ABIOTIC STRESS HORMESIS: HORMETIC STRESSES TO MAINTAIN QUALITY AND ENHANCE GLUCOSINOLATES AND PHENOLIC COMPOUNDS IN BROCCOLI (*BRASSICA OLERACEA* VAR. *ITALICA*) DURING STORAGE

Thèse

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Résumé

Le brocoli (Brassica oleracea var. italica) est un légume devenu populaire grâce à ses caractéristiques nutritionnelles et bioctives qui sont associées entre autres à la prévention de certaines maladies chroniques. L'utilisation de stress abiotiques tels que UV-C comme traitement de pré-entreposage a montré un grand potentiel pour l'induction de la résistance aux maladies et la préservation de la qualité des produits frais, et il est de plus en plus évident qu'il existe un potentiel pour améliorer les métabolites secondaires. L'objectif de ce travail a été, d'abord, d'établir si divers stress abiotiques, UV-B, UV-C, la chaleur, l'ozone, le peroxyde d'hydrogène, l'éthanol, et méthyl jasmonate (MeJA), induisent le phénomène d'hormèse. L'effect de ces traitements sur certains paramètres associés à la qualité des fleurons de brocoli tels que: la couleur, la perte de poids, la teneur en glucosinolates et en composés phénoliques. La chaleur et l'éthanol ont été les meilleurs traitements pour le retarder le jaunissement des fleurons, mais UV-C et UV-B étaient également efficaces pour maintenir la couleur verte de fleurons dans l'entreposage. D'autre part, la capacité antioxydant des fleurons a été principalement renforcée par les traitements d'UV-B et de chaleur. Le paramètre le plus important dans cette recherche était la teneur en glucosinolates de fleurons qui a été influencé positivement par le traitement à l'ozone et au peroxyde d'hydrogène, et dans une moindre mesure par le traitement d'UV-B. Il a été conclu que les stress abiotiques peuvent influencer favorablement soit la qualité ou l'augmentation de glucosinolates dans les fleurons pendant l'entreposage, mais pas le deux. Parmi les agents stressants utilisés, la lumière UV-B a été le plus efficace à maintenir la qualité et à induire une augmentation des composantes phytochimiques dans le broccoli.

Abstract

Broccoli (*Brassica oleracea* var. Italica) has become popular thanks to its health properties that are associated with the prevention of certain chronic diseases. The use of abiotic stresses such as UV-C as pre-storage treatment has shown great potential for induction of disease resistance in and preservation of quality of fresh produce, and it is becoming increasingly clear that there is potential for enhancing secondary metabolites. The objective of this work was, first, to establish whether various abiotic stresses, UV-B UV-C, heat, ozone, hydrogen peroxide, ethanol, and the plant signalling molecule, methyl jasmonate (MeJA), may induce hormesis in broccoli florets on color retention response; and second, to determine the effect of various abiotic stresses on quality, mainly color retention and weight loss; the contents of glucosinolates and phenolic compounds in florets during storage. Heat and ethanol were the best treatments for delaying yellowing florets, but UV-C and UV-B were also effective at a lower extent. On the other hand, the antioxidant capacity of the florets was mostly enhanced by UV-B and heat treatments. The most important enquiry in this research was the augmentation of glucosinolates titers, which was influenced by the treatment with ozone and hydrogen peroxide, and to a less extent by UV-B. It was concluded that abiotic stresses could influence favourably either the quality or the enhancement of glucosinolates in broccoli during storage and not both. Among the considered stressing factors, UV-B was the most effective for maintenance of quality as well as to elevate the levels of phytochemicals in broccoli.

Résumé long

Le brocoli est un légume très apprécié pour son contenu en nutriments et composés potentiellement bioactifs, cependant il est sensible à des détériorations rapides pendant l'entreposage post-récolte. Le principal paramètre de qualité qui est affecté lors de cette période est la couleur, les fleurons changeant de vert foncé à jaune en un temps relativement court. Ce changement de la couleur est fortement influencé par la température et l'humidité relative, qui sont ainsi les principaux outils utilisés pour ralentir la sénescence du brocoli pendant le période post-récolte.

Les stress abiotiques à des niveaux élevés peuvent être nocifs pour la survie d'une plante mais, à de faibles doses, ils peuvent induire des processus adaptatifs ou des réactions bénéfiques, phénomènes connus sous le nom d'hormèse. L'apparition de ce phénomène a été démontrée avec l'application de doses hormétiques d'UV-C à plusieurs produits horticoles, lesquels ont développé une résistance aux maladies en plus de présenter un ralentissement de la maturation ou un retard de la sénescence. L'existence du phénomène d'hormèse en post récolte a été mise en évidence seulement avec les UV-C. Cependant, il n'est pas connu si l'effet hormétique de divers facteurs de stress abiotiques peut améliorer le contenu en composés phytochimiques bénéfiques, même si les plantes accumulent naturellement des métabolites secondaires pour se protéger de stress environnementaux.

L'objectif de cette étude a été, dans un premier temps, d'établir si des stress abiotiques tels que UV-B, UV-C, chaleur, ozone, peroxyde d'hydrogène, éthanol et jasmonate de méthyle, appliqués après la récolte provoquent le phénomène d'hormèse chez le brocoli, notamment quant à la rétention de la couleur des fleurons, et deuxièmement de caractériser l'effet des stress, à des doses hormétiques et des doses élevées, sur la qualité, notamment rétention de la couleur et perte de poids, et sur le contenu en glucosinolates et composés phénoliques des fleurons pendant l'entreposage.

La dose de 1,2 $kJ.m^{-2}$ de lumière UV-C a montré un effet hormétique pour la rétention de la couleur. Tant la dose hormétique (1,2 $kJ.m^{-2}$) que la dose élevée (3,0 $kJ.m^{-2}$) d'UV-C ont diminué les titres d'acides aminés, méthionine, tryptophane et phénylalanine qui sont les précurseurs de glucosinolates, et les titres d'acides hydroxycinnamiques. L'épuisement des titres d'acides aminés a été associé à une surexpression de gènes phenylalanine N-hydroxylase (CYP79A2), tryptophan N-hydroxylase 2 (CYP79B3), dihomomethionine N-hydroxylase (CYP79F1) et flavanone 3-hydroxylase

(F3H1) chez les brocolis exposés. De la même façon, une augmentation significative de glucoraphanine et des glucobrassicines totaux, particulièrement de 4-hydroxyglucobrassicine, et des acides hydroxy cinnamiques a été observée dans les fleurons de brocoli traités avec 1,2 et 3,0 $kJ.m^{-2}$ respectivement.

Les traitements thermiques impliquent une combinaison de température et de durée, et la superposition temps-température peut permettre des équivalences dans une certaine gamme de températures pour obtenir une valeur spécifique de la chaleur, soit une dose. La fuite d'électrolytes (FE) produite dans le tissu a été utilisée comme un indicateur d'endommagement de la membrane cellulaire après l'exposition du brocoli à la vapeur à des températures comprises entre 32 et 52 °C pendant 5 à 1440 min. Bien que des équivalences temps-températures aient été déterminées à partir de la cinétique de fuite d'électrolyte, un choix de température de traitement thermique au-dessus de la température critique (Tc) de 43 °C peut entraîner des conditions anaérobiques. Toutefois, des températures supérieures à Tc ont provoqué la meilleure rétention de couleur à la suite de la probable altération de la surface et des propriétés de réflexion suite à l'élimination de l'air entre les cellules.

Les fleurons de brocoli ont été traités avec la dose hormétique de chaleur (41 °C/180 min) ou avec son équivalent à 47 °C/12 min (HDHT). Les deux traitements à la vapeur ont augmenté significativement les taux de respiration de fleurons après traitements. Les titres de chlorophylle ont été maintenus avec les deux traitements pendant 21 jours d'entreposage par rapport aux fleurons non traités qui ont jauni. La concentration de glucosinolates de type indole a augmenté après l'exposition du brocoli à l'un ou l'autre des traitements, cependant la concentration de glucoraphanine a été renforcée seulement avec HDHT. Aussi, HDHT a amélioré la concentration des acides hydroxy cinnamiques par rapport à la dose hormétique. Néanmoins des mauvaises odeurs ont été détectées à la fin de la durée d'entreposage avec HDHT.

L'effet du stress oxydatif sur les fleurons de brocoli a été étudié via des traitements avec UV-B, ozone (O_3) ou peroxyde d'hydrogène (H_2O_2). Les trois facteurs de stress (ou agents stressants) ont provoqué le phénomène d'hormèse quant à la rétention de la couleur dans les fleurons, cependant ce paramètre a été mieux préservé dans les fleurons traités avec UV-B. Les doses hormétiques étaient 1,5 $kJ.m^{-2}$ (UV-B), 5 ppm/60 min (O_3) et 1,25 mM/180 min (H_2O_2). L'ozone a semblé être la cause du stress oxydatif le plus intense quant au niveau des taux de respiration et de perte de poids. Cependent, cette perte de poids n'est pas entièrement attribuable à la perte

d'humidité. La capacité antioxydante des fleurons a diminué avec l'application des trois stress après 21 jours d'entreposage par rapport aux brocolis non traités.

Les doses hormétiques d'O_3 et H_2O_2 étaient plus efficaces pour élever les niveaux de glucoraphanine et glucobrassicines par apport à UV-B, alors que ce dernier était plus efficace dans l'amélioration des niveaux d'acides hydroxy cinnamiques. Toutefois, la dose élevée d'O_3 a réduit les niveaux des glucosinolates et des acides hydroxy cinnamiques. En conséquence, UV-B semble être le stress le plus efficace en ce qui concerne la préservation de la qualité et l'amélioration des phytocomposés des fleurons de brocoli.

L'expression du gène CYP79B3 a été particulièrement surexprimée avec l'application des stress oxydatifs et a été reliée à l'augmentation des titres de glucosinolates de type indoles. Ceci suggère que la cible d'UV-B, O_3 et spécialement H_2O_2, est probablement la voie des glucosinolates de type indole. Il semble que l'accumulation de glucosinolates de type indole peut être préférentielle parmi les glucosinolates ou autres métabolites secondaires tels que les composés phénoliques en raison de leur état plus réduit.

Finalement, l'effet de la fumigation des fleurons de brocoli avec l'éthanol et le jasmonate de méthyle (MeJA) a été étudié. Il a été démontré que le jaunissement des fleurons a été retardé en utilisant 10,000 ppm d'éthanol pendant 30 ou 120 min par rapport aux fleurons non traités, bien que l'éthanol ait eu un effet modéré negative sur la perte de poids des fleurons. Les titres de chlorophylle étaient également supérieurs avec les deux doses par rapport au témoin. En revanche, l'exposition des fleurons à 1 ppm de MeJA pendant 45 min (dose hormétique) a conduit à un changement de couleur comparable à celle du témoin. Cependant, dans les fleurons traités avec 1 ppm de MeJA pour 45 ou 180 min, le contenu en glucosinolates de type indole et de 4-hydroxyglucobrassicine était supérieur au témoin. D'autre part, le contenu en glucosinolates de type indole et de type aliphatique ainsi qu'en acides hydroxy cinnamiques a été augmenté de façon conséquente par les traitements avec l'éthanol.

La chaleur et l'éthanol ont été les traitements les plus efficaces pour retarder le jaunissement des fleurons pendant l'entreposage. La capacité antioxydante des fleurons a été légèrement augmentée par la chaleur ainsi que par les UV-B. L'ozone et le H_2O_2 ont été jugés efficaces pour l'augmentation des glucosinolates de type indole, ainsi que pour la glucoraphanine. Néanmoins, l'ozone n'est pas avantageux du point de vue de la perte de poids des fleurons pendant le traitement et l'entreposage. Aucun des agents de stress abiotiques évalué n'a permis d'améliorer à la fois la

qualité des fleurons et leur contenu en métabolites secondaires d'intérêt pendant l'entreposage. Cependant, les UV-B constituent le stress le plus efficace pour le maintien de la qualité, ainsi que pour l'augmentation du contenu de composés phytochimiques dans les fleurons. Ce traitement peut être envisagé comme complément à l'entreposage à basse température des brocolis et d'autres produits végétaux frais.

Extended abstract

Broccoli is a highly valued vegetable for its nutrient and bioactive contents, but it is also susceptible to rapid deterioration during post-harvest storage. The main quality factor that is affected during storage is color, also an indicator of senescence florets changing from dark green to yellow in a relatively short time. This change of color is strongly influenced by temperature and relative humidity; and hence, low temperature-high relative humidity storage is the main method used to delay the senescence of broccoli. Postharvest treatments, mainly the use of abiotic stresses, along with ideal temperature and relative humidity storage conditions can help maintain the quality of broccoli.

Abiotic stresses at high levels can be harmful to plant life, but they can induce adaptive processes or beneficial reactions at low doses, a phenomenon known as hormesis. The occurrence of this phenomenon has been shown with UV-C radiation in postharvest systems, where the produce treated with hormetic doses of UV-C showed induced disease resistance and delay ripening and senescence. However, hormetic phenomenon has not been shown to exist with other abiotic stresses in postharvest produce. In addition, it is not known whether hormetic doses of stresses could indeed enhance secondary metabolites, although plant bodies accumulate secondary products to protect themselves when subject to environmental stresses.

Thus the objective of this study was, first, to establish whether various abiotic stresses, UV-B, UV-C, heat, ozone, hydrogen peroxide, ethanol, and the plant signalling molecule, methyl jasmonate (MeJA), cause hormesis in broccoli florets on color retention response. Secondly, to determine the effect of various abiotic stresses, both at hormetic and high doses, on quality, mainly color retention and weight loss; the contents of glucosinolates and phenolic compounds in florets during storage.

UV-C light showed hormetic effect for color retention at 1.2 $kJ.m^{-2}$. Both the hormetic dose (1.2 $kJ\ m^{-2}$) and the high dose (3.0 $kJ\ m^{-2}$) of UV-C caused a decrease in the titers of amino acids, methionine, tryptophan and phenylalanine, the precursor amino acids of glucosinolates or phenolic compounds. The depletion of amino acid titers was linked to overexpression of phenylalanine N-hydroxylase (CYP79A2), tryptophan hydroxylase (CYP79B3), dihomomethionine N-hydroxylase (CYP79F1) and flavonone 3-hydroxylase (F3H1) in broccoli florets exposed to UV-C. Similarly, a significant increase in glucoraphanin and glucobrassicins, especially 4-hydroxy-glucobrassicin was observed in broccoli florets treated with 1.2 and 3.0 kJm^{-2}, respectively.

Heat treatment involves a combination of temperature and time, and time-temperature superposition can allow to determine time-temperature equivalences for a range of temperature to obtain a specific heat value or dose. Electrolyte leakage (EL) was used as an indicator of cell membrane damage in response to exposure of broccoli florets to hot humidified air at temperatures from 32 to 52 °C for periods ranging from 5 to 1440 min. Although equivalent exposure time/temperatures were found from the kinetics of electrolyte leakage, there existed a critical temperature (Tc) of 43 °C, above which the heat treatment caused severe anaerobic conditions and off-odors with increase in temperature, although color was maintained, presumably, by alteration in the surface reflection properties of tissue due to the removal of air between the cells. Heat treatment at 41 °C for 180 min was considered optimal to deliver the hormetic heat dose

Broccoli florets were treated with the hormetic heat dose (41 °C/180 min) and the same dose at 47 °C/12 min (HDHT), and both treatments significantly increased the initial respiration rate or stress respiration. Chlorophyll titers were maintained with both treatments over 21 days of storage, compared with non-treated florets that turned yellow. The concentration of indole-type glucosinolates increased after the exposure to both doses; but the concentration of glucoraphanin was only enhanced by the HDHT, which improved the concentration of hydroxyl-cinnamic acids, but off-odors were detected in florets exposed to this temperature (47 °C).

The effect of oxidative stresses, UV-B light, ozone (O_3) and hydrogen peroxide (H_2O_2), on broccoli florets was studied. All the three stress factors showed hormesis with respect to color retention in broccoli florets, but the color retention was superior with UV-B; and hormetic doses were 1.5 $kJ.m^{-2}$ (UV-B); 5ppm /60 min (O_3); and 1.25mM /180min, (H_2O_2). Ozone appeared to be the most intense stress factor among the stresses, as seen by the level of respiration rate, as well as the significant weight of weight loss of florets during the treatment, although this last may not be entirely due to moisture loss. The antioxidant capacity of florets decreased with the application of O_3 and UV-B after 21 days of storage compared with the non-treated florets.

Hormetic doses of O_3 and H_2O_2 were effective in elevating the levels of glucoraphanin and glucobrassacins compared with UV-B; whereas UV-B was more effective in the enhancement of hydoxycinnamic acids than ozone and H_2O_2. Furthermore, high ozone dose depressed the levels of both glucosinolates and hydroxycinnamic acids. Thus UV-B appears to be the stress exhibiting balanced effects with respect to quality preservation and enhancement of phyto-compounds in broccoli florets. Results showed a good correlation between gene expression of CYP79B3, and the

titers of indole glucosinolates in the treated broccoli florets, suggesting that the target of UV-B, O_3 and specially H_2O_2 is likely to be the branch pathway of indole glucosinolates. It appears that the accumulation of indole glucosinolates may be preferential among the glucosinolates or among the other secondary metabolites such as phenolic compounds because of their most reduced state.

Finally, the effect of fumigation of broccoli florets with ethanol and methyl jasmonate (MeJA) was studied. It was found that yellowing was delayed using 10,000 ppm of ethanol for 30 min (hormetic dose), and for 120 min (high dose). Chlorophyll titer was also superior for both doses compared with the control, although ethanol had a moderate effect on florets weight loss. In contrast, the exposure of florets to 1 ppm of MeJA for 45 min (hormetic dose) led to color change that was similar to that of the control. However, the amount of the indole-type glucosinolate, 4-hydroxyglucobrassicin in florets treated with the hormetic dose (1 ppm of MeJA/45 min) and high dose (1 ppm/180 min) was superior compared with the control. On the other hand, the content of indole and aliphatic-type glucosinolates, as well as hydroxy-cinnamic acids were enhanced after ethanol exposure with both doses.

Heat and ethanol were found to be the most effective treatments for delaying yellowing florets, as were UV-C and UV-B, but the effects of the later were less pronounced. The antioxidant capacity of the florets was moderately enhanced by heat as well as by UV-B. Ozone and hydrogen peroxide were found to be effective efficient stresses to enhance indole glucosinolates as well as glucoraphanin. But ozone, the most intense oxidative stress may not be advantageous from the standpoint of severe weight loss during treatment as well as during storage. There was no single stress that can improve storability of broccoli florets and be equally effective in the enhancement of phytochemicals in broccoli. UV-B was found to be the balanced stress for maintenance of quality as well as to elevate the levels of phyto-compounds in broccoli. This work shows that there is potential for the use of abiotic stresses as treatment to improve quality, delayed senescence as well as to enhance phytochemical content, as adjunct to low temperature storage of fresh produce.

Foreword

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Exposure of vegetal tissues to abiotic stresses previous to the postharvest storage is an approach to delayed senescence, improve quality and enhance their phytochemical composition.

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To my Parents, María and Sofía

Index

Figure list

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

Chapter VI

Chapter VII

Annexes

Table list

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

Chapter VI

Annexes

Abbreviation list

Abbreviation	Meaning
13-HPOT	13(S)-hydroxy lonolenic acid
1-MCP	1-methyl-cyclopropene
ABA	absicic acid
ACC	1-aminocyclopropane-1-carboxylic acid
acetyl CoA	acetyl coenzyme A
ANR	leucocyanidin deoxygenase
AOA	amino oxyacetic acid
AOC	allene oxide cyclase
AOS	Allene oxide synthase
AOXs	alternative oxidases
APX	ascorbate peroxidase
ATP	adenosine triphosphate
AVG	aminoethoxy vinylglycine
BA2H	benzoic acid 2-hydroxyase
BR	brassinosteroids
bZIP	basic-domain leucine-zipper
CA	controlled atmosphere
Ca^{2+}	calcium
CAT	catalase
CDPKs	calcium-dependent protein kinases
CHI	chalcone isomerase
CHS	chalcone synthase
CK	cytokinins
CYP79A2	phenylalanine N-hydroxyase
CYP79B3	tryptophan N-hydroxyase
CYP79F1	dihomo-methionine N-hydroxyase
DFR	dehydroflavonol reductase
DHA	dehydroascorbate
DNA	deoxyribonucleic acid
ERS	ethylene response sensor
ET	ethylene
EtOH	ethanol
ETR	ethylene response
F3H	flavanone 3-hydroxyase
F3H1	flavonoid monooxygenase
FLS	flavonol synthase

GA	gibberellins
GLS	glucosinolates
GPCRs	G-proteins-coupled receptors
GRAS	Generally Regarded as Safe
GSH	reduced glutathione
GSSG	oxidized glutathione
GTP	guanosine-5'-triphosphate
H_2O_2	hydrogen peroxide
HKs	histidine kinases
HSPs	heat-shock proteins
IAA	indole-3-acetic acid
IFS	isoflavone synthase
JA	jasmonates
JMP	jasmonic acid carboxyl methyltransferase
LAR	leucocyanidin 4-reductase
LDOX	leucocyanidin deoxynase
LOX	lipooxygenase
LP	lipid peroxidation
MAP	modified atmospheres
MAP	mitogen-activated protein
MDHA	monodehydroascorbate
MDHAR	monodehydroascorbate reductase
MeJA	methyl jasmonate
MTA	methylthioadesine
NADH	nicotinamide adenine dinucleotide
NO	nitrous oxide
O_2^-	Superoxide
O_3	ozone
OPDA .	12-oxo-phytodienoic acid
PAL	phenylalanine ammoniolyase
PCD	programed cell death
PPO	Polyphenol oxidase
PR	pathogenesis-related
PUFA	Polyunsaturated fatty acids
Q_{10}	temperature coefficient
RH	relative humidity
RLKs	receptor-like kinases
RNA	ribonucleic acid
ROS	reactive oxygen species

SA	salicylic acid
SAM	S-adenosylmethinine
SAR	systemic acquired resistance response
SMs	Secondary metabolites
SO_2	sulfur dioxide
SOD	superoxide dismutase
TCA	tricarboxylic acid cycle
TFs	transcription factors
UV	ultraviolet light
WVP	water vapor pressure
WVPD	water vapor pressure deficit

General introduction

"Let food to be thy medicine and medicine be thy food" said the Greek philosopher Hippocrates many centuries ago, and it still rings true in the general perception of our global society towards the food that we eat. In particular fruits and vegetables are generally considered as "good" food as they are excellent sources of vitamins, minerals, fiber and phytochemicals with health-promoting properties. Certain phytochemicals present in fruits and vegetables have been found to have anti-oxidant, anti-cancer and cardiovascular protection properties. However, fruits and vegetables are perishable once they are harvested since physiological processes such as respiration, transpiration and hormone activity are still active and may even favor decay caused by microorganisms as a consequence. In order to delay degradation of produce, postharvest techniques and treatments have been applied since ancient time. Among them, temperature and relative humidity control are the most important techniques used to delay senescence. These treatments are important for two reasons: 1) produce can retain its quality and phytochemical content of for longer periods of time, 2) economical profit is generated since larger quantities of commodities can be commercialized.

Brassica is the most economically important genus within the *Brassicaceae* family, including three diploids: *Brassica nigra*, *Brassica oleracea* and *Brassica rapa* and three amphidiploids: *Brassica carinata*, *Brassica juncea* and *Brassica napus.* Along with cereals, brassica crops represent the basis of world foods and their usage can be classified as oilseed, forage, condiment and vegetable crops (Cartea et al., 2011). Domestication of *Brassicas* probably can be traced back to Neolithic man; ancient Greeks, Romans and Chinese valued and cultivated them significantly. Production of brassicas has been in continuous expansion worldwide. By 1961, 26.8 million tons were produced increasing by 300 % in 2007 with 88.3 million tons. Of particular importance, production of cauliflower and broccoli has increased six times and that of cabbage and other brassicas has increased three-times in the last 5 decades. The principal producers are China, India, Russian Federation, Republic of Korea, Japan and the United States (Cartea et al., 2011).

Broccoli (*Brassica oleracea var. italica*), sometimes referred to as 'the crown jewel of nutrition', combines high levels of micronutrients and phytochemical compounds. Broccoli is a low source of fat and energy and has several vitamins (A, C, E), calcium, carotenoids, terpenes, indoles, isothyocianates, flavonoids and glucosinolates (Nestle, 1998). The most value portions of broccoli plants are the heads, which are inflorescences consisting of immature fully differentiated flower buds

and tender upper stems. Broccoli is available commercially as fresh or frozen florets and is used raw in salads or as vegetable crudités. It is also frequently cooked and served by itself as well as being a component of many cooked and stir-fried dishes. There are over 100 commercial hybrid cultivars of broccoli, derived from a limited number of landraces or open pollinated cultivars that include purple sprouting, purple cape, purple Sicilian, white sprouting, and green sprouting broccoli. Impetus for the dramatic increase in consumption over the past 10 years or so have been driven by the reported the putative positive benefits on the human health related with broccoli. In fact, it is regularly identified as the vegetable eaten most often for health reasons, including cancer prevention (Schouten et al., 2009b). However, broccoli is also a highly perishable vegetable with a shelf life of only a few days.

Broccoli has a very low rate of ethylene production but it is very sensitive to this gas and the most visible symptom is yellowing of florets. Moreover, harvested broccoli is composed by immature tissue which deteriorates very quickly due to very high respiration rates (Finger et al., 1999). Also there are other physiological and pathological problems that can significantly reduce the shelf life of this vegetable. The most common is the head yellowing produced by inappropriate storage temperatures and ethylene exposure. On the other hand *Botrytis cinerea* and *Erwinia carotovora* are the most common mold and bacteria found in stored broccoli (Toivonen and Forney, 2004). In order to control these effects, appropriate precooling and storage conditions must be used. The most common storage conditions are cold storage at 0 °C and 90 % of relative humidity. Controlled and modified atmospheres are also used to delay senescence of fresh-broccoli (Thompson, 2008). In addition to these techniques, postharvest treatments have been recently applied to broccoli in order to enhance phytochemical compounds.

Broccoli is an excellent dietary source of glucosinolates and glucosinolate-breakdown products, phenolic acids, hydroxy-cinnamic acids and flavonoids (Moreno et al., 2006). Biosynthesis of glucosinolates and flavonoids which are the most important phytochemicals in broccoli, is derived from amino acids through different pathways. The first step in glucosinolate biosynthesis is the conversion of amino acids to aldoximes which is catalyzed by cytochromes P450 from CYP79 family (Mikkelsen et al., 2002). On the other hand, flavonoids are derived from phenylalanine through the phenylpropanoid or acetate-malonate pathway catalyzed by three important enzymes, phenylalanine ammoniolyase (PAL), chalcone synthase (CS) and flavonoid monooxygenase (F3H1) (Weston and Mathesius, 2013). In broccoli the most important phytochemical compounds are glucoraphanin, glucobrassicin quercetin and kaempferol (Moreno et al., 2006). These compounds function as

defenses, and their concentration can be increased under the pressure of external factors such as insects, microorganisms or unfavorable environmental conditions (Mewis et al., 2012; Textor and Gershenzon, 2009). This characteristic has been employed in postharvest storage as a tool to enhance the natural content of phytochemical compounds of broccoli and other commodities.

Different postharvest treatments have been applied during the storage of broccoli in order to delay senescence. UV light has been found to delay chlorophyll degradation in broccoli florets (Aiamla-or et al., 2010; Aiamla-or et al., 2012; Aiamla-or et al., 2009; Costa et al., 2006; Duarte-Sierra et al., 2012b). Heat and ethanol are two abiotic stresses known to arrest yellowing in broccoli (Corcuff et al., 1996; Duarte-Sierra et al., 2012a; Forney, 1995; Fukasawa et al., 2010; Tian et al., 1996). These are examples of positive effects that were produced by appropriate doses of harmful stresses. Hormesis is the term that refers to this phenomenon in which moderate stress can produce adaptive responses of cells and organisms (Calabrese and Baldwin, 2002). Similarly low amount of stress during short times can produce beneficial effects on phytochemical content of postharvest commodities. For instance, UV-B was found to increase titers of kaempferol, quercetin and glucosinolates in broccoli sprouts (Mewis et al., 2012). The exposure of seeds to exogenous elicitors such as methionine, tryptophan, chitosan, salicylic acid and methyl jasmonate, have shown a great potential to induced flavonoids and indole glucosinolates in broccoli (Pérez-Balibrea et al., 2011).

The present work is n exploratory study conducted in order to gain insights of the postharvest physiology of broccoli florets responding to various stresses applied before simulated commercial storage. Results have shown that the use hormetic doses can be used in order to enhance the phytochemical content of broccoli without compromising its quality. Since broccoli was a model of study, the effect of the applied stresses might be equivalent in other produce responding to appropriate hormetic doses.

The thesis is organized in eight chapters starting with a review of the state of art on treatments used to maintain quality and induce phytochemicals in broccoli. The effect of UV-C light on glucosinolates and amino acids was analyzed in the second chapter. Effect of heat treatment on broccoli florets was extensively studied in the third chapter. Electrolyte leakage of broccoli florets heated at different temperatures was used as a reference to established equivalences among tested temperatures. In the fourth chapter different temperatures were applied to florets in order to distinguish their effect on quality and nutraceutical characteristics. In the fifth chapter, oxidative stresses: UV-B, ozone and hydrogen peroxide, were analyzed simultaneously in order to gain

insights on the expression of enzymes related with glucosinolates biosynthesis as well as quality and nutraceutical parameters of florets. In the sixth chapter the influence of ethanol and methyl jasmonate on quality, phenolic compounds and glucosinolate content. The chapter seven is a general summary, in which all the stresses were compared in order to gain knowledge of specific effects of abiotic stresses on quality, glucosinolate content and antioxidant activity. The final chapter is a general conclusion, including some perspectives for further work.

Chapter I: Literature Review

1.1 Fruits and vegetables

There are more than 2000 plant species, of which only about 1 % represent the most important fruits and vegetable crops in the world. The classification of these crops can be done by botanical systematics, by plant organ (flower, leaf, tuber, etc.) or on by their end-uses and economic considerations (Haard, 1984). Fruits and vegetables have been part of our diet since ancient time, probably because of their delightful taste and aroma. However, their contribution to health and general well-being, through a sciencific approach, has been recognized over the last century. One of the most illustrative examples is the identification of vitamin C as an essential nutrient present in fruits by Albert Szent-Gyorgyi, for which he was honored with the Nobel Prize in medicine in 1932 (Terry and Thompson, 2011). The American National Academy of Sciences emphasized the importance of fruit and vegetables in the diet in 1982, highlighting carotene-rich fruits and cruciferous (*Brassicas*) vegetables for reducing risk of cancer (Liu, 2003). Consumers have been increasing eager to adopt a healthier diet since the National Cancer Institute (NCI) of the U.S. Department of Health and Human Services promoted in 1991 the "5-a-Day" consumption of fruit and vegetables as a measure to prevent cancer (Bartz and Brecht, 2002). Lower risk of coronary artery disease and stroke has been correlated with the increased dietary intake of plant-based foods such as fruits and vegetables (Hu, 2003).

Phytochemicals are non-nutrients exhibiting bioactive properties, present in fruits, vegetables and other plant foods. They can be classified into major classes, including carotenoids, saponins, glucosinolates and polyphenols (Liu, 2003; Watzl and Leitzmann, 1999). Consumption of fruits and vegetables with enhanced levels of phytochemicals can be a proactive approach to ensure a healthier population, since daily servings of phytochemical-enriched produce can ensure adequate exposure of the consumer to health-beneficial compounds. In addition, producers can add value to fruits and vegetables emphasizing their health functionalities such as "high lycopene/antioxidant content" that has shown increased sales (Kubota et al., 2006).

During the postharvest phase, fruits and vegetables are perishables and are subject to physiological, biochemical and microbial changes that can be influenced by postharvest handling and storage conditions (Arul, 1994; Terry and Thompson, 2011). Appropriate handling and storage are of vital importance for preserving quality, nutritional and phytochemical content of fresh produce

throughout storage, transportation and distribution. Therefore, research to develop new technologies to improve postharvest handling of fresh fruits, vegetables, and minimally-processed products is critical (Sitbon and Paliyath, 2011).

1.2 Factors limiting the storability and quality of fresh produce

Commodities are perishable and keep an active metabolism in the postharvest phase (Arul, 1994). Biochemical and metabolic events such as the loss of chlorophyll, the solubilization of pectin and starch degradation will continue until the complete degradation of fruits and vegetables. Respiratory activity, transpiration, maintenance of membrane integrity and ethylene biosynthesis are the most important physiological factors to control senescence (Phan, 1987). In addition, with the advance in senescence, the tissue becomes susceptible to decay caused by fungal and bacterial infection, which is also a major factor to be considered during the postharvest stage of fresh produce. Finally, non-controlled storage conditions such as high temperature and low humidity can negatively influence the postharvest storage of produce. Thus, understanding the physiological and metabolic changes during postharvest storage of produce would enable adopting appropriate strategies to delay the senescence process.

1.2.1 Respiration

In the senescing produce the stored resources are the only source of energy, since the capacity to produce new storable materials by photosynthesis is minimal (Solomos, 1981). Cellular respiration is a metabolic process where sugars are oxidized to simpler molecules such as CO_2 and H_2O with the release of energy as adenosine triphosphate (ATP). This process can be aerobic or anaerobic. The aerobic respiration involves three metabolic pathways: glycolysis, tricarboxylic acid cycle (TCA) and electron transport system. Briefly, glycolysis is the breakdown of glucose into pyruvate which occurs in the cytoplasm. Pyruvate is later converted into CO_2 in the TCA located in the mitochondrial matrix, along with reduced nicotinamide adenine dinucleotide (NADH). The electron transport system occurs in the mitochondrial cristae where NADH is oxidized to produce a proton gradient to synthetize ATP, and water by the addition of two protons to exogenous oxygen. The total amount of energy produced by aerobic respiration is of 38 ATP molecules produced by 1 glucose molecule. On the other hand, anaerobic respiration is defined as the conversion of carbohydrates into

ethanol and CO_2 in the absence of oxygen or when there is a lack of oxygen, where pyruvate is produced by glycolysis, which is converted into acetaldehyde and ethanol. The amount of energy produced by anaerobic respiration is 2 ATP, from one molecule of glucose, which is much less than compared with the aerobic respiration (Kader and Saltveit, 2002).

Besides the generation of energy for cellular function, respiration and more specifically respiration rate, can be used as an indicator for the metabolic activity of produce during the postharvest storage (Wills et al., 2007). Respiration rate can be measured as the oxygen consumed or the carbon dioxide generated by a specific vegetable during storage. Generally, there is an inverse relationship between respiration rate and the time that produce can be stored.

Fruits and vegetables can be classified into five different classes according to their relative rates of respiration and degree of perishability as very low, low, moderate, high and very high. Examples of very low respiration rate commodities at low temperature (5 °C) are cassava, garlic, potato, and other tubercles, where respiration rate are of less than 5 mL $CO_2.kg^{-1}.h^{-1}$. On the opposite side at the same temperature, very high respiration rates are observed in asparagus, broccoli and mushrooms, where the values are superior to 30 mL $CO_2.kg^{-1}.h^{-1}$ (Kader and Saltveit, 2002). Another classification that is often used during the senescence of fruits, cut flowers and leaves is related to ethylene action. Those fruits that produce high levels of ethylene are considered as climacteric, and the non-climacteric fruits are those unable to produce enough quantities of this phytohormone (Solomos, 1981).

Respiration can be affected by internal and external factors. Internal factors include the type of commodity, stage of development and surface area-to-volume ratio. Generally, bulbs are vegetables with low respiration rates but floral meristematic tissues exhibit very high respiration rates; and mature vegetables respire less compared with the immature vegetables (Kader and Saltveit, 2002). External factors that affect respiration of fruits and vegetables include temperature, oxygen and carbon dioxide concentration, ethylene as well as physical, mechanical and biological stresses (Kader and Saltveit, 2002). Temperature is without any doubt the most important factor that affects respiration, as respiration rate increases with temperature.

