam, A.A., 2010. Jasmonic acid induces resistance to economically important insect pests in winter wheat. *Pest Management Science* 66:549-554.

Fahey, J.W., Haristoy, X., Dolan, P.M., Kensler, T.W., Scholtus, I., Stephenson, K.K., Talalay, P., and Lozniewski, A., 2002. Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of *Helicobacter pylori* and prevents benzo[a]pyrene-induced stomach tumors. *Proc Natl Acad Sci U S A* 99:7610-7615.

Fahey, J.W., Zalcmann, A.T., and Talalay, P., 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56:5-51.

Falk, R.H., Guggenheim, R., and Schulke, G., 1994. Surfactant-induced phytotoxicity. *Weed Technology* 8:519-525.

Fan, X. and Mattheis, J.P., 2000. Yellowing of broccoli in storage is reduced by 1-methylcyclopropene. *Hortscience* 35:885-887.

Farnham, M.W. and Kopsell, D.A., 2009. Importance of genotype on carotenoid and chlorophyll levels in broccoli heads. *American Society for Horticultural Science* 44:6.

Farnham, M.W., Wilson, P.E., Stephenson, K.K., and Fahey, J.W., 2004. Genetic and environmental effects on glucosinolate content and chemoprotective potency of broccoli. *Plant Breeding* 123:60-65.

Fritz, V.A., Justen, V.L., Bode, A.M., Schuster, T., and Wang, M., 2010. Glucosinolate enhancement in cabbage induced by jasmonic acid application. *Hortscience* 45:1188-1191.

Gajewski, M., PrzybyŁ, J.L., Bajer, M., and JarienĖ, E., 2011. The influence of controlled atmosphere storage on phytosterols in broccoli heads. *Journal of Food Processing and Preservation* 35:722-728.

Galati, G. and O'Brien, P.J., 2004. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radic Biol Med* 37:287-303.

Gamet-Payrastre, L., Li, P., Lumeau, S., Cassar, G., Dupont, M.A., Chevolleau, S., Gasc, N., Tulliez, J., and Terce, F., 2000. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. *Cancer Res* 60:1426-1433.

Gang, M., Ran, W., Cheng-Rong, W., Kato, M., Yamawaki, K., Fei-Fei, Q., and Hui-Lian, X., 2009. Effect of 1-methylcyclopropene on expression of genes for ethylene biosynthesis enzymes and ethylene receptors in post-harvest broccoli. *Plant Growth Regulation* 57:223-232.

Gaofeng, Y., Bo, S., Jing, Y., and Qiaomei, W., 2010. Effect of 1-methylcyclopropene on shelf life, visual quality, antioxidant enzymes and health-promoting compounds in broccoli florets. *Food Chemistry* 118:774-781.

Gapper, N.E., Coupe, S.A., McKenzie, M.J., Scott, R.W., Christey, M.C., Lill, R.E., McManus, M.T., and Jameson, P.E., 2005. Senescence-associated down-regulation of 1-aminocyclopropane-1-carboxylate (ACC) oxidase delays harvest-induced senescence in broccoli. *Functional Plant Biology* 32:891-901.

Gates, M.A., Tworoger, S.S., Hecht, J.L., De Vivo, I., Rosner, B., and Hankinson, S.E., 2007. A prospective study of dietary flavonoid intake and incidence of epithelial ovarian cancer. *Intl. J. Cancer* 121:2225-2232.

Gill, S.S. and Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem* 48:909-930.

Gliszczynska-Swiglo, A., Kaluzewicz, A., Lemanska, K., Knaflewski, M., and Tyrakowska, B., 2007. The effect of solar radiation on the flavonol content in broccoli inflorescence. *Food Chemistry* 100:241-245.

Gomez-Lobato, M.E., Civello, P.M., and Martínez, G.A., 2012a. Effects of ethylene, cytokinin and physical treatments on *BoPaO* gene expression of harvested broccoli. *Journal of the Science of Food and Agriculture* 92:151-158.

Gomez-Lobato, M.E., Hasperué, J.H., Civello, P.M., Chaves, A.R., and Martínez, G.A., 2012b. Effect of 1-MCP on the expression of chlorophyll degrading genes during senescence of broccoli (*Brassica oleracea* L.). *Scientia Horticulturae* 144:208-211.

Gong, Y. and Mattheis, J.P., 2003. Effect of ethylene and 1-methylcyclopropene on chlorophyll catabolism of broccoli florets.33-38.

González-Aguilar, G.A., Buta, J.G., and Wang, C.Y., 2001. Methyl jasmonate reduces chilling injury symptoms and enhances colour development of 'Kent' mangoes. *Journal of the Science of Food and Agriculture* 81:1244-1249.

González-Aguilar, G.A., Fortiz, J., Cruz, R., Baez, R., and Wang, C.Y., 2000. Methyl jasmonate reduces chilling injury and maintains postharvest quality of mango fruit. *Journal of Agricultural and Food Chemistry* 48:515-519.

González-Aguilar, G.A., Tiznado, H., ndez, M., and Wang, C.Y., 2006. Physiological and biochemical responses of horticultural products to methyl jasmonate. *Stewart Postharvest Review* 2:1-9.

Griffiths, D.W., Bain, H., Deighton, N., Botting, N.P., and Robertson, A.A.B., 2000. Evaluation of liquid chromatography-atmospheric pressure chemical ionisation-mass spectrometry for the identification and quantification of desulphoglucosinolates. *Phytochemical Analysis* 11:216-225.