The temperature coefficient or Q_{10} is a common measure to relate the reaction rate at two different temperatures and it is generally used to assess the impact of temperature on the respiration of produce. Between 5 to 25 °C, the Q_{10} value for respiration is 2.0 and 2.5 for many fresh products

(Nunes do Nascimento and Emond, 2002). The Q_{10} value for broccoli is 3.9-4.1 from 0-10 °C and 3.6-3.7 from 10-20 °C (Hardenburg et al., 1986).

Oxygen concentration in the atmosphere affects the respiration rate of vegetables as well. While significant reductions in the respiration rate are observed when O_2 is reduced below 10 %, the respiration rate in some commodities increases significantly with oxygen concentrations above 80 %, due to toxicity (Kader and Saltveit, 2002). Carbon dioxide, on the other hand, can reduce respiration by the inhibition of the oxidation of succinate, due in part by the inhibition of succinate dehydrogenase (Gonzalez-Meler et al., 1996; Kays, 1991). The optimal levels of oxygen and carbon dioxide for broccoli are 1-2 % and 5-10 % respectively (Toivonen and Forney, 2004).

1.2.2 Transpiration

Transpiration is the evaporation of water from the intercellular spaces of plants, fruits, and vegetables to the atmosphere. This flux is proportional to partial pressure gradient between the vegetal structure and its surroundings, as well as the surface area, and inversely proportional to the resistance of the barrier to diffusion as it can be represented by Fick's Law:

$$J = \frac{(P_i - P_a)A_t}{(R_D T)r}$$

Where, J is the water vapor flux ($g.sec^{-1}.cm^{-2}$); P_i and P_a are the partial pressures (atm) in the intercellular spaces and in the ambient atmosphere, respectively; A_t is the organ's surface area (cm^2); R_D is the gas constant per gram; T is the absolute temperature (K) and r is the resistance to the flux of water vapor ($sec.cm^{-1}$)(Ben-Yehoshua and Rodov, 2002).

Transpiration in plants can be described by means of water relations. The partial water vapor pressure in the air at specified temperature is known as a water vapor pressure (WVP) (van den Berg, 1987). The difference between the WVP of the air and that of the vegetable at the same temperature is referred as water vapor pressure deficit (WVPD) or (P_i–P_a) and is the driving force in the process of evaporation (Ben-Yehoshua, 1987). The presence of high WVPD in the storage space can lead to an increase of evaporation resulting in water loss (Laurin et al., 2005).

Evaporation of water is one of the most important causes of deterioration in fresh produce after harvest. There are physical and physiological effects of moisture loss on vegetables. Among the physical effects, water loss induces wilting, shriveling, loss of firmness, crispness and succulence, which are related to the freshness and the overall quality of produce. In most of the cases

commodities become unsalable after losing 3 to 10 % of their weight (Ben-Yehoshua, 1987). Softening of leaves may occur at lower moisture loss compared with solid vegetables such as potatoes, where higher moisture loss may be required. On the other hand, absorption of water can occur leading to positive effects in quality such as an increase in color and brightness; but also some root vegetables may split, because of the increase in turgor pressure (van den Berg, 1987). At physiological level, water loss can affect plant tissue by the increased production of ethylene, adverse membrane changes, increased skin tissue breakdown as well as changes in the cuticle (van den Berg, 1987). For instance, the most evident physiological effect of water loss in vegetables is yellowing caused by increased ethylene production (Isenberg, 1979). In addition, postharvest pathogens survive longer at high humidity and temperature. *Botrytis cinerea* and *Sclerotinia sclerotiorum* had maximum growth rates at 98-99 % RH (van den Berg, 1987).

There are several factors affecting transpiration and water loss. Among the produce characteristics, the specific surface area is the major factor determining the rate of water loss from produce. For example, the ratio for edible leaves is situated between 50-100 $cm^2.cm^{-3}$, whereas tubers have ratios of about 1.5 $cm^2.cm^{-3}$ (Wills et al., 2007). The differences in surface structures have a pronounced impact on the variation of respiration rates between commodities and cultivars. Water loss may occur through stem scars, lenticels, stomata, wounds, epidermal hairs and cracks in the cuticle (Herman et al., 2003). Respiration is another inherent factor that contributes to transpiration. The mass balance between the intake of oxygen and the release of carbon dioxide can generate a total weight loss of 3 to 5 %. The heat produced by respiration can increase WVPD and evaporation (Ben-Yehoshua and Rodov, 2002).

The magnitude of the transpirational driving force is determined by environmental factors such as temperature, relative humidity, pressure and air movement (Ben-Yehoshua and Rodov, 2002). Transpiration is affected by a difference in temperature between produce and the surrounding air, which ultimately impacts on the water vapor pressure equilibrium. Also, evaporation increases when air currents disturb the boundary layer of produce (Ben-Yehoshua and Rodov, 2002).

Transpiration and evaporation of fresh fruits and vegetables can be reduced by minimizing WVPD, which is generally achieved by lowering the temperature and/or raising the relative humidity of the air. These conditions are normally achieved during the storage by refrigeration systems and by mechanical devices such as sprayers (Herman et al., 2003). Evaporation can also be reduced by physical barriers around the produce. Coating of produce is a common practice to enhance the

natural water vapor barrier function, however, is gradually being replaced with plastic-films which appear to be more effective to reduce transpiration (Ben-Yehoshua and Rodov, 2002)

1.2.3 Membrane integrity

Cell membranes are essential for quality maintenance of fruits and vegetables during storage, since they are critical for life functions such as energy transfer, hormone binding, signal transduction, plant pathogen interactions and transport (Whitaker, 2012). Cell membranes are composed mostly of lipids and a small portion of proteins. Most of the lipids are amphipathic phospholipids (PL) which contain a saturated fatty acid, an unsaturated fatty acid, normally linoleic or linolenic acid and a phosphate containing group on a glycerol backbone and forms bilayers in aqueous environments (Hopkins et al., 2007; Stanley and Parkin, 1991). The predominant PL in cell membranes are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Whitaker, 2002). Proteins, on the other hand, are of amphipathic nature, are extended across the bilayer or peripherally attached to the membrane by hydrophilic interactions (Hopkins et al., 2007). One important characteristic of cell membranes is their fluidity, and membranes are composed generally of unsaturated fatty acid containing lipids are more fluid compared with saturated fatty-acid membranes (Marangoni et al., 1996). When fluidity decreases, the membrane becomes more rigid, a condition that can modify the activity of the enzymes associated with the membrane as well as the receptors (Kays, 1991). For instance, the possible involvement of lipid-phase transition in chilling injury, reduces the fluidity of cell membranes (Whitaker, 2002).

During senescence, the balance of membrane metabolism is shifted in favor of PL catabolism. Free fatty acids will be oxidized by lipoxygenase (LOX), which produces free radicals leading to cellular damage (Marangoni et al., 1996). Additionally, reactive oxygen species (ROS) that are generated in the course of aerobic respiration pose a major threat to membrane lipids, since antioxidant compounds are eventually depleted during the course of storage (Kays, 1991; Whitaker, 2002). Lipid peroxidation is one of the most important events which accelerate senescence. The accumulation of lipid peroxidation products in fruits and vegetables has significant effects on plant organelles. The simple fact of membrane breakdown will be catastrophic for the cell since the contents will leak out, promoting cell death. Similarly, in energy-transducing membranes in chloroplast and mitochondria, where electron transport occurs, coupling factors are very susceptible

to lipid peroxidation products (Mazliak, 1987). Thus, membrane breakdown is probably the most important factor that has to be slowed down in order to delay senescence.

1.2.4 Tissue softening

The plasma membrane plays a major role in water movement through osmosis which is responsible for the cell pressure or turgor. Combined with turgor, cell walls outside the membrane are key players to determine the mechanical properties of cells (Smith et al., 2002). Primary cell walls are composed of cellulose micro fibrils held by hemicellulose and pectins as well as some structural proteins and phenols. During fruit ripening polysaccharides are modified by a variety of enzymes including polygalacturonase and cellulase, that affect the structure of the cell wall and eventually bring about fruit softening (Toivonen and Brummell, 2008). Furthermore, the free radical generated by aerobic respiration may contribute to loosening the cell wall structure and exposing pectin to endogenous enzymes, leading to tissue softening (Reddy et al., 2000). Significant delays in softening have been reported for the maintenance of firmness in fresh-produce by calcium (calcium chloride or calcium lactate) by the formation of ionic bridges between pectin molecules that improve mechanical strength, and also because calcium may delay senescence processes (Toivonen and Brummell, 2008).

1.2.5 Phytohormones

Plant hormones are small molecules that can influence physiological processes at low concentrations (Davies, 2010). Plants produce hormones which include auxins, gibberellins (GA), abscisic acid (ABA), cytokinins (CK), salicylic acid (SA), ethylene (ET), jasmonates (JA), brassinosteroids (BR), peptide hormones, and strigolactones which have been recently identified (Bari and Jones, 2009). Phytohormones are fundamental in the development of plants, but also appear to control physiological events in stored commodities. All the known phytohormones such as auxins, gibberellins, cytokinins, abscisic acid (ABA), ethylene, polyamines and jasmonic acid are present in postharvest commodities (Table 1.1). Moreover, endogenous hormones tend to increase after wounding, since they may stimulate the necessary synthetic activity to heal a wound, and exogenous applications can boost their activities. Exogenous methods have been used in plants or commodities in order to have insights into the endogenous hormonal responses (Ludford, 2002).

Hormones can be divided into two groups: 1) growth promotors: auxins, gibberellins and cytokinins; and 2) growth inhibitors: ABA and ethylene. Auxins generally prevent leaf and fruit drop, while ABA and ethylene promote senescence, ripening and abscission (Klein and Goldschmidt 2005). Another important group of phytohormones are polyamides and jasmonates which are compounds with signal functions in plant responses, particularly to biotic and abiotic stresses.

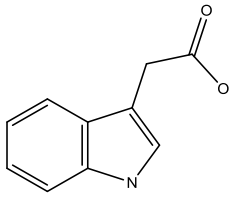
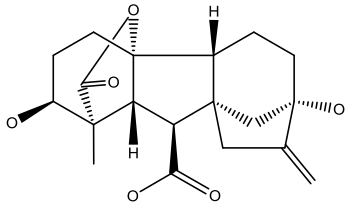
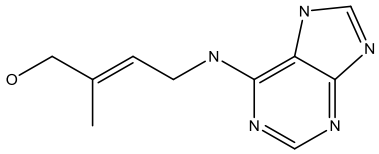
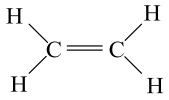
Auxins are involved in many plant responses, such as cell enlargement and differentiation, the most important auxin in plants is indole-3-acetic acid (IAA). It has been found that auxin levels increase during the senescence in leaves, and exogenous application of auxins repressed the transcription of senescence-associated genes (SAGs) (Lim et al., 2007; Ludford, 2002). The decreased rate of senescence in detached leaves has been attributed to the alteration of cytokinins (Kays and Paull, 2004a). Cytokinins can also delay senescence of vegetables (Downs et al., 1997; Zaicovski et al., 2008). Gibberellins can retard the typical respiration response in climacteric fruits as well as the suppression of some enzymatic activity related to fruit softening, in particular polygalacturonase (Ludford, 2002). Treatments with ABA have shown to advance ripening in climacteric and non-climacteric fruits (Glasson, 1978). To date, it is assumed that ABA has a crucial role in fruit maturation and senescence (Zhang et al., 2009). Among all hormones, perhaps, ethylene effects are the most evident in produce during postharvest storage, and will be discussed separately.

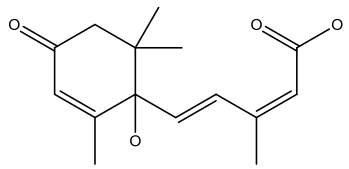
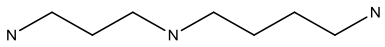
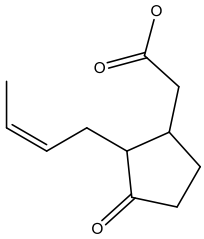
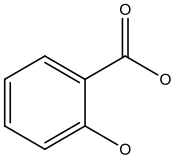
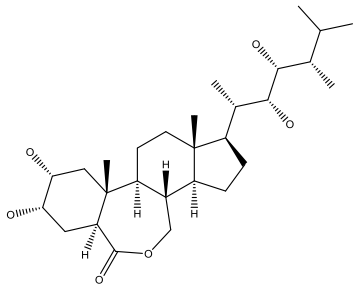
1.2.5.1 Polyamines and jasmonates

Polyamines (PAs) are organic compounds with two or more primary amino groups. In plants, the main palyamines are putrescine (Put), spermidine (Spd) and spermine (Spm), which are positively associated with growth as well as in stress responses (Valero and Serrano, 2010). Polyamines, especially Spd and Spm share, *S*-adenosylmethinine (SAM), a common precursor with ethylene, and competitive effects between both molecules have been demonstrated in fruit development and ripening (Liu et al., 2006). Application of exogenous polyamines, is thus responsible for decreased ethylene production, increase firmness and amelioration of mechanical damage (Olusola, 2002).

Jasmonic acid (JA) and methyl jasmonate (MeJA) are considered candidates as intracellular or intercellular messengers and as signal transducers leading to gene expression (Ludford, 2002). In plants, the role of jasmonates on senescence is the down-regulation of housekeeping proteins and the up-regulation of defense-related genes (Wasternack, 2007). In postharvest, jasmonates has been

Table 1.1 Plant hormones: Their structures, biosynthesis and actions

Hormone	Biosynthesis and actions
Auxins: Indole-3-acetic acid (IAA) 	IAA is synthesized from tryptophan or indole, and their actions include: Inhibition of leaf and fruit abscission, stimulation of flower parts growth, and delay of leaf senescence and fruit ripening.
Gibberellins: Gibberellin A1 	Gibberellins (GAs) are synthesized from glyceraldehyde-3-phosphate, via isopentyl diphosphate, and their most relevant actions are: Climacteric respiration in fruits is delayed. Color and fruit softening are retarded by the application of GA_3.
Cytokinins: Zeatin 	Cytokinins (CKs) is synthesized *via* biochemical modification of adenine. The most common actions in produce are: Delay in senescence, and maintenance of green color and fresh appearance in leafy vegetables.
Ethylene 	Ethylene (ET) is synthetized from methionine and is the fruit ripening hormone. Its effects are: Triple response (decrease, thickening and lateral growth of stems), stimulation of defense responses, shoot and root growth, leaf and fruit abscission, flower induction, flower opening, flower and leaf senescence, and fruit ripening.

Abscisic acid (ABA) 	ABA is synthesized from glyceraldehyde-3-phosphate *via* isopentenyl diphosphate and carotenoids in response to water stress. The effects of this hormone are: Advanced ripening in climacteric and non-climacteric fruits. Plays a crucial role in fruit maturation and senescence.
Polyamines: Spermidine 	Polyamines are derived from the decarboxylation of arginine or ornithine. Decrease ethylene production, increase firmness and amelioration of mechanical damage.
Jasmonic acid (JA) 	Jasmonic acid is synthesized from linolenic acid, their effects in plants are: Plant defense by inducing the synthesis of proteinase inhibitors, inhibition of growth and seed germination, senescence promotion, abscission, tuber formation, fruit ripening, pigment formation, and tendril coiling.
Salicylic acid (SA) 	SA is synthesized from phenylalanine, the effects of SA are mainly in the resistance to pathogens. It is also involved in the systemic acquired resistance (SAR) response, inhibition of ethylene biosynthesis, blocking of wound response, and in the reversal of the effects of ABA.
Brassinosteroids: Brassinolide 	Brassinosteroids are biosynthesized from campesterol. Most relevant effects are: Cell division, cell elongation, vascular differentiation, inhibition of root growth and development and promotion of ethylene biosynthesis.

applied exogenously to protect vegetables against microbial infection and chilling injury, and to enhance phytochemical compounds (Rohwer and Erwin, 2008).

1.2.5.2 Ethylene

Ethylene is a two-carbon compound with a double bond and molecular weight of 28.05 Da and exhibits full biological activity at 1 µL/L. Fruits and vegetables may be intentionality or unintentionally exposed to ethylene during postharvest storage. The importance of ethylene in postharvest related to its impact on senescence of harvested horticultural crops (Saltveit, 1999). Common effects of ethylene on produce include: increased respiration activity; increased activity on enzymes such as polygalacturonase, peroxidase, lipoxygenase, α-amylase, polyphenol oxidase and phenylalanine ammonia-lyase (PAL); increased permeability and loss of cellular compartmentalization (Kader, 1985). The characteristics having direct impact on quality attributes of fruits and vegetables such as color, texture and flavor are affected by this molecule. Ethylene can enhance the appearance of many climacteric fruits by stimulating their ripening; however, this effect can be either positive or negative depending on the produce. For instance, chlorophyll degradation promoted by ethylene in lettuce is considered a detrimental effect; but degreening of lemons is considered a positive effect (Saltveit, 1999).

Ethylene is biosynthesized from methionine *via* S-Adenosyl methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang and Hoffman, 1984). The first step involves the conversion of methionine (Met) to SAM by incorporation of ATP, and SAM is converted to ACC, while methionine is recycled within the Yang's cycle. Ethylene is produced by the oxidation of ACC. The key enzymes in ethylene synthesis are ACC synthase (ACS) and ACC oxidase (ACO) (Pech et al., 2002). Ethylene perception is achieved by a family of five proteins: ethylene response 1 (ETR1), ETR2, ethylene response sensor 1 (ERS1), ERS2 and ethylene-insensitive 4 (EIN4). These receptors are located in the endoplasmic reticulum (ER), where ethylene binds to a copper cofactor associated with a hydrophilic pocket on the receptors (Wang et al., 2013).

Synthesis of ethylene is produced during ripening of climacteric fruits as well as in diseased, wounded or stressed tissue. Ethylene produced after infection by either fungi or bacteria is synthesized by the plant and not by the pathogen (Barkai-Golan and Kopeliovitch, 1983; Imaseki et al., 1968; Zaat et al., 1989). Similarly, ethylene production increases in produce exposed to abiotic stresses such as extreme temperatures, chemicals, mechanical wounding and radiation. In sensible tropical and subtropical fruits stored at cold temperatures (<12 °C), ethylene is produced once the

tissue is returned to warm temperatures. On the other hand, vegetable tissue exposed to supra-optimal temperatures also produces ethylene, reaching maximum concentrations at 35 °C, but the production is inhibited by temperatures above 40 °C (Abeles et al., 1992).

Chemicals belonging to the category of hormones, elicitors and phytotoxic compounds also increase ethylene production in plants. It has been found that methyl jasmonate stimulated ethylene formation at different stages of ripening of tomatoes (Saniewski et al., 1987). Ozone and other phytotoxic air pollutants such as sulfur dioxide also lead to stress ethylene production (Morgan and Drew, 1997).

Because several factors can induce the production of ethylene in vegetable tissues with its undesirable consequences in most cases, efforts have been directed to control ethylene biosynthesis by chemicals means. Among them, aminoethoxy vinylglycine (AVG) and amino oxyacetic acid (AOA) inhibit ACC synthase. Metal ions such as as Co^{2+} and Ni^{2+} as well as free radical scavengers such as n-propyl gallate, acetyl salycilate and sodium benzoate inhibit ACC oxidase. Ethylene action inhibitors are those molecules which interfere with ethylene perception. The most common is CO_2 which is largely used in controlled and modified atmospheres. Silver is another powerful inhibitor of ethylene action; however, it can not be applied in foods. Nonetheless, the most powerful inhibitor of ethylene action is 1-methyl-cyclopropene (1-MCP), since it tightly binds to the ethylene receptor in plants (Pech et al., 2002).

1.2.6 Disease and decay

Vegetables and fruits are more susceptible to microbial attack during the postharvest storage compared with their development stage on the plant. This is due to the inability of many pathogens to penetrate intact cuticle during the development of the produce. (Wills et al., 2007). However, once harvested, produce become more susceptible to decay due to a decreased ability of the tissue to synthesize inhibitors to combat infection (Arul, 1994).

Physical barriers to infection include waxes and cuticle, for instance, produce with thicker cuticles may be less susceptible to those with thinner skins (Barkai-Golan, 2001). The preformed inhibitors are chemical substances constitutively present in their biologically active form. Examples of these compounds are saponins such as α-Tomatine, avenacins, avenacosides, cyanogenic glycosides, glucosinolates and phenols (Osbourn, 1996). These compounds are referred as

phytoanticipins, to distinguish them from phytoalexins which are synthesized and accumulated after exposure to microorganisms (VanEtten et al., 1994).

Phytoalexins are typically stress-response compounds elicited by fungal, bacteria, mechanical damage, low temperature, irradiation and other stresses. The most common in the *Brassicaceae* family are camalexin, spirobrassicin, rutalexin, brassilexin. Resveratrol, and ε-viniferin, are typical phytoalexins of the *Vitaceae* family, while capsidiol and scopoletin are the most common in the and *Solonaceae* family (Ahuja et al., 2012). In addition, the host is capable of forming protective barriers composed of tightly packed cells to intensify the defense response in response to infection. In this process, phenolic compounds participate by either being converted into lignin mediated by peroxidase, or combined with aliphatic compounds for the suberization of cells at the wound area (Barkai-Golan, 2001). Pathogenesis-related (PR) proteins are also inducible responses to the resistance against pathogens. These proteins exhibit antifungal properties and have the ability to degrade β-1,3-glucan and chitin which are fugal cell wall constituents (Charles et al., 2009).

Infection occurs in the postharvest phase and it is influenced by the microorganism itself, the host and the environment. For a pathological disorder to occur, the pathogen must be in contact with the produce and a successful infection may depend on a large quantity of inoculum. The vulnerability of the host depends on its constitutive defenses as well as the environment, which is probably the most important factor that may influence pathogenesis during storage (Barkai-Golan, 2001). For instance, the exposure of produce to high temperatures after harvest can increase the susceptibility to diseases. On the other hand, low temperatures delay diseases by the inhibition of ripening of the plant tissue and also by providing unfavorable growth conditions for the pathogen.

There are several fungi and some bacteria that can cause spoilage of produce; however, the most important fungi are *Alternaria, Aspergillus, Botrytis, Fusarium, Geotrichum, Monilinia, Penicillium, Rhizopus* and *Sclerotinia*. *Erwinia* and *Psudomonas* are among the most significant (Wills et al., 2007). Although most pathogens are inhibited at temperatures near 0 °C, certain psychrotolerant fungi such as *Botrytis* and *Penicillium expansum* may grow slowly at this temperature. Hence, additional treatments are necessary to control them (Eckert, 1978).

1.3 The role of postharvest management on the quality of produce

Fruits and vegetables are highly perishable during storage mainly because of senescence, transpiration and decay caused by microorganisms. There are three main strategies to cope with

postharvest losses: 1) use of genotypes that have longer postharvest life; 2) use of good agricultural practices to maintain the quality of the commodities; and 3) use of proper postharvest handling practices to keep the quality and safety of the produce (Kader and Rolle, 2004). These strategies are of equal importance; however, the first two are beyond the scope of this review. The appropriate postharvest handling of produce include: 1) postharvest management procedures that are critical to maintain the quality and safety of commodities; 2) postharvest treatments designed to manipulate the environment around the produce; and 3) postharvest treatments to minimize decay.

Temperature and relative humidity are by far the most critical factors to manage to maintain the quality of produce. Good temperature management is the simplest and most important procedure to retard senescence, softening, and changes in the texture and color. Furthermore, low temperatures decrease both the physiological activity of the produce and the activity of microorganisms causing spoilage (Nunes do Nascimento and Emond, 2002). Lower the storage temperature, within limits, is generally associated with longer storage life. Most perishables commodities have an optimal shelf-life at temperatures near 0 °C. However, the lowest safe temperature can be different for other commodities. For instance, the lowest safe temperature for avocado, pineapple, pomegranate and okra is 7 °C, while for tropical fruits such as banana, mango, breadfruit and jicama is 13 °C (Kader and Rolle, 2004). Relative humidity (RH) on the other hand, can influence water loss, due to evaporation and also decay development caused by condensation of moisture on the commodity. Reduced weight loss at high humidity results in firmer, crisper and higher quality vegetables (Van den Berg, 1981). Proper relative humidity storage of fruits is 85-95 %, while for most vegetables the range of RH varies from 90-98 % (Kader and Rolle, 2004).

The second strategy to reduce postharvest losses during the storage is by using postharvest treatments that modify the environment, achieved by controlled atmosphere (CA) and modified atmospheres (MAP). The principle of CA/MA consists of altering the concentrations of oxygen (O_2) and carbon dioxide (CO_2) in the storage room or in the package. Low levels of O_2 decrease the respiration rate and high levels of CO_2 inhibit the development of fungal growth. CA is mostly used on large volumes and long-term storable commodities. MAP, on the other hand, is used on fresh cut and ready-to-eat highly perishables fruits and vegetables. The concentration of the gases in MAP can be altered passively or actively. In the first case, the modification of the atmosphere inside the package is created by respiration rate of the produce and the gas transfer capacity of the packing material. In the second case, levels of O_2 and CO_2 will be altered by replacing the atmosphere in the package

with a desired gas mixture. The composition of the atmosphere can be further adjusted by the addition of gas scavengers or absorbers to establish a desired concentration (Caleb et al., 2012; Kader and Watkins, 2000). In addition, since many horticultural produce are sensitive to ethylene, mixture of ethylene-producing commodities with ethylene-sensitive produce should be avoided and scrubbing units based on the catalytic oxidation of ethylene are often used in some commercial facilities as well (Kader and Rolle, 2004).

Postharvest treatments to minimize decay are the third strategy to reduce spoilage of produce during storage. When necessary, the use of clean water with appropriate concentration of sanitizers is important to reduce the transition of pathogens from water to the produce. Curing is a method that can be used to prolong the storage life of certain commodities such as tuber and bulb crops. Heat treatments at relatively high temperature, i.e. 50 °C, are used to reduce decay in mangoes. Also, fungicides, such as imazalil and/or thiabendazole are utilized to reduce spoilage. Biological control alone or in combination with fungicides is an effective method to reduce decay by microorganisms (Kader and Rolle, 2004). Recently, decay has been controlled by the induction of natural defenses of produce by biological, chemical or physical elicitors. Induced resistance consists in the activation of different defense mechanisms, such as strengthening of cell wall, activation of pathogenesis-related (PR) proteins, the *de novo* synthesis of phytoalexins as well as increased levels of constitutive secondary metabolites (Dann, 2003). Examples of elicitors are, chitosan (Reddy et al., 2000), salicylic acid (SA) (Yalpani et al., 1991), and UV-C (Arul et al., 2001b).

1.4 Physiological characteristics during the postharvest storage of broccoli

Broccoli is an Italian word from the Latin *brachium*, meaning branch or arm which refers to an edible green plant whose large flowering head is used as a vegetable (Gray, 1982). Broccoli (*Brassica oleracea*) is an important vegetable due of its health-promoting attributes (Bhattacharjee and Singhal, 2010). Broccoli is highly perishable, which is due to its high respiration rate which derives into a rapid deterioration (Page et al., 2001). It has a respiration rate of 20 - 22 mg $CO_2.kg^{-1}h^{-1}$ at 0 °C and a Q_{10} value of 3.9 - 4.1 between 0 – 10 °C (Nunes do Nascimento and Emond, 2002; Toivonen and Forney, 2004). The most evident indicator of senescence of broccoli also an important quality factor is yellowing, (King and Morris, 1994b; Tian et al., 1997; Toivonen and Forney, 2004). Nutrient and phytochemical contents are also affected with progress in senescence. Physical changes such as weight loss, yellowing and chlorophyll degradation are found to occur with

concomitant reduction in ascorbic acid and total phenolic content in cold stored broccoli (Serrano et al., 2006). Given the high respiration and transpiration rates of broccoli temperature, high relative humidity and low oxygen atmosphere are very important factors to delay the senescence of broccoli during postharvest storage (Jones et al., 2006).

Even with appropriate temperature and relative humidity to delay the deterioration of broccoli, physiological and biochemical events continue, and senescence will progress at a slower rate. Broccoli senescence proceeds similarly as in leaf, where an orderly dismantling of cellular constituents occurs. Yellowing is the major indicator of broccoli senescence as chlorophyll is catabolized, leading to the appearance of carotenoids and the yellow coloration (Figure 1.1) (Costa et al., 2006; Funamoto et al., 2002; Nunes do Nascimento 2009; Tian et al., 1997). Changes in lipids, proteins, chlorophyll and gene expression patterns occurred in tissue before any signs of senescence are detected (Page et al., 2001). Sucrose and organic acids are largely consumed in all section of the florets. The protein content of the floral sections decline and the free amino acid content increase (King and Morris, 1994a). Polyunsaturated fatty acids (PUFA) are prone to attack by lipoxygenases leading to lipid peroxidation (LP). There is evidence that LP preceded chlorophyll losses in broccoli, indicating a clear relationship between LP and broccoli senescence (Zhuang et al., 1995).

Decay is another factor that limits the postharvest storage of broccoli. Broccoli is susceptible to molds, especially to *Botrytis cinerea* and bacterial decay caused produced *by Erwinia carotovora* and *Pseudomonas* spp. that have been identified in stored broccoli (Toivonen and Forney, 2004). Tissue breakdown of commodities is caused by a range of macerating enzymes secreted by the microorganisms leading to the degradation of the middle lamella and the cell wall (Ragaert et al., 2007). Infection by *Pseudomonas maculicoca* is characterized by a small purple-gray to black spot located on the florets, petioles, stems and leaves of broccoli. The grey mold rot caused by *Botrytis cinerea* is characterized by the greenish-brown to grey-brown water-soaked lesions on the leaves and the presence of fine mycelia on florets (Tournas, 2005).

Figure 1.1 Yellowing of broccoli stored at 20 °C. Modified from Nunes do Nascimento (2009).

1.4.1 Preservation of fresh broccoli

The most important quality aspects associated with freshness of broccoli florets are appearance and color. Attractive appearance can stimulate purchase, while unattractive products repel consumers. Broccoli heads and florets are expected to have a dark green color, an indicator of appropriate quality; otherwise, the produce is usually rejected. Thus, the yellowing of florets is probably the major limiting factor in the marketing of broccoli. At optimum temperature and relative humidity of 0 °C and 98 %, broccoli can be kept for 2 to 3 weeks (Toivonen and Forney, 2004). Another important factor for broccoli quality is water loss by transpiration because water content affects tissue turgor and freshness of the florets. Retention of water is one of the most important factors to avoid rapid senescence of broccoli. It has been suggested that the maximum permissible weight loss in broccoli sprouts is of 4 % (Ben-Yehoshua, 1987).

After harvest, broccoli is generally pre cooled by forced-air cooling, vacuum cooling or top icing, and stored at 0-1 °C, and optimal high relative humidity fluctuates between 90 and 98 %. Controlled atmosphere (CA) and modified atmosphere (MAP) are also used as adjuvant to low temperature storage to extend further the storability of broccoli. The recommended atmospheres are 1-2 % O_2 + 5-10 % CO_2 when storage temperature is 0-5 °C. Modified atmosphere packaging (MAP), generally maintain both O_2 and CO_2 at 10 % using low density polyethylene films (Thompson, 2008; Toivonen and Forney, 2004). Other postharvest treatments that have been investigated to preserve broccoli quality include heat treatment at temperatures between 41-52 °C (Duarte-Sierra et al., 2012a; Forney, 1995; Funamoto et al., 2002); fumigation with ethanol vapor at 2,500 ppm (Asoda et al., 2009; Corcuff et al., 1996; Fukasawa et al., 2010); UV-C light with doses of 1.2-10 $kJ.m^{-2}$ (Costa et al., 2006; Duarte-Sierra et al., 2012b; Lemoine et al., 2007), and UV-B light with doses of 4.4-19 $kJ.m^{-2}$ (Aiamla-or et al., 2010; Aiamla-or et al., 2012). In addition to the quality maintenance, treatments such as UV-B light, exposure to signal molecules such as methyl jasmonate and salicylic acid have been shown to enhance the phytochemical composition of broccoli (Mewis et al., 2012; Pérez-Balibrea et al., 2011).

1.5 Biosynthesis of secondary metabolites in plants

Secondary metabolites (SMs) are a vast and diverse collection of organic compounds which don't act directly on growth and development of plants. These compounds are not produced under all conditions, while the majority of their function is not yet known, some SMs provide defense against

predators and pathogens. Thousands of SMs have been isolated and their structures have been identified. Secondary metabolites can be classed as: 1) Nitrogen-containing SMs, and 2) SMs without nitrogen. The nitrogen-containing SMs include: alkaloids, non-protein amino acids (NPAAs), amines, cyanogenic glycosides, glucosinolates, alkamides, lectins, peptides and polypeptides. The SMs without nitrogen include: terpenoids, steroids, saponins; the phenolic acids, flavonoids, coumarins, lignin; and acetate-derived fatty acids, waxes and polyketides (Wink, 2010).

The building blocks for SMs are derived from primary metabolism (Figure 1.2). The compounds employed in the synthesis of SMs are derived from the acetyl coenzyme A (acetyl CoA), shikimic acid, mevalonic acid, and 1-deoxyxylulose 5-phosphate. They are used in the acetate, shikimate, mevalonate and deoxyxylusose phosphate (non-mevalonate) pathways. Acetyl-CoA is synthesized by the oxidative decarboxylation of pyruvic acid and also by the β-oxidation of fatty acids. Shikimic acid is produced from phosphoenolpyruvate and erythrose 4 phosphate. Mevalonate is formed from the condensation of three molecules of acetyl-CoA, and deoxyxylulose phosphate is produced by the condensation of pyruvic acid and glyceraldhyde-3-phosphate. Both pathways are responsible for the biosynthesis of terpenoids and steroids (Dewick, 2009).

In broccoli, the most important pathway for the biosynthesis of phytochemicals is the shikimate pathway that along with amino acids from citric acid cycle, are responsible for the production of glucosinolates and phenolic compounds.

1.5.1 Phenolic compounds biosynthesis

Phenolic compounds are biosynthesized from phosphoenolpyruvate and erythrose 4-phosphate *via* shikimate in the shikimic acid pathway, where phenylalanine ammonia- lyase (PAL) is the key enzyme for the conversion of aromatic amino acids into phenolic compounds (Cartea et al., 2010). These molecules can be classified into flavonoids (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others) and non-flavonoids (phenolic acids, hydroxycinnamates, stilbenes and others), and in most of the cases they are stored as reserves in the form of glucosides or as organic acid esters (Crozier et al., 2007). Phenolic acids are the major source of dietary phenols, and usually are present as complexes linked to various plant compounds through ether, ester or acetal bonds (Ignat et al., 2011). The phenolic acids are either derived from benzoic acid or cinnamic acid. The most important benzoate-derived compounds are: ρ-hydroxybenzoic, vanillin, syringic and gallic acid with a common C6-C1 structure.

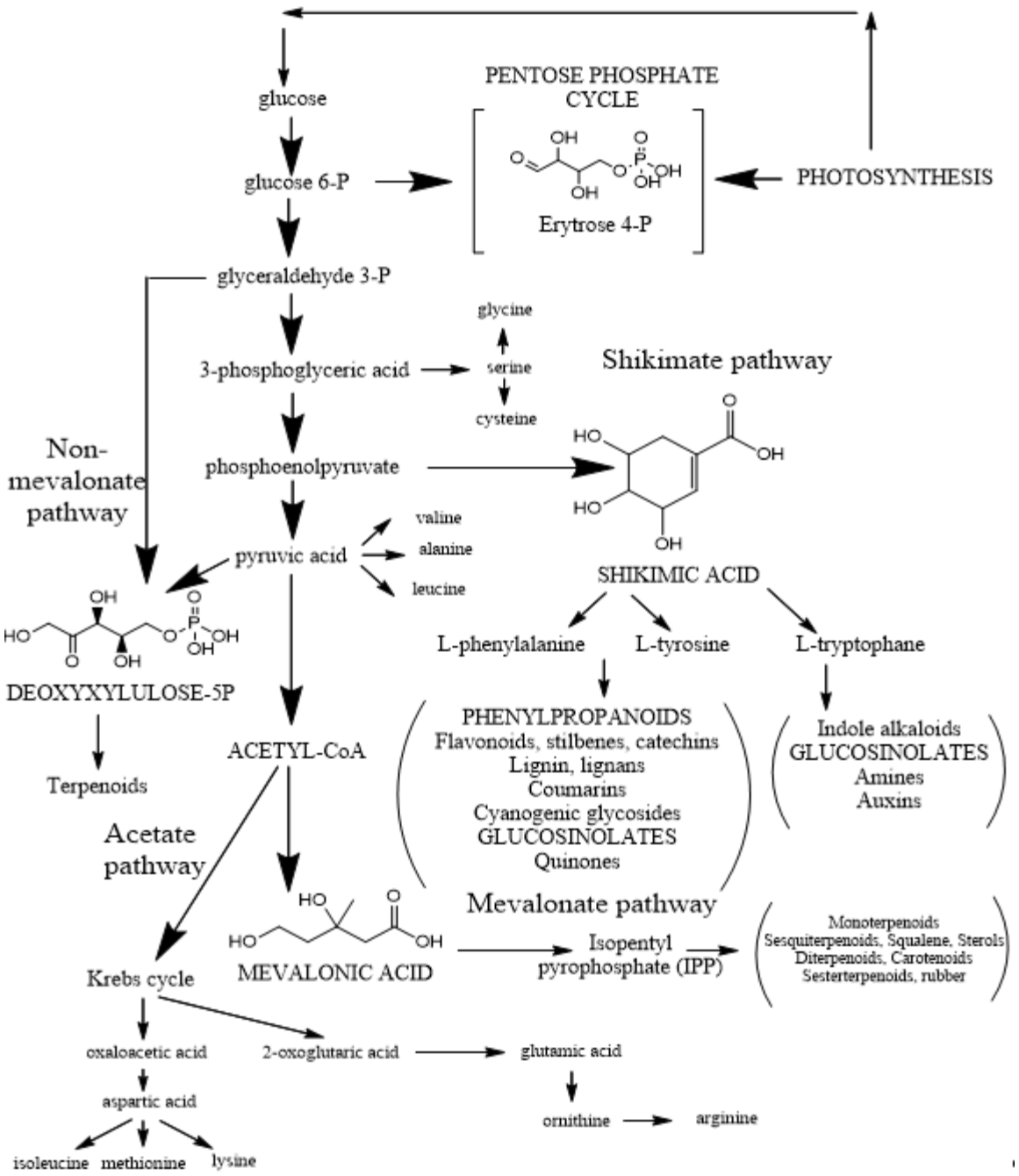


Figure 1.2 General overview of secondary metabolism in plants. Secondary metabolites are synthesized from primary metabolism through four main pathways: Shikimate, acetate, mevalonate and non-mevalonate. Adapted from (Dewick, 2009).

Caffeic, ρ-coumaric, ferulic and sinapic acids are the most important compounds derived from cinnamic acid (Figure 1.3) with a C6-C3 construction (Butt and Sultan, 2010).

Conversion from aromatic amino acids to specific phenolic acids occurs in several steps. PAL catalyzes the conversion of phenylalanine or tyrosine to trans-cinnamate and *p*-hydroxy cinnamate. Cinnamic acid hydrolase (C4H) catalyzes successive methylation and hydroxyation, and the synthesis of phenolic acid thioesters is completed with ligase/*p*-coumaric acid/CoA (4CL) (Amarowicz et al., 2009).

Hydroxycinnamic acids are commonly derived from *p*-coumaric, caffeic and ferulic acids, and to a lesser extent from sinapic acid (Fig. 1.3) (Herrmann and Nagel, 1989). These compounds are very rarely found in the free form in fruits and vegetables; and they are typically attached to other molecules by two types of bonds: 1) ether (e.g., chlorogenic acid or 2) glycosidic bound (e.g., ρ-cinnamic acid O-glucoside) (Murkovic, 2003).

Flavonoids are low molecular weight secondary metabolites from the phenylpropanoid pathway (Weston and Mathesius, 2013). The basic flavonoid structure is the flavan nucleus of 15 carbons organized in three rings (C6-C3-C6), which are labeled A, B and C (Fig. 1.3) (Pietta, 2000). The flavan structure is the product of two different pathways, where the bridge (ring C) and the aromatic B-ring are phenylpropanoid units derived from *p*-coumaryl CoA, while the six carbons of ring-A is synthetized from the condensation of three acetate units. The fusion of these two pathways involves the condensation of p-coumaryl–CoA with three malonyl-CoA residues in a reaction catalyzed by chalcone synthase (CHS), and the production of naringenin-chalcone as a result (Fig. 1.3) (Crozier et al., 2009). The subsequent step is the conversion of naringenin-chalcone to naringenin, which is the central intermediate of this pathway by chalcone isomerase (CHI). From this point, the biosynthetic pathway diverges into different classes of flavonoids including isoflavones, flavonones, flavones, flavonols, flavan-3-ols and anthocyanins (Figure 1.3).

Several enzymes are involved in the formation of different intermediates in the biosynthesis of isoflavonoids, flavanones, flavonols, flavan-3-ols, anthocyanins and proanthocyanidins. Isofalvonoids, are synthesized from naringenin, which is converted into isoflavonoids by isoflavone synthase (IFS). Flavanones are derived form a chalcone-like product by chalcone isomerase (CHI). Flavanones are then converted to dehydroflavonols by flavanone 3-hydroxyase (F3H). Dehydroflavonols are further transformed into flavonols by flavonol synthase (FLS). Dehydroflavonols are then converted to leucoanthocyanidins by dehydroflavonol reductase (DFR). These compounds

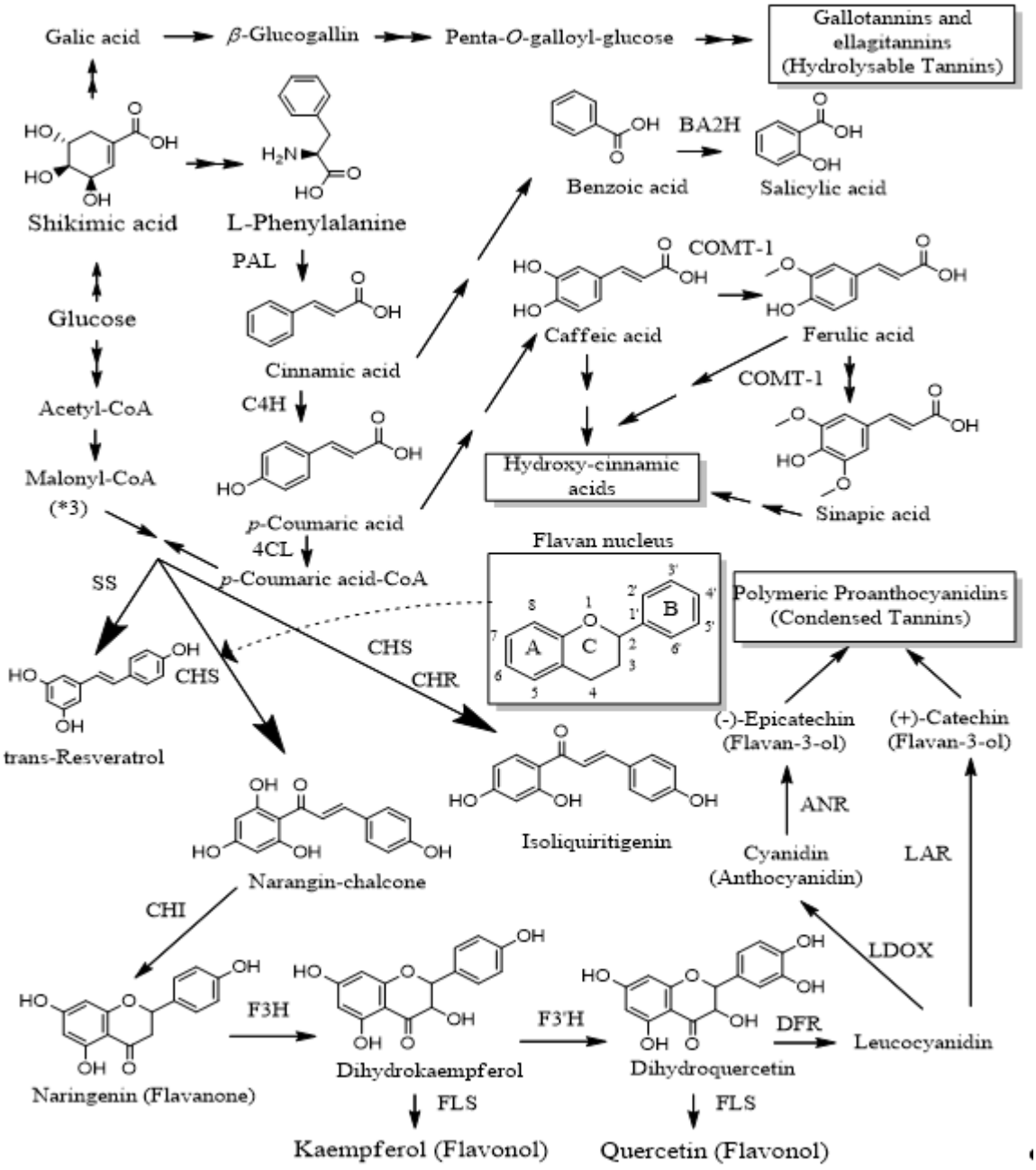


Figure 1.3 Biosynthesis of phenolic compounds. Several enzymes participates in the flavonoid biosynthesis: phenylalanine ammonia-lyase (4.3.1.5); naringenin-chalcone synthase (2.3.1.74); chalcone isomerase (5.5.1.6); dihydrokaempferol 4-reductase (1.1.1.219); flavone synthase (1.14.11.22); 2-hydroxyisoflavanone synthase (1.14.13.86); 2'-hydroxyisoflavone reductase (1.3.1.45); flavonol synthase (1.14.11.23). Modified from Weston and Mathesius (2013).

are the key intermediates in the synthesis of flavan-3-ols, proanthocyanidins and anthocyanidins by leucocyanidin dioxygenase (LDOX), leucocyanidin 4-reductase (LAR) and leucocyanidin dioxygenase (ANR).

1.5.2 Glucosinolate biosynthesis and regulation

Glucosinolates are (Z)-cis-N-hydroximinosulfate esters, with a thio β-D-glucopyranose moiety and an amino acid-derived side chain (Holst et al., 2003). Glucosinolates (GLS) are SMs synthesized from amino acids. Aliphatic GLS are synthesized from methionine, isoleucine, leucine and valine; aromatic GLS are produced from phenylalanine and tyrosine; and indole GLS from tryptophan. The biosynthesis of glucosinolates proceeds in three steps: (1) side-chain elongation of amino acids, (2) development of the core structure, and (3) secondary side-chain modifications (Figure 1.4) (Sønderby et al., 2010).

Amino acid elongation requires an initial and final transamination, acetyl-CoA condensation, isomerization and oxidative decarboxylation (Figure 1.4). The synthesis of the glucosinolate core structure is accomplished in five reactions steps, beginning with the oxidation of the elongated precursor amino acids to aldoximes by side chain-specific cytochrome P450 monooxygenases (cytochromes P450) of the CYP79 gene product family. The aldoximes are further oxidized by cytochromes P450 of the CYP83 gene family to nitrile oxides; being strong electrophiles, react spontaneously with thiols to form S-alkylthiohydroximate conjugates (Bak et al., 2001; Hansen et al., 2001). S-alkylthiohydroximate conjugates are cleaved by a C-S lyase into thiohydroximates, pyruvate and ammonia. Given that thiohydroximates are reactive and unstable compounds, the remainder of the core pathway (i.e., glycosylation followed by sulfatation) may be considered a detoxification process (Mikkelsen et al., 2002). The final step in glucone formation is 3'phosphoadenosine 5'-phosphosulfate-dependent sulfation of desulfoglucosinolates (Piotrowski et al., 2004) (Figure 1.4). Side chain modification is generally considered to be the final stage in glucosinolate synthesis. Side chain decorations entail various kinds of oxidations, eliminations, alkylations and esterifications (Figure 1.4) (Grubb and Abel, 2006).

The first two steps in the synthesis of GLS, i.e., amino acids to aldoximes and nitrile oxides to S-alkylthiohydroximate, are the key steps in the production of each class GLS. In the first step of synthesis, the cytochrome P450 (CYP) gene products, CYP79F1 and CYP79F2 are responsible for the formation of aliphatic GLS (Hansen et al., 2000), although CYP79F1 metabolizes homomethionine, resulting in both short and long-chain aliphatic glucosinolates.

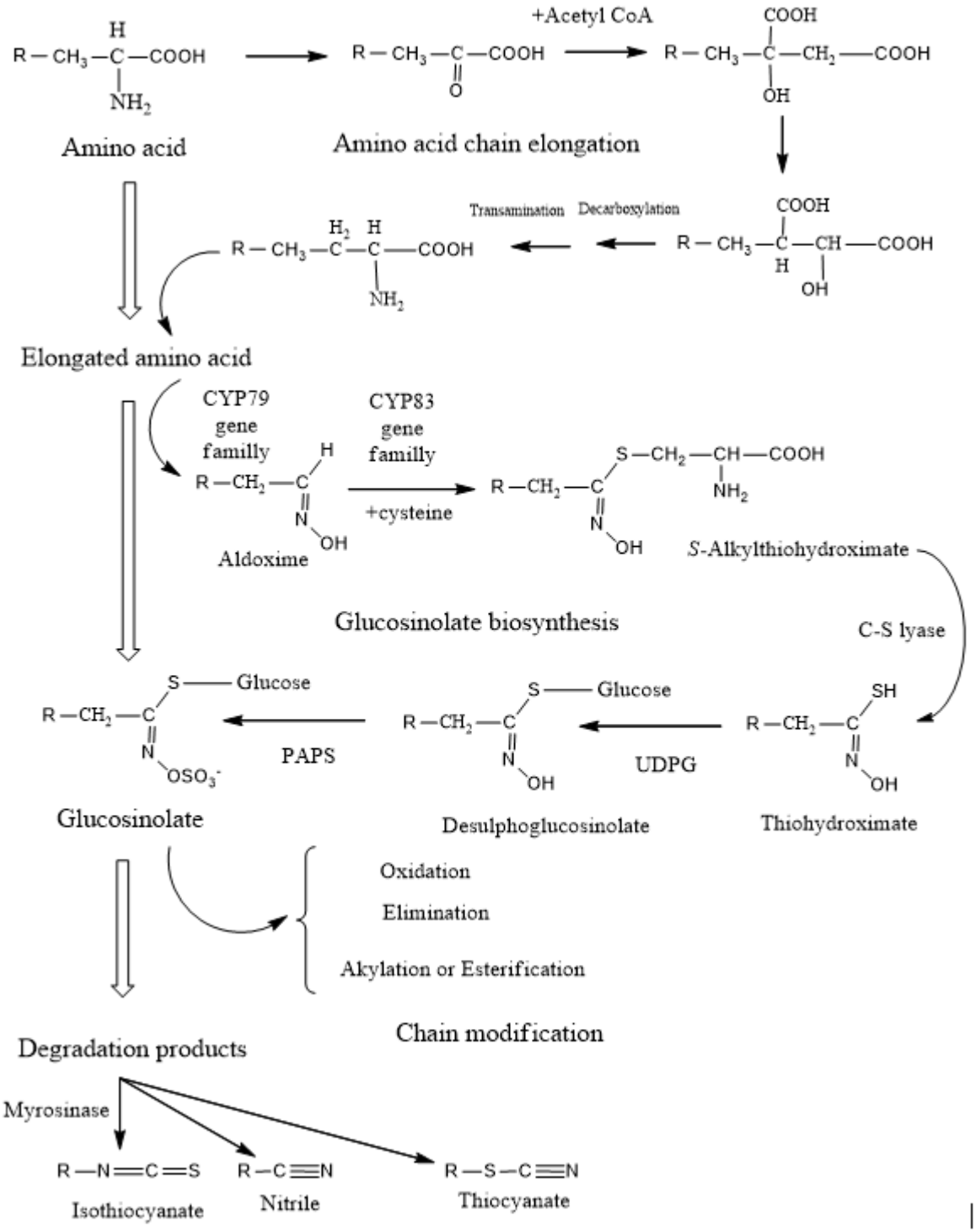


Figure 1.4 Overview of glucosinolate biosynthesis.

In contrast, CYP79F2 exclusively metabolizes the long-chain elongated penta- and hexahomomethionines (Chen et al., 2003). On the other hand, CYP79A2 gene product synthesizes aromatic GLS, phenylalanine being its substrate (Wittstock and Halkier, 2000). The conversion of tryptophan to indole-3-acetaldoxime is catalyzed by cytochrome P450 CYP79B2 (Mikkelsen et al., 2000), and the thiohydroxymate formation is catalyzed by members of the CYP83 gene product family (Bak and Feyereisen, 2001), where the level of indole-3-acetic acid is regulated by the flux of indole-3-acetaldoxime through a cytochrome P450, CYP83B1, to the glucosinolate pathway (Bak et al., 2001). Aliphatic oximes derived from chain-elongated homologues of methionine are efficiently metabolized by CYP83A1, whereas CYP83B1 gene product metabolizes these substrates with very low efficiency. Aromatic oximes derived from phenylalanine, tryptophan, and tyrosine are metabolized by both CYP83A1 and CYP83B1, although CYP83B1 gene product has higher affinity for these substrates than CYP83A1 (Naur et al., 2003b). The remaining steps of glucosinolate biosynthesis involve enzymes that are thought to accommodate nearly all glucosinolate precursors regardless of their side chain (Redovnikovic et al., 2008).

1.6 Phytochemicals in Brassicas and their bioactivities

Glucosinolates (GLS) and flavonoids are considered the most important health-related compounds in broccoli. A large number of hydroxy-cinnamic acid esters of kaempferol and quercetin glucosides, as well as glucosinolates are constitutive compounds in broccoli (Figure 1.5). Nonetheless, glucosinolates can be induced upon damage and may serve as one of the principal chemical barriers to deter a broad spectrum of potential pathogens (Chen and Andreasson, 2001). Flavonoids, on the other hand, can act as screens of high light intensities and reduce the generation of reactive oxygen species due to oxidative stresses (Ferdinando et al., 2012). Like glucosinolates, flavonoids can be induced by environmental stresses as well as challenges by pathogens and herbivores. It is conceivable that this capacity can be used as a means of enhancing specific secondary metabolites in plants.

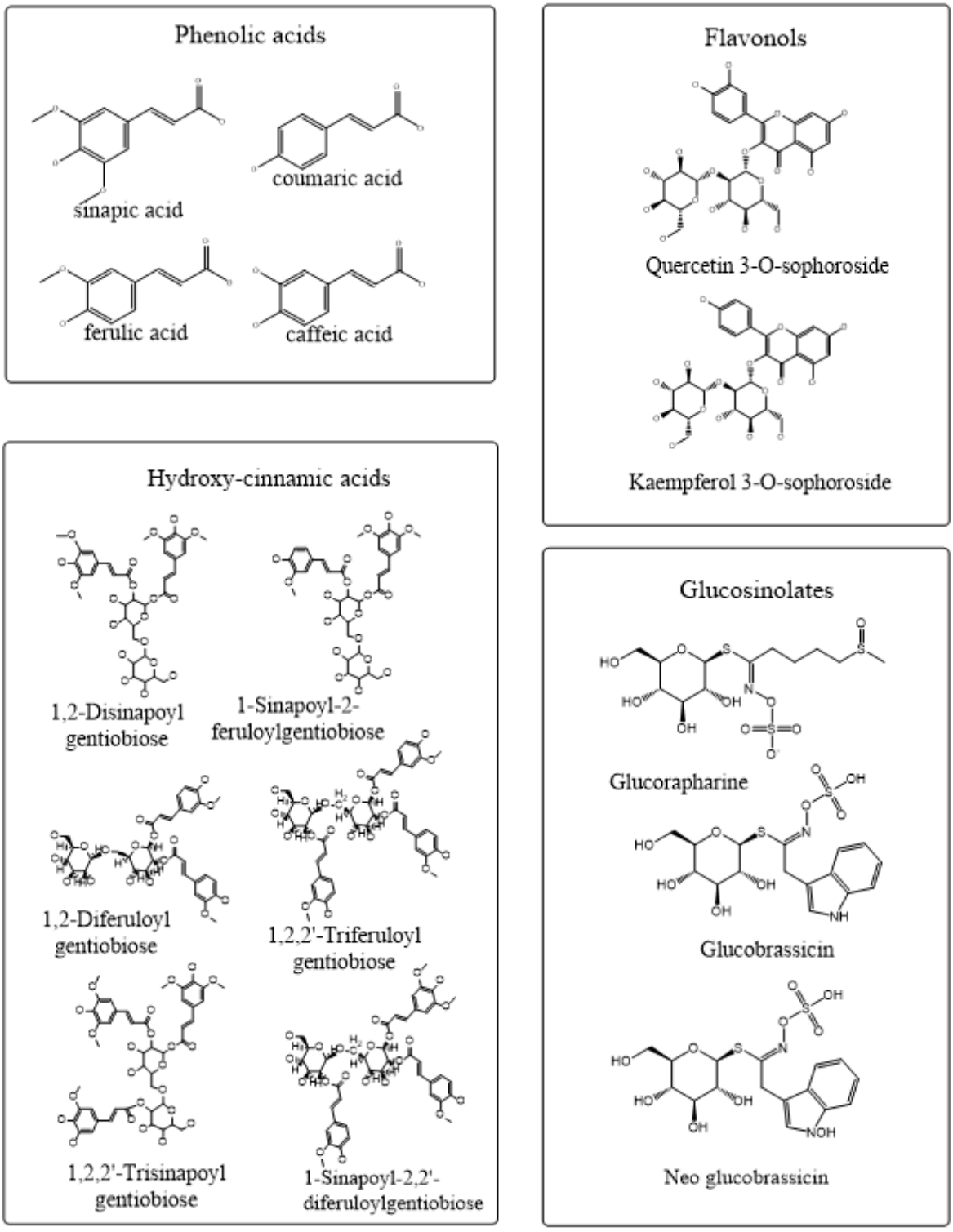


Figure 1.5 Principal phytochemical compounds in broccoli.

1.6.1 Constitutive phyto-compounds

The main phenolic acids or phenylpropanoids in broccoli are caffeic, p-coumaric, sinapic and ferulic (Gliszczynska-Swiglo et al., 2006). The most predominant hydroxy-cinnamic acid conjugates found in broccoli are sinapic acid and ferulic acid ester forms of gentiobiose (Price et al., 1997). Hydroxycinnamic acids are antioxidant as well as antimicrobial, anti-inflamatory and anticancer compounds found in *Brassicas* (Nićiforović and Abramovič, 2014; Rice-Evans et al., 1996). Most common esters are: 1,2-disinapoylgentiobiose; 1-sinapoyl-2-feruloylgentiobiose; 1,2-diferuloylgentiobiose; 1,2,2-trisinapoylgentiobiose; 1,2-disinapoyl-2-feruloylgentiobiose; 1-sinapoyl-2,2'-diferuloylgentiobiose; 1,2,2'-trisinapoylgentiobiose and 1,2,2'- triferuloylgentiobiose (Fig. 1.5) (Vallejo et al., 2004).

Flavonols are the most important subclass of flavonoids, even though, other subclasses as anthocyanins are also present. The flavonols present in broccoli are quercetin 3-O-sophoroside and kaempferol 3-O-sophoroside (Figure 1.5); other minor glucosides being, iso-quercetin, kaempferol 3-O-glucoside and a kaempferol diglucoside. The levels of quercetin and kaempferol glycosides expressed as aglycone are 43 and 94 ug.g^{-1}fresh weight, respectively (Price et al., 1998). Quercetin is frequently the most studied flavonoid because of its biological properties such as antiviral and carcinostatic activities, and its potential role in ischemic heart disease and stroke (Perez-Vizcaino et al., 2006). Also, there is evidence that quercetin can protect low-density lipoproteins form oxidation, prevent platelet aggregation and promote relaxation of cardiovascular muscles (Formica and Regelson, 1995). Kaempferol and some of its glycosides are also active compounds exhibiting a number of biological properties including antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective and neuroprotective activities (M. Calderon-Montano et al., 2011).

Glucosinolates and their derivatives have many biological functions for plants as well as for humans because of their cancer-preventing properties, and other applications such as bio pesticides and flavor compounds (Halkier and Gershenzon, 2006). Isothyocianates and indoles may regulate target enzymes controlling apoptosis and blocking the cell cycle (Cartea and Velasco, 2008).

At least 120 different glucosinolates have been identified in *Brassica* plants, although closely related taxonomic groups typically contain only a small number of such compounds. Glucosinolates have been grouped in different chemical classes based on structural similarities. These classes are: aliphatic, methylthioalkyl, aromatic and heterocyclic (indole) (Fahey et al., 2001). The main glucosinolates in broccoli are of indole and aliphatic types, 1-methoxy-3-indolylmethyl-glucosinolate

(neoglucobrassicin) and 3-indolylmethyl-glucosinolate (glucobrassicin), although 4-methylsulfinylbutyl-glucosinolate (glucoraphanin) is also present (Vallejo et al., 2003a).

1.6.2 Inducible compounds in response to stresses

Like many defense secondary metabolites, glucosinolates are constitutively present in plants but they may be induced. The amount of glucosinolates as well as polyphenols in *Brassicas* varies in relationship with the organ, developmental stage and environmental factors. They are usually present in all parts of the plant and can be detected with relative ease. But when an external stimulus is perceived by the plant, the level of secondary metabolites can be highly enhanced (Textor and Gershenzon, 2009). In the case of glucosinolates, induction is not generalized to all the glucosinolates in the tissue, but to specific compounds, mostly the indole class (Bennett and Wallsgrove, 1994). Some of the factors affecting glucosinolate profiles and concentrations are of biotic or abiotic nature. Among the biotic factors, the most important are the presence of fungi, bacteria, insects, weeds and other competing plants and plant density. The abiotic factors include temperature, light (ultraviolet radiation), water supply, fertilizer, harvested conditions, postharvest and storage conditions and processing conditions (Holst et al., 2003). Phenylpropanoid compounds can be also induced by various biotic and abiotic stresses. Many of these compounds are induced in response to wounding or feeding by insects, while the levels of anthocyanins and flavones may increase in response to high UV light levels (Dixon and Paiva, 1995).

1.6.2.1 Phenolic compounds

The phenolic contents in *Brassica* can be affected by insect attack, pathogen infection and environmental factors such as light, temperature, nutrient supply, water availability, and more importantly UV radiation (Cartea et al., 2010; Dixon and Paiva, 1995). In most cases, plants that express high levels of phenols are less susceptible to the attack of herbivores, pathogens and nematodes. On the other hand, it has been shown that most of the constitutively present phenols are also inducible. For instance, upon pathogen challenges, the increase in phenolic compounds provide adequate substrates to oxidative reactions, leading to oxygen consumption and synthesis of quinones that are unfavorable for further pathogen development (Lattanzio, 2003).

A common feature between stresses is the rapid induction of phenylalanine ammonia lyase enzyme (PAL) (Bennett and Wallsgrove, 1994). Induction of phenolic compounds in *Brassica* tissue

has been observed to be pathogen-dependent. The phenolic content of *Brassica rapa* varied with bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium* and *Shigella flexneri;* but in all cases, the concentration of sinapoyl-malate and caffeoyl-malate increased (Jahangir et al., 2008). Upon the infection of roots of *Arabidopsis thaliana* with *Phytium sylvaticum*, the 4-hydroxybenzoic acid level increased (Tan et al., 2004). Environmental stresses also induce phenolic compounds, especially heat stress. In *Brassica rapa* the hydroxy-cinnamic acids, sinapic acid and 1-sinapoyl-2-feruloylgentiobiose were negatively affected by cold temperature. Temperature also have a negative effect on the flavonoid content of *Brassica rapa*, especially kaempferol-3,7-di-O-glucoside and isorhamnetin-3,7-di-O-glucoside (Francisco et al., 2012).

1.6.2.2 Glucosinolates

Glucosinolates are major amino acid-derived compounds related to plant defense responses in the *Brassicas* which are induced after wounding and/or pathogen attack (Clay et al., 2009), phytohormones, such as methyl jasmonate and salicylic acid (Doughty et al., 1995; Kiddle et al., 1994), herbivore attack (Textor and Gershenzon, 2009) and abiotic stresses (Mewis et al., 2012). When hydrolysis of GLS by thioglucosidases (myrosinases) occurs; isothyocyanates, thyocyanates and nitriles are synthetized.

The glucosinolate-myrosinase (EC 3.2.3.1) system 'mustard oil bomb' (Figure 1.6) is thought to be a binary chemical defense system that is activated upon tissue damage and may serve at the front line of chemical defense to deter potential pathogens (Chen and Andreasson, 2001). Myrosinase isoenzymes and glucosinolates are located in all cells but are compartmentalized; glucosinolates are stored in the vacuole, while myrosinases are located in the cytoplasm. Following tissue damage, the enzyme and the substrate come into contact, causing hydrolysis of the S-glucose bond, and thereby, yielding an unstable aglycone that undergoes spontaneous rearrangement to either isothiocyanates, thiocyanates, oxazolidine-2-thiones, epithionitriles or nitriles (Textor and Gershenzon, 2009). Rearrangement of aglycone at neutral pH results in the formation of isothyocinates, while acidic pH is responsible for the development of nitriles. In the presence of epi-thiospecifier protein and ferrous ions, glucosinolates containing a terminal double bound produce epi-thionitriles. On the other hand, glucosinolates that contain hydroxyated side chains yield oxazolidine-2-thione. Indole, benzy and allyl glucosinolates rearrange to produce thiocyanates (Chen and Andreasson, 2001; Cole, 1976). Broccoli contains glucobrassacin, and after hydrolytic action of

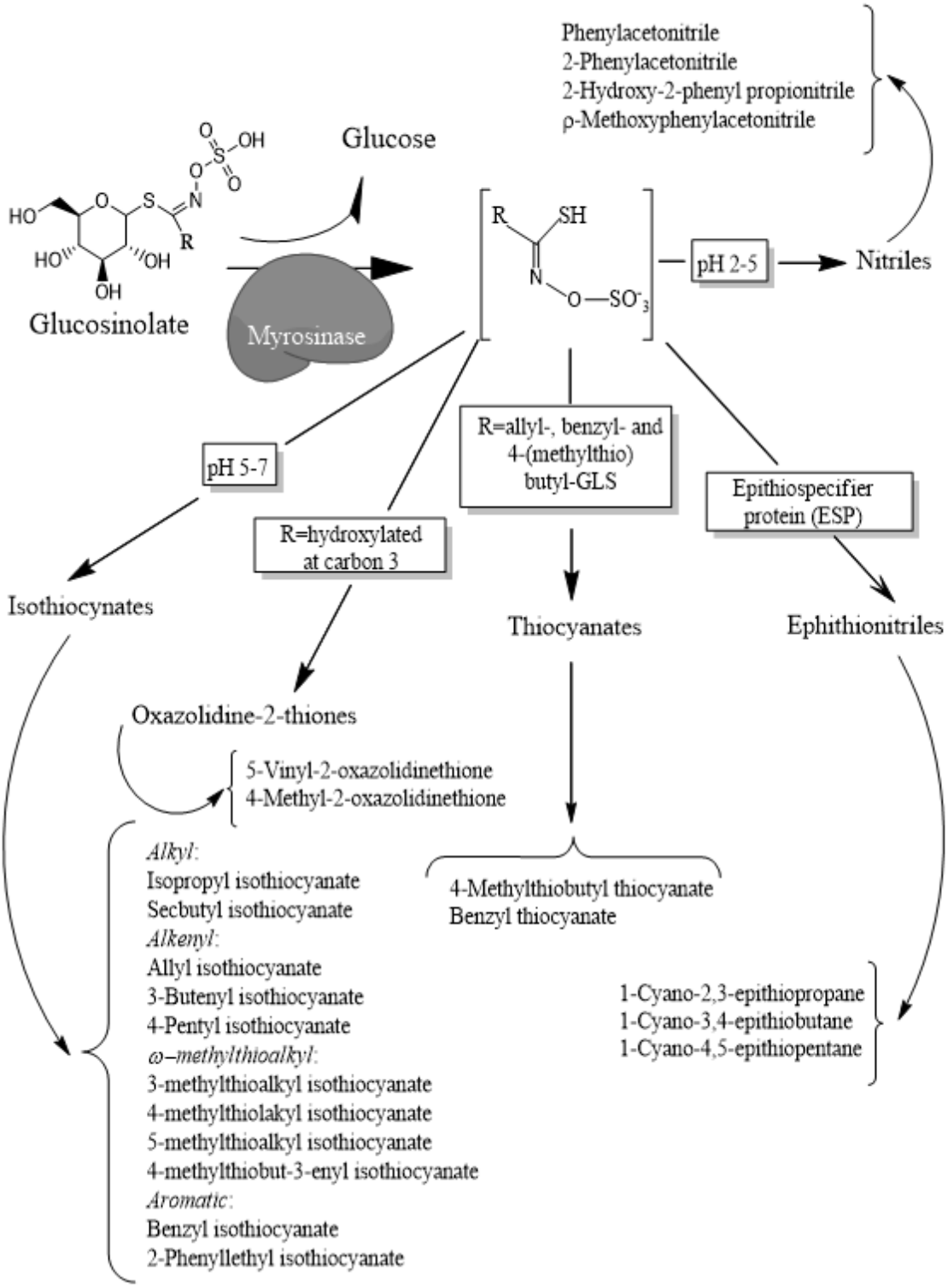


Figure 1.6 Glucosinolate-myrosinase system. Glucosinolates are hydrolyzed by the removal of the glucose by myrosinase (3.2.1.147) to form isothyocianates, oxazolidine-2-thiones, thiocyanates and ephithionitriles.

Table 1.2 Examples of inducible glucosinolates and their derivatives by pathogens and herbivores.

Plant	Biotic agent	Type	Inducible glucosinolate and/or derivative
Brassica rapa	*Alternaria brassicae*	Pathogen	3-butenyl and 4-pentenyl isothiocyanates
Brassica crops	*Brevicoryne brassicae*	Aphid	4-pentenylglucosinolate
Brassica campestris ssp. *pekinesis*	*Plasmodiophora brassicae*	Protist (parasite)	Aliphatic and aromatic glucosinolates
Arabidopsis thaliana	*Myzus persicae* and *Brevicoryne brassicae*	Aphid	Methylsulfinyl glucosinolate
B. napus, B.rapa, B. juncea	*Phyllotreta cruciferae* and *Psylliodes chrysocephala*	Insect	Indole glucosinolates

* Modified from (Jahangir et al., 2009).

myrosinase and rearrangement, the main products are isothiocyanates, but thiocyanates and nitriles are also formed (Selmar, 2010). The liberated thiocyanates have goitrogenic potential, i.e., they interfere with iodine uptake by the thyroid gland and may cause goiter (Burel et al., 2001). However, their presence in the diet at reasonable amounts does not seem to affect thyroid function in humans (Verhoeven et al., 1997).

Members of *Brassicaceae* family have the ability to control the growth of phytopathogenic fungi mainly by the accumulation of isothiocyanates, the products of hydrolysis of glucosinolates. A comparative in *vitro* study on fungicidal activity of 11 glucosinolates from various cruciferous seeds with that of their hydrolysis products against *Fusarium culmorum*, showed that fungicidal activity of glucosinolates was very low compared with that of the isotiocyanates (Manici et al., 1997). In *Brassica rapa* seedlings infected with *Alternaria brassicae*, the release of isothiocyanates correlated with the catabolism of glucosinolates and this was a prerequisite for resistance to infection (Doughty et al., 1996). The putative mechanisms of the antimicrobial activity of glucosinolates-derived products are the oxidative breakdown of –S – S– bridges present in enzymes as well as the inhibition of ATP synthesis of the pathogens (Kojima and Oawa, 1971).

Glucosinolates are induced by herbivore, pathogen and parasite attack. Innoculation of *Brassica rapa* seedlings with the fungal pathogen *Alternaria brassicae* induced the catabolism of glucosinolates to isothyocianates and dimethyl disulfide. Partial resistance of *Brassica* crops to *Brevicoryne brassicae* (cabbage aphid) is provided by the increase in the production of 4-pentenylglucosinolate. The susceptibility of *Brassica campestris* to *Plasmodiophora brassicae* increases with the accumulation of aliphatic and aromatic glucosinolates. Short-chain aliphatic methylsulfinyl glucosinolates have been observed to accumulate in *Arabidopsis thaliana* in response to generalist insects (*Myzus persicae* and *Brevicoryne brassicae*). Mechanical injury by insect feeding on *B.rapa and B. juncea* increases the concentration of indole glucosinolates (Table 1.2) (Jahangir et al., 2009).