Haack, M., Lowinger, M., Lippmann, D., Kipp, A., Pagnotta, E., Iori, R., Monien, B.H., Glatt, H., Brauer, M.N., Wessjohann, L.A., and Brigelius-Flohe, R., 2010. Breakdown products of neoglucobrassicin inhibit activation of Nrf2 target genes mediated by myrosinase-derived glucoraphanin hydrolysis products. *Biol Chem* 391:1281-1293.

Halkier, B.A. and Gershenzon, J., 2006. Biology and biochemistry of glucosinolates. *Annual Review of Plant Biology* 57:303-333.

Hall, B.P., Shakeel, S.N., Amir, M., Ul Haq, N., Qu, X., and Schaller, G.E., 2012. Histidine kinase activity of the ethylene receptor ETR1 facilitates the ethylene response in Arabidopsis. *Plant Physiol* 159:682-695.

Hasperué, J.H., Gómez-Lobato, M.E., Chaves, A.R., Civello, P.M., and Martínez, G.A., 2013. Time of day at harvest affects the expression of chlorophyll degrading genes during postharvest storage of broccoli. *Postharvest Biology and Technology* 82:22-27.

Heil, M. and Baldwin, I.T., 2002. Fitness costs of induced resistance: emerging experimental support for a slippery concept. *Trends in plant science* 7:61-67.

Hofseth, L.J., Hussain, S.P., Wogan, G.N., and Harris, C.C., 2003. Nitric oxide in cancer and chemoprevention. *Free Radic Biol Med* 34:955-968.

Hopkins, R.J., van Dam, N.M., and van Loon, J.J., 2009. Role of glucosinolates in insect-plant relationships and multitrophic interactions. *Annu Rev Entomol* 54:57-83.

Horbowicz, M., Wiczkowski, W., Koczkodaj, D., and Saniewski, M., 2011. Effects of methyl jasmonate on accumulation of flavonoids in seedlings of common buckwheat (*Fagopyrum esculentum* Moench). *Acta Biologica Hungarica* 62:265-278.

Hou, D.-X. and Kumamoto, T., 2010. Flavonoids as protein kinase inhibitors for cancer chemoprevention: direct binding and molecular modeling. *Antioxidants & Redox Signaling* 13:691-719.

Hou, D.X., Fukuda, M., Fujii, M., and Fuke, Y., 2000. Transcriptional regulation of nicotinamide adenine dinucleotide phosphate: quinone oxidoreductase in murine hepatoma cells by 6-(methylsufinyl)hexyl isothiocyanate, an active principle of wasabi (*Eutrema wasabi* Maxim). *Cancer Letters* 161:195-200.

Howe, G.A. and Jander, G., 2008. Plant immunity to insect herbivores. *Annual Review of Plant Biology* 59:41-66.

Hwang, E.S. and Jeffery, E.H., 2003. Evaluation of urinary N-acetyl cysteinyl allyl isothiocyanate as a biomarker for intake and bioactivity of Brussels sprouts. *Food Chem Toxicol* 41:1817-1825.

Ibrahim, K.E. and Juvik, J.A., 2009. Feasibility for improving phytonutrient content in vegetable crops using conventional breeding strategies: case study with carotenoids and tocopherols in sweet corn and broccoli. *Journal of Agricultural and Food Chemistry* 57:4636-4644.

James, D.C. and Rossiter, J.T., 1991. Development and characteristics of myrosinase in *Brassica napus* during early seedling growth. *Physiologia Plantarum* 82:163-170.

Jin, P., Wang, K., Shang, H., Tong, J., and Zheng, Y., 2009. Low-temperature conditioning combined with methyl jasmonate treatment reduces chilling injury of peach fruit. *Journal of the Science of Food and Agriculture* 89:1690-1696.

Jun, B.-K., Seo, S.-G., Kim, J.-S., Lee, Y., Shin, M.-R., Choi, H.-S., Yi, B.-Y., and Kim, S.-H., 2011. Molecular cloning and expression analysis of *Bro-GS-elong* and *Bro-myro* from Brassica oleracea. *Genes & Genomics* 33:299-305.

Jung, S., 2004. Effect of chlorophyll reduction in *Arabidopsis* thaliana by methyl jasmonate or norflurazon on antioxidant systems. *Plant Physiol Biochem* 42:225-231.

Justen, V.L. and Fritz, V.A., 2013. Temperature-induced glucosinolate accumulation Is associated with expression of *BrMYB* transcription factors. *Hortscience* 48:47-52.

Kai, K., Takahashi, H., Saga, H., Ogawa, T., Kanaya, S., and Ohta, D., 2011. Metabolomic characterization of the possible involvement of a cytochrome P450, CYP81F4, in the biosynthesis of indolic glucosinolate in *Arabidopsis*. *Plant Biotechnology* 28:379-385.

Kang, Y.-H. and Pezzuto, J.M., 2004. Induction of quinone reductase as a primary screen for natural product anticarcinogens, p. 380-414. In: Helmut, S. and Lester, P. (eds.), Methods in Enzymology. Academic Press.

Kasim, R., Kasim, M.U., and Erkal, S., 2007. The effect of packaging after 1-MCP treatment on color changes and chlorophyll degradation of broccoli (*Brassica oleracea var italica cv* Monopoly). *Journal of Food, Agriculture & Environment* 5:48-51.

Kato, M., Kamo, T., Ran, W., Nishikawa, F., Hyodo, H., Ikoma, Y., Sugiura, M., and Yano, M., 2002. Wound-induced ethylene synthesis in stem tissue of harvested broccoli and its effect on senescence and ethylene synthesis in broccoli florets. *Postharvest Biology and Technology* 24:69--78.

Katsir, L., 2008. Jasmonate signaling: a conserved mechanism of hormone sensing. *Current opinion in plant biology* 11:428.

Keum, Y.S., Jeong, W.S., and Kong, A.N., 2005. Chemopreventive functions of isothiocyanates. *Drug News Perspect* 18:445-451.

Kim, D.J., Shin, D.H., Ahn, B., Kang, J.S., Nam, K.T., Park, C.B., Kim, C.K., Hong, J.T., Kim, Y.B., Yun, Y.W., Jang, D.D., and Yang, K.H., 2003. Chemoprevention of colon cancer by Korean food plant components. *Mutat Res* 523-524:99-107.

Kim, H.-J., Chen, F., Wang, X., and Choi, J.-H., 2006a. Effect of methyl jasmonate on phenolics, isothiocyanate, and metabolic enzymes in radish sprout (*Raphanus sativus* L.). *Journal of Agricultural and Food Chemistry* 54:7263-7269.

Kim, H.-J., Chen, F., Wang, X., and Rajapakse, N.C., 2006b. Effect of methyl jasmonate on secondary metabolites of sweet basil (*Ocimum basilicum* L.). *Journal of Agricultural and Food Chemistry* 54:2327-2332.

Kim, H.J., Park, K.J., and Lim, J.H., 2011. Metabolomic analysis of phenolic compounds in buckwheat (*Fagopyrum esculentum* M.) sprouts treated with methyl jasmonate. *J Agric Food Chem* 59:5707-5713.

Kim, H.S., 2011. Functional studies of lignin biosynthesis genes and putative flowering gene in *Miscanthus x giganteus* and studies on indolyl glucosinolate biosynthesis and translocation in *Brassica oleracea*. University of Illinois at Urbana-Champaign, Urbana-Champaign, Ph. D. Dissertation (Ph. D.).

Kim, H.S. and Juvik, J.A., 2011. Effect of selenium fertilization and methyl jasmonate treatment on glucosinolate accumulation in broccoli florets. *J. Amer. Soc. Hort. Sci.* 136:239-246.

Kim, J.H., Lee, B.W., Schroeder, F.C., and Jander, G., 2008. Identification of indole glucosinolate breakdown products with antifeedant effects on *Myzus persicae* (green peach aphid). *The Plant Journal* 54:1015-1026.

King, G.A. and Morris, S.C., 1994a. Early compositional changes during postharvest senescence of broccoli. *J. Am. Soc. Hort. Sci.* 119:1000-1005.

King, G.A. and Morris, S.C., 1994b. Physiological changes of broccoli during early postharvest senescence and through the preharvest–postharvest continuum. *J. Am. Soc. Hort. Sci.* 119:270-275.

Koh, E., Wimalasiri, K.M.S., Chassy, A.W., and Mitchell, A.E., 2009. Content of ascorbic acid, quercetin, kaempferol and total phenolics in commercial broccoli. *Journal of Food Composition and Analysis* 22:637-643.

Koroleva, O.A., Davies, A., Deeken, R., Thorpe, M.R., Tomos, A.D., and Hedrich, R., 2000. Identification of a new glucosinolate-rich cell type in *Arabidopsis* flower stalk. *Plant Physiol* 124:599-608.

Krinsky, N.I., Landrum, J.T., and Bone, R.A., 2003. Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. *Annual Review of Nutrition* 23:171-201.

Kronbak, R., Duus, F., and Vang, O., 2010. Effect of 4-methoxyindole-3-carbinol on the proliferation of colon cancer cells in vitro, when treated alone or in combination with indole-3-carbinol. *Journal of Agricultural and Food Chemistry* 58:8453-8459.

Kroymann, J., Donnerhacke, S., Schnabelrauch, D., and Mitchell-Olds, T., 2003. Evolutionary dynamics of an Arabidopsis insect resistance quantitative trait locus. *Proc Natl Acad Sci U S A* 100 Suppl 2:14587-14592.

Ku, K.M., Choi, J.-H., Kushad, M.M., Jeffery, E.H., and Juvik, J.A., 2013. Pre-harvest methyl jasmonate treatment enhances cauliflower chemoprotective attributes without a loss in postharvest quality. *Plant Foods for Human Nutrition* 68:113-117.

Ku, K.M. and Juvik, J.A., 2012. Optimum methyl jasmonate application to enhance glucosinolate concentration in broccoli florets. *Hortscience* 47:S311.

Ku, K.M., Kim, J., Park, H.J., Liu, K.H., and Lee, C.H., 2010. Application of metabolomics in the analysis of manufacturing type of pu-erh tea and composition changes with different postfermentation year. *J Agric Food Chem* 58:345-352.

Ku, V.V.V. and Wills, R.B.H., 1999. Effect of 1-methylcyclopropene on the storage life of broccoli. *Postharvest Biology and Technology* 17:127-132.

Kuhlmann, F. and Müller, C., 2009. Independent responses to ultraviolet radiation and herbivore attack in broccoli. *J. of Expt. Bot.* 60:3467-3475.