Season, temperature, light and water availability can influence the synthesis of total and specific glucosinolates (GLS). Generally, at intermediate temperature with high light intensity during the spring, plants develop higher total GLS content compared with autumn/winter season plants when the temperature and light intensity are low. The contents of ascorbic acid, lutein and alkyl glucosinolates, in particular glucoraphanin, increase when broccoli is grown under mild temperature conditions with moderate light radiation (Schonhof et al., 2007). Some investigations have shown that

the plants exposed to high or low temperature had higher content of glucosinolates compared with mild conditions. In fact, seedlings cultivated at 30/15 °C (day/night) temperature had significantly higher glucosinolate levels compared with sprouts at 22/15 or 18/12 °C (Pereira et al., 2002). UV-B radiation induces glucosinolates, especially 4-methylsulfinylbutyl GLS and 4-methoxy-indol-3-ylmethyl GLS (Mewis et al., 2012). Finally, the effect of water deficiency in *Brassicas* is generally associated with higher glucosinolate content, due to the increased concentration per unit dry weight and/or due in part to the increased synthesis of amino acids and sugars under dry conditions (Björkman et al., 2011).

1.6.2.3 Phytoalexins

Phytoalexins are low molecular mass secondary metabolites with antimicrobial activity which are synthesized *de novo* after the perception biotic and abiotic stresses. The concept derives from the finding that potato which was infected with an incompatible race of *Phytophthora infestans* developed resistance to another compatible race of *P. infestans*, the reason being the accumulation of phytoalexin (Müller and Börger, 1940 cited by Ahuja et al., 2012). Phytoalexins from *Brassicas* share two common structural features: an indole ring and at least one sulfur atom. The first *Brassica* phytoalexins reported were brassicin, 1-methoxybrassicin and cyclobrassicin, elicited by *Psudomonas cichorii* or ultraviolet radiation (UV-B). Similarly, camalexin is induced by pathogens and by abiotic stress that generate reactive oxygen species (ROS) (Kliebenstein, 2004). L-Tryptophan is the biogenic precursor of these phytoalexins which has an obvious structural similarity to indole glucosinolates (Pedras et al., 1997). Most of the phytoalexins have antifungal activity against pathogenic fungi, which can cause serious economic losses such as *L. maculans*, *R. sonali* and *S. sclerotiorum.* Phytoalexins can also be induced by abiotic stresses such as copper chloride and UV light (Jahangir et al., 2009).

1.7 Abiotic stresses in plant bodies

Stress can be defined as the altered physiological condition in plants caused by different factors that modify their biochemical equilibrium (Gaspar et al., 2002). There are various natural and anthropogenic factors that can reduce plant's ability to survive. Among the natural stress factors, the most important are high light irradiance, heat, low temperature or chilling, water shortage, mineral deficiency, long rainy periods and flooding, insects and pathogens. Anthropogenic stress factors

include herbicides, air pollutants, ozone, acid rain, heavy metal load in the soil, increased UV radiation and increased level of carbon dioxide (Lichtenthaler, 1998). Among these stresses, temperature, light and water availability are the primary causes for disorders during the preharvest and postharvest handling of produce (Toivonen and Hodges, 2011). To cope with these factors, plants are able to set forth several mechanisms by which they can increase their tolerance through physical adaptations as well as molecular and cellular changes (Knight and Knight, 2001).

It is established that after the onset of the abiotic stress, the first step in the signal transduction pathway is the perception of a signal with a concomitant generation of secondary signals such as Ca^{2+} and reactive oxygen species (Agarwal and Zhu, 2005; Apel and Hirt, 2004; Knight and Knight, 2001). The second messengers can modulate intracellular Ca^{2+}, which is often accompanied by a protein phosphorylation cascade targeting transcription factors controlling stress-regulated genes as well as proteins involved in cellular protection (Figure 1.8) (Xiong and Zhu, 2001).

1.7.1 Sensors

The signal transduction process is initiated by sensors that create a cascade of signals for the activation of transcription factors, leading to the activation of genes and the modification of plant's metabolism. Although, there are specific receptors for abiotic stresses such as UV-B (Rizzini et al., 2011), the best-characterized plasma membrane receptors are of two kinds: transmembrane receptor enzymes including receptor-like kinases (RLKs) as well as two-component histidine kinases (HKs), and G-proteins-coupled receptors (GPCRs) (Tuteja, 2009; Xiong and Zhu, 2001).

Kinases are enzymes that phosphorylate proteins using ATP. The addition of phosphate groups to proteins is known as phosphorylation, and it has a great impact on their activity, stability and/or localization within the cell (Jonak and Hirt, 2003). Phosphorylation is a reversible process where the conformational state of proteins changes due to the addition or subtraction of phosphate making them to be activated or deactivated. This capacity, make RLKs able to participate in diverse range of processes including disease resistance, regulation of development as well as hormone perception. RLKs are transmembrane proteins having amino-terminal extracellular domains and carboxyl-terminal intracellular kinase domains, which represents almost 2.5 % of the annotated protein-coding genes in *Arabidopsis* (Shiu and Bleecker, 2001). HKs also use ATP as the phosphate donor to catalyze their own phosphorylation or other protein substrates at specific histidine residues. Besides their involvement in signaling, HKs participate in ethylene signaling, osmo-sensing, cold

perception as well in drought response (Nongpiur et al., 2012; Urao et al., 2001). GPCRs are also transmembrane proteins that convert signals from outside to responses inside the cell. The activation of these proteins is completed by the binding of one their subunits with guanosine-5'-triphosphate (GTP), instead of ATP which is the common activator of kinases (Jones, 2010). GPCRs have structural similarities to the corresponding proteins in animals and they transmit signals to control growth, cell proliferation, hormonal responses, stomata movements and defense (Urano et al., 2013). Since GPCRs are defense associated proteins, G-proteins may participle in the perception of environmental stresses (Xiong and Zhu, 2001).

1.7.1 Stress-induced changes of Ca^{2+}

The influx of Ca^{2+} from outside the cell or the release of this ion from intracellular stocks produces alterations in the cytosolic concentrations that are responsible for downstream responses involved in plant's protection via calmodulin, and calcium-protein kinases (Knight, 1999). Abiotic stresses are responsible for temporary increases of cytosolic calcium (Bowler and Fluhr, 2000; Chinnusamy et al., 2004; Kaur and Gupta, 2005; Knight and Knight, 2001; Tuteja and Sopory, 2008). Calcium is one of the most versatile ions in eukaryotic organisms since it can exhibit different coordination numbers and it can also form reversible complexes with proteins, membranes and organic acids (Gong et al., 2013). Calcium is stored in different vesicular compartments and can be released to cytoplasm under stress conditions. For instance, the concentration of Ca^{2+} in apoplast is close to 0.1mM; 1.0 mM in vacuoles; and 1.0 mM in the endoplasmic reticulum, where it can reach 50 mM during stress events (Tuteja and Sopory, 2008).

It has been hypothesized that the magnitude of calcium elevations can encode specific information and that plants decode this signatures to give specific gene responses (Whalley and Knight, 2013). Additionally, one of the important features of calcium as a signal is the presence of repetitive Ca^{2+} transients. The primary increase of Ca^{2+} facilitates the generation of secondary signaling molecules, which can also stimulate a second round of transient Ca^{2+} increase that have different outputs compared with the original one (Xiong et al., 2002).

1.7.2 Secondary signals

Secondary signals include second messengers and hormones that are generated after the perception of abiotic stresses and which can modulate intracellular Ca^{2+} levels; although secondary

signaling molecules can also regulate signal transduction without Ca^{2+} (Xiong et al., 2002). Reactive oxygen species (ROS), phospholipids and nitric oxide are well established second messengers (Smékalová et al., 2014).

1.7.2.1 Reactive oxygen species

Reactive oxygen species (ROS) play a significant role in plant growth, development, and interaction with biotic and abiotic factors (Shulaev and Oliver, 2006). Plant cells produce ROS as a consequence of aerobic metabolism. This is a normal process due to the unavoidable escape of electrons from the transport activities of chloroplast, mitochondria and plasma membranes (Sharma et al., 2012). Superoxide ($O_2^{\cdot-}$), and particularly, hydrogen peroxide (H_2O_2) acts as a secondary messenger in many processes associated with plant growth and development such as senescence, photorespiration and photosynthesis, stomatal movement as well as growth and development (Quan et al., 2008). Also they are important in the signal transduction cascades of phenomena as diverse as mitosis, tropisms and cell death, and their accumulation is crucial to plant defense, i.e., the reinforcement of cell wall (Foyer and Noctor, 2005).

Under normal growth conditions, the production of ROS in the cells is low (240 μM $O_2^{\cdot-}$ s^{-1} and a steady-state level of 0.5 μM H_2O_2 in the chloroplasts). However, many stresses that disrupt the cellular homeostasis enhance the production of ROS (240–720 μM $O_2^{\cdot-}$ s^{-1} and a steady-state level of 5–15 μM H_2O_2) (Polle, 2001). These stresses include drought and desiccation, salt, chilling, heat shock, heavy metals, ultraviolet radiation, air pollutants such as ozone (O_3) and sulfur dioxide (SO_2), mechanical stress, nutrient deprivation, pathogen attack and high light stress. The enhanced production of ROS during stress can pose a threat to cells, but at the same time ROS act as signals for the activation of stress-response and defense pathways (Desikan et al., 2001).

Upon ROS production, there is an induction of the antioxidant and scavenging enzymes to modulate the redox status of the cells. There are two main antioxidant systems in cells: the enzymatic and the non-enzymatic. The enzymatic systems includes: superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Asada, 2006; Bowler et al., 1992; Willekens et al., 1997). Furthermore, ROS levels may be mitigated by the alternative channeling of electrons in the electron transport chain of the chloroplasts and mitochondria by a group of enzymes called alternative oxidases (AOXs) (Mittler, 2002).

Non enzymatic antioxidants can be classified into two general groups: lipid soluble membrane-associated antioxidants and water soluble reductants. The first group includes α-tocopherol and β-carotene; and the second group includes glutathione and ascorbate. (Lurie, 2003). Among the water-soluble antioxidants, the oligopeptide glutathione is the major source of non-protein thiols in most plant cells (Mahmood et al., 2010). Reduced glutathione (GSH) and its oxidized form (GSSG) play a significant role in the redox balance of the cellular compartments, and in the fine tuning of cellular redox environment under normal conditions as well as under stress conditions (Blokhina et al., 2003). Ascorbate is a potent reducing agent, it is oxidized in two-steps: production of monodehydroascorbate (MDHA) radical and its further oxidation to produce dehydroascorbate (DHA). One of the key features of ascorbate as antioxidant is the high stability of MDHA. It has long life and can be regenerated back to ascorbate by monodehydroascorbate reductase (MDHAR) avoiding the propagation of more free radicals (Smirnoff, 2011).

The role of ROS during abiotic stresses appears to be different from that during biotic stresses. For instance, during pathogen attack in plants, ROS is produced *via* plasma-membrane-bound NADPH-oxidases, cell wall-bound peroxidases and amine oxidases in the apoplast (Grant and Loake, 2000) and the levels of detoxifying enzymes are suppressed by salicylic acid (SA) and nitrous oxide (NO). This process is crucial for the generation of programed cell death (PCD) which limits the spread of disease. On the other hand, during abiotic stresses, the enhancement of ROS is produced by chloroplasts and mitochondria and ROS scavenging enzymes are induced to decrease the concentration of intracellular ROS levels (Apel and Hirt, 2004; Mittler, 2002). Downstream signal cascades of ROS are thought to be activated *via* Ca^{2+} or sensed directly by proteins such as tyrosine phosphatase (Xiong et al., 2002). A hypothetical model states that hydrogen peroxide (H_2O_2) is sensed by a two-component histidine kinase receptor followed by the activation of calmodulin, and a cascade of mitogen-activated protein (MAP) kinase resulting in the activation or suppression of several transcription factors (TFs) (Mittler, 2002).

1.7.2.2 Phytohormone signals

Hormones are fundamental elements during the signal transduction pathways involving stresses. While phytohormones including salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play central roles in biotic stress signaling; and abscisic acid (ABA) is extensively involved in responses to abiotic stresses such as drought, low temperature, and osmotic stress (Atkinson and

Urwin, 2012; Fujita et al., 2006). Under water stress, ABA induces stomatal closure which reduces water loss and growth rate, also it enhances cold tolerance in plants (Gong et al., 2013). ABA interacts with other phytohormones as well. For instance, synthesis of MeJA is accompanied by ABA production, on the other hand, ABA strongly down regulates isopentenyl transferase which is the key enzyme for cytokinin biosynthesis (Kohli et al., 2013). Other hormones, such as salicylic acid, ethylene and jasmonic acid have direct or indirect effects in abiotic stress responses.

Salicylic acid (SA), jasmonic acid (JA) and ethylene serve as the backbone of the induced defense signaling network in biotic stress signaling, (Fujita et al., 2006), but also in abiotic stress signaling. Salicylic acid is one of the most important signal for the systemic adquired resistance (SAR), and for the activation of PR-proteins. The induced systemic resistance (ISR) is commonly regulated by jasmonic acid and ethylene dependent signaling pathways (Pieterse et al., 2009). Besides the activation of defense-related genes including those for PR-proteins, SA signaling is associated with the accumulation of reactive oxygen species which are the causal effector for hypersensitive response (Eyidogan et al., 2012; Wang et al., 2002a).

Ethylene is also a stress hormone since it can be induced by different abiotic conditions, such as chemicals, metals and extreme temperatures. For instance, in *Arabidopsis*, ethylene synthesis is one of the earliest responses to ozone. Moreover, compared with jasmonic acid and salicylic acid which are also involved in ozone response, ethylene reaches its maximum one hour after exposure, while JA and SA reach their maximum after 5 hours (Wang et al., 2002a). Jasmonic acid can also be triggered by mechanical wounding, water deficit as well as pathogens; and it is generally accepted that JA helps to control the conversion of energy to defense and growth processes in plants (Eyidogan et al., 2012). Jasmonates are synthetized *via* the octadecanoic pathway in chloroplasts and peroxisomes (Figure 1.7), where α-linolenic acid is oxygenated by lipoxygenase (LOX), producing 13(S)-hydroxy linolenic acid (13-HPOT). Allene oxide synthase (AOS) and allene oxide cyclase (AOC) converts (13-HPOT) into 12-oxo-phytodienoic acid (OPDA). Jasmonic acid is derived from OPDA through β-oxidation, and methyl jasmonate by the action of jasmonic acid carboxyl methyltransferase (JMP) (Cheong and Choi, 2003).

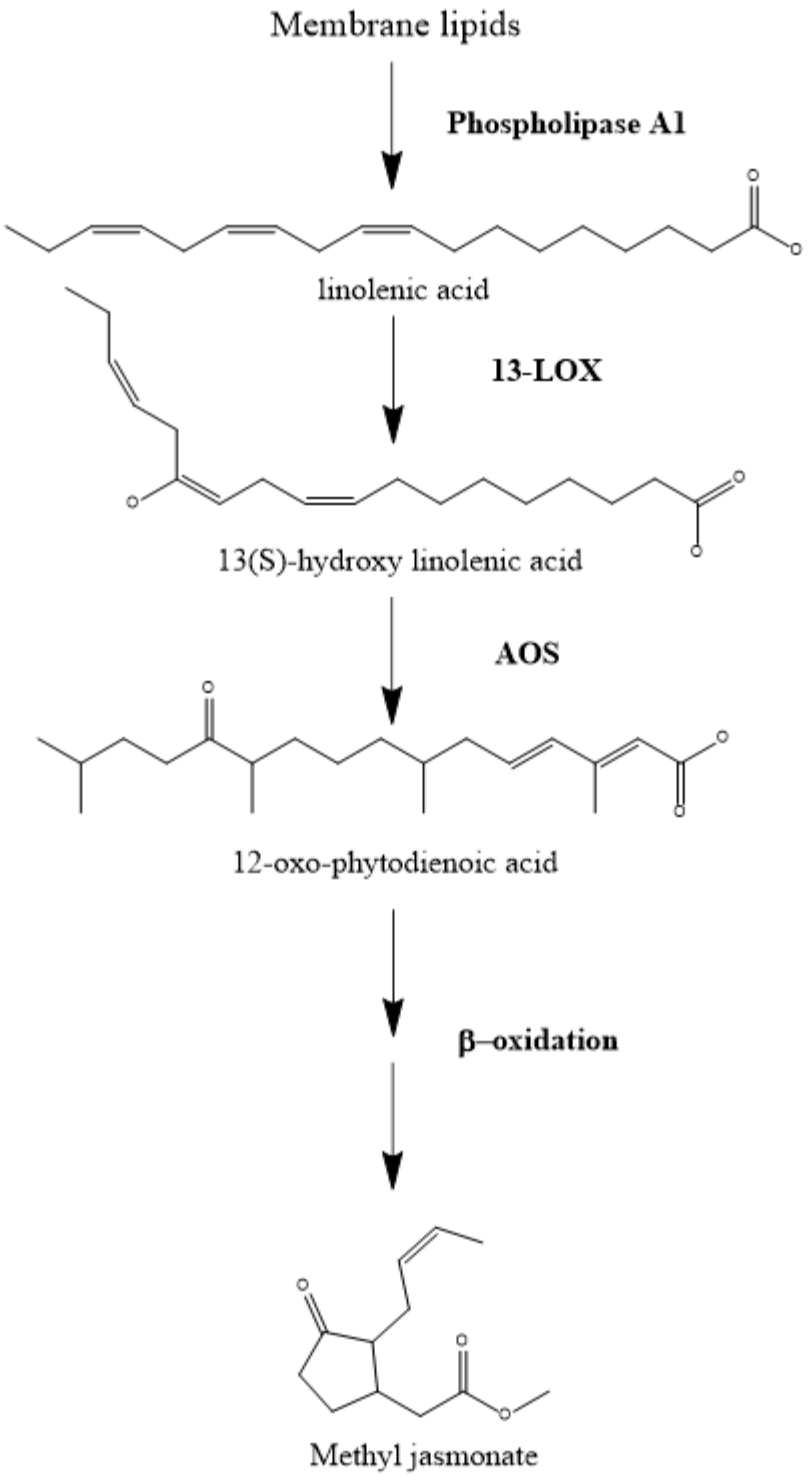


Figure 1.7 Biosynthesis of jasmonate. Modified from (Cheong and Choi, 2003).

1.7.2.3 Cross-talk

Plant adaptation to abiotic stresses often requires the activation of multiple responses including crosstalk with many molecular pathways (Duque et al., 2013). Phytohormones share signaling pathways where they often "cross-talk" each other. This relationship exists between many hormones, although only the interaction between ethylene, jasmonic and salicylic acid have been extensively explored compared with other phytohormones. There is evidence that positive and negative cross-talk occurs among SA, JA and ET. For instance, ethylene can interact positively with JA in defense related gene expression of *Arabidopsis*, and generally they are thought of as collaborative signals. On the other hand, a negative interaction between SA and JA has been demonstrated in several species in disease and pest resistance (Bostock, 2005). As mentioned before, ABA is the most representative phytohormone in abiotic stresses and massive production of this molecule is generated under drought stress. Moreover, the stomatal closure which was thought to be a specific characteristic of ABA, is also regulated by cytokinins, ethylene, brassinosteroids, jasmonic acid, salycilic acid and nitric oxide (Gong et al., 2013). ABA and gibberellins played a central role in regulating abiotic stress responses through protein receptors called DELLA (named after the first five amino acids). These receptors allow the interaction among auxins, ethylene and cytokinins, which is critical for plant adaptation under stress conditions (Kohli et al., 2013).

Cross-talk is not unique to plant-hormones; in fact, this phenomenon is frequently persistent in plants that are exposed to more than one environmental stress. Due to the combination of stress-conditions that plants may face, an integrated response is necessary, and evidence suggests that this response may be different from the response to single stresses (Atkinson and Urwin, 2012; Knight and Knight, 2001). There are some major points of signaling cross-talk between biotic and abiotic stresses, and the most important are Ca^{2+} and mitogen activated proteins kinases (MAPK) (Chinnusamy et al., 2004). Biotic and abiotic stress signaling pathways can be controlled by calcium-dependent protein kinases (CDPKs), which can sense fluctuation of the cystolic Ca^{2+} (Li et al., 2008a). Cascades of MAPK can be activated by several types of stresses, including salt stress, cold, wonding, ROS and biotic stresses (Chinnusamy et al., 2004). For instance, one MAPK gene (OsMAPK5) is induced by ABA, pathogens (Cauliflower mosaic virus) and abiotic stresses (wounding, drought, salt and cold) (Xiong and Yang, 2003).

1.7.3 Protein kinases in signal transduction

Mitogen activated proteins (MAPK) cascades are involved in abiotic stresses, transducing signals from cell surfaces to the nucleus. These proteins are the universal mechanism that connects receptors/sensors to cellular and nuclear responses in eukaryotes, and are involved in hormonal responses, cell cycle regulation, abiotic stress signaling and defense mechanisms (Pitzschke et al., 2009; Tena et al., 2001). All eukaryotes share a conserved MAPK cascade of three-kinase modules. Mitogen-activated protein kinase in plant signaling compromises of MAP kinase kinase kinase (MAP3Ks); MAP kinase kinase (MAP2Ks) and MAP kinase (MAPK). MAP3K are divided into two large subfamilies, MEKK and RAF-like kinases. On the other hand, MAP2Ks and MAPK are divided into four subgroups A-D (Rodriguez et al., 2010). MAPK, is the last element in the cascade, and it's activated by the phosphorylation mediated by MAPK kinase (MAPKK or MEC), which in turn, is activated by MAPKK kinase (MAPKKK or MEKK) (Zhang and Klessig, 2001).

Despite the diversity of stresses that plants must overcome during its life span, the generation of reactive oxygen species and MAPKs cascades are likely to be converging points in the defense signaling network (Zhang and Klessig, 2001). In oxidative stresses such as ozone, one of the essential MAPK pathways corresponds to the MEKK1-MOK4 cascade (Nakagami et al., 2006). When plants have been exposed to salt, drought and cold, the induction of MAPK genes and increased MAPK kinase activity has been detected. In *Arabidopsis,* MKK2 was activated by cold and salt as well as by the stress-induced MAPK kinase kinase MEKK1 (Teige et al., 2004).

1.7.4 Transcription factors

Transcription factors (TFs) are proteins which control the transcription, thus determining which genes are transcribed into primary RNA transcript (Latchman, 1997). There are more than 1500 transcription factors in *Arabidopsis* (Riechmann et al., 2000). Nonetheless, there are only three families of transcription factors which have been related

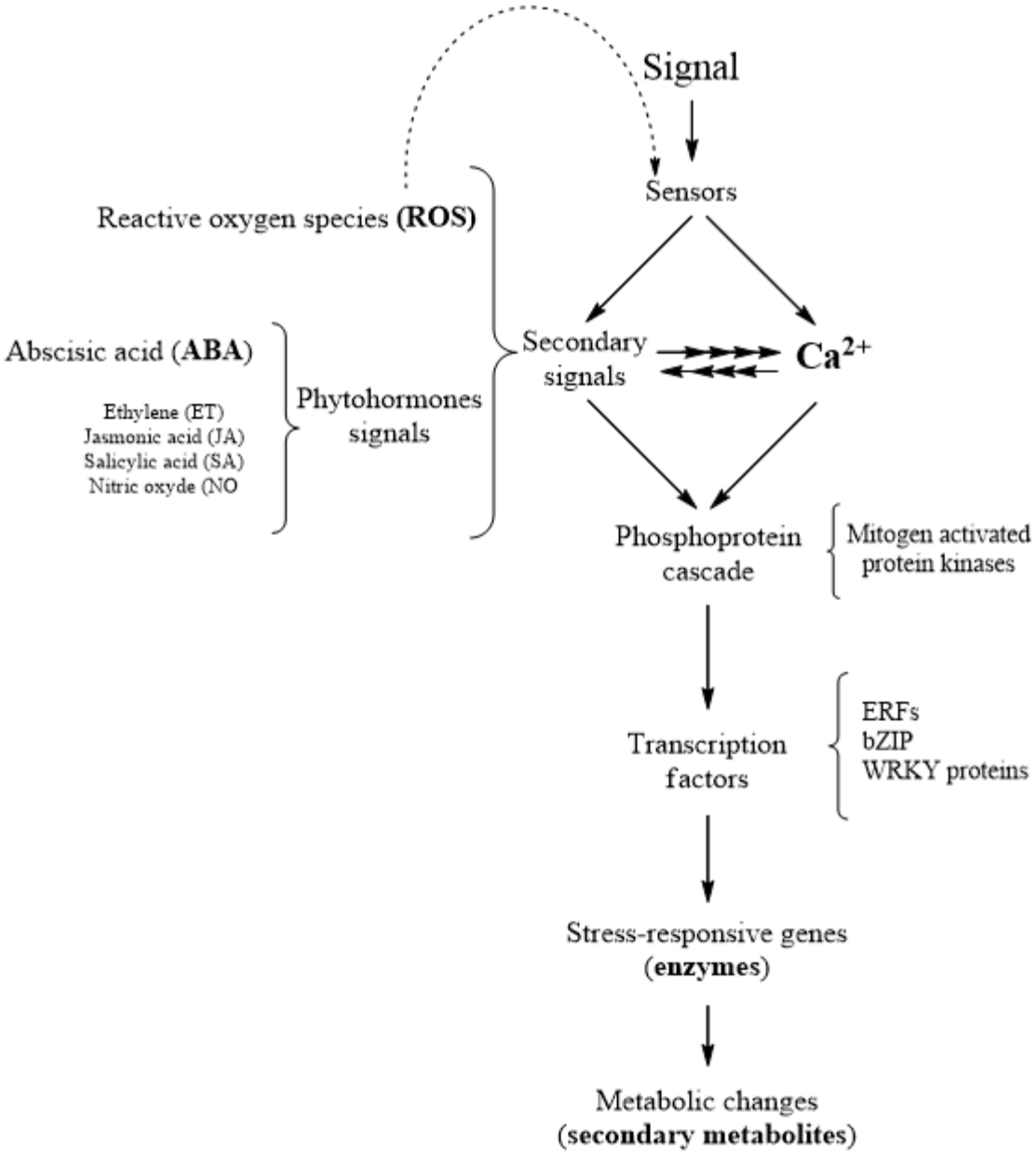


Figure 1.8 Generic pathway for the transduction of stress signals in plants. Abiotic stresses act as elicitor of cell responses towards defense reactions. Production of reactive oxygen species (ROS) is generally the first reaction to further signaling as well as cell wall reinforcement. Temporary increase of cytoplasmic ions such as calcium as well as the acidification of cytoplasm and phytohormones are all signaling components. These signals are finally translated into enzymes which are responsible of the biosynthesis of secondary metabolites (SMs). Modified from:Xiong and Zhu, 2001

to defense and stress responses in plants: ethylene-responsive-element-binding factors (ERFs); basic-domain leucine-zipper (bZIP) and WRKY (named after the amino acids) proteins (Singh et al., 2002). ERFs can regulate a variety of stresses including disease-related stimuli as well as wounding and abiotic stresses; and the expression of several ERFs is regulated by plant hormones (Gutterson and Reuber, 2004). The bZIP factors can also regulate pathogen defense processes as well as abiotic stresses such as light and other events including seed maturation and flower development (Jakoby et al., 2002). WRKY proteins trigger strong immune responses, for instance, promotion of SA-dependent signals, and suppression of JA-dependent signals is controlled by *AtWRKY70* (Eulgem and Somssich, 2007). Despite the specificity of some TFs related to biotic stresses, there is no general rule for the activation of specific TFs with respect to abiotic stresses. Instead, several TFs can cooperatively activate one gene (Xiong and Zhu, 2001).

1.8 Abiotic stresses and elicitors for postharvest preservation of fresh produce

An abiotic stress is a condition that reduces growth and yield below optimum levels. While it is difficult to get accurate estimates, it is clear that environmental factors have a great impact based on the affected land area as well as with the number of reports related to abiotic stresses (Cramer et al., 2011). The most important and common point in plants exposed to abiotic stresses is a decrease in the energy production and/or an increase demand on energy to overcome the stress (Duque et al., 2013). Inhibition of growth, protein synthesis and generation of ROS in the apoplast, mitochondria, cytoplasm, chloroplast and endoplasmic reticulum are the earliest responses to abiotic stresses (Good and Zaplachinski, 1994; Jaspers and Kangasjärvi, 2010). To cope with these conditions, plants have developed tolerance mechanisms for protection. The most shared are ion transporters, osmoprotectants, free-radical scavengers, late embryogenesis abundant (LEA) proteins and heat-shock proteins (HSPs) (Wang et al., 2003). Abiotic stresses can also influence secondary metabolites in plants; common examples include the increase of flavonoids with light and the synthesis of cryoprotectants for low temperature tolerance (Ramakrishna and Ravishankar, 2011).

Abiotic stresses have a great influence on fruits and vegetables during preharvest period. On one hand, extreme stresses such as high light exposure and temperature influence the development of physiological disorders such as watercore in apples and chilling injury in avocado (Ferguson et al., 1999). On the other hand, low abiotic stresses can positively affect the content of nutrients in crops

such as ascorbic acid, which is generally enhanced in plants exposed to high light intensity and during relatively low water stress conditions (Lee and Kader, 2000).

During the postharvest phase, produce is also exposed to abiotic stresses; the most recurrent are extreme temperatures, desiccation, mechanical injury, low O_2 and high CO_2 (Toivonen and Hodges, 2011). High temperature exposure can generate external and internal damages in the produce such as peel browning, yellowing of green vegetables, tissue damage produced by decay development, abnormal softening, lack of starch breakdown and development of internal cavities (Lurie, 1998). In contrast, low temperatures are responsible for chilling injury development in tropical and sub-tropical commodities, where the main symptoms are pitting, discoloration, water soaked appearance, internal breakdown, failure to ripen, loss of flavor and aroma (Wang, 1992). The main physical and physiological adverse effects of water stress in commodities have been previously described elsewhere in this review. Mechanical injury is produced in commodities during harvest, loading, transport, unloading and cleaning. Some of the effects of the mechanical injury include microbial colonization, textural and cell-wall changes and transitory increases in respiration, ethylene and phenolics production (Miller, 2002). Very low concentration of O_2 can create uncontrolled oxygen burst when tissue is re-aerated resulting in lipid peroxidation and membrane injury. On the other hand, high CO_2 concentration can affect the tricarboxylic acid (TCA) cycle and respiration by inhibiting succinate dehydrogenase (Toivonen and Hodges, 2011).

While abiotic stresses are harmful conditions for the produce, postharvest treatments have been evaluated for enhancing the resistance of fruits and vegetables to overcome these circumstances. The most important are temperature modulation, extreme atmospheres (high O_2, CO_2 and low O_2) and growth regulators (Toivonen, 2003). Gradual cooling can induce defense responses via the activation of phenylalanine-ammonia lyase (PAL) and increase resistance to chilling injury resistance (Galvez et al., 2010). Modified atmospheres can alleviate chilling injury in certain vegetables such as cucumbers by indirectly inducing high levels of polyamines, especially putrescine (Wang and Qi, 1997). The application of growth regulators is also important in produce which senescence occurs fast. Since ethylene is often related senescence, application of anti-ethylene aminovinylglycine (AVG) or 1-methylcyclopropene (1-MCP) can enhance the storage life of produce (Toivonen and Hodges, 2011).

Postharvest treatments are not only used to enhance the resistance of produce to abiotic stresses. Low or also called-hormetic doses of harmful abiotic stresses have been deliberately

applied to induce defense-related responses, quality and phytochemical content of commodities. For instance, UV light induces enzymes such as PAL, chalcone synthase (CHS), and the synthesis of several chemical compounds, including phenylpropanoids (Pombo et al., 2011) and delayed yellowing during storage of broccoli (Costa et al., 2006). Likewise, phytohormones such as methyl jasmonate can improve the phytochemical composition of vegetables during the postharvest storage (Pérez-Balibrea et al., 2011). Even if positive effects can be achieved when applying abiotic stresses to produce, the amount and exposure time of the stresses are generally low and this is a biological phenomenon known as hormesis.

1.8.1 Hormesis

Hormesis is a biological phenomenon, and it has been used by toxicologist to indicate a biphasic dose-response to an environmental agent that is harmful, which is described by a low dose stimulation or beneficial effect and a high dose inhibitory or toxic effect (Mattson, 2008). Graphically it can be represented by a biphasic or U-shaped inverted dose-response. U and inversed U-shaped dose-responses illustrate positive effects compared with a reference, followed by an increase of toxicity. In public health domain, U-shaped dose-response is used to illustrate incidence of disease, while inversed U-shaped dose-response is typically used to represent growth or longevity (Calabrese and Baldwin, 2002). At high doses, the endpoints indicate cellular damage, the low dose below the threshold, represents a manifestation of an adaptive response that can be measured as a biological performance (Calabrese et al., 2012). Hormesis is fundamental for life and evolution and hormetic responses involve complex mechanisms to handle environmental stresses. Some of these mechanisms include the production of proteins such ion channels, kinases deacetylases, heat shock proteins, and antioxidant enzymes such as superoxide dismutases and gluthathione peroxidases (Mattson, 2008).

Hormesis has been extensively investigated in UV-C stress for the preservation of fresh fruits and vegetables (Arul et al., 2001b; Charles and Arul, 2007; Shama and Alderson, 2005; Stevens et al., 1996). The hormetic phenomena of induced resistance in postharvest crops and delay ripening of fruits by UV-C have been well documented. Disease resistance has been observed in UV-C treated tomato below 3.7 $kJ.m^{-2}$ (Charles et al., 2008a; Charles et al., 2008c; Charles et al., 2008d). Similarly, UV-C treatments have delayed the yellowing of broccoli florets with doses between 1.2-10 $kJ.m^{-2}$ (Costa et al., 2006; Duarte-Sierra et al., 2012b). In addition to these parameters, low doses of UV-B

light have affected the secondary metabolism of broccoli florets (Mewis et al., 2012). Although, the hormesis concept has been investigated extensively for UV light, it has not been applied to other stresses or treatments such as ozone.

1.8.2 Visible Light

Visible light is part of the electromagnetic spectrum (400-720nm) compromising 40 % of the solar radiation energy (Koutchma et al., 2009). The molecule or pigment responsible of light harvesting in photosynthesis is chlorophyll. This is a non-polar molecule formed by four modified pyrrole rings attached to a magnesium atom and a long hydrocarbon tail found inside the chloroplast (Malkin and Niyogi, 2000). The breakdown of chlorophyll during senescence is a detoxification mechanism which is important for plant development in order to prevent accumulation of phototoxic intermediaries (Hörtensteiner, 2006). It has been also shown that thylakoid membrane is the first membrane to be affected by senescence. This is probably due to the fact that thylakoid membrane is the principal source of carbon inside the cell, that is possibly used for ATP generation through β-oxidation (Hopkins et al., 2007).

High light intensities are harmful for plants, due to the inactivation of electron transport in the photosystem II which lead to oxidative damage in the cell (Aro et al., 1993). During the postharvest phase, visible light can produce a stress condition, and chlorophyll can be affected by high light. Also, low light intensity can decrease the activity of enzymes and alter the chloroplast structure (Kays and Paull, 2004b). Recent reports, however, had shown positive effects of visible light delaying senescence in postharvest storage of broccoli. Exposure to a dose of 12 $\mu mol.m^{-2}.s^{-1}$ during broccoli storage delayed yellowing and maintained chlorophyll, while reducing sugar and starch contents decreased (Büchert et al., 2011b). Similarly, light exposure at the rate of 24 $\mu mol.m^{-2}.s^{-1}$ preserved chlorophyll a and b, antioxidant capacity, total phenols and ascorbic acid during 10 days storage at 7 °C of broccoli florets (Zhan et al., 2012). Another study showed that light increased total glucosinolate and phenolic content by 33 and 61 %, respectively, compared with dark growth conditions (Pérez-Balibrea et al., 2008). Thus, appropriate white light doses are probably an interesting means of enhancing quality and nutraceutical compounds in stored broccoli.