Kumar, A., D'Souza, S.S., Tickoo, S., Salimath, B.P., and Singh, H.B., 2009. Antiangiogenic and proapoptotic activities of allyl isothiocyanate inhibit ascites tumor growth in vivo. *Integr Cancer Ther* 8:75-87.

Kurilich, A.C., Jeffery, E.H., Juvik, J.A., Wallig, M.A., and Klein, B.P., 2002. Antioxidant capacity of different broccoli (*Brassica oleracea*) genotypes using the oxygen radical absorbance capacity (ORAC) Assay. *Journal of Agricultural and Food Chemistry* 50:5053-5057.

Larson, A.J., Symons, J.D., and Jalili, T., 2012. Therapeutic potential of quercetin to decrease blood pressure: review of efficacy and mechanisms. *Adv Nutr* 3:39-46.

Lee, Y.-J., Kim, K.-J., Park, K.-J., Yoon, B.-R., Lim, J.-H., and Lee, O.-H., 2013. Buckwheat (*Fagopyrum esculentum* M.) Sprout Treated with Methyl Jasmonate (MeJA) Improved anti-adipogenic activity associated with the oxidative stress system in 3T3-L1 adipocytes. *Intl. J. of Mol. Sci.* 14:1428-1442.

Li, X. and Kushad, M.M., 2005. Purification and characterization of myrosinase from horseradish (*Armoracia rusticana*) roots. *Plant Physiology and Biochemistry* 43:503-511.

Lichtenthaler, H.K. and Wellburn, A.R., 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochemical Society Transactions* 11:591 - 592.

Liechti, R., Gfeller, A., and Farmer, E.E., 2006. Jasmonate signaling pathway. *Sci STKE* 2006:cm2.

Lin, W., Wu, R.T., Wu, T., Khor, T.-O., Wang, H., and Kong, A.-N., 2008. Sulforaphane suppressed LPS-induced inflammation in mouse peritoneal macrophages through Nrf2 dependent pathway. *Biochemical Pharmacology* 76:967-973.

Ma, G., Wang, R., Wang, C.R., Kato, M., Yamawaki, K., Qin, F.F., and Xu, H.L., 2009. Effect of 1-methylcyclopropene on expression of genes for ethylene biosynthesis enzymes and ethylene receptors in post-harvest broccoli. *Plant Growth Regulation* 57:223-232.

Ma, G., Zhang, L., Kato, M., Yamawaki, K., Asai, T., Nishikawa, F., Ikoma, Y., and Matsumoto, H., 2010. Effect of 1-methylcyclopropene on the expression of genes for ascorbate metabolism in postharvest broccoli. *Postharvest Biology and Technology* 58:121-128.

Manesh, C. and Kuttan, G., 2003. Effect of naturally occurring allyl and phenyl isothiocyanates in the inhibition of experimental pulmonary metastasis induced by B16F-10 melanoma cells. *Fitoterapia* 74:355-363.

Manson, M.M., Ball, H.W., Barrett, M.C., Clark, H.L., Judah, D.J., Williamson, G., and Neal, G.E., 1997. Mechanism of action of dietary chemoprotective agents in rat liver: induction of phase I and II drug metabolizing enzymes and aflatoxin B1 metabolism. *Carcinogenesis* 18:1729-1738.

Matusheski, N.V., Swarup, R., Juvik, J.A., Mithen, R., Bennett, M., and Jeffery, E.H., 2006. Epithiospecifier protein from broccoli (*Brassica oleracea* L. *ssp. italica*) inhibits formation of the anticancer agent sulforaphane. *J Agric Food Chem* 54:2069-2076.

McEwen, C.L., 2011. Two entomological studies: 1. The potential of methyl jasmonate applications as a pest management method on cruciferous crops 2. contributions to the biology of *Disholcaspis quercusmamma* (Walsh) (*Hymenoptera: Cynipidae*). Colorado State University, M.S. dissertation Dissertation.

Mewis, I., Schreiner, M., Nguyen, C.N., Krumbein, A., Ulrichs, C., Lohse, M., and Zrenner, R., 2012. UV-B irradiation changes specifically the secondary metabolite profile in broccoli sprouts: induced signaling overlaps with defense response to biotic stressors. *Plant and Cell Physiology* 53:1546-1560.

Michaud, D.S., Spiegelman, D., Clinton, S.K., Rimm, E.B., Willett, W.C., and Giovannucci, E.L., 1999. Fruit and vegetable intake and incidence of bladder cancer in a male prospective cohort. *J Natl Cancer Inst* 91:605-613.

Michnovicz, J.J. and Bradlow, H.L., 1991. Altered estrogen metabolism and excretion in humans following consumption of indole-3-carbinol. *Nutr Cancer* 16:59-66.

Michnovicz, J.J., Adlercreutz, H., and Bradlow, H.L., 1997. Changes in levels of urinary estrogen metabolites after oral indole-3-carbinol treatment in humans. *J Natl Cancer Inst* 89:718-723.

Mikkelsen, M.D., Petersen, B.L., Glawischnig, E., Jensen, A.B., Andreasson, E., and Halkier, B.A., 2003. Modulation of CYP79 genes and glucosinolate profiles in Arabidopsis by defense signaling pathways. *Plant Physiol* 131:298-308.

Misiewicz, I., Skupinska, K., and Kasprzycka-Guttman, T., 2003. Sulforaphane and 2-oxohexyl isothiocyanate induce cell growth arrest and apoptosis in L-1210 leukemia and ME-18 melanoma cells. *Oncol Rep* 10:2045-2050.

Mithen, R.F., Dekker, M., Verkerk, R., Rabot, S., and Johnson, I.T., 2000. The nutritional significance, biosynthesis and bioavailability of glucosinolates in human foods. *Journal of the Science of Food and Agriculture* 80:967-984.

Moon, J., Lee, S.M., Do, H.J., Cho, Y., Chung, J.H., and Shin, M.J., 2012. Quercetin up-regulates LDL receptor expression in HepG2 cells. *Phytother Res* 26:1688-1694.

Moon, Y.J., Wang, X., and Morris, M.E., 2006. Dietary flavonoids: Effects on xenobiotic and carcinogen metabolism. *Toxicology in Vitro* 20:187-210.

Morton, L.W., Caccetta, R.A.-A., Puddey, I.B., and Croft, K.D., 2000. Chemistry and biological effects of dietary phenolic compounds: relevance to cardiovascular Disease. *Clinical and Experimental Pharmacology and Physiology* 27:152-159.

Müller, R., Vos, M., Sun, J.Y., Sønderby, I.E., Halkier, B.A., Wittstock, U., and Jander, G., 2010. Differential effects of indole and aliphatic glucosinolates on *Lepidopteran* herbivores. *Journal of Chemical Ecology* 36:905-913.

Murakami, A., Ashida, H., and Terao, J., 2008. Multitargeted cancer prevention by quercetin. *Cancer Lett.* 269:315-325.

Muntane, J. and la Mata, M.D., 2010. Nitric oxide and cancer. *World J Hepatol* 2:337-344.

Nath, A., Bagchi, B., Misra, L.K., and Deka, B.C., 2011. Changes in post-harvest phytochemical qualities of broccoli florets during ambient and refrigerated storage. *Food Chemistry* 127:1510-1514.

Neave, A.S., Sarup, S.M., Seidelin, M., Duus, F., and Vang, O., 2005. Characterization of the N-methoxyindole-3-carbinol (NI3C), induced cell cycle arrest in human colon cancer cell lines. *Toxicological Sciences* 83:126-135.

Nestle, M., 1998. Broccoli sprouts in cancer prevention. *Nutr Rev* 56:127-130.

Neuhouser, M.L., 2004. Dietary flavonoids and cancer risk: evidence from human population studies. *Nutr. Cancer* 50:1-7.

Nilprapruck, P., Pradisthakarn, N., Authanithee, F., and Keebjan, P., 2008. Effect of exogenous methyl jasmonate on chilling injury and quality of Pineapple (*Ananas comosus* L.) cv. Pattavia. *Silpakorn University Science and Technology Journal* 2.

Nothlings, U., Murphy, S.P., Wilkens, L.R., Henderson, B.E., and Kolonel, L.N., 2007. Flavonols and pancreatic cancer risk: the multiethnic cohort study. *Am. J. Epidemiol.* 166:924-931.

Oh, M.-M., Carey, E.E., and Rajashekar, C.B., 2010. Regulated water deficits improve phytochemical concentration in lettuce. *J. Amer. Soc. Hort. Sci.* 135:223-229.

Okubo, T., Washida, K., and Murakami, A., 2010. Phenethyl isothiocyanate suppresses nitric oxide production via inhibition of phosphoinositide 3-kinase/Akt-induced IFN-gamma secretion in LPS-activated peritoneal macrophages. *Mol Nutr Food Res* 54:1351-1360.

Oostendorp, M., Kunz, W., Dietrich, B., and Staub, T., 2001. Induced disease resistance in plants by chemicals. *European Journal of Plant Pathology* 107:19-28.

Osbourn, A.E., 1996. Preformed antimicrobial compounds and plant defense against fungal attack. *The Plant Cell Online* 8:1821-1831.

Page, T., Griffiths, G., and Buchanan-Wollaston, V., 2001. Molecular and biochemical characterization of postharvest senescence in broccoli. *Plant Physiology* 125:718-727.

Paliyath, G., 2008. *Postharvest biology and technology of fruits, vegetables, and flowers*. Wiley-Blackwell, Ames, Iowa.

Palozza, P., Serini, S., Torsello, A., Di Nicuolo, F., Maggiano, N., Ranelletti, F.O., Wolf, F.I., and Calviello, G., 2003. Mechanism of activation of caspase cascade during beta-carotene-induced apoptosis in human tumor cells. *Nutr Cancer* 47:76-87.

Pang, Q., Chen, S., Li, L., and Yan, X., 2009. Characterization of glucosinolate-myrosinase system in developing salt cress *Thellungiella halophila*. *Physiol Plant* 136:1-9.

Paré, P.W. and Tumlinson, J.H., 1999. Plant volatiles as a defense against insect herbivores. *Plant Physiology* 121:325-332.

Penas, E., Frias, J., Martinez-Villaluenga, C., and Vidal-Valverde, C., 2011. Bioactive compounds, myrosinase activity, and antioxidant capacity of white cabbages grown in different locations of Spain. *J Agric Food Chem* 59:3772-3779.

Pereira, F.M., Rosa, E., Fahey, J.W., Stephenson, K.K., Carvalho, R., and Aires, A., 2002. Influence of temperature and ontogeny on the levels of glucosinolates in broccoli (*Brassica oleracea Var. italica*) sprouts and their effect on the induction of mammalian phase 2 enzymes. *J Agric Food Chem* 50:6239-6244.