1.8.3 UV light

The sun is the principal source of UV light. Without the protection of the atmosphere, UV light would probably be lethal to most living organisms. Ozone layer prevents wavelengths ≤ 290 nm from reaching the earth's surface, thus the terrestrial environment is exposed to UV-B and UV-A light from 290 nm to 400 nm (World Health Organization, 1994). The action spectra of UV light is grouped into three wavelength ranges: UV-A from 315 to 400 nm (photon energy of 3.10-3.94 eV) accounts for 95 % of UV radiation, UV-B from 280-315 nm (photon energy of 3.94-4.43 eV) and UV-C, the shortest and highest energy per volt from 100 to 280 nm (photon energy of 4.43-12.4 eV) (Koutchma et al., 2009). The depth of penetration of UV light is wavelength-dependent, the longer the wavelength the deeper the penetration (Bruls et al., 1984). The absorption spectra of human skin ranges from 290 to 341 nm, thus UV-A is able to penetrate deeply in the skin compared with UV-B (Meinhardt et al., 2008). Similarly the penetration of UV-B light into the cell is limited and has a strong effect on surface or near-to-surface area in plant cells (Kovács and Keresztes, 2002).

1.8.3.1 UV-B light

UV-B light is of particular importance in plant life, because plants are often exposed to these wavelengths and which may generate considerable damage to agricultural crops. UV-B has the highest energy of the day light spectrum and can damage the cell (Nawkar et al., 2013). The most common damages caused by UV-B light in plants are the reduced leaf and stem growth, low total dry weight and low photosynthetic activity (Tevini, 1988). The photosynthetic machinery is particularly affected by the inactivation of photosystem II, the degradation of proteins, the loss of membrane integrity in thylakoid, the reduction of Rubisco activity, and lower levels of chlorophyll and carotenoids accumulation. Membranes are similarly affected by lipid peroxidation. In contrast, there is an up-regulation of genes from the phenylpropanoid pathway which leads to the accumulation of flavonoids and anthocyanins, which are used as blockers of these wavelengths. There is also an increase in glutathione and ascorbate contents as well as an increase in the activities of superoxide dismutase, glutathione reductase and peroxidase in chloroplasts (Jansen et al., 1998).

In postharvest, UV-B light has been used mainly to increase the antioxidant capacity of produce. For instance, postharvest radiation at 0.17 $W.m^{-2}$ during a period of 10 days, enhanced the total phenolic contents of apple fruits. The augmentation of phenols was accompanied by improved skin color as well (Hagen et al., 2007). An increase in the antioxidant capacity has also been

observed in other horticultural produce such as carrots (Avena-Bustillos et al., 2012) and tomato (Castagna et al., 2014). In addition, yellowing of broccoli has been successfully delayed with UV-B light by the suppression of chlorophyll-degrading enzymes such chlorophyllase and Mg-dechelatase at a dose of 8.8 kJ.m-2 (Aiamla-or et al., 2010). Furthermore, UV-B light enhanced the phytochemical levels in some *Brassica* crops. In nasturtium, the levels of glucosinolates, especially glucotropaeolin, and the total phenol content were enhanced by UV-B (Schreiner et al., 2009). UV-B at 0.3-1 $kJ.m^{-2}.d^{-1}$ mediated the induction of 4-methylsulfinylbutyl and 4-methoxy-indol-3ylmethyl glucosinolates in broccoli sprouts; this induction was also associated by the increased expression of genes associated with salicylate and jasmonic acid defense signaling pathways (Mewis et al., 2012).

1.8.3.2 UV-C light

UV-C constitutes a part of the electromagnetic spectrum between 100-280 nm with a peak emission at 254 nm and has been primary related to germicidal activity (Maharaj et al., 1999; Shama, 2007; Stevens et al., 1998). The germicidal effect of UV-C arises from photochemical lesions to nuclei, including the dimerization of nucleic acids and the laddering and fragmentation of DNA (Danon and Gallois, 1998; Shama, 2007). UV irradiation also promotes photo-oxidation reactions in plants *via* production of activated oxygen species. The free radicals target cell membranes, nucleic acids, cell wall polymers, enzymes, and chlorophyll degradation resulting in the acceleration of plant senescence (Foyer and Noctor, 2005). During postharvest storage, high UV doses generate undesirable changes, including skin discoloration, browning and drying, increasing susceptibility to brown rot and premature ripening.

However, UV-C light can also induce biochemical responses in plants that are very similar to those induced by pathogens such as the increase in salicylic acid and increased of the activity of benzoic acid 2-hydroxyase (BA2H) that catalyzes SA biosynthesis (Yalpani et al., 1994). One of the first responses of plants exposed to UV-C light is the synthesis of phytoalexins. These compounds vary among different crops exposed to UV-C light, including δ-viniferin in grapes (Pezet et al., 2003), scoparone and scopoletin in oranges (D'Hallewin et al., 1999) and rishitin in tomato (Charles et al., 2008d). Similarly, UV-C light can enhance the production of pathogenesis-related (PR) proteins in exposed plants (Yalpani et al., 1994), and the induction of β-1,3-glucanases and chitinases in tomato fruit (Charles et al., 2009). There is evidence that UV-C induced resistance in produce is a chronological process involving multiple defense mechanisms. This observation has been made

principally in tomato exposed to UV-C light were the accumulation of phytoalexins is the earliest defense response, followed by the formation of physical barriers, and ultimately by increased production of constitutive and inducible β-1,3-glucanases and chitinases (Charles et al., 2011).

UV light has been employed in postharvest crops to induce disease defense compounds as well as to delay senescence (Charles and Arul, 2007; Shama, 2007). UV-C at 3.7 $kJ.m^{-2}$ induced resistance to *Botrytis cinerea* in tomato fruit by the accumulation of phytoalexins, phenolic compounds, ultrastructural modification in the pericarp and the induction of PR proteins (Charles et al., 2008a; Charles et al., 2008b; Charles et al., 2008c; Charles et al., 2008d; Charles et al., 2009). UV-C doses of 2.4 - 4.9 $kJ.m^{-2}$ maintained the overall appearance, decreased the decay percentage and increased the shelf life of mango (González-Aguilar et al., 2007). Treatments with UV-C delayed yellowing, chlorophyll a and b degradation, reduced tissue damage and disruption, and maintained antioxidant capacity of broccoli (Costa et al., 2006). UV-C treatment contributed to the preservation of tissue integrity, as indicated by lower electrolyte leakage and respiratory activity in treated broccoli florets (Lemoine et al., 2007).

Synthesis of health-related compounds in plants exposed to UV-C has been also explored. For instance, UV-C irradiated grapes have the potential to produce wine with high content of resveratrol and piceatannol (Cantos et al., 2003). During postharvest storage, the trend has been focused to elevate the antioxidant capacity of commodities by the enhancement of phenolic compounds (Costa et al., 2006; Lemoine et al., 2007; Perkins-Veazie et al., 2008). The induction of these compounds is due to increased activity of PAL, chalcone synthase (CHS), which catalyze the synthesis of phenylpropanoids, coumarins and flavonoids (Pombo et al., 2011)(Charles et al., 2008d; Mercier et al., 1994).

The physiological age of the produce and UV-C dose are important factors since younger tissue appears to exhibit active responses (Arul et al., 2001b). UV doses found optimal to achieve beneficial effects in produce range from 0.5 $kJ.m^{-2}$ in strawberry to 10 $kJ.m^{-2}$ in broccoli (Baka et al., 1999; Costa et al., 2006). It also appears that the induced disease resistance and the accumulation of some phytoalexins by UV light action is a characteristic localized response (Arul et al., 2001a). UV treatments at postharvest stage must take into consideration these factors to attain prolonged shelf-life, and to increase valuable health-beneficial compounds.

1.8.4 Ozone

Ozone (O_3) is an allotrope of oxygen and is constantly present in the air. The formation of this gas in the stratosphere occurs when oxygen is dissociated to atoms in the presence of intense ultraviolet solar radiations (Roshchina and Roshchina, 2003a). Ozone generates a variety of free radicals in water solution in a cycle of seven steps (Figure 1.9). The effects of O_3 on living organisms depend on the dose; the minimal dose required to induce damage in the tissue is the threshold dose. Ozone dose can be: acute, using high concentration for a short period of time; and chronic, using low concentration for long period of time (Roshchina and Roshchina, 2003d).

Chronic exposure of plants to ozone results in visual and physiological changes such as changes in the pigmentation, chlorosis and premature senescence. In addition, physiological effects include reduced photosynthesis, turnover of antioxidant systems, and increased dark respiration occur (Felzer et al., 2007).

Biological effects of ozone depends on how far it penetrates into the cell. In plants, ozone first has to interact with both, cuticule or stomata to enter the extracellular space, cell wall, outer cytoplasmic membrane (plasmalemma), and finally to the cytoplasmic matrix and organelles. The actual physical process of O_3 transport is diffusion (concentration gradient). Long exposure to high O_3 concentration can damage the cuticle as well as the stomata (Laisk et al., 1989). Stomata are probably the main barriers to prevent the entrance of ozone, but cuticle may absorb part of the gas. Composition of the cuticles in broccoli consists of alkenes, nonacosane and secondary alcohols nonacosan-15-ol (Latimer and Severson, 1997). Damage of cuticle by ozone occurs by lipid peroxidation but plant absorption of ozone under normal conditions through cuticle is about 1/10000 compared with open stomata (Roshchina and Roshchina, 2003d). The entrance of ozone into the tissue affects apoplastic antioxidants, damages membranes, and generates stress responses, mainly ROS, and visual responses such as chlorosis or necrosis (Heath, 2008).

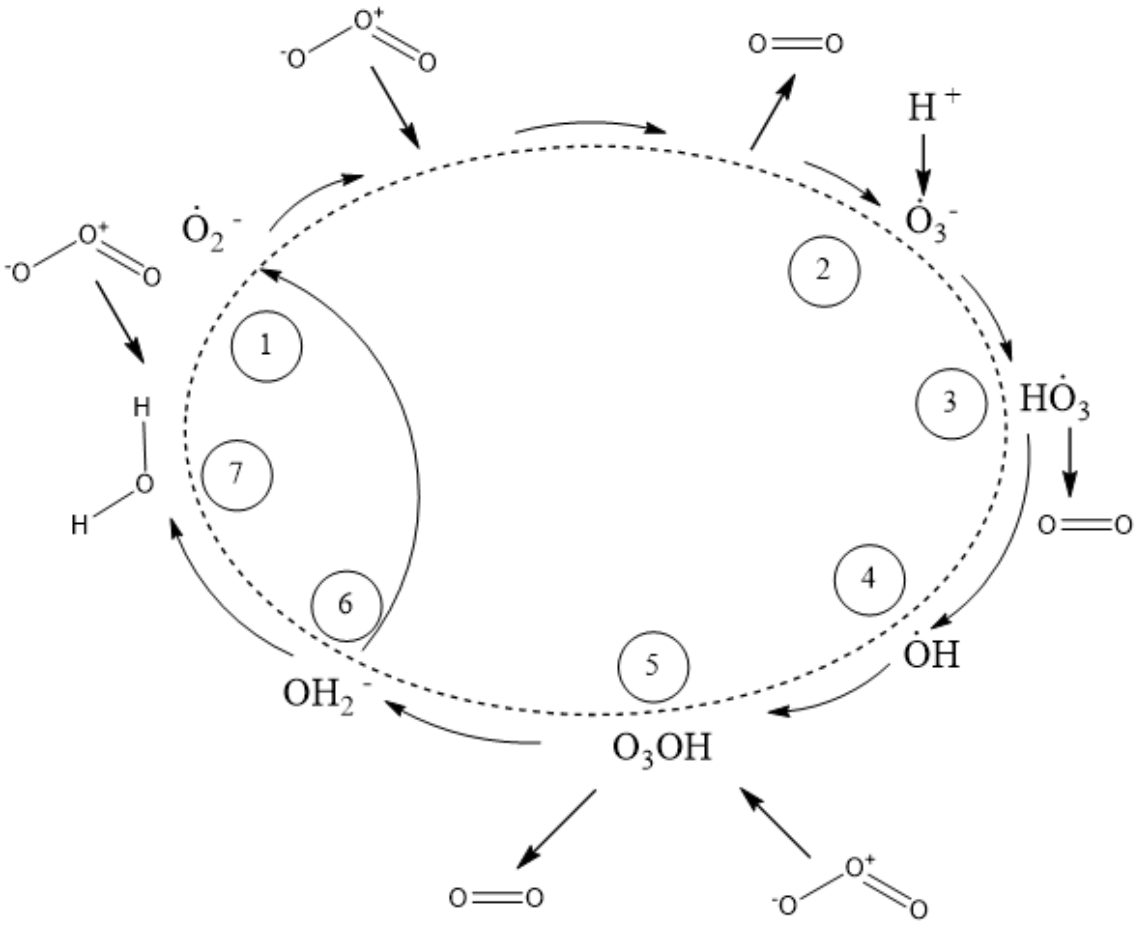


Figure 1.9 Ozone cycle in water. Modified from (Roshchina and Roshchina, 2003b). Ozone cycle involves several steps: ozone can react with hydroxy to produce superoxide radical $\dot{O}_2$. which reacts with ozone (1), resulting in trioxide radical (ozonide) (2). Ozonide reacts with hydrogen ion (3), and hydrotrioxide ion radical is formed. The hydrotrioxide ion decomposes (4) leading to hydroxy radical and molecular oxygen. A new molecule of ozone (5) reacts with hydroxy radical producing hydroxy trioxide. This molecule decomposes to form hydrodioxyion and oxygen (6). The hydrodioxyion can react with the hydroxy ion giving superoxide anion radical and water (7).

As ozone diffuses into the intercellular space, ascorbic acid may limit the amount of the gas penetrating through cell wall, avoiding the contact with more vulnerable structures inside the plasmalemma (Chameides, 1989). Evidence suggests that plants protect themselves from ozone by accumulating ascorbic acid in the cell walls that ultimately limits the entrance of the gas to more vulnerable structures in the cell (Chameides, 1989). Along with ascorbic acid, other antioxidants present in the apoplast are phenols, sulfhydryl amino acids (Roshchina and Roshchina, 2003d). And certainly, ozone influences the antioxidant system of the cell including superoxide dismutase, catalase, glutathione peroxidase as well as ascorbate peroxidase (Sandermann, 1998).

Changes at membrane level are important for signaling purposes. Ozone appears to enter through the stomata and it decomposes in the apoplast (Roshchina and Roshchina, 2003b). The putative signal pathways include seven possible mechanisms (Baier et al., 2005). The oxidative activation of Ca^{2+} channels which triggers the influx of calcium as a secondary messenger. A second model includes the possibility of a specific ozone receptor transducing the signal from the apoplast to the cytoplasm. A third model is the possible lipid derived-signaling, consequent enzymatic or non-enzymatic oxidation of unsaturated lipids to peroxides. The fourth model includes the generation of hydrogen peroxide that can diffuse into the cytoplasm. The fifth and sixth models involve the oxidation and regeneration of ascorbic acid at the expense of glutathione. The last possibility is that ozone diffuses directly into the cytosol generating reactive oxygen species (ROS).

The biochemical responses induced by ozone include the induction of polyamine and ethylene biosynthesis (Langebartels et al., 1991). Induction of stilbene biosynthesis has also been observed in conifer species after exposure to ozone. An increase in the activities of phenylalanine ammonia-lyase and chalcone synthase activity was observed prior to the biosynthesis of stilbenoids pinosylvin and pinosylvin 3-methyl ether (Rosemann et al., 1991). Exposure of plants to ozone has been found to cause similar responses as pathogen attack. For instance, the induction of β-1,3-glucanase and chitinase in leaf cells, and the production of necrotic spots by ozone have been observed (Schraudner et al., 1992). It seems that ozone can also act as inducer of different defense pathways at the same time. Enhancement of flavones glycosides (that are normally induced by UV exposure) and phytoalexins that are related to fungal attack were produced at the same time after the exposure of parsley to ozone (Eckey-Kaltenbach et al., 1994). On the other hand, the oxidative burst caused by ozone indirectly induces glutathione-S-transferase, PR-1-type proteins, and generates

phytohormone signals such as ethylene, salicylic acid and methyl jasmonate (Rao et al., 2000; Sandermann Jr et al., 1998).

Although ozone is mainly used for disinfection, its capacity to destroy ethylene makes this gas ideal for storage of fresh produce when appropriate doses are used. Ozone is known to produce antioxidants and phytoalexins in plants but it can also induce ethylene (Forney, 2003). Very little information is available regarding the induction of beneficial effects of ozone exposure in postharvest. For instance, broccoli exposed to continuous 0.04 $\mu L.L^{-1}$ of ozone for 21 days at 4 °C resulted in a significantly less pronounced color change compared with control stored without ozone (Skog and Chu, 2001). Ozone exposure increased the total flavan-3-ol content, maintained the levels of hydroxycinnamates and increased the total phenolics in table grapes (Artés-Hernández et al., 2007). However, special attention is necessary in selecting doses, due to the oxidative power of this molecule that can react with practically every component in the cells causing damages (Roshchina and Roshchina, 2003b).

1.8.5 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a non-radical ROS having an intermediate oxidation number which can be converted into other ROS by various means such as Fenton reaction and enzymes (Bienert et al., 2006). It is a fundamental component of signal transduction responses in biotic an abiotic stresses, since it is a small molecule with a relatively long half-life (1mS) compared with other ROS (Petrov and Van Breusegem, 2012). H_2O_2 is generated in normal metabolism in chloroplasts *via* the Mehler reaction, electron transport in mitochondria and photorespiration in peroxisomes (Quan et al., 2008). Stresses enhance its production *via* these routes, and also *via* enzymes such as membrane- localized NADPH oxidase, cell wall peroxidases, xanthine oxidase and amine oxidase (Neill et al., 2002). At low concentrations, it can act as a signal molecule in the resistance responses to biotic and abiotic stress, but higher concentrations can be toxic to the cells leading to programmed cell death (Gechev and Hille, 2005).

When high concentrations of H_2O_2 are not scavenged, hydroxy radicals can be produced by Haber-Weiss reaction with transition metals (Asada, 1992). These compounds are among the most highly reactive molecules known (Babs, 1989). The effect of hydroxy radicals include the attack on amino acid residues in proteins, protein degradation, formation of carbonyl derivatives, -S-S- bonding and damages to membranes by lipid peroxidation (Shen et al., 1997). On the other hand, hydrogen

peroxide is involved in the resistance mechanisms such as reinforcement of cell walls (lignification, cross-linking of cell wall proteins) and phytoalexin production (Quan et al., 2008). Endogenous H_2O_2 production leads to ethylene accumulation, and in contrast, methyl jasmonate induces hydrogen peroxide accumulation (Quan et al., 2008).

Exogenous H_2O_2 treatments are normally used for sterilization and disinfection of fresh produce as an alternative to chlorine (Abadias et al., 2011). The obvious advantage of H_2O_2 over chlorine is the lack of residues since it is rapidly decomposed into oxygen and water. Thus, it has been recognized as GRAS (Generally Regarded as Safe) (Bayoumi, 2008). Most postharvest reports of H_2O_2 in the literature focus on disinfection at relatively high concentrations. Hydrogen peroxide at 0.4 M reduced the microbial population on tomato slices by 5 log, although, carotenoids and total antioxidant capacity decreased as a consequence (Kim et al., 2007). Similarly, vapor phase hydrogen peroxide significantly reduced the number of *Botrytis* spores on grapes without affecting their quality (Forney et al., 1991b). Induction of antioxidant enzymes and stabilization of membranes against chilling-injury were also mediated by hydrogen peroxide (Zhou et al., 2012).

1.8.6 Heat

One of the most important physical conditions that affect life is temperature, and understandably many organisms share signal pathways to sense changes in temperatures in order to adjust their metabolism. It is possible that all the structures in the cell can perceive heat because of the increase in the thermal energy which causes physical changes such as membrane fluidity, partial melting of DNA and RNA strands and protein modifications (Mittler et al., 2012).

In most crop species, the margin of thermal tolerance fluctuates from 0 °C to 40 °C, temperatures above or under this range can cause damage to plant tissue (Źróbek-Sokolnik, 2012). Low temperatures can cause chilling (≤20 °C) or freezing injury (≤0 °C). Chilling injury effects include anomalous respiratory behavior, metabolic and membrane changes. At cellular level, chilling injury is responsible for the conversion of flexible membrane structure into a solid gel which makes difficult the exchange of essential ions, osmosis and diffusion processes (Hasanuzzaman et al., 2013; Lyons, 1973). High temperatures can also affect physiological processes in plants. Cellular injury or death can be produced by the denaturation and/or aggregation of proteins at very high temperatures. Morphological symptoms of high temperature stress include scorching and sunburns on leaves, branches and stems, leaf senescence, fruit discoloration and damages and reduced yields (Źróbek-

Sokolnik, 2012). High temperature also increases respiration and affect photosynthesis by alterations in chloroplasts, as well as injuries to cell membrane including swelling and production of ROS (Bita and Gerats, 2013). High temperature stresses have also been implicated in the generation of off-odors and ethanol, suggesting hypoxic stress (Duarte-Sierra et al., 2012a; Forney and Jordan, 1998).

There are specific proteins that are induced upon high and low temperature exposure of tissue, known as heat shock proteins (HSPs). They represent 1 % of the total proteins of eukaryotic and prokaryotic cells, but can easily increase to as much as 6 % of total proteins under heat stress, and they are also able to intervene in other abiotic and biotic stresses in plants (Xu et al., 2012b). HSPs includes HSP100, HSP90, HSP70, HSP60 and small-HSP (sHSP) (Kotak et al., 2007). Among HSPs, the HSP90 is the most important, because it can mediate stress signal transduction. Under normal physiological conditions, HSPs are located in the cytoplasm. But once stress conditions manifest, they can be transferred to the nucleus. In non-stress conditions, HSPs take part in cellular signaling control, protein folding translocation and degradation, while under stress conditions, they prevent protein aggregation and protect membrane (Mittler et al., 2012). Gradual exposure to low or high temperatures may enhance the resistance of plants to extreme conditions, such as chilling injury by the accumulation of HSPs. The synthesis HSPs can also protect plants from other stresses, such anoxia which is a phenomenon known as cross-protection or cross-tolerance (Banti et al., 2008; Sabehat et al., 1998).

Even though HSPs are of vital importance to maintain cell integrity under heat stress conditions, most of the induced transcripts in the cells in response to heat stress may not be translated for heat shock proteins. The heat shock response is driven by multiple temperature sensors. These sensors include, cytoplasmic streaming (distribution of heat throughout the cell), membrane fluidity, lipid rafts, activation of ion channels, protein stability, cytoskeleton disassembly, chromatin changes, DNA-protein interactions, histone displacement, ROS and redox changes (Mittler et al., 2012). Membrane fluidity might represent a potential site of perception as fluidity is sensitive to temperatures. Evidence suggests that the temperature sensing mechanisms are probably found in the thylakoid membrane due to its highly unsaturated fatty acid profile (Sung et al., 2003). Changes in membrane fluidity lead to the influx of calcium into the cell by the opening of a specific calcium channel, and activate several calcium-dependent protein kinases. Thus, plasma membrane and calcium signaling are the master sensors for the heat shock response in plants (Mittler et al., 2012).

In postharvest, heat treatments can be performed using hot water (>40 °C), steam, forced hot air, high-temperature controlled atmospheres and solar energy, infrared, microwave and radio frequency (Hansen and Johnson, 2007). Among the positives effects, high temperature treatments can delay the ripening of fruits by its influence on ethylene activity. Ethylene is inhibited at high temperatures (30 to 40 °C) and the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to C_2H_4 is also vulnerable to high temperatures (Yu et al., 1980). Also fruits exposed to high temperatures (45 °C) soften more slowly than un-heated fruits, due to the reduced activities of hydrolytic cell wall enzymes as polygalacturonase, β-galactosidase, endo-1,4-β-d-glucanase (EGase) and β-xylosidase (Martínez and Civello, 2008). Heat treatments have also delayed yellowing of broccoli. For instance, the immersion of broccoli at 50 °C in water for two minutes was found to be the most effective treatment to slow-down yellowing and decay (Forney, 1995), which may be due, in part, to reduced chlorophylase activity (Funamoto et al., 2002).

Heat treatment can have a negative impact when high temperatures and long exposure times are used. High respiration rates can lead to anaerobic conditions due to depleted tissue oxygen as well as membrane disruption and progressive electrolyte leakage (Duarte-Sierra et al., 2012a). Off-odor development and increased production of ethanol were observed in broccoli after hot water treatment at 52 °C for 3 min that, presumably, creates anaerobic conditions in the tissue (Forney and Jordan, 1998). Finally, higher water loss in heat-treated commodities has been also observed, likely due to a higher respiration and transpiration rates (Schirra et al., 2000).

1.8.7 Ethanol

Ethanol is a 2-carbon alcohol (CH_3CH_2OH). It is often used as a topical disinfectant, solvent and preservative in pharmaceutical preparations as well as the primary ingredient in alcoholic beverages (PubChem, Ethanol-Compound Summary). Ethanol is also considered as a drug for humans with toxicological effects. The generation of free radicals, cellular injury and the resulting liver disease are the most studied effects of this compound (Lieber, 2000). Free radical generation can occur through the cytosolic xanthine/aldehyde oxidases as well as through the mitochondrial respiratory chain (Albano, 2006; Nordmann et al., 1992). The oxidative stress is associated with lipid peroxidation and membrane dysfunction (Miller, 2013). Ethanol can easily disturb fluid membranes such as those with low cholesterol content (Goldstein, 1986).

In plants, the biosynthesis of ethanol generally occurs by anaerobic respiration as a consequence of water logged soils (Waters et al., 1989). Fermentation pathways lead to the lactate and ethanol production. Ethanol is formed by the decarboxylation of pyruvate to acetaldehyde followed by the reduction of aldehyde to ethanol by alcohol dehydrogenase (Perata and Alpi, 1993). Exogenous ethanol is also metabolized by plant tissue, the main metabolites synthetized after the application of ethanol are acetaldehyde, acetone, acetic acid, arginine, aspartic acid, glutamic acid, glycine, methionine, serine and malic acid (Cossins and Beevers, 1963). Thus, it can be hypothesized that exogenous ethanol can be used by plants as a carbon source (Janeczko, 2011).

Exposure of plants to ethanol in normal environmental conditions is not common, however, crops can be pre-exposed to ethanol before storage in order to alleviate decay, delay senescence and maintain quality of the produce. Ethanol has been successfully used to control decay in small fruits specially grapes (Candir et al., 2012; Lichter et al., 2002; Lurie et al., 2006). Exposure to ethanol vapor inhibited ripening in pericarp discs of mature green tomato fruit (Saltveit, 1989). Other studies have shown a delay in the senescence of broccoli. Broccoli exposed to an atmosphere containing 2,500 ppm of ethanol at 13 °C for 12-24 hours reduced chlorophyll loss compared with the control, and prevented the production of off-odors (Corcuff et al., 1996). Yellowing in broccoli florets was also inhibited by placing broccoli florets with 12 g of encapsulated alcohol over 5-day storage at 20 °C (Suzuki et al., 2004). These results are also supported by the effect of ethanol on the chlorophyll catabolic enzymes in broccoli. Mg-dechelase and Chl-degrading peroxidase activities did not increase during storage in ethanol-treated florets (Fukasawa et al., 2010). In addition, ethanol vapor, decreased the enzyme activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase (Asoda et al., 2009).

1.8.8 Application of elicitors: methyl jasmonate

Methyl jasmonate (MeJA) and its free-acid jasmonic acid are fatty acid-derived cyclo pentanones which act as signal transduction intermediates when plants are subjected to biotic and abiotic stresses (Cohen and Flescher, 2009). Jasmonates are important cellular signal components involved in development processes such as germination, root growth, fruit ripining and senescence (Cheong and Choi, 2003). The main deleterious biological activity of jasmonates in plants is the senescence-promoting effect. Jasmonates are related with senescence-associated genes (SAGs) in the down regulation of house keeping photosynthetic proteins (Wasternack, 2007). Jasmonates

mediate systemic wound responses in plant defense which are generally accompanied by other hormones (Koo and Howe, 2009). For instance, methyl jasmonate and salicylic acid treatments in Chinese seed rape breeding lines produced an increase in leaf indole and aromatic glucosinolates, respectively. However, the extent of such increases differed widely between lines (Schreiner and Huyskens-Keil, 2006).

Sasaki-Sekimoto et al. (2005) underscore the importance of JA as a signal in plant defense. They found that several types of stress conditions, such as wounding and pathogen infection cause endogenous JA accumulation and the expression of jasmonate-responsive genes. Jasmonates activate the coordinated gene expression of factors belonging to two functionally related groups: (i) ascorbate and glutathione metabolic pathways, which are important in defense responses to oxidative stress, and (ii) biosynthesis of indole glucosinolate, which is a defense compound occurring in the *Brassicaceae* family. These results suggest that the coordinated activation of the metabolic pathways mediated by jasmonates provides resistance to environmental stresses (Sasaki-Sekimoto et al., 2005).

Treatments with MeJA at the postharvest stage have been focused on the protection of commodities from microbial development, chilling injury and the enhancement of secondary metabolites. The treatments are generally applied as vapor in micro molar concentrations, although sprays are also used. Resistance of produce to decay has been correlated with the induction of polyphenols, alkaloids or pathogenesis-related proteins (Ding et al., 2002; Droby et al., 1999; Yao and Tian, 2005). Reduced chilling injury in produce has been reported after MeJA application by a mechanism involving the regulation of ABA and polyamine levels (Lee et al., 1996; Wang and Buta, 1994). In recent years, however, the use of methyl jasmonate has been focused towards the accumulation of phytochemicals and plant nutraceuticals such as resveratrol (Rohwer and Erwin, 2008). The antioxidant activities, anthocyanin and β-carotene contents of several fruits and vegetables including apples, potatoes, tomatoes, guava, mango, banana and papaya have been shown to increase with the application of MeJA (Schreiner and Huyskens-Keil, 2006).

1.10 Enhancement of phytochemicals in fresh produce and broccoli

The development of postharvest treatments has long been focused on longevity, maintenance of quality of produce and recently on the preservation of health-beneficial compounds they contain. Evidence indicates that postharvest conditions such as low temperature, heat, modified

atmosphere, and elicitors such as jasmonate and ethylene induce the synthesis of phytochemicals belonging to different classes of compounds in fresh fruits and vegetables (Alasalvar et al., 2005; Jones, 2007; Matusheski et al., 2004).

There is emerging interest on the use of abiotic stresses as treatments to enhance desirable health-beneficial compounds. Controlled abiotic stress treatments can be used as a tool by the fresh produce industry to enhance health-beneficial compounds in fruits and vegetables (Cisneros-Zevallos, 2003; Schreiner and Huyskens-Keil, 2006; Terry and Joyce, 2004).

Along with vitamin C and vitamin E, plant phenolics, terpenes, phytosterols and glucosinolates are the main groups of bioactives sensitive to abiotic stresses (Burritt, 2013). UV light is one of the most common treatments evaluated to enhance the phenolic content of fruits due to the increased activity of enzymes such as phenylalanine ammonia-lyase and 4-coumarate ligase, involved in the synthesis of these compounds (Kuhn et al., 1984). Methyl jasmonate has also been used in fruits before their storage, as a means to induce anthocyanin biosynthesis by the induction of chalcone synthase and dehydroflavonol-4-reductase (Schreiner and Huyskens-Keil, 2006). Besides UV light and methyl jasmonate, other treatments such as ionizing radiation, electric currents and low temperatures have shown positive effects on fruit phenolic contents (Girennavar et al., 2008; Rapisarda et al., 2008; Zhao et al., 2011).

Postharvest treatments using abiotic stresses has been applied to different produce and positive results are commonly observed with exposition to UV-C on small fruits such as grapes, berries and strawberries, as well as mangoes and tomatoes (Burritt, 2013). For instance, total anthocyanin levels increase in grapes exposed to UV-C ranging from 0.8 to 2.4 $kJ.m^{-2}$ (Crupi et al., 2014). Similarly phytochemicals in blueberries, including flavonoids and anthocyanins, are enhanced after the exposure to doses between 2.15 $kJ.m^{-2}$ and 4.30 $kJ.m^{-2}$ of UV-C light (Wang et al., 2009). The total anthocyanin content is increased in strawberries after the treatment at 0.25 $kJ.m^{-2}$ and 1.0 $kJ.m^{-2}$ of UV-C light (Baka et al., 1999). UV-C light treatments with doses of 2.46 $kJ.m^{-2}$ and 4.93 $kJ.m^{-2}$ were also effective in increasing the total phenols and flavonoids in mangoes (González-Aguilar et al., 2007). Besides UV-C, electron-beam ionizing radiation at 3.1 kGy enhanced the flavonol constituents of mangoes (Reyes and Cisneros-Zevallos, 2007). In tomatoes treated with 3.7 $kJ.m^{-2}$ and 24.4 $kJ.m^{-2}$ of UV-C light the total phenol content was enhanced (Maharaj et al., 2014). Furthermore, moderate salt stress increases the concentration of lycopene in the tomato fruit (Kubota et al., 2006).

The effective means of preserving phytochemicals including glucosinolates and phenolic compounds in broccoli are refrigeration at 4 °C and freezing (Rodrigues and Rosa, 1999). However, enhancement of phytochemicals in broccoli during the postharvest storage has been also observed with UV light and methyl jasmonate treatments. Broccoli heads treated with 10 $kJ.m^{-2}$ of UV-C light enhanced total phenols (Costa et al., 2006). Elicitors such as MeJA at 10 and 25 μM increased the concentrations of total flavonoids and indole glucosinolates (Pérez-Balibrea et al., 2011). Despite being less explored alternatives for the elicitation of health-related secondary metabolites, ozone, hydrogen peroxide, ethanol and heat are all oxidative stresses, against which the activation of protection mechanisms is expected. Thus, it is plausible that the application of these stresses can lead to the enhancement of glucosinolates and flavonoids in broccoli florets.

1.11 Recapitulation

Broccoli is susceptible to rapid senescence which is characterized by the yellowing of florets during the postharvest storage. Storage temperature and relative humidity are the two main factors that influence the rate of senescence in stored produce, including broccoli, during the postharvest period. These two factors have great impact on its physiological characteristics such as respiration and transpiration rates as well as decay caused by pathogens. Respiration and transpiration rates are slowed down by low temperatures and high relative humidity. After harvest, broccoli is generally pre-cooled and stored at 0-1 °C, and optimal high relative humidity fluctuating between 90 and 98 %. As a consequence, quality factors such as color and firmness are maintained for longer periods of time, compared with the produce handled without proper conditions. Nonetheless, temperature and relative humidity control are not the only means to maintain quality of broccoli during the postharvest.

Postharvest treatments aimed at maintaining quality of broccoli often involve abiotic factors such as heat, ethanol and UV-B light. Abiotic stresses are conditions that reduce plant growth and yield during the preharvest phase, and are associated with physiological disorders and diseases during the postharvest period. For instance, high light intensities can lead to the inactivation of electron transport in the photosystem of plants, UV-B light can affect membrane integrity leading to cell death, and others such as ozone can induce premature senescence. Despite these deleterious effects, abiotic stresses can be used to maintain quality on fruits and vegetables including broccoli, and this is possible by a phenomenon known as hormesis. Low or hormetic of an agent or stress

considered toxic induces beneficial effects (adaptive responses) while high doses can generate detrimental effects.

In addition to quality attributes, nutriments such as carbohydrates, vitamins and minerals as well as phytochemical compounds such as phenolic compounds, terpenes and sulfur compounds can also be preserved using hormesis. Broccoli is a rich source of nutriments and phytochemical compounds. It contains two flavonol compounds related with the prevention of cardio-vascular illness: quercetin and kaempferol. Additionally, broccoli contains sulfur-compounds, the glucosinolates, which are an essential part of the defense system of *Brassicaceae* family. Moreover, glucosinolates such as glucoraphanin are the precursor molecules of bioactive anticancer-related isothiocyanates such as sulforaphane. These compounds are part of the plant secondary metabolism, and may be induced by biotic or abiotic stresses. Abiotic stresses can be applied as pre-storage postharvest treatments in order to induce targeted secondary metabolites. Induction of secondary metabolites may depend on the type of stress and its dose. The use of postharvest treatments to modify the secondary metabolism of broccoli during the postharvest storage of broccoli is relatively new. Abiotic stresses such as UV-C and UV-B light have been used largely to delay yellowing by modifying chlorophyll catabolism. However, the effect of these treatments on the phytochemical content has not been explored, and there is a window opportunity to test the effects of abiotic stresses.

1.11 Hypothesis

Secondary metabolites accumulate in plants in response to abiotic stresses and, therefore it can be anticipated that that their levels can be enhanced by the application of stresses at hormetic doses. The postharvest phase is ideally suited for the application of stresses in a controlled manner. It is hypothesized that the application of hormetic/controlled doses of stresses such as UV-B, UV-C, heat, ozone, hydrogen peroxide, ethanol and elicitors such as methyl jasmonate would enhance the amount of glucosinolates and phenolic compounds in broccoli while maintaining its sensory acceptability.

1.12 Objectives

The main objective of this thesis was to increase the levels of the prominent secondary metabolites in broccoli florets, glucosinolates and hydroxycinnamic acids, using hormetic doses of

abiotic stress without compromising the quality during the postharvest storage. The specific objectives were: 1) To establish a hormetic doses for each of the applied stress using color change response during storage; 2) To determine the effects of abiotic stress doses on different quality factors; 3) To evaluate expression of genes of key enzymes involved in the biosynthetic pathways for glucosinolates and phenolic compounds in broccoli; and 4) To assay the concentrations of secondary metabolites: glucosinolates and hydroxycinnamic acids after the exposure of broccoli florets to abiotic stresses during the postharvest storage.

Chapter II: UV-C Hormesis in Broccoli: Preservation of Quality, Glucosinolates, Phenolic acids, their Amino Acid Precursors and Gene Expression.

2.1 Résumé

L'effet de l'exposition à la lumière ultraviolette (UV-C) de fleurons de brocoli (*Brassica oleraceae* var. Italica) avant leur entreposage à 4 °C et 90 % HR a été étudié. Le jaunissement des fleurons de brocoli a été retardé par la dose hormétique de 1,2 $kJ.m^{-2}$. La respiration initiale (jour 0) des fleurons exposés à 1,2 $kJ.m^{-2}$ et à 3,0 $kJ.m^{-2}$ d'UV-C était significativement plus élevée par rapport à celle des fleurons non exposés. Les valeurs moyennes de l'acide ascorbique et de l'ORAC (capacité d'absorption des radicaux oxygénés) du tissu exposé à des doses UV-C de 1,2 ou 3,0 $kJ.m^{-2}$ étaient plus bas sur la période d'entreposage comparé aux fleurons qui n'ont pas reçu de traitement de lumière UV-C. La surexpression de la chalcone synthase (CHS) et de la 4-coumarate-CoA ligase (4CL) a été observée aux jours 0, 2 et 4 dans les fleurons exposés aux UV-C. En outre, la surexpression de la phénylalanine N-hydroxylase (CYP79A2), la tryptophane N-hydroxylase (CYP79B3), la dihomo-méthionine N-hydroxylase (CYP79F1) et de la flavonoïde monoxygénase (F3H1) a été observée dans les fleurons exposés à l'UV-C au jour 0. Les concentrations des acides aminés méthionine, tryptophane et phénylalanine,, qui servent de source pour la biosynthèse de glucosinolates dans les tissus végétaux, ont été trouvées dépendantes de la dose. Les doses d'UV-C de 1,2 et 3,0 $kJ.m^{-2}$ ont provoqué une diminution de leur concentration par rapport au témoin. En conséquence les doses d'UV-C ont augmenté significativement la concentration totale de glucobrassicines totales, ainsi que la 4 hydroxyglucobrassicine. De même, les fleurons traités à l'UV-C, soit à 1,2 ou 3,0 kJm^{-2} contenaient un niveau plus élevé d'acides hydroxy cinnamiques par rapport au témoin. Les résultats suggèrent que l'application de la dose hormétique d'UV-C peut être bénéfique pour la conservation de la qualité et des composés phytochimiques dans les fleurons de brocoli pendant l'entreposage à basse température.

2.2 Abstract

The effect of pre-storage exposure to ultraviolet light (UV-C) on broccoli (*Brassica oleraceae* var. Italica) florets stored at 4 °C and 90 % HR was investigated. Hormetic dose of 1.2 $kJ.m^{-2}$ was found to delay yellowing in broccoli florets. The initial respiration rate (day 0) of broccoli exposed to 1.2 kJm^{-2} and 3.0 kJm^{-2} of UV-C light was significantly higher than for the unexposed florets. The time-averages over the storage period of both ascorbic acid titer and ORAC (oxygen radical absorbance capacity) of the tissue exposed to UV-C doses of 1.2 or 3.0 $kJ.m^{-2}$ were lower as well. Overexpression of chalcone synthase (CHS) and 4-coumarate-CoA ligase (4CL) in florets was observed on days 0, 2 and 4 in florets exposed to UV-C. Furthermore, overexpression of phenylalanine N-hydroxyase (CYP79A2), tryptophan N-hydroxyase (CYP79B3), dihomo-methionine N-hydroxyase (CYP79F1) and flavonoid monoxygenase (F3H1) was observed in UV-C exposed broccoli on day 0. The titers of glucosinolate-precursor amino acids, methionine, tryptophan and phenylalanine in tissue were dose-dependent, where the doses of 1.2 and 3.0 $kJ.m^{-2}$ UV-C caused a decrease in their concentrations compared with the control. Hormetic doses of UV-C significantly increased the concentration of total glucobrassicins and 4-hydroxyglucobrassicin. In addition, UV-C treated florets at 1.2 or 3.0 kJm^{-2} contained a higher level of hydroxycinnamic acids compared with the control. The results suggest that the application of hormetic doses of UV-C can be beneficial in maintaining the quality in broccoli florets and phytochemical-compounds during the low-temperature storage.
Keywords: UV-C light, broccoli, amino acids, glucosinolates phytochemicals.

2.3 Introduction

Longevity and quality of postharvest horticultural crops are important issues for both producers and consumers. The emphasis of producers is on the preservation of produce quality and reducing postharvest losses; whereas the consumer is not only conscious about the quality of produce, but also about the chemical residues that could be potentially harmful to human health (Tiznado-Hernández and Troncoso-Rojas, 2006). In order to avoid or reduce the use of chemicals to extend the marketable life of produce, novel approaches are being sought. Ultraviolet C radiation (UV-C) obtained artificially from germicidal lamps emitting in the range of 200-280 nm with peak emission at 254 nm (Bintsis et al., 2000; Fredericks et al., 2011) has been shown to cause the biological phenomenon known as hormesis in postharvest crops (Arul et al., 2001a; Stevens et al., 2006). Although it is a harmful agent to living organisms, it has been shown to elicit beneficial effects in postharvest crops at low doses. The hormetic or beneficial effects of UV-C in fresh crops include induced resistance to diseases (Charles et al., 2011), and delayed ripening and senescence (Costa et al., 2006). Multiple mechanisms appear to be involved in UV-C induced disease resistance: the accumulation of phytoalexins and phenolic compounds (Ben-Yehoshua et al., 1992; Charles et al., 2008d; Mercier et al., 1993a; Mercier et al., 1993b); the elicitation of pathogenesis-related (PR) proteins (Charles et al., 2009); and the formation of physical and biochemical barriers (Charles et al., 2008b). UV-C has also been shown to delay color development in a variety of crops (Baka, 1997; Maharaj et al., 1999), and delay yellowing and chlorophyll degradation in broccoli (Baka, 1997; Costa et al., 2006; Lemoine et al., 2007).

Broccoli is an excellent source of nutrients such as vitamin C, folic acid, vitamin K, and essential minerals. It also contains powerful health-promoting phytochemicals and antioxidants including phenolic acid derivatives, flavonols, and glucosinolates (Figure 2.1), the latter also play a defense role against infection (Robbins et al., 2005). Glucosinolates (GLS) are anionic and sulphur rich compounds found in vegetables with a common structure of a β-thioglucosyl linked to a α carbon of an amino acid (Cabello-Hurtado et al., 2012), and are derived from various amino acids (Sønderby et al., 2010). They are grouped into classes based on their structural similarity: alkyl, derived from methionine; aromatic resulting from phenylalanine; and indole from tryptophan (Clarke, 2010). The final GLS core structure relays on the conversion of amino acids to aldoximes by cytochromes p450 of CYP79 family: CYP79A2 uses phenylalanine as substrate; CYP79B2 and CYP79B3 uses tryptophan; and CYP79F1 converts all chain-elongated methionine derivatives (Sønderby et al.,

2010). Similarly, phenolic compounds are produced by plants via the shikimic acid or phenylpropanoid pathway from the aromatic amino acids, tryptophan, tyrosine and phenylalanine (Stewart and Stewart, 2008). Phenylalanine ammonia-lyase (PAL) is the key enzyme for the conversion of aromatic amino acids into phenolic compounds (Cartea et al., 2010). Chalcone synthase (CHS) is the enzyme responsible for the biosynthesis of flavonoid (Agati et al., 2012). UV treatment has been shown to induce over-expression of PAL in strawberries at a dose of 4.1 $kJ.m^{-2}$ (Pombo et al., 2011), as well as increase of its activity in mangos with doses ranging from 2.46 - 4.98 $kJ.m^{-2}$ (González-Aguilar et al., 2007). The effect of UV-C on glucosinolates has been less explored, however enhancement of glucosinolate content has been observed in broccoli following UV-B light exposure (Mewis et al., 2012).

The objective of this work was first to determine the hormetic UV-C dose for maintaining broccoli quality; and second, to determine the levels of the secondary metabolites glucosinolates and phenols in response to UV-C. In an effort to understand the underlying events, free amino acid profile and the expression of target genes in the relevant biosynthetic pathways were also evaluated.

2.4 Materials and Methods

2.4.1 Broccoli

Freshly harvested broccoli (*Brassica oleraceae* L. var. Italica 'Diplomat') heads were obtained from a commercial farm (Ile d'Orléans, Québec, Canada). Florets (300g) of uniform size (approximately, 7 cm) were separated from heads and randomly arranged in small plastic punnets of 500 mL. The punnets were placed in plastic containers of 5 L with perforations for ventilation, and containing a layer of water at the bottom to maintain high humidity (98-100%), and the containers were stored inside a controlled chamber overnight at 4 °C.

2.4.2 UV-C Treatment

The florets were irradiated with UV-C doses ranging from 0.0 (control) to 6 $kJ.m^{-2}$. UV-C radiation was emmited from fluorescent germicidal lamps with peak emission at 254 nm (TUV G30T8, 30W, Philips). Broccoli florets were arranged in transparent racks where the head of the floret was in direct contact with light (15 cm of distance). To ensure uniform exposure of florets, racks were settled into a closed chamber covered with aluminum foil. Exposure of florets to the light was done at

room temperature. Radiation intensity was measured with a digital radiometer (Cole-Parmer Instrument Company, Vernon Hills, IL, USA). After treatment, broccoli florets were loosely covered with black PVC film to prevent photo-reactivation (a repair mechanism taking place in exposed tissue to UV light by photolyases that requires visible light), and stored at 4 °C for 27 days in darkness. About 9 florets were disposed in plastic punnets inside plastic containers of 5L with ventilation and water that maintained high humidity and stored inside a controlled chamber at 4 °C/90%RH. Florets were weighed at regular intervals of time (0, 7, 14 and 21 days) for weigh loss determination.

2.4.3 Color Measurement

Color of florets was invariably measured in the middle part of the head (buds). Color was determined by measuring parameters L*, a*, and b* with a colorimeter (Minolta CR200, Osaka, Japan) on 9 florets from each treatment daily during 27 days of storage. The total color change (ΔE) was determined from L*, a* and b* values: $\sqrt{(L_0 * - L_t *)^2 + (a_0 * - a_t *)^2 + (b_0 * - b_t *)^2}$, where L, a and b represent the color coordinates, lightness of the color, its position between red/magenta and green and its position between yellow and blue respectively, at the start of the storage (t_0) and the specified period of storage time (t_t).

2.4.4 Respiration rate

Respiration rate of broccoli florets was determined by measuring the accumulation of CO_2 and the depletion of O_2. The florets (ca. 30 g) were kept in an air tight glass container of 17 0mL (the void volume was filled with small plastic spheres) stored at room temperature for 1h and the concentrations of CO_2 and O_2 in the container atmosphere were measured using a gas analyzer (CheckMate 9900, Cambridge, ON, Canada). Respiration rate was monitored at regular intervals (0, 7, 14 and 21 days) during storage, and all measurements were performed in triplicates (three glass containers per treatment).

2.4.5 Chemical assays

2.4.5.1 Total phenolic content

Determination of the total phenolic content in the samples was carried out using Folin-Ciocalteau method (Ainsworth and Gillespie, 2007). Samples were prepared in a similar way as samples for color analysis and respiration rate experiments. Flower buds were cut from florets using sharp blades and buds were immediately submerged in liquid nitrogen to avoid tissue degradation and glucosinolate conversion. Samples were ground using liquid nitrogen and subsequently lyophilized. Extraction was carried out from lyophilized broccoli samples of (ca.0.01g) previously frozen at -80 °C, and made with 1mL of deionized water at 70 ºC in a sonication bath for 5 minutes followed by 1 minute of vortexing. The suspension was centrifuged at 11,270 g at 4ºC for 1 min. The pellets were re-extracted using deionized water and sonication for 5 min and the supernatants of each extraction were pooled together. The solution (0.1 mL) was transferred to an assay tube and mixed with 0.5 mL of deionized water, 20 µL of Folin–Ciocalteau reagent, and 400 µL of 700 mM sodium carbonate (Na_2CO_3). After incubation at room temperature for 30 min, 200 µL of sample, standard (Gallic acid) or blank (water) from the assay tube were transferred to a clear 96-well micro plate, and the absorbance was read at 765 nm on a spectrophotometer (Benchmark Plus, Bio-Rad, Philadelphia, USA). Using a 5 data points standard curve (0 – 250 mg/L) the level of total phenolic content in broccoli was determined in triplicates, and the phenolic content was expressed as mg equivalent gallic acid/ g dry weight (DW) of the sample.

2.4.5.2 Total flavonoid content

Total flavonoid content in the sample was determined by aluminum chloride-colorimetric method (Lin and Tang, 2007). The extract used in this experiment was the same for total phenolics determination. The solution (0.1 mL) in an assay tube was mixed with 300 µL of ethanol, 20 µL of 10 % aluminum chloride ($AlCl_3$), 20 µL of 1 M potassium acetate and 600 µL of water. After incubation at room temperature for 30 min, 200 µL of sample, standard (quercetin) or blank (water) were transferred to a clear 96-well micro plate, and the absorbance was read at 415 nm on a spectrophotometer (Benchmark Plus, Bio-Rad, Philadelphia, USA). Using a 5 data point standard curve (0-100 mg/L quercetin) the level of total flavonoid content in broccoli was determined in triplicate; and value was expressed as mg equivalent quercetin/ g dry weight (DW) of the sample.

2.4.5.3 Assay of reduced and total ascorbic acid

The reduced and total ascorbic acid contents were determined using a previously reported method (Gillespie and Ainsworth, 2007). Extractions were carried from lyophilized broccoli samples (ca. 0.01g) with 1 mL of 6 % (w/v) thrichloroacetic acid (TCA). The suspension was centrifuged at 11,270 g at 4 ºC for 5 min. The pellets were re-extracted using 1 mL of TCA, and the supernatants of each extraction were pooled together. For total ascorbic acid (AA) content, 200 µL of the samples were incubated at room temperature for 10 min with 100 µL of 75 mM phosphate buffer (pH 7) and 100 µL of 10 mM dithiothreitol (DTT). The excess DTT was removed using 100 µL of 0.5 % (w/v) N-ethylmaleimide (NEM) and incubating at room temperature for at least 30 sec.

Reduced ascorbic acid content was determined on 200 µL of the extract mixed with 100 µL of 75 mM phosphate buffer (pH 7.0) and 200 µL of deionized water. To the solution, 500 µL of 10 % (w/v) TCA, 400 µL of 43 % (v/v) of phosphoric acid (H_3PO_4), 400 µL of 4 % (w/v) α-α'- bipyridine and 200 µL of 3 % (w/v) $AlCl_3$ were added in an assay tube. The assay tubes were incubated at 37 °C for 1h, and 200 µL of the samples or standards (AA) or blank (6 % (w/v) TCA were transferred to a clear 96-well micro plate, and the absorbance was read at 525 nm.

Using a 5 data points standard curve (0-5 mM), the levels of reduced and total ascorbic acid were determined in triplicate, and the values were expressed in mg equivalent of ascorbic acid/g dry weight (DW) of the sample.

2.4.5.4 Total antioxidant capacity

ORAC (Oxygen Radical Absorbance Capacity) assay was performed following a previously reported method by Gillespie et al. (2007). Extractions were carried out from lyophilized broccoli samples (ca. 0.02 g) with 1.5 mL of 50 % (v/v) acetone. The suspension was centrifuged at 1,585 g at 4 ºC for 30 min. The pellets were re-extracted using 1.5 mL of 50 % acetone and supernatants of each extraction were pooled together, and the extract was diluted 80 times. Along with 150 µL of 0.08 µM fluorescein, an aliquot of 25 µL of the diluted extract or 25 µL of 75 mM phosphate buffer (pH 7.0) as a blank or 25 µL of 20 mM Trolox as standard was added to each well of a clear micro plate. The solution of 25 µL of 2,2'-Azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) was added to the samples, blanks and standards and ORAC measurements were performed at 37 °C on a micro plate reader (FluoStar Galaxy DMG, Vienna, VA, USA) with excitation wavelength of 485 nm and emission wavelength of 530 nm. The ORAC values were calculated using the regression

equation relating Trolox concentration and the net area under the curve (AUC) [Net AUC = AUC (extract) – AUC (blank)]. Using a five points standard curve (0-50 µM Trolox) the total antioxidant capacity was determined in triplicates, and the values were expressed as mg Trolox equivalent/ g dry weight (DW) of sample.

2.4.6 Glucosinolates and hydroxycinnamic acid analysis

Separation of glucosinolates (GLS) and hydroxycinnamic acids (HCA) was carried out following a developed method by Nadeau et al. (2012). Broccoli inflorescence samples in triplicate were drawn from stored florets at 0, 3, 7 and 14 days after the treatment, frozen in liquid nitrogen, followed by lyophilization. The dried samples were pulverized, packed in plastic laminate pouches under vacuum, and stored at -30 ºC until extraction. GLS and HCA were extracted from the dried sample (0.5 g) containing 800 µg of sinigrin (internal standard) with 70 % aqueous methanol at 70 ºC in a sonication bath for 10 min. The suspension was centrifuged at 4,528 g at 4ºC. The pellets were re-extracted twice using 70 % methanol solution and sonication for 10 min, and the supernatants of each extraction were pooled together. The extract was concentrated to dryness by evaporation under vacuum using a rotavapor (Büchi R215, Flawil, Switzerland), and was recovered in the mobile phase (ammonium acetate 10 mM / formic acid pH 4.4). Compounds were separated on HPLC equipped with MS detector (HP series 1100 LC/MSD quadruple) using Phenomenex Synergi Hydro-RP column (250 mm x 2mm, 80Å) at 30ºC. The analytes were eluted using a gradient mobile phase (A: Ammonium acetate 10 mM / formic acid pH 4.4 - B: Acetonitrile). Separation of GLS and HCA was achieved using retention times (Table 2.1) and molecular weights (Figure 2.2). Identification of compounds was achieved by electrospray ionization MS on negative ion mode. Quantification of the GLS was carried out with UV detection at 224 nm and HCA at 330nm using a diode array detector.

2.4.7 Free Amino Acid Profile

The broccoli samples were drawn 24 h after UV-C treatment, and pulverized in liquid nitrogen using pestle and mortar. Free amino acids were extracted from broccoli florets according to the method of Lee et al. (2005). The frozen powder (200 mg) was mixed with 5 mL of distilled water under vortex for 3 min. The suspension was then centrifuged at 6,500 g at 4 °C for 10 min, and the supernatant collected. The pellet was re-extracted twice, each time with 2.5 mL of distilled water, and

the extracts were pooled together. An amino acid analysis kit (EZ: faast ® Kit Phenomenex, Torrence, CA, USA) was used for separation and identification of the amino acids: 200 µL of broccoli extract and 200 µL of internal standard (norvaline at a concentration of 200 µmol/L) were placed in glass vials and the instructions on the kit were followed. A Hewlett-Packard (HP) 6890 Network GC system (Wilmington, DE, USA) coupled to a quadrupole HP 5973 mass spectrometer (MS) was used to confirm the identification of the free amino acids present in the standard as well as in the samples. Helium carrier gas flow rate was 1.8 mL/min. The following oven temperature program was used: holding at 90 °C for 1 min, increasing to 140 °C at 15 °C/min, and subsequently increasing to 320 °C at 35 °C/min, followed by holding at that temperature for 1 min. The temperature of injection port was 250 °C. The MS temperatures were set at 250 °C for the ion source, 180 °C for the quadrupole, and 310°C for the auxiliary. The scan range was set to 30-500 (3.15 scans s^{-1}). The injection volume was 2 µL, and injections were performed in split mode (15:1, v/v).

2.4.8 Gene expression

RNA was extracted from lyophilized broccoli inflorescences samples (0.02 g) at 0, 48 and 96 h. using RNeasy plant minikit (Qiagen, Germany). The clean-up of RNA was done with deoxyribonuclease (DNase) (TURBO DNA-free Kit, Ambion, USA). The integrity of RNA was verified by running 1µg of RNA sample on to a 1 % denaturing agarose gel (formaldehyde 37 %; formamide; and 10X MOPS (pH 7)). Subsequently cDNA synthesis was carried out using 3µg of the DNase treated RNA; oligo dT; and SuperScript Reverse Transcriptase II (Invitrogen, Carlsbad, CA, USA) following the kit instructions. Polymerase chain reaction (PCR) was prepared using a commercial kit (*Taq* PCR) (New England Biolabs, Ipswich, MA, USA) with 0.25 mM forward and reverse primers shown in Table 2.2, 1 unit of polymerase and 3,000 ng of cDNA in a total volume of 20 mL. PCR program was set in a thermo-cycler (TPersonal, Biometra, Goettingen, Germany) to perform an initial denaturation for 3 min at 95 °C and n cycles (depending on primer used): [30 s 95 °C; 45 s (temperature depending on primers); and 1 min-68 °C] and a final extension step of 5 min at 68 °C. PCR products were run on 1 % agarose gel at 160V/60 min using 1x Borax buffer (pH 8.0), normalized with actin and analyzed by densitometry with ImageJ (Wayne Rasband, NIH, USA).

2.4.9 Statistical analysis

Color analysis was performed on three replicates per treatment (punnet containing nine florets). Three different treatments were used: non exposed, exposed to a hormetic dose and the third to a high dose. Sample periods corresponded to 0, 3, 7, 14 and 21 days. Respiration and weight loss were carried out in a similar manner. For biochemical and secondary metabolite quantification, three samples were used per treatment (flower buds in dry powder form from nine different florets). Sample days corresponded to 0, 3, 7 and 14 days for secondary metabolites as well as for biochemical measurements. No time*treatment interaction was considered for time average values. Data analysis was carried out based on a complete randomized design by one-way analysis of variance (one-way ANOVA) using a significant level of 0.05. Least significant difference test at the same significant level was done when the analysis of variance found significant differences. The statistical analysis was executed using the statistical analysis system version 9.3 (SAS Institute Inc. 2011. Base SAS® 9.3 Procedures Guide. Cary, NC, USA). PCA analysis was performed with the statistical package unscrambler version 10.0 (CAMO, Trondheim, Norway). The time average value for total phenols, flavonoids, total ascorbic acid, and ORAC assay was calculated from days 0, 7, 14 and 21, and the result was used to compare UV-C exposed florets with non-treated broccoli.

2.5 Results and discussion

2.5.1 Determination of hormetic dose of UV-C

The color of broccoli and other green vegetables is conferred to, mainly, chlorophyll pigments in tissue, and can be expressed in a set of variables, such as brightness, green-red and blue-yellow values that correspond with the color perception of humans (Tijskens et al., 2001b). This set of variables is known as CIE-Lab system and can be effectively correlated with sensory evaluation when significant color changes occurred such as the yellowing of the florets which terminates the marketable life of broccoli (Gnanasekharan et al., 1992; Makhlouf et al., 1990). UV-C treatment affected the color of broccoli florets in a dose-dependent manner throughout the storage period of 27 days at 4 °C. The effect of UV-C on color development was biphasic, where the retention of green color of the control florets and those treated with either low doses of UV-C (≤0.6 $kJ.m^{-2}$) or elevated doses (≥3.0 $kJ.m^{-2}$) was significantly lower compared with the dose range of 0.9-1.5 $kJ.m^{-2}$. The best level of color retention was observed with the UV-C dose of 1.2 $kJ.m^{-2}$. (Figure 2.3). Florets treated

with 1.2 kJ.m^{-2} showed the least total color change (ΔE) value of 5.47. Doses of 0.9 and 1.5 kJ.m^{-2} were not significantly different on broccoli color retention with ΔE values of 5.97 and 6.45, respectively (Figure 2.3). From these results, 1.2 kJ.m^{-2} was considered as the hormetic dose of UV-C for broccoli florets from the point of color retention. Furthermore, by comparing florets treated with 1.2 kJ.m^{-2} and florets without any UV-C treatment, it was determined that the storage time can be prolonged by at least 7 days at 4 °C, considering an acceptable ΔE value of 5 (Figure 2.4). For further investigations on the effect of UV-C on broccoli, the doses of 1.2 kJ.m^{-2} and 3.0 kJ.m^{-2} were used and designated as hormetic and high dose, respectively.

UV-C doses ranging from 0.125 to 9 kJ.m^{-2} have been reported as hormetic for a variety of fruits and vegetables (Charles and Arul, 2007), the lowest being for table grapes (Nigro et al., 1998), and the highest for apples (Manzocco et al., 2011). A preliminary work of Baka (1997) attributed a hormetic dose of 0.9 kJ.m^{-2} for green color retention of broccoli florets, which is somewhat in agreement with this study. However, a higher UV-C dose of 10.0 kJ.m^{-2} has been reported to be beneficial for chlorophyll retention in broccoli florets (Costa et al., 2006), where they examined doses greater than 4.0 kJ.m^{-2}. Martínez-Hernández et al. (2011) observed a bimodal chlorophyll retention response to UV-C doses in the range of 1.5–15 kJ.m^{-2}; where a high retention of chlorophyll occurred at 1.5 kJ.m^{-2} followed by a greater retention at a higher UV-C doses between 9.0 and 15.0 kJ.m^{-2}. However, these doses also contributed to other quality deteriorations such as off-odor, leading to shorter shelf life of fresh-cut broccoli, whereas the quality retention was superior with 1.5 kJ.m^{-2} at the end of storage of 19 days at 5 °C, in contrast to an apparent storability of 27 days at 4 °C with a dose of 1.2 kJ.m^{-2} observed in this study. Although from the standpoint of chlorophyll retention and other quality attributes the beneficial dose was 1.5 kJ.m^{-2} among the doses tested by these authors (Martínez-Hernández et al. (2011), they suggested a dose of 4.5 kJ.m^{-2} as being useful for improved microbial quality and health-promoting bioactive compounds. Thus the beneficial dose for color as well as quality retention appears to be in the low dose range, as observed in this study. Nevertheless, it raises the question with respect to the protective effect of high UV-C doses, such as 10 kJ.m^{-2} in the color retention of broccoli. It is presumable that chlorophyll may accumulate in response to high stresses and act as antioxidants (Lanfer-Marquez et al., 2005).

It also raises the question whether color retention and/or chlorophyll content alone could serve as an indicator of senescence of broccoli florets. Other indicators such as membrane integrity and lipid oxidation, tissue softening and loss of proteins should also be monitored to follow the real

progress of senescence. Zhuang et al. (1995) observed the interrelationship between lipid peroxidation, chlorophyll and protein contents in cold stored broccoli.

The preservation of chlorophyll by UV-C treatment may be attributed to reduced activities of the enzymes involved in chlorophyll catabolism such as chlorophyllase, chlorophyll peroxidase and magnesium-dechelatase. A significant decrease in chlorophyllase activity was observed in broccoli treated with a high dose of UV-C (10 $kJ.m^{-2}$) and stored at 20 °C (Costa et al., 2006). Although detailed assessment of senescence in the UV-C treated broccoli was not the scope of this study, literature data suggest that hormetic dose of UV-C appears to delay various aspects of senescence in tomato, including color development and chlorophyll degradation (Maharaj et al., 2010), membrane leakage and lipid oxidation (Ait-Barka et al., 2000a), protein loss (Charles et al., 2009) and tissue softening (Ait-Barka et al., 2000b).

2.5.2 Physiological and Biochemical Characteristics

2.5.2.1 Respiration rate

Generally, there is an inverse relationship between respiration rate of a produce and its storability. Yet external factors such as physical stresses can stimulate the respiration rate of fresh vegetables (Kader and Saltveit, 2002). The exposure of broccoli florets to UV-C presumably, caused stress in broccoli as seen by elevated respiration rate (CO_2 production) following its exposure to UV-C dose of 1.2 or 3.0 $kJ.m^{-2}$ on day 0 (Figure 2.5). The increase in respiration rate was significantly different ($p<0.05$) between UV-C treatment and the control: 33.5, 75.8 and 72.9 mL $CO_2.kg^{-1}h^{-1}$ for the control, 1.2 and 3.0 $kJ.m^{-2}$, respectively. After 7 days of storage, CO_2 production reached a steady level for all treatments with CO_2 production rates of 12.8, 16.7 and 15.6 mL $CO_2.kg^{-1}h^{-1}$, for control, hormetic and high dose respectively. There was no change in the respiration rate thereafter until the end of the storage period of 21 days; although the lot treated with the hormetic dose of 1.2 kJm^{-2} showed 2 mL $CO_2.kg^{-1}h^{-1}$ lower production (14 %) at the end of the storage compared with the lot treated with 3.0 $kJ.m^{-2}$.

The increase in the respiration due to a stress may be related with an overall cell acclimation process which demands a higher energy supply compared with a non-stress situation. It is known that oxidative stresses increase respiration rate and generate reactive oxygen species (ROS) as well as ATP depletion leading to programmed cell death (Tiwari et al., 2002). The generation of ROS was

indicated by the depletion of total ascorbic acid content as well as the reduction in ORAC level (Table 2.3).

The initial high respiration rate of UV-C treated broccoli at 4 °C observed is consistent with initial high respiration rate of tomato fruit treated with a hormetic UV-C dose of 3.7 $kJ.m^{-2}$ at 16 °C (Maharaj et al., 1999), but it is not consistent with the report of Costa et al. (2006) on the initial respiration rate of UV-C treated broccoli at a high dose of 10.0 kJm^{-2} (that retained color as mentioned above) at 20 °C. The latter reported that the initial respiration rate of UV-C treated broccoli was lower, though not significant, than that of the untreated broccoli. The lower initial respiration of UV-C treated with such a high dose could possibly a result of mitochondrial dysfunction, a dose that also caused off-flavor, although the color was retained. Furthermore, Yang et al. (2014) reported identical initial respiration rate for both UV-C treated peaches with a dose of 3.0 kJm^{-2} and the untreated fruits, although there was significant increases in the activities of superoxide dismutase and ascorbate peroxidase in the treated fruit, indicative of ROS generation in the treated fruits. It is difficult to reconcile with this observation, unless the authors had assumed it to be the same for both. The rise in the respiratory activity following exposure to stresses reflects the high energy requirement of acclimation process leading to the synthesis of compounds that possess protective and defense functions, albeit at the loss of substrate reserves.

2.5.3.2 Phenylpropanoid compounds, ascorbic acid and ORAC

Flavonoids, hydroxycinnamic acids and sinaptate esters absorb UV-B light and act as free radical scavengers (Ueda and Nakamura, 2011), and UV-C light also induce these compounds (Crupi et al., 2013; González-Aguilar et al., 2007). The content of total phenols on broccoli florets exposed to either 1.2 or 3.0 kJm^{-2} of UV-C light was higher than the control throughout the storage. However, the amount of total flavonoids in broccoli florets was not significantly affected by the UV-C treatments compared with the control (Table 2.3). The exposure of broccoli florets to UV-C light caused a reduction in the ascorbic content and antioxidant capacity of broccoli. The total ascorbic acid decreased by 5.7 and 11.9 % during the storage after the exposure to 1.2 and 3.0 kJm^{-2} of UV-C light, respectively, compared with the control, where the reduction was significant ($p<0.05$) at the high dose of 3.0 $kJ.m^{-2}$ (Table 2.3). Oxygen radical absorption capacity (ORAC) also declined after the exposure to UV-C light. The hormetic dose decreased ORAC capacity by 8 %, whereas the decrease was of 9.3 % with the high UV-C dose compared with the non-treated broccoli (Table 2.3).

The decrease in ORAC was also accompanied by a reduction in ascorbic acid content with increase in UV-C dose, whereas the content of total phenols increased with UV-C dose (Table 2.3). Despite the discrete accumulation of phenylpropanoid compounds, the decrease on total ascorbic acid and ORAC capacity, suggest the intensification of reactive oxygen species (ROS) production in treated broccoli. The major cellular redox buffers in plant cells are ascorbate and glutathione as well as tocopherols, carotenoids, alkaloids and flavonoids; and a high ratio of reduced to oxidized ascorbate appears to be essential for ROS scavenging (Apel and Hirt, 2004). We observed a reduction in the average ratio of reduced to oxidized ascorbate during storage from of 2.2 for non-exposed florets to 2.0 for the hormetic dose and to 1.9 for the high dose (Table 2.3). On the same basis, the total phenols increased by 13.6 % and 25.1 % with hormetic and high doses, and flavonoids increased by 12.1 % and 14.6 %, respectively.

The lower ORAC values of UV-C treated broccoli indicate that the redox status of the treated tissue is oxidized due, in part, to the depletion of antioxidants. The overall reduction in ascorbic acid supports that observation, in part, while total phenols and flavonoids increased during storage. Maharaj et al. (2014) observed significant decreases in ascorbic acid, α-tocopherol, and glutathione, the principal antioxidants, in UV-C treated tomato fruit, while also observing increases in phenols. The latter metabolites possess multiple activities as antioxidants and antimicrobials as well as UV light absorbers or blockers (Stewart and Stewart, 2008), and they generally accumulate later during storage. Following exposure to UV-C and the establishment of an oxidized-state in the tissue, ethylene stress becomes apparent (Severo et al., 2015; Tiecher et al., 2013) along with a high respiration rate, resulting in the depletion of primary antioxidants. An inverse relationship between the respiration rate of broccoli and its content in ascorbic acid was highlighted by Techavuthiporn et al. (2008). In addition, ethylene appears to be involved in the control of the contents of antioxidants such as ascorbic acid in senescing leaves (Gergoff et al., 2010)

2.5.4 Free amino acid assay

Twelve free amino acids were present in broccoli samples, namely, L-serine (Ser), L-Aspartic acid (Asp), L-glutamic acid (Glu), L-valine (Val), L-isoleucine (Ile), L-leucine (Leu), L-methionine (Met), L-Lysine (Lys), L-tyrosine (Tyr), L-Glutamine (Gln) and L-tryptophan (Thr). Serine, aspartic and glutamic acid had the highest titers among the amino acids (>100 µmol g^{-1} FW) after 24 hours of storage at 4 °C following the treatment (Figure 2.6). The levels of branched chain amino acids

(BCAAs) were registered between 30 and 65 μmol g^{-1}; and among them, valine was found at a higher level, followed by isoleucine and leucine. The lowest levels were found for the glucosinolate precursor amino acids, phenylalanine, methionine and tryptophan, ranging from 23 to 1.6 μmol g^{-1}. The UV-C dose-response relationships (Figure 2.6) showed that all amino acids, with the exception of pyruvate-derived branched-chain amino acids (leucine, isoleucine and valine), exhibited a similar pattern. The levels of serine, aspartate, glutamate and the phosphoenolpyruvate-shikimate-derived aromatic amino acids (tryptophan, and phenylalanine), as well as those derived from aspartate (methionine) decreased with increasing UV-C dose, and their levels remained steady above the dose of 1.2 $kJ.m^{-2}$. On the other hand, the levels of BCAAs recorded a minimum at 1.2 $kJ.m^{-2}$, but increased above that dose.