Pérez-Balibrea, S., Moreno, D.A., and Garcia-Viguera, C., 2011. Improving the phytochemical composition of broccoli sprouts by elicitation. *Food Chemistry* 129:35-44.

Pfalz, M., Mikkelsen, M.D., Bednarek, P., Olsen, C.E., Halkier, B.A., and Kroymann, J., 2011. Metabolic engineering in *Nicotiana benthamiana* reveals key enzyme functions in Arabidopsis indole glucosinolate modification. *The Plant Cell Online* 23:716-729.

Piironen, V., Toivo, J., Puupponen-Pimiä, R., and Lampi, A.-M., 2003. Plant sterols in vegetables, fruits and berries. *Journal of the Science of Food and Agriculture* 83:330-337.

Pogson, B.J., Downs, C.G., and Davies, K.M., 1995. Differential expression of two 1-aminocyclopropane-1-carboxylic acid oxidase genes in broccoli after harvest. *Plant Physiology* 108:651-657.

Poulsen, H.E., Prieme, H., and Loft, S., 1998. Role of oxidative DNA damage in cancer initiation and promotion. *European J. of Cancer Prevention* 7:9-16.

Prestera, T., Zhang, Y., Spencer, S.R., Wilczak, C.A., and Talalay, P., 1993. The electrophile counterattack response: Protection against neoplasia and toxicity. *Advances in Enzyme Regulation* 33:281-296.

Prochaska, H.J. and Santamaria, A.B., 1988. Direct measurement of NAD(P)H:quinone reductase from cells cultured in microtiter wells: a screening assay for anticarcinogenic enzyme inducers. *Anal Biochem* 169:328-336.

Prochaska, H.J., Santamaria, A.B., and Talalay, P., 1992. Rapid detection of inducers of enzymes that protect against carcinogens. *Proc Natl Acad Sci U S A* 89:2394-2398.

Riby, J.E., Chang, G.H., Firestone, G.L., and Bjeldanes, L.F., 2000. Ligand-independent activation of estrogen receptor function by 3, 3'-diindolylmethane in human breast cancer cells. *Biochem Pharmacol* 60:167-177.

Rodrigues, A.S. and Rosa, E.A.S., 1999. Effect of post-harvest treatments on the level of glucosinolates in broccoli. *Journal of the Science of Food and Agriculture* 79:1028-1032.

Rose, P., Faulkner, K., Williamson, G., and Mithen, R., 2000. 7-Methylsulfinylheptyl and 8-methylsulfinyloctyl isothiocyanates from watercress are potent inducers of phase II enzymes. *Carcinogenesis* 21:1983-1988.

Sapone, A., Affatato, A., Canistro, D., Pozzetti, L., Broccoli, M., Barillari, J., Iori, R., and Paolini, M., 2007. Cruciferous vegetables and lung cancer. *Mutat Res* 635:146-148.

Schnee, C., Köllner, T.G., Held, M., Turlings, T.C.J., Gershenzon, J., and Degenhardt, J., 2006. The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores. *Proceedings of the National Academy of Sciences of the United States of America* 103:1129-1134.

Schonhof, I., Kläring, H.P., Krumbein, A., Claußen, W., and Schreiner, M., 2007. Effect of temperature increase under low radiation conditions on phytochemicals and ascorbic acid in greenhouse grown broccoli. *Agr., Ecosystems & Environ.* 119:103-111.

Schreiner, M., Krumbein, A., Schonhof, I., Widell, S., and Huyskens-Keil, S., 2003. Quality determination of red radish by nondestructive root color measurement. *Journal of the American Society for Horticultural Science* 128:397-402.

Schreiner, M.C., Peters, P.J., and Krumbein, A.B., 2006. Glucosinolates in mixed-packaged mini broccoli and mini cauliflower under modified atmosphere. *Journal of Agricultural and Food Chemistry* 54:2218-2222.

Scognamiglio, J., Jones, L., Letizia, C.S., and Api, A.M., 2012. Fragrance material review on methyl jasmonate. *Food and Chemical Toxicology* 50, Supplement 3:S572-S576.

Sepkovic, D.W., Bradlow, H.L., Michnovicz, J., Murtezani, S., Levy, I., and Osborne, M.P., 1994. Catechol estrogen production in rat microsomes after treatment with indole-3-carbinol, ascorbigen, or beta-naphthaflavone: a comparison of stable isotope dilution gas chromatography-mass spectrometry and radiometric methods. *Steroids* 59:318-323.

Simmonds, N.W., 1976. *Evolution of crop plants*.

Singh, G., Kawatra, A., and Sehgal, S., 2001. Nutritional composition of selected green leafy vegetables, herbs and carrots. *Plant Foods for Human Nutrition* 56:359-364.

Sisler, E.C. and Serek, M., 1997. Inhibitors of ethylene responses in plants at the receptor level: Recent developments. *Physiologia Plantarum* 100:577-582.

Sisler, E.C., Serek, M., and Dupille, E., 1996. Comparison of cyclopropene, 1-methylcyclopropene, and 3,3-dimethylcyclopropene as ethylene antagonists in plants. *Plant Growth Regulation* 18:169-174.

Somerset, S.M. and Johannot, L., 2008. Dietary flavonoid sources in Australian adults. *Nutr Cancer* 60:442-449.

Sønderby, I.E., Geu-Flores, F., and Halkier, B.A., 2010. Biosynthesis of glucosinolates - gene discovery and beyond. *Trends in plant science* 15:283-290.