Pools of amino acids were generally depleted by high UV-C dose, presumably they are expended for the biosynthesis of metabolites deriving from amino acids. However, the BCAAs increased with the high dose of UV-C (Figure 2.6). The increase in the titers of BCAAs above the hormetic dose may relate to stress, and they may be markers of stress caused by high doses of UV-C. It has been hypothesized that elevated free amino acids can act as osmolytes that protect plants from dehydration (Joshi and Jander, 2009). Osmolytes are synthetized in plants as a general conserved response to abiotic stresses, and proline is the most common osmolyte and reliable biochemical marker of salt stress (Boscaiu et al., 2013). Yet, plants under salt stress accumulate other organic osmolytes such as betaine, glucose, fructose, sucrose, fructans and BCAAs: valine and isoleucine (Burg and Ferraris, 2008).

In this experiment it was clear that water loss occurred during the storage of broccoli. The most affected florets were those treated with 3.0 $kJ.m^{-2}$, followed by non-treated florets and lastly, those treated with the hormetic dose of 1.2 kJm^{-2} (Figure 2.7). BCAAs participate in the generation of aroma volatiles in fruits (Gonda et al., 2010), and under stress conditions they can generate defense related compounds such as cyanogenic glycosides which are effective compounds against pathogens (Kaplan et al., 2004). BCAAs can also provide electrons to the electron transfer flavoprotein (ETF) complex or feed the TCA cycle by their catabolic products to generate ATP under stress conditions (Obata and Fernie, 2012). Thus, the difference on weight loss observed on the broccoli exposed to different doses could be due to the activation of different metabolic pathways by UV-C. On one hand, the hormetic dose enhanced levels of osmoprotectants, and on the other hand,

high dose could also have enhanced the production of stress volatiles resulting in moderate weight loss, in addition to the initial high respiration rate.

The function of BCAAs is not only related with osmotic stresses, volatile generation or as energy backup for respiration. It has been also reported that a significant correlation exists between senescence development and leucine accumulation in *Arabidopsis thalina* (Diaz et al., 2005). A principal component analysis (PCA) was performed with all analyzed amino acids correlated with Hunter LAB parameters L*, a*, b* and H°. Correlation loadings between color and amino acid levels in PCA analysis showed that leucine content was related to yellowing of broccoli, and it appears to be a marker for yellowing in broccoli florets (Figure 2.8).

2.5.5 Glucosinolates and Hydroxycinnamic acid analysis

Phenylpropanoids, and glucosinolates in particular, are important compounds in *Brassicaceae* for defense against biotic and abiotic stresses (Agati et al., 2012; Textor and Gershenzon, 2009). Defense responses are initiated *via* the oxidative stress generated by ROS in plant cells (Apel and Hirt, 2004). In the present study, the production of ROS was indirectly confirmed by the depletion of ascorbic acid and ORAC as well as the depletion of amino acids other than branched-chain amino acids at and above the hormetic dose would seem to relate to either increased synthesis of secondary metabolites that are constitutively expressed or induced in response to UV-C stress.

Analysis of gene expression included three genes related in the glucosinolate pathway, phenylalanine N-hydroxyase (CYP79A2), tryptophan N-hydroxyase (CYP79B3) and dihomo-methionine N-hydroxyase (CYP79F1). Relative gene expression of enzymes coding for glucosinolate biosynthesis in florets was affected. This was evident immediately after the exposure of florets with UV-C on day 0 (Figure 2.9). Genes coding for CYP79A2 and CYP79B3 were mainly enhanced by the high dose of 3.0 kJm^{-2}, increasing by 2.6 and 3.7 folds, respectively, compared with the control. Overexpression of 3.1 folds on CYP79B3 was also observed on florets exposed to the hormetic dose of UV-C light (Figure 2.9). A similar pattern was observed on the following days after exposure, where significant overexpression of CYP79B3 were measured on days 2 and 4 (Figure 2.9). Changes in the expression of CYP79B3 were concomitant with the quantification of glucosinolates (Table 2.4). The titers of the aliphatic-glucosinolate, glucoraphanin in the treated broccoli florets remained modestly higher with both doses of UV-C compared with the untreated florets, although significant

differences were found at the beginning of the storage (Figure 2.10). The amount of total glucobrassicin content of florets was significantly (p<0.05) increased by both UV-C doses and the titers remained constant throughout the storage period compared with the control; where in the latter, a depletion of 13 % was observed after the storage of 14 days (Figure 2.11). Likewise, the titers of 4-hydroxyglucobrassicin in broccoli treated with the hormetic and high doses were higher by the end of the storage after 14 days compared with the control; but in this case, the concentration of 4-hydroxyglucobrassicin also increased in non-exposed florets during the storage time (Figure 2.12).

Gene expression was also determined for genes of the phenylpropanoid pathway encoding for coumarate ligase (CoL), chalcone synthase (CHS), flavonoid monoxygenase (F3H1) and phenylalanine ammonia-lyase (PAL). Both UV-C treatments induced an overexpression of these genes, which was maintained during the 4 days of storage (Figure 2.9). The expression of CHS and CoL genes were the most prominent genes by both UV-C treatments, where the overexpression was in the order of 2.9 - 4.2 folds (Figure 2.9). Besides these two genes, F3H1, a phenylpropanoid-related gene was also overexpressed in response to UV-C treatment, to the order of 3 folds, followed by PAL (Figure 2.9). The overexpression of these genes in florets due to the UV-C treatment correlated to the amount of total phenols previously described. Hydroxycinnamic acids (HCA) were also affected by UV-C light. Among the HCAs, 1-sinapoyl-2-feruloyl gentiobiose, was found at the highest level of 5.7 mg eq SIN/g DW, followed by 1,2 disinapoyl gentiobiose: 3.2 mg eq SIN/g DW; 1,2-disinapoyl-2-feruloyl gentiobiose: 3.2 mg eq SIN/g DW; 1,2,2'–trisinapoyl gentiobiose: 2.4 mg eq SIN/g DW; 1,2-diferuloyl gentiobiose: 1.4 mg eq SIN/g DW and sinapolyl-diferuloyl gentibionse: 0.7 mg eq SIN/g (DW) (Table 2.5). The total hydroxycinnamic acids content in florets was enhanced by UV-C light at both doses, increasing by 10 % and 13 % with the hormetic and high dose, respectively, compared with the control (Figure 2.13).

UV-C induced disease resistance in tomato has been shown to be a multicomponent and time-dependent system (Charles et al., 2011). In this system, the accumulation of phytoalexins account for the early defense response, followed by the formation of cell wall stacked zone, reinforced with phenols, and *de novo* synthesis of PR proteins and the enhancement of constitutive PR proteins (Charles et al., 2011). The *Brassicacaceae* utilize glucosinolate-myrosinase defense system distributed throughout the organs of the plant, where glucosinolates accumulate and their conversion to isothyocianates is the first response to biotic stresses (Textor and Gershenzon, 2009). This response appears to be mediated by jasmonic acid that activates the transcription factors and

expression of the genes involved in the synthesis of indole glucosinolates, such as CYP79B2 and CYP79B3 (Halkier and Gershenzon, 2006). Synthesis of indole glucosinolates has been also observed in *Brassicas* exposed to UV-B. Recently, exposure of broccoli sprouts to UV-B light (0.3 $kJ.m^{-2}d^{-1}$), induced the accumulation of glucosinolates and increased the expression of genes related to salicylic and jasmonic acid signal pathways (Mewis et al., 2012). Although, Stapleton (1992) pointed out that plants may respond differently to UV in the B or C ranges, the expression of the gene *OsOPR1*, encoding one of the final reactions for the synthesis of jasmonic acid, was observed in rice after 60 min of exposition to UV-C light at 254 nm (Agrawal et al., 2003).

The accumulation of glucosinolates, especially those derived from glucobrassicin may relate to jasmonate signaling, and the response might be similar to herbivore attack, where ROS is also a component in signal transduction (Textor and Gershenzon, 2009). Moreover, accumulation of glucosinolates has also been observed in *Brassicas* infected with fungi. For instance, 4-hydroxyglucobrassicin accumulated in *B. rapa* upon challenge with *F. oxysporum*; and its accumulation was significantly higher in response to *L.maculans* inoculation, where the accumulation of glucobrassicanapin an aliphatic glucosinolate was higher with *L.maculans* (Abdel-Farid et al., 2010). Our results show that UV-C light may be less specific on the induction of both indole and aliphatic glucosinolates, since both classes were enhanced. However, it was clear that the enhancement of indole-type glucosinolates was more important than aliphatic GLS. In addition, the concentration of indole-types seems to be dose-dependent, where the high dose of UV-C appears to enhance the accumulation of this class of compounds. Further investigation is needed to verify this hypothesis, since this assumption is based on only two UV-C doses.

Glucosinolates are not antimicrobial compounds *per se*, to be active they are hydrolyzed by thioglucosidase enzymes known as myrosinases, to form isothyocianates along with other molecules such as nitriles, epithionitriles and thyocianates (Textor and Gershenzon, 2009). In broccoli, sulforaphane is derived from glucoraphanin and 3-indolylmethyl-isothiocyanate from glucobrassicin. Isothiocyanates are toxic volatile compounds that possess demonstrated inhibitory activity against fungi, nematodes, bacteria, insects and weeds (Tiznado-Hernández and Troncoso-Rojas, 2006). In our study, the accumulation of indole-glucosinolates reached a maximum at 4 and 7 days for glucoraphanin; and from that point onward, they started to deplete, presumably through conversion to isothiocynates. Since the exposure of vegetable tissue to excessive UV-C doses can increase lipid peroxidation and disruption of cell membranes (Civello et al., 2006). The production of

isothiocyanates in florets can likely be initiated due to the interaction of glucosinolates with myrosinases as a result of membrane disruption caused by UV radiation as well as the senescence process. The membrane disruption process appears to be more severe with the high dose of UV-C as seen by the weight loss observed in florets (Figure 2.7).

2.6 Conclusion

The exposure of broccoli florets to hormetic dose of UV-C delayed yellowing during 21 days compared with either a high dose or the untreated florets. The implication of hormetic dose seems not only relates to the preservation of broccoli, but also to changes in free amino acid pools, which are precursors of secondary metabolites of interest in plant protection and human health. The effect of UV-C on secondary metabolites seems to be mediated by the oxidative stress caused by UV-C, and it also appears to have specific impact on indole glucosinolate branch pathway. In addition, there is some indication that branched chain-amino acids can serve as indicators of stress levels in plant tissue, and that leucine is possibly a marker of yellowing or senescence in broccoli.

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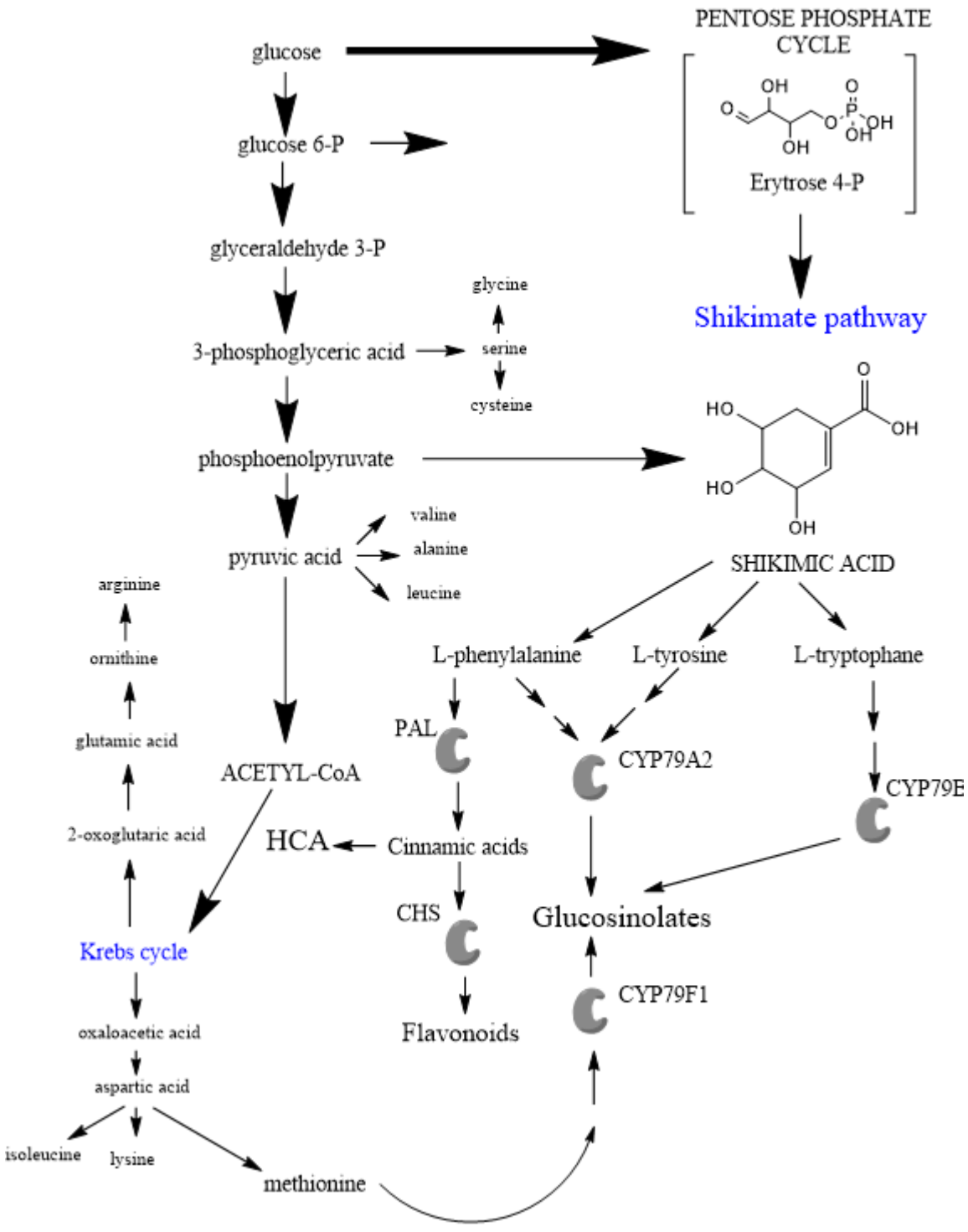

Figure 2.1 Biosynthesis of amino acids derived compounds.

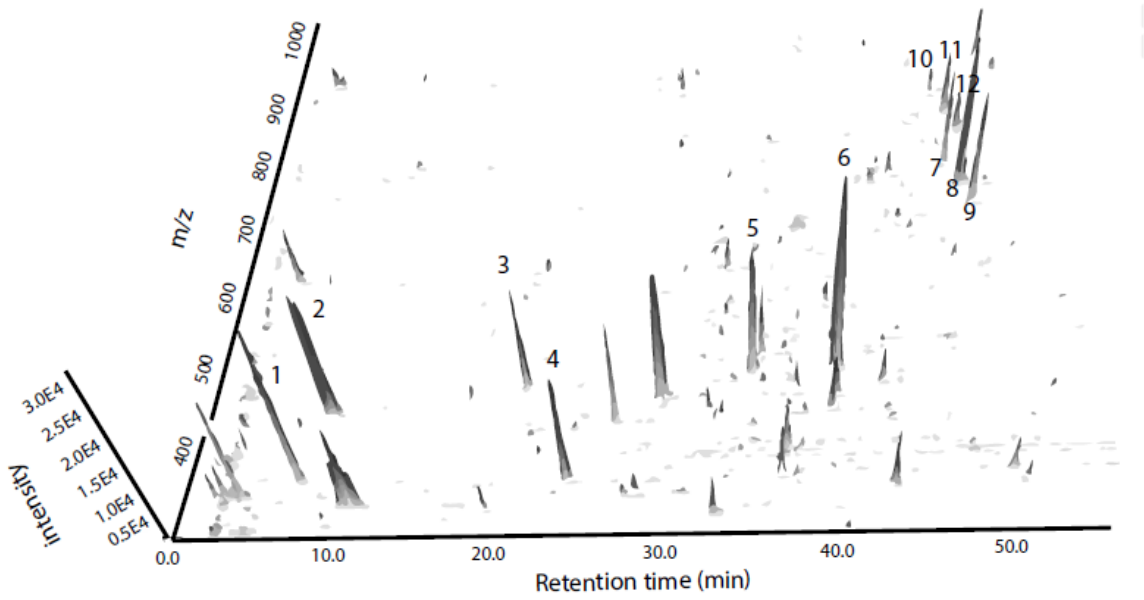

Figure 2.2 Separation profile of glucosinolates (GLS) and hydroxy-cinnamic acids (HCA) in broccoli florets by RP-HPLC and detected by MS. 1=sinigrin (8.31 min, 358.85M); 2=glucoraphanin (9.93 min, 436.91M); 3=4-hydroxyglucobrassicin (23.02 min, 463.91M); 4=chlorogenic acid (31.25 min, 447.93M); 5=glucobrassicin (36.91 min, 477.99M); 6=4-methoxyglucobrassicin (41.81 min, 477.95M); 7=neoglucobrassicin (50.52 min, 754.21M); 8=1,2 disinapoyl gentiobiose (51.26 min; 724.2M); 9=1-sinapoyl-2-feruloyl gentiobiose (51.85 min, 694.21M); 10=1,2-diferuloyl gentiobiose (53.63 min, 960.23M); 11=1,2,2'–trisinapoyl gentiobiose (54.36min, 930.22M); 12=1,2-disinapoyl-2-feruloyl gentiobiose (55.19 min, 900.27M).

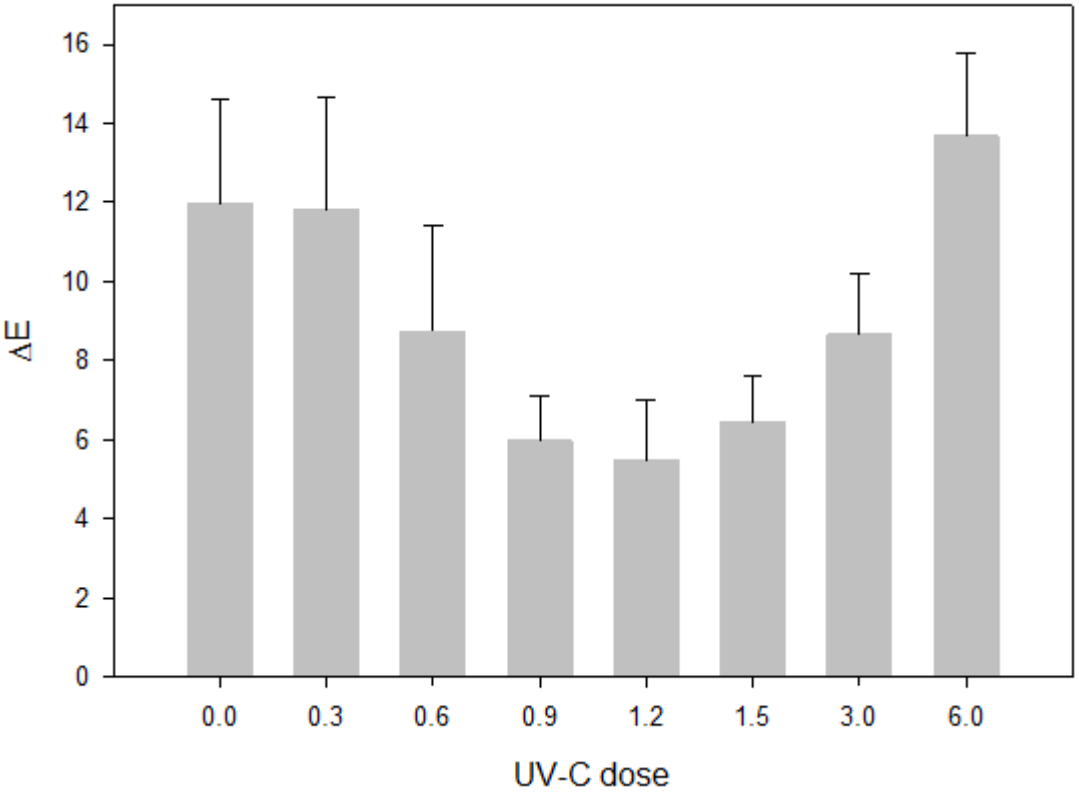

Figure 2.3 Determination of hormetic dose of UV-C for color retention in broccoli florets. Total color change of eight UV-C doses (0 -6.0 kJm^{-2}) was evaluated at the end of the storage of 27 days at 4 °C. Bars are the mean of 9 observations with ±SD.

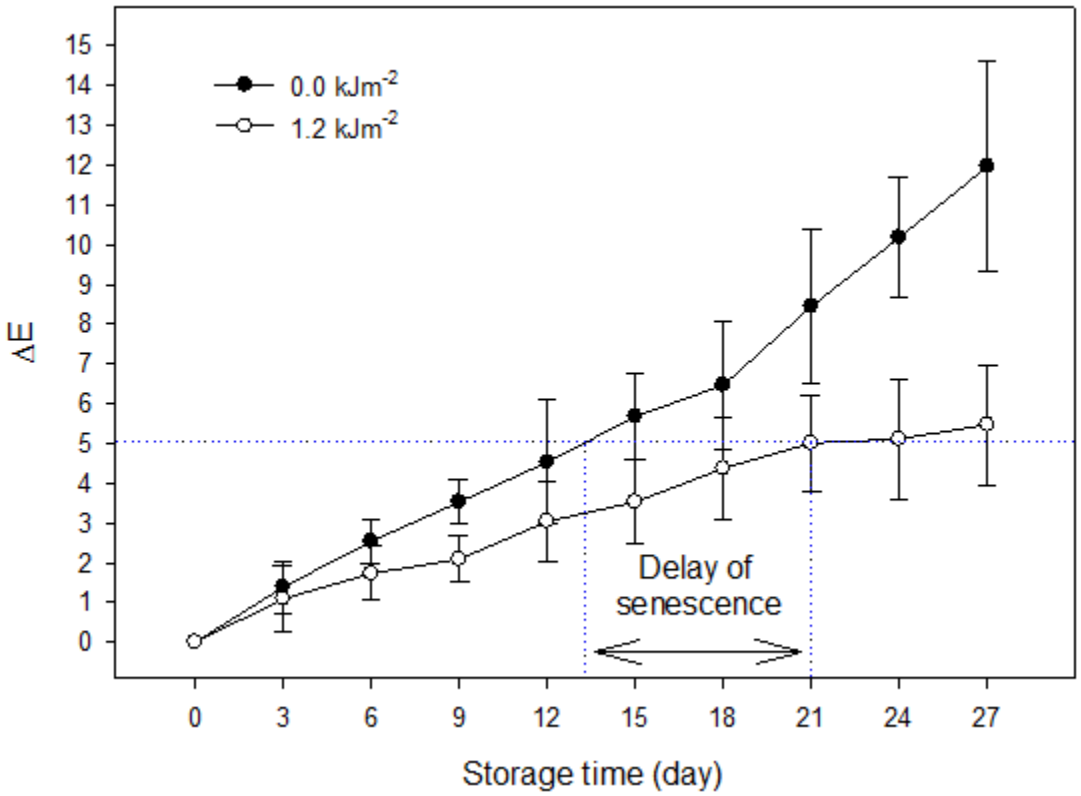

Figure 2.4 Evolution of total color change (ΔE) of broccoli florets treated with UV-C light during storage at 4 °C for 27 days. Total color change of untreated florets compared with treated florets at 1.2 kJ.m^{-2} (hormetic dose) was followed during storage. Yellowing of broccoli florets was delayed by about 8 days with the hormetic dose. Data point is mean of 9 measurements, and the vertical bar represents standard deviation of the mean.

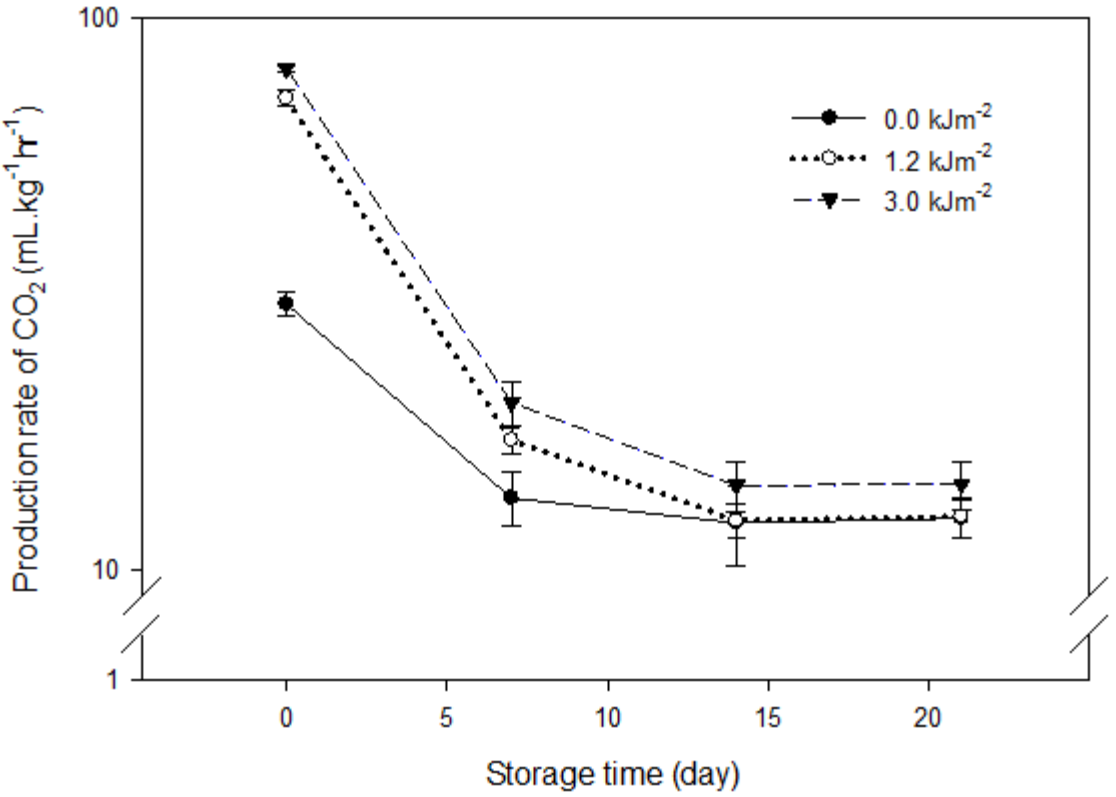

Figure 2.5 Evolution of respiration rate of UV-C treated broccoli florets. Production of carbon dioxide (CO_2) was measured for three UV-C doses: control, (0 kJm^{-2}); hormetic, (1.2 kJm^{-2}); and high, (3.0 kJm^{-2}) dose during 21 days of storage in darkness at 4 °C. Each point is the mean of 3 observations and vertical bars represent standard deviation.

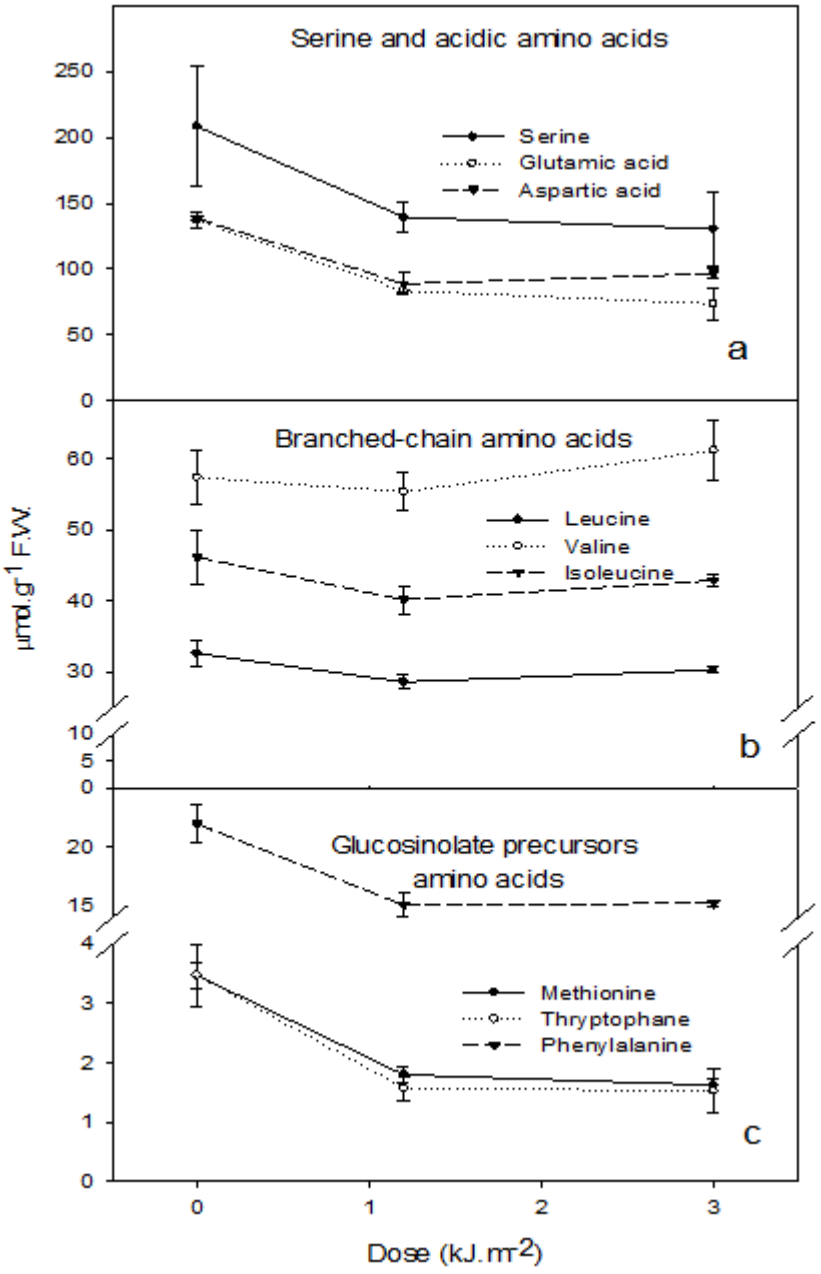

Figure 2.6 Amino acid profile of UV-C treated broccoli florets after 24 hours of storage at 4 °C. Amino acids were classified into three groups: (a) serine and acidic amino acids, (b) branched-chain, and (c) glucosinolate precursor amino acids. Each point represents the mean of three replicates with standard deviation.

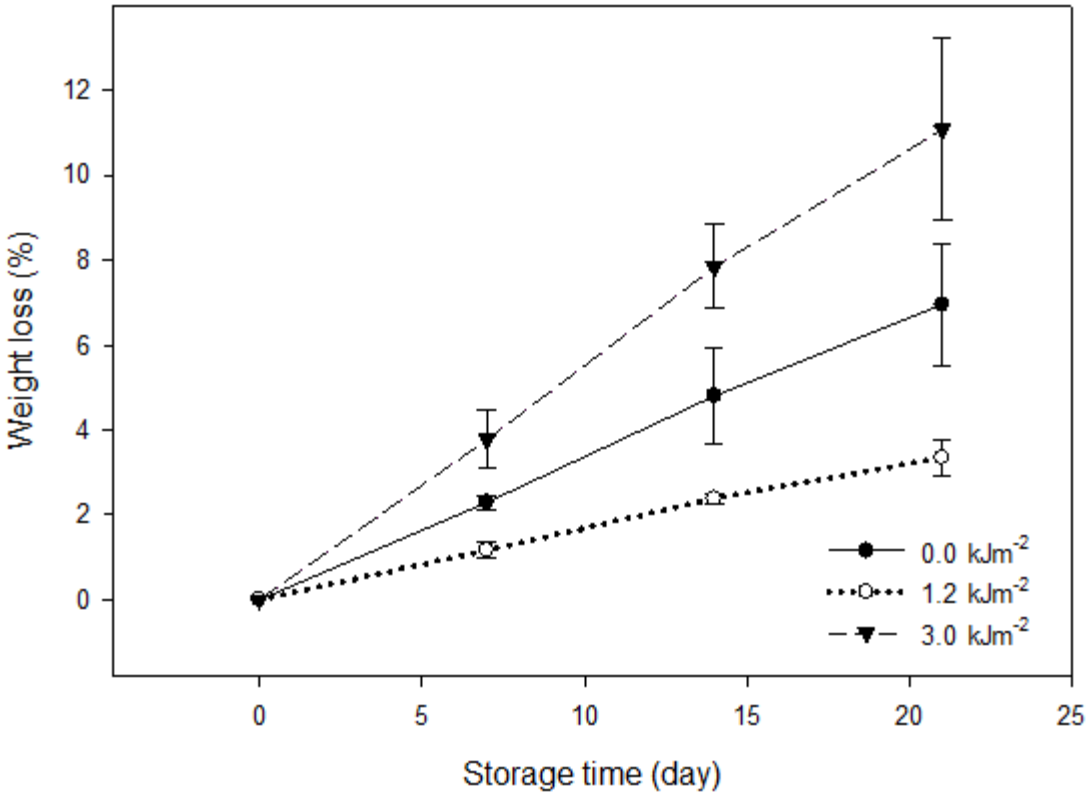

Figure 2.7 Evolution of weight loss of UV-C treated broccoli florets during storage at 4 °C for 21 days. Weight loss (%) was monitored in broccoli florets treated with three UV-C doses: control, (0 kJm^{-2}); hormetic, (1.2 kJm^{-2}); and high, (3.0 kJm^{-2}) during storage in darkness. Each point is the mean of 3 observations and vertical bars represent standard deviation.