Stotz, H.U., Pittendrigh, B.R., Kroymann, J., Weniger, K., Fritsche, J., Bauke, A., and Mitchell-Olds, T., 2000. Induced plant defense responses against chewing insects. Ethylene signaling reduces resistance of Arabidopsis against Egyptian cotton worm but not diamondback moth. *Plant Physiol* 124:1007-1018.

Sun, J.Y., Sønderby, I.E., Halkier, B.A., Jander, G., and Vos, M., 2009. Non-volatile intact indole glucosinolates are host recognition cues for ovipositing *Plutella xylostella*. *Journal of Chemical Ecology* 35:1427-1436.

Suzuki, A., Yamamoto, N., Jokura, H., Yamamoto, M., Fujii, A., Tokimitsu, I., and Saito, I., 2006. Chlorogenic acid attenuates hypertension and improves endothelial function in spontaneously hypertensive rats. *J Hypertens* 24:1065-1073.

Talalay, P., Fahey, J.W., Holtzclaw, W.D., Prestera, T., and Zhang, Y., 1995. Chemoprotection against cancer by phase 2 enzyme induction. *Toxicol Lett* 82-83:173-179.

Tamogami, S., Rakwal, R., and Agrawal, G.K., 2008. Interplant communication: airborne methyl jasmonate is essentially converted into JA and JA-Ile activating jasmonate signaling pathway and VOCs emission. *Biochem Biophys Res Commun* 376:723-727.

Tattini, M., Guidi, L., Morassi-Bonzi, L., Pinelli, P., Remorini, D., Degl'Innocenti, E., Giordano, C., Massai, R., and Agati, G., 2005. On the role of flavonoids in the integrated mechanisms of response of *Ligustrum vulgare* and *Phillyrea latifolia* to high solar radiation. *New Phytol* 167:457-470.

Textor S., Bartram, S., Kroymann, J.r., Falk, K., Hick, A., Pickett, J., and Gershenzon, J., 2004. Biosynthesis of methionine-derived glucosinolates in *Arabidopsis thaliana*: recombinant expression and characterization of methylthioalkylmalate synthase, the condensing enzyme of the chain-elongation cycle. *Planta* 218:1026-1035.

Tian, M.S., Downs, C.G., Lill, R.E., and King, G.A., 1994. A role for ethylene in the yellowing of broccoli after harvest. *Journal of the American Society for Horticultural Science* 119:276-281.

Tian, Q., Rosselot, R.A., and Schwartz, S.J., 2005. Quantitative determination of intact glucosinolates in broccoli, broccoli sprouts, Brussels sprouts, and cauliflower by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry. *Anal Biochem* 343:93-99.

Truscott, R.J.W., Minchinton, I., and Sang, J., 1983. The isolation and purification of indole glucosinolates from brassica species. *Journal of the Science of Food and Agriculture* 34:247-254.

U.S. Department of Agriculture, A.R.S., 2011a. USDA Database for the flavonoid content of selected foods, Release 3.0.

U.S. Department of Agriculture, A.R.S., 2011b. USDA National nutrient database for standard reference.

Valko, M., Izakovic, M., Mazur, M., Rhodes, C.J., and Telser, J., 2004. Role of oxygen radicals in DNA damage and cancer incidence. *Mol. and Cellular Biochem.* 266:37-56.

Van Dam, N.M., Witjes, L., and Svatoš, A., 2004. Interactions between aboveground and belowground induction of glucosinolates in two wild *Brassica* species. *New Phytologist* 161:801-810.

van Dam, R.M., Naidoo, N., and Landberg, R., 2013. Dietary flavonoids and the development of type 2 diabetes and cardiovascular diseases: review of recent findings. *Curr Opin Lipidol* 24:25-33.

van Poppel, G., Verhoeven, D.T., Verhagen, H., and Goldbohm, R.A., 1999. Brassica vegetables and cancer prevention. Epidemiology and mechanisms. *Adv Exp Med Biol* 472:159-168.

Velasco, P., Cartea, M.E., Gonzalez, C., Vilar, M., and Ordas, A., 2007. Factors affecting the glucosinolate content of kale (*Brassica oleracea* acephala group). *J Agric Food Chem* 55:955-962.

Velasco, P., Soengas, P., Vilar, M., Cartea, M.E., and del Rio, M., 2008. Comparison of glucosinolate profiles in leaf and seed tissues of different *Brassica napus* Crops. *Journal of the American Society for Horticultural Science* 133:551-558.

Velasco, P., Francisco, M., Moreno, D.A., Ferreres, F., Garcia-Viguera, C., and Cartea, M.E., 2011. Phytochemical fingerprinting of vegetable *Brassica oleracea* and *Brassica napus* by simultaneous identification of glucosinolates and phenolics. *Phytochem Anal*.

Verkerk, R., Dekker, M., and Jongen, W.M.F., 2001. Post-harvest increase of indolyl glucosinolates in response to chopping and storage of *Brassica* vegetables. *Journal of the Science of Food and Agriculture* 81:953-958.

Volden, J., Bengtsson, G.B., and Wicklund, T., 2009. Glucosinolates, L-ascorbic acid, total phenols, anthocyanins, antioxidant capacities and colour in cauliflower (*Brassica oleracea* L *ssp botrytis*); effects of long-term freezer storage. *Food Chemistry* 112:967-976.

Wagner, A.E. and Rimbach, G., 2009. Ascorbigen: chemistry, occurrence, and biologic properties. *Clin Dermatol* 27:217-224.

Wallig, M.A., Kingston, S., Staack, R., and Jefferey, E.H., 1998. Induction of rat pancreatic glutathione S-transferase and quinone reductase activities by a mixture of glucosinolate breakdown derivatives found in Brussels sprouts. *Food Chem Toxicol* 36:365-373.

Wang, C.Y., 1977. Effect of aminoethoxy analog of rhizobitoxine and sodium benzoate on senescence of broccoli. *Hortscience* 12:54-56.

Wang, L., Liu, D., Ahmed, T., Chung, F.L., Conaway, C., and Chiao, J.W., 2004. Targeting cell cycle machinery as a molecular mechanism of sulforaphane in prostate cancer prevention. *Int J Oncol* 24:187-192.

Wang, S.Y., Bowman, L., and Ding, M., 2008. Methyl jasmonate enhances antioxidant activity and flavonoid content in blackberries (*Rubus sp.*) and promotes antiproliferation of human cancer cells. *Food Chemistry* 107:1261-1269.

Watanabe, K., Kamo, T., Nishikawa, F., and Hyodo, H., 2000. Effect of methyl jasmonate on senescence of broccoli florets. *Journal of the Japanese Society for Horticultural Science* 69: 605-610.

Wathelet, J.P., Marlier, M., Severin, M., Boenke, A., and Wagstaffe, P.J., 1995. Measurement of glucosinolates in rapeseeds. *Natural Toxins* 3:299-304.

Williamson, G., Plumb, G.W., Uda, Y., Price, K.R., and Rhodes, M.J., 1996. Dietary quercetin glycosides: antioxidant activity and induction of the anticarcinogenic phase II marker enzyme quinone reductase in Hepalclc7 cells. *Carcinogenesis* 17:2385-2387.

Wilson, E.l., Ennahar, S.Ø., Zhao, M., Bergaentzle, M., Marchioni, E., and Bindler, F.o., 2011. Simultaneous determination of various isothiocyanates by RP-LC following precolumn derivatization with mercaptoethanol. *Chromatographia* 73:137-142.

Wolucka, B.A., Goossens, A., and Inze, D., 2005. Methyl jasmonate stimulates the de novo biosynthesis of vitamin C in plant cell suspensions. *Journal of Experimental Botany* 56:2527-2538.

Yannai, S., Day, A.J., Williamson, G., and Rhodes, M.J.C., 1998. Characterization of flavonoids as monofunctional or bifunctional inducers of quinone reductase in murine hepatoma cell lines. *Food and Chemical Toxicology* 36:623-630.

Yao, H. and Tian, S., 2005. Effects of a biocontrol agent and methyl jasmonate on postharvest diseases of peach fruit and the possible mechanisms involved. *J Appl Microbiol* 98:941-950.

Yudina, L.N., Korolev, A.M., Reznikova, M.I., and Preobrazhenskaya, M.N., 2000. Investigation of neoascorbigen. *Chemistry of Heterocyclic Compounds* 36:144-151.

Zang, Y.-X., Kim, D.-H., Park, B.-S., and Hong, S.-B., 2009. Metabolic engineering of indole glucosinolates in Chinese cabbage hairy roots expressing Arabidopsis CYP79B2, CYP79B3, and CYP83B1. *Biotechnology and Bioprocess Engineering* 14:467-473.

Zang, Y.X., Kim, J.H., Park, Y.D., Kim, D.H., and Hong, S.B., 2008a. Metabolic engineering of aliphatic glucosinolates in Chinese cabbage plants expressing Arabidopsis MAM1, CYP79F1, and CYP83A1. *BMB Rep* 41:472-478.

Zang, Y.X., Lim, M.H., Park, B.S., Hong, S.B., and Kim, D.H., 2008b. Metabolic engineering of indole glucosinolates in Chinese cabbage plants by expression of Arabidopsis CYP79B2, CYP79B3, and CYP83B1. *Mol Cells* 25:231-241.

Zhang, J., Satterfield, M.B., Brodbelt, J.S., Britz, S.J., Clevidence, B., and Novotny, J.A., 2003. Structural characterization and detection of kale flavonoids by electrospray ionization mass spectrometry. *Analytical Chemistry* 75:6401-6407.

Zhang, Y., Kensler, T.W., Cho, C.G., Posner, G.H., and Talalay, P., 1994. Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. *Proc Natl Acad Sci U S A* 91:3147-3150.

Zhang, Y., Talalay, P., Cho, C.G., and Posner, G.H., 1992. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc Natl Acad Sci U S A* 89:2399-2403.

Zhang, Z., Ober, J.A., and Kliebenstein, D.J., 2006. The gene controlling the quantitative trait locus *EPITHIOSPECIFIER MODIFIER1* alters glucosinolate hydrolysis and insect resistance in Arabidopsis. *Plant Cell* 18:1524-1536.

Zangerl, A.R. and F.A. Bazzaz. 1992. Theory and pattern in plant defense allocation., p. ix, 590 p. In: Fritz, R. S. and Simms, E. L. (eds.), Plant resistance to herbivores and pathogens : ecology, evolution, and genetics. University of Chicago Press, Chicago.

Zhu, C.Y. and Loft, S., 2003. Effect of chemopreventive compounds from *Brassica* vegetables on NAD(P)H:quinone reductase and induction of DNA strand breaks in murine hepa1c1c7 cells. *Food Chem Toxicol* 41:455-462.