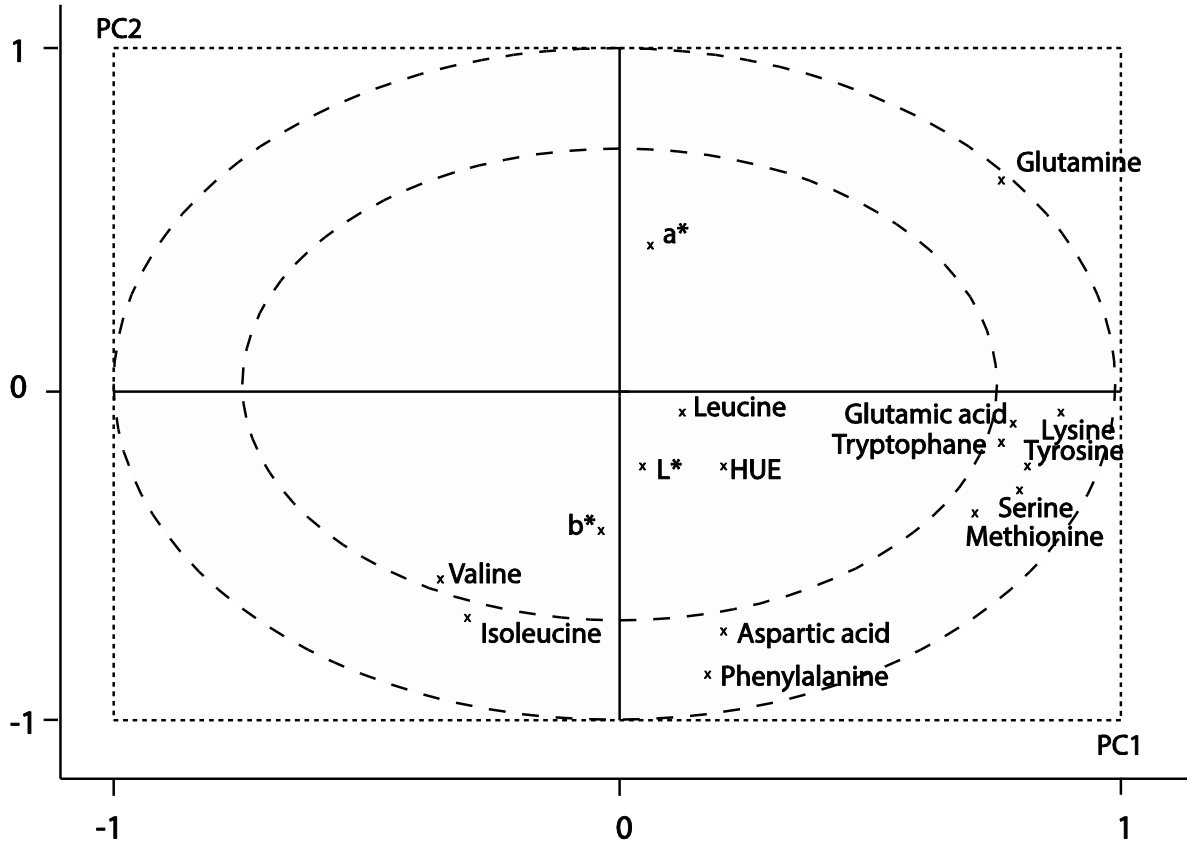

Figure 2.8 Correlation loadings between amino acids and color coordinates. A principal component analysis (PCA) was performed on the titers of amino acids in broccoli florets and correlated to color parameters of CIE-Lab system: *L*, a* and b*.*

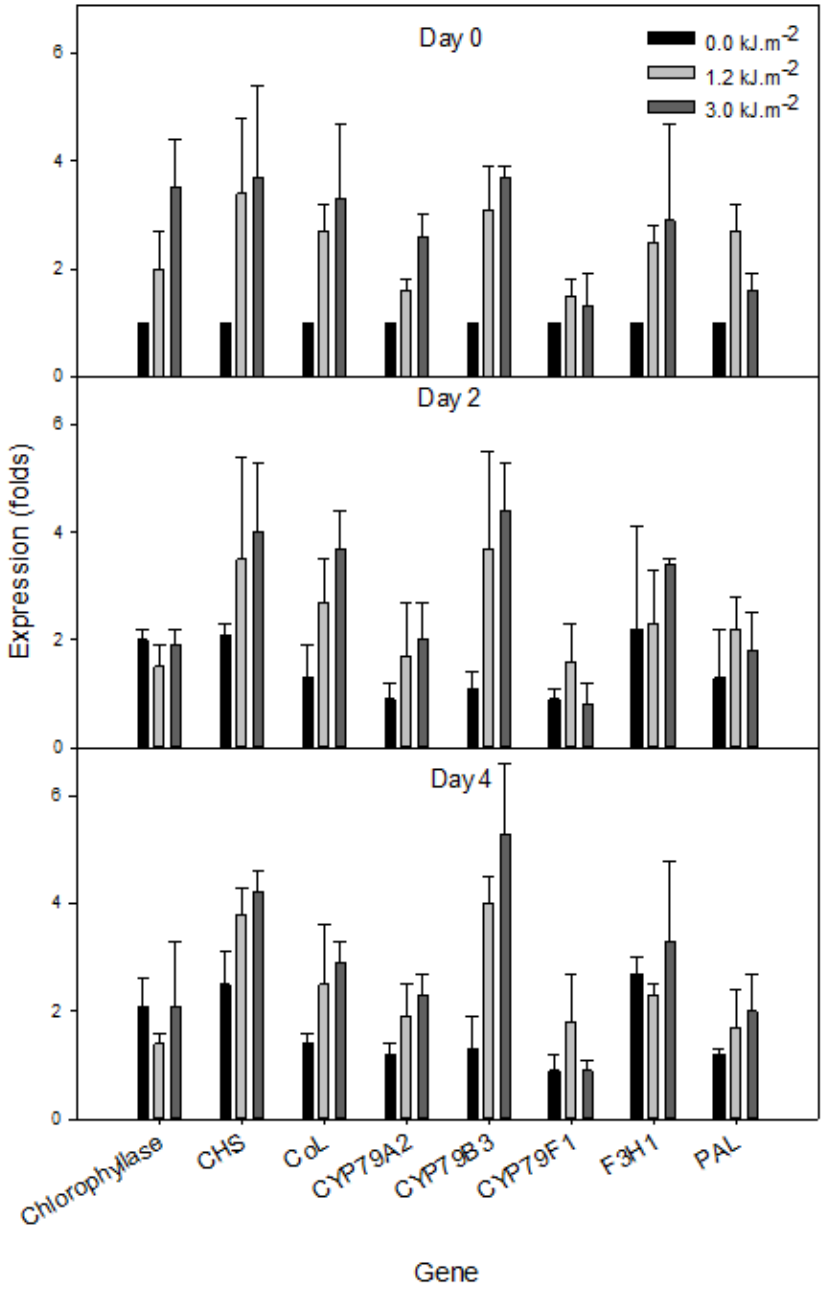

Figure 2.9 Gene expression on UV-C treated broccoli florets. Seven genes were analyzed after UV-C treatment on day 0, chlorophyllase (Chlo), chalcone synthase (CHS), phenylalanine N-hydroxyase (CYP79A2), tryptophan N-hydroxyase (CYP79B3), dihomo-methionine N-hydroxyase (CYP79F1), flavonoid monoxigenase (F3H1) and phenylalanine ammonia-lyase (PAL). Florets were treated with UV-C light at 0 kJm^{-2}; 1.2 (hormetic dose); and 3.0 kJm^{-2} (high dose) and stored up to four days at 4 °C and the values were normalized against actin. Each point is the mean of 3 observations and vertical bars represent standard deviation.

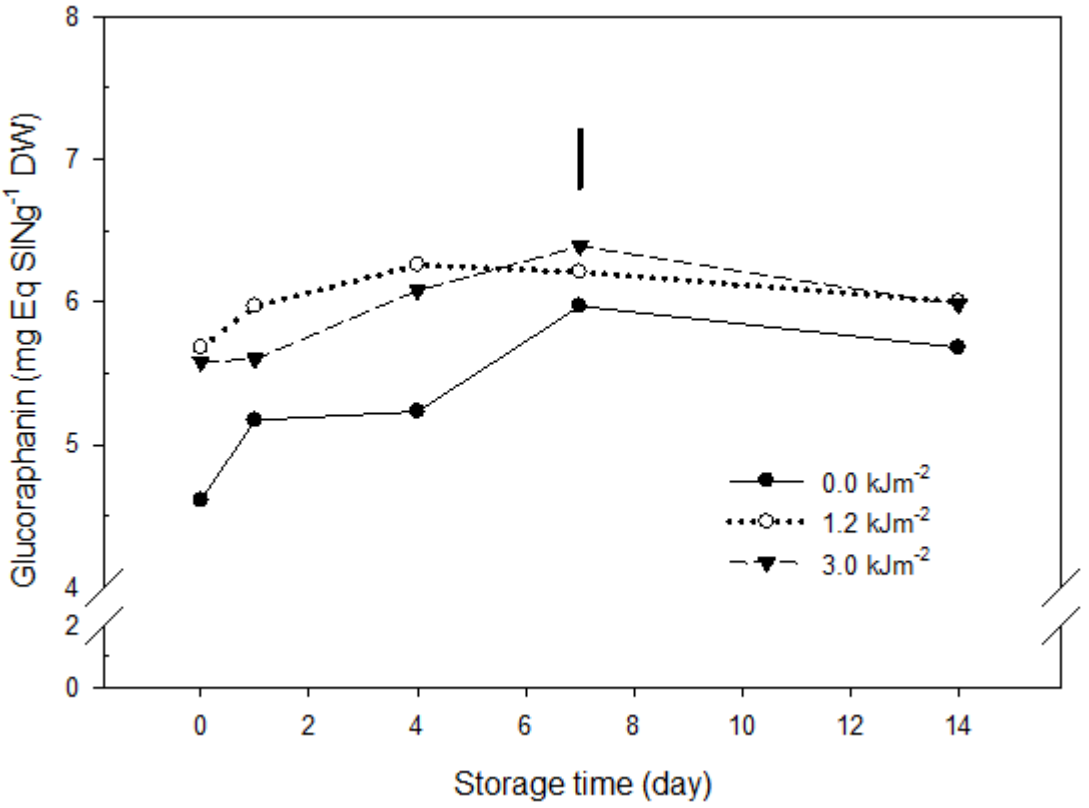

Figure 2.10 Impact of UV-C on glucoraphanin in broccoli florets. Profile of glucoraphanin in broccoli florets exposed to three doses of UV-C, 0, 1.2 and 3.0 kJm^{-2} during storage in darkness at 4 °C for 14 days. Each point is the mean of 3 observations and differences between the treatments were found from LSD (0.05) = 0.40.

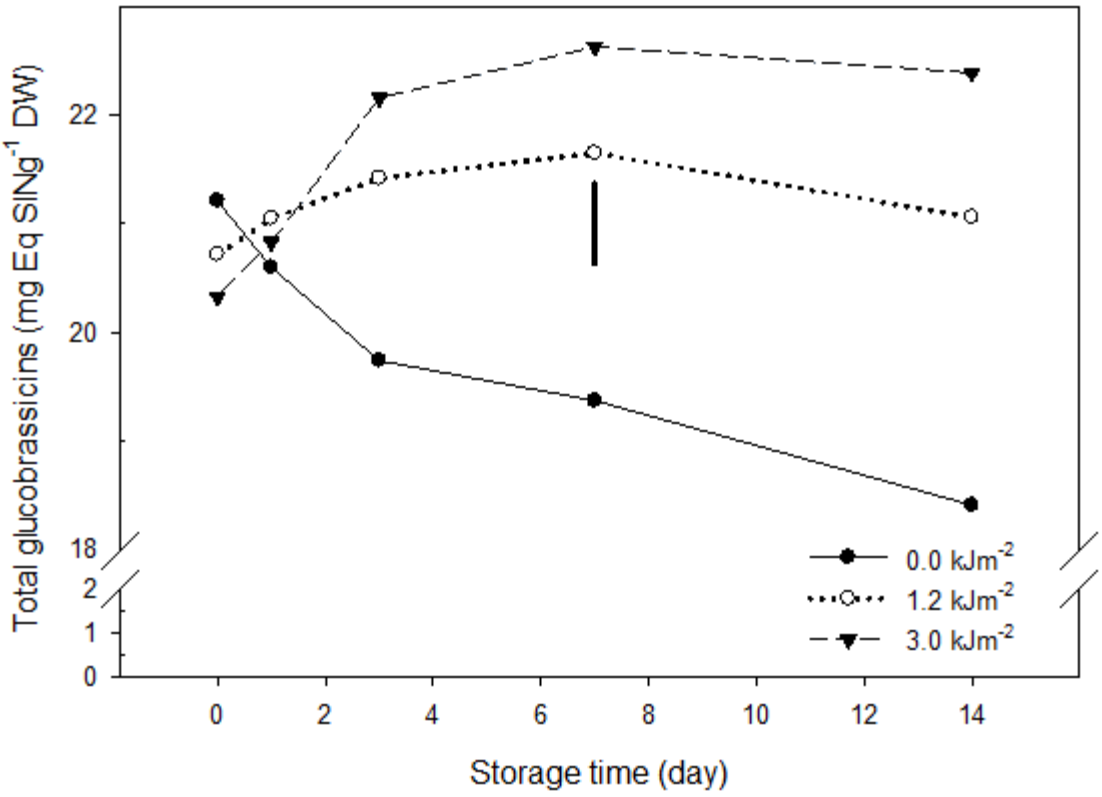

Figure 2.11 Impact of UV-C on total glucobrassicins in broccoli florets. Profile of total glucobrassicins in broccoli florets exposed to three doses of UV-C, 0, 1.2 and 3.0 kJm^{-2} during storage in darkness at 4 °C for 14 days. Each point is the mean of 3 observatons and differences between the treatments were found from LSD (0.05) = 0.74.

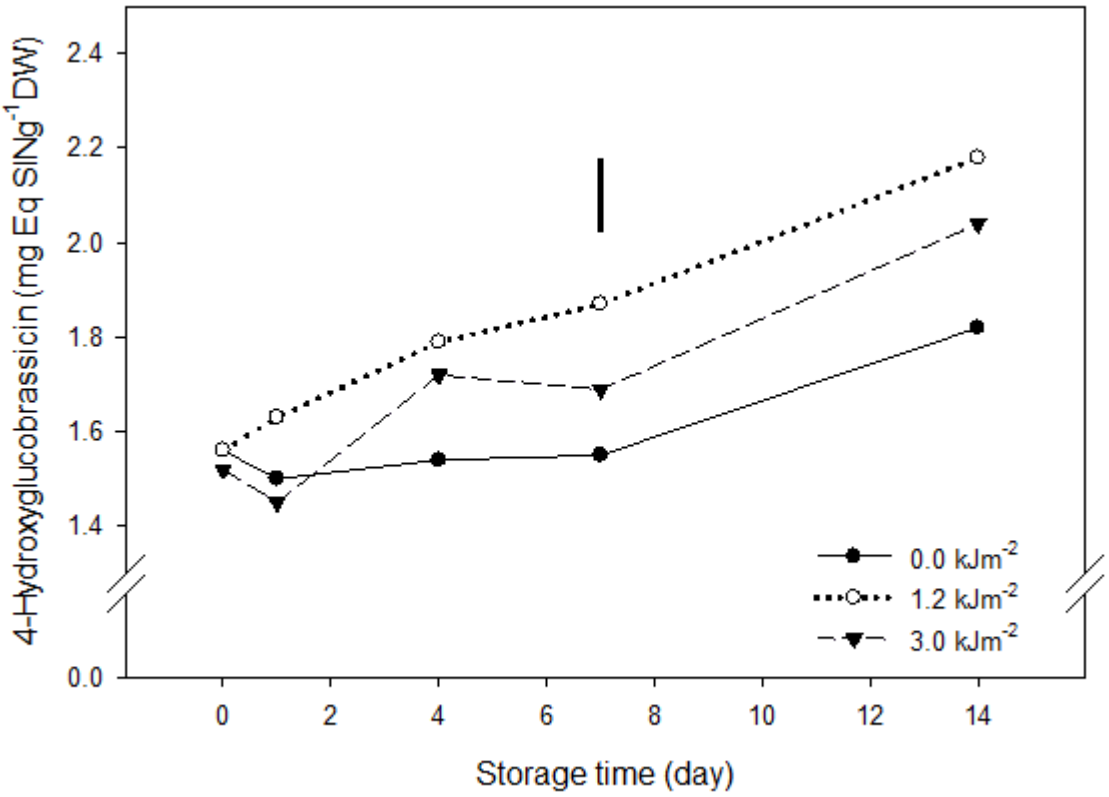

Figure 2.12 Impact of UV-C on 4-hydroxyglucobrassicin in broccoli florets. Profile of 4-hydroxyglucobrassicin in broccoli florets exposed to three doses of UV-C, 0, 1.2 and 3.0 kJm^{-2} during storage in darkness at 4 °C for 14 days. Each point is the mean of 3 observations and differences between the treatments were found from LSD (0.05) = 0.16.

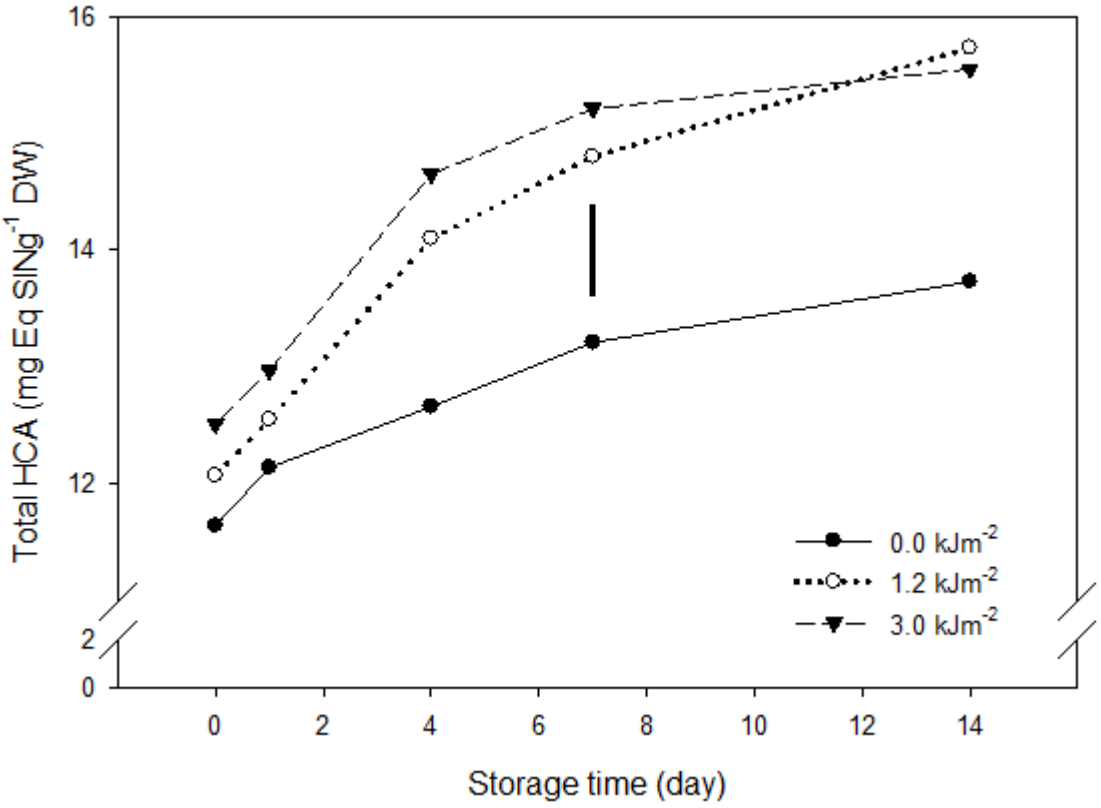

Figure 2.13 Impact of UV-C on total hydroxycinnamic-acid (HCA) content of broccoli florets. Profile of total HCA (1,2-disinapoyl gentibiose + 1-sinapoyl-2-feruloyl gentibiose + 1,2-diferuloyl gentibiose + 1,2,2-trisinalpoyl-gentibiose + 1,2-disynalpoyl-2-feruloyl gentibiose) in broccoli florets exposed to three doses of UV-C, 0, 1.2 and 3.0 kJm^{-2} during storage in darkness at 4 °C for 14 days. Each point is the mean of 3 observations and differences between the treatments were found from LSD (0.05) = 0.74.

Table 2.1 Glucosinolates and hydroxy-cinnamic acids in broccoli (in order or elution with LC-MS)

Compound	Retention time (min)	Mass
1. *Sinigrin (propenyl glucosinolate)	8.31	358.85
2. Glucoraphanin (4-Methylsulfinylbutyl glucosinolate)	9.93	436.91
3. 4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl-glucosinolate)	23.02	463.91
4. Chlorogenic acid (3-(3,4-Dihydroxycinnamoyl) quinate)	31.25	447.93
5. Glucobrassicin (Indol-3-ylmethyl glucosinolate)	36.91	477.99
6. 4-methoxyglucobrassicin (4-Methoxy-3-indolylmethyl glucosinolate)	41.81	477.95
7. Neoglucobrassicin (1-Methoxy-3-indolylmethyl glucosinolate)	50.52	754.21
8. 1,2 disinapoyl gentiobiose	51.26	724.2
9. 1-sinapoyl-2-feruloyl gentiobiose	51.85	694.21
10. 1,2-diferuloyl gentiobiose	53.63	960.23
11. 1,2,2'–trisinapoyl gentiobiose	54.36	930.22
12. 1,2-disinapoyl-2-feruloyl gentiobiose	55.19	900.27

* Internal Standard

Total glucobrassicins (GBS) = Total of compound numbers (3,5,6,7)

Total hydroxy-cinnamic acids (HCA) = Total of compound numbers (8,9,10,11,12)

Compound	Retention time (min)	Mass
13. *Sinigrin (propenyl glucosinolate)	8.31	358.85
14. Glucoraphanin (4-Methylsulfinylbutyl glucosinolate)	9.93	436.91
15. 4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl-glucosinolate)	23.02	463.91
16. Chlorogenic acid (3-(3,4-Dihydroxycinnamoyl) quinate)	31.25	447.93
17. Glucobrassicin (Indol-3-ylmethyl glucosinolate)	36.91	477.99
18. 4-methoxyglucobrassicin (4-Methoxy-3-indolylmethyl glucosinolate)	41.81	477.95
19. Neoglucobrassicin (1-Methoxy-3-indolylmethyl glucosinolate)	50.52	754.21
20. 1,2 disinapoyl gentiobiose	51.26	724.2
21. 1-sinapoyl-2-feruloyl gentiobiose	51.85	694.21
22. 1,2-diferuloyl gentiobiose	53.63	960.23
23. 1,2,2'–trisinapoyl gentiobiose	54.36	930.22
24. 1,2-disinapoyl-2-feruloyl gentiobiose	55.19	900.27

* Internal Standard

Total glucobrassicins (GBS) = Total of compound numbers (3,5,6,7)

Total hydroxy-cinnamic acids (HCA) = Total of compound numbers (8,9,10,11,12)

Table 2.2 Primers for gene expression analysis

Gene	GenBank AN	Primers	Tm (°C)	Cycles
*Actin	AF044573	Sense: 5'-GGCATCACACTTTCTACA-3'	49	28
		Antisense: 5'-CCTTAATCTTCATGCTGC-3'		
Chlorophyllase	AF337544	Sense: 5'-AAAACCTCAAAGCTCACC-3'	49	36
		Antisense: 5'-ATCCATATGTCCGTAATCC-3'		
Chalcone synthase (CHS)	AY228486	Sense: 5'-ACTTCCGCATCACCAACA-3'	62	36
		Antisense: 5'-CACTCCAACCCTTCTCCT-3'		
4-coumarate:CoA ligase (CoL)	AF207572	Sense: 5'-GAGTTATCAGAAGATGATG-3'	61	45
		Antisense: 5'-CTCTTGTAAAACACAACC-3'		
Phenylalanine N-hydroxylase (CYP79A2)	EU877074	Sense: 5'-AGAACCGAAGAGGCTGAT-3'	60	36
		Antisense: 5'-CCTTCTCATGGCCTTCCA-3'		
Tryptophan N-hydroxylase (CYP79B3)	FJ376047	Sense: 5'-GGCTGCTCCAGACAATCCATCG-3'	61	45
		Antisense: 5'-GTTCCTCCGACAACCTCAATTCTCC-3'		
Dihomomethionine N-hydroxylase (CYP79F1)	GU385846	Sense: 5'-TCTCGAGGGTTTATGGTT-3'	62	36
		Antisense: 5'-CCATGTTATTTGCCGGATT-3'		
Flavanone 3-hydroxylase (F3H1)	EU402420	Sense: 5'-GACAGGAAGAGGTTGGAA-3'	57	36
		Antisense: 5'-TGAAGGTAAGGAAGCTGA-3'		
Phenylalanine amoniolyase PAL	HM623311	Sense: 5'-TCAACACTCTCCTCCAAG-3'	49	36
		Antisense: 5'-GAAGTTACCACCGTGAAT-3'		

*Housekeeping gene (for normalization)

Table 2.3 ORAC and contents of ascorbic acid (oxidized, reduced, total), total phenols, total flavonoids, and rutin in UV-C exposed broccoli florets. Florets were treated with UV-C light with 0 kJm^{-2}; 1.2 (hormetic dose); and 3.0 kJm^{-2} (high dose) and stored for 14 days at 4 °C. The values were time-averaged (0, 7, 14 and 21 days).

Total phenols (mg eq GA.g^{-1} DW)			
0 kJm^{-2}		13.9±0.5	
1.2 kJm^{-2}		15.8±0.6	
3.0 kJm^{-2}		17.4±0.9	
Total flavonoids (mg eq QE.g^{-1} DW)			
0 kJm^{-2}		4.1±0.4	
1.2 kJm^{-2}		4.6±0.3	
3.0 kJm^{-2}		4.7±0.2	
Ascorbic acid (mg Eq AA. g^{-1} DW)			
	Oxidized	**Reduced**	**Total**
0 kJm^{-2}	3.2±0.3	7.3±0.4	10.5±0.4
1.2 kJm^{-2}	3.3±0.3	6.6±0.6	9.9±0.7
3.0 kJm^{-2}	3.2±0.7	6.1±0.7	9.2±0.4
ORAC (mg Eq trolox g^{-1}DW)			
0 kJm^{-2}		152.6±17.3	
1.2 kJm^{-2}		140.3±6.3	
3.0 kJm^{-2}		138.3±6.0	
Rutin (mg eq SIN.g^{-1} DW)			
0 kJm^{-2}		0.5±0.1	
1.2 kJm^{-2}		0.6±0.1	
3.0 kJm^{-2}		0.7±0.1	

Table 2.4 Impact of UV-C on glucosinolates. Florets were treated with UV-C light with 0 kJm^{-2}; 1.2 (hormetic dose); and 3.0 kJm^{-2} (high dose), and stored for 14 days at 4 °C. The values were time-averaged (0, 7, 14 days).

	Glucobrassicin	**4-methoxyglucobrassicin**	**Neoglucobrassicin**
		Day 0	
0.0 $kJ.m^{-2}$	11.3±0.5	2.6±0.3	5.9±0.3
1.2 $kJ.m^{-2}$	10.8±0.8	2.4±0.0	6.0±0.3
3.0 $kJ.m^{-2}$	10.4±0.3	2.3±0.0	6.0±0.6
		Day 7	
0.0 $kJ.m^{-2}$	10.0±0.6	2.6±0.0	5.3±0.5
1.2 $kJ.m^{-2}$	10.7±0.2	2.8±0.1*	6.3±0.1*
3.0 $kJ.m^{-2}$	11.4±0.6	2.8±0.1*	7.0±0.7*
		Day 14	
0.0 $kJ.m^{-2}$	9.3±0.1	2.2±0.2	5.1±0.3
1.2 $kJ.m^{-2}$	10.5±0.6*	2.6±0.1	5.8±0.4
3.0 $kJ.m^{-2}$	11.5±0.9*	2.5±0.2	6.3±0.2

The asterisk indicates that the value is significantly different from the corresponding control at $p < 0.05$.

Table 2.5 Impact of UV-C on hydroxycinnamic acids (HCA). Florets were treated with UV-C light with 0 kJm^{-2}; 1.2 (hormetic dose); and 3.0 kJm^{-2} (high dose), and stored for 14 days at 4 °C. The values were time-averaged (0, 7, 14 days).

	Sinapolyl-diferuloyl gentibionse	**1,2-disynalpoyl-2-feruloyl gentibiose**	**1,2,2-trisinalpoyl-gentibiose**	**1,2-diferuloyl gentibiose**	**1-sinapoyl-2-feruloyl gentibiose**	**1,2-disinapoyl gentibiose**
Day 0						
0.0 $kJ.m^{-2}$	0.6±0.01	2.1±0.2	1.8±0.1	1.1±0.1	4.1±0.1	2.5±0.2
1.2 $kJ.m^{-2}$	0.6±0.02	2.3±0.1	1.9±0.1	1.1±0.1	4.3±0.4	2.5±0.2
3.0 $kJ.m^{-2}$	0.6±0.05	2.3±0.1	2.1±0.1	1.2±0.1	4.5±0.5	2.4±0.2
Day 7						
0.0 $kJ.m^{-2}$	0.6±0.1	2.5±0.1	2.1±0.2	1.1±0.16	4.7±0.5	2.9±0.2
1.2 $kJ.m^{-2}$	0.7±0.03	2.8±0.1*	2.3±0.2	1.4±0.04*	5.2±0.3	3.1±0.2
3.0 $kJ.m^{-2}$	0.7±0.1	3.0±0.2*	2.4±0.3	1.4±0.07*	5.2±0.5	3.2±0.0
Day 14						
0.0 $kJ.m^{-2}$	0.7±0.1	2.7±0.1	2.1±0.2	1.2±0.07	5.1±0.3	2.6±0.3
1.2 $kJ.m^{-2}$	0.7±0.01	3.1±0.1*	2.4±0.2	1.3±0.05*	5.7±0.2*	3.2±0.2
3.0 $kJ.m^{-2}$	0.7±0.04	3.2±0.2*	2.4±0.3	1.4±0.04*	5.7±0.0*	2.9±0.1

The asterisk indicates that the value is significantly different from the corresponding control at $p < 0.05$.

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Chapter III: Methodology for the Determination of Hormetic Heat Treatment in Broccoli Florets Using Humidified Hot Air: Temperature-Time Relationships

3.1 Résumé

Le brocoli (*Brassica oleracea*) est l'un des produits les plus consommés parmi les cultures des *Brassicacées* à cause de sa teneur en composés bioactifs tels que les glucosinolates et les flavonoïdes. La conservation de ce légume est une tâche difficile en raison de sa sénescence rapide, qui se manifeste par le jaunissement des fleurons. Afin de retarder cet aspect indésirable, plusieurs traitements post-récolte ont été explorés pour prolonger sa vie commercialisable, y compris le traitement thermique. Bien que les traitements thermiques utilisant des combinaisons arbitraires de température et de temps aient été trouvés efficaces pour ralentir le jaunissement des fleurons de brocoli, il n'y aucune méthodologie claire pour la sélection des variables température-temps pour l'application de chaleur. L'objectif de ce travail était d'établir une relation température-temps en utilisant la réponse de fuite d'électrolyte de la membrane cellulaire comme un indicateur de la gravité de la chaleur. Les fleurons de brocoli ont été traités avec de l'air chaud humidifié à des températures de 32 à 52 ° C pendant des périodes allant de 5 à 1440 min. La fuite d'électrolyte a été déterminée en mesurant la conductivité électrique des éluants de tiges de brocoli traitées dans une solution de mannitol 0.4 M. Le pourcentage de fuite d'électrolyte a augmenté avec le temps d'exposition à chaque test de température montrant une cinétique d'ordre zéro. Le taux de fuite d'électrolyte augmentait avec la température, mais le graphique d'Arrhenius a montré un motif linéaire clairement interrompu avec une zone de transition ou zone de température critique entre 42-45 °C. Bien que les temps équivalents pour le chauffage à des températures différentes peuvent être estimés à partir de la cinétique de la fuite de électrolytes, la sélection de la température de traitement doit être inférieure à 42 °C, pour laquelle la couleur de fleurons varie avec la progression de la sénescence sans causer des conditions anaérobies excessives et / ou des lésions tissulaires. Les traitements thermiques des fleurons à des températures dans la zone critique ont conduit à l'accumulation excessive d'éthanol en raison de la respiration anaérobie, tandis que des traitements avec des températures supérieures à la zone critique (> 45 ° C) conduisent à des conditions anaérobies graves ainsi que des dommages aux tissus, en dépit de leurs effets sur l'amélioration de la rétention de la couleur dans les fleurons de brocoli traités avec des températures supérieures à 42 °C. Le traitement thermique de 41 °C pendant 180 minutes comme dose de chaleur hormétique pour les fleurons de brocoli est suggéré. Les résultats de ces travaux suggèrent que la sélection de la température de traitement est une considération primordiale pour le traitement thermique de produits frais.

3.2 Abstract

Broccoli (*Brassica oleracea*) is one of the most consumed produce among *Brassica* crops because of its content in bioactive compounds such as glucosinolates and flavonoids. Preservation of this vegetable is a challenging task because of its rapid senescence, manifested as floret yellowing. In order to delay this undesirable aspect, several postharvest treatments have been explored including heat treatment, to extend its marketable life. Although heat treatments using arbitrary combinations of temperature and time have been found effective in slowing down the yellowing of broccoli florets, there is no clear methodology for the selection of temperature-time variables for heat application. The objective of this work was to establish a temperature-time relationship using membrane electrolyte leakage response as an indicator of heat severity. Broccoli florets were treated with hot humidified air at temperatures from 32 to 52 °C for periods ranging from 5 to 1,440 min. Electrolyte leakage was determined by measuring the conductivity of cell eluate from broccoli stems in 0.4 M mannitol solution. The percentage of electrolyte leakage increased with exposure time at each temperature test following zero order kinetics. The electrolyte leakage rate increased with temperature, but the Arrhenius plot showed a clear broken linear pattern with a break with a transition or critical temperature zone of 42-45 °C. Although equivalent times for heating at different temperatures can be estimated from the kinetics of electrolyte leakage, the selection of treatment temperature had to be below 42 °C, where the color of florets changes with the progress of senescence without causing excessive anaerobic conditions and/or tissue damage. Heat treatment of florets at temperatures in the critical zone led to excessive accumulation of ethanol as a result of anaerobic respiration, while treatments with temperatures above the critical zone (> 45 °C) led to severe anaerobic conditions as well as tissue damage, despite enhanced color retention of broccoli florets treated with temperatures above 42 °C. Heat treatment of at 41 °C for 180 min as hormetic heat dose for broccoli florets is suggested. The results of this work suggest that the selection of treatment temperature is of primary consideration for heat treatment of fresh produce.

Key words: broccoli, heat treatment, postharvest, membrane leakage, temperature-time superposition, senescence

3.3 Introduction

Fruits and vegetables are an important part of a healthy diet because they are sources of vital nutrients such as vitamin C, thiamine, niacin, pyridoxine, folic acid and minerals, including zinc, calcium, potassium, phosphorus and other phytochemicals which decreases the risk of chronic diseases (Oguntibeju et al., 2013). However, fruits and vegetables are highly perishable, susceptible to physiological disorders such as chilling injury and internal discoloration as well as to microbial attack throughout storage. In order to reduce fungal and bacterial diseases; chemical methods are employed, including sulfur dioxide (Rivera et al., 2013), chemical fungicides such as azoxystrobin, fludioxinil and pyrimethanil (Kanetis et al., 2007). Nonetheless, the use of chemicals to extend the postharvest life of fruits and vegetables has become less and less acceptable by consumers, as they may contribute to environmental pollution and/or may be harmful to human health.

Recently, new physical treatments are gaining interest and increasingly being considered for prolonging the postharvest life of fruits and vegetables. Among them, heat treatment has received much interest as a pre-storage treatment for the postharvest preservation of fruits and vegetables (Klein and Lurie, 1992; Lurie, 1998; Lurie and Pedreschi, 2014). Heat treatment can be performed using hot water (>40 °C), forced hot air, high-temperature controlled atmospheres using steam, solar energy, infrared, microwave and radio frequency (Hansen and Johnson, 2007). Among the beneficial effects, heat treatment has been shown to delay the ripening of fruits by reducing ethylene production and hydrolytic cell wall enzymes. Likewise, chilling injury and diseases in commodities are reduced by heat treatments. Ethylene production is reduced at temperatures between 30 to 40 °C as the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to C_2H_4 is vulnerable to heat treatment (Yu et al., 1980). Strawberries exposed to hot-air at 45 °C for 3 h soften more slowly than the non-heated fruits, presumably due to the inactivation of hydrolytic cell wall enzymes such as polygalacturonase, β-galactosidase, endo-1,4-β-d-glucanase and β-xylosidase (Martínez and Civello, 2008). Heat treatment has also been shown to delay the yellowing of broccoli; attributed, due in part, to reduced chlorophyllase activity (Funamoto et al., 2002).

The prevention of chilling injury by heat treatment has been associated with the presence of heat shock proteins (HSPs) and polyamines stimulated (Lurie and Pedreschi, 2014; Mirdehghan et al., 2007). These responses can condition or protect plants against subsequent exposure to low temperature, preventing chilling injury. Furthermore, heat treatment controls postharvest diseases

either by direct inhibition of pathogens or by the induction of host-defenses such as pathogenesis-related (PR) proteins (Lurie, 1998; Pavoncello et al., 2001).

Heat treatment can have negative impacts as well, when high temperatures or long exposure times are used, including changes in membrane fluidity, lipid rafts and the activation of ion channels (Mittler et al., 2012). High respiration rates can lead to anaerobic conditions due to depleted tissue oxygen as well as membrane disruption and progressive leakage (Duarte-Sierra et al., 2012a). Off-odor development and increased production of ethanol were observed in broccoli after, presumably severe hot water treatment at 52 °C for 3 min that could create anaerobic conditions in the tissue (Forney and Jordan, 1998). Moderate water loss observed in heat-treated commodities is likely due to a higher respiration and transpiration rates (Lydakis and Aked, 2003; Schirra et al., 2000).

The severity of heat treatment involves two factors: temperature (heat intensity) and heating time. Heat processing of foods is a well-known operation and is designed to inactivate targeted microbial spores, cells or enzymes (Karel and Lund, 2003). The microbial resistance to heat is expressed by the time required to obtain a one 1 log reduction in the microbial count (*D value*) at a specific temperature, and it decreases with increase in temperature. The effect of temperature on microbial resistance is expressed by z value, characteristic of the organisms; where z value is the change in temperature required to affect a response of 1 log change in *D value*. For commercial sterilization of low-acid foods, the assigned heat or sterilization value is 3 min at the reference temperature of 121 °C (Rees and Bettison, 1991). The relationship between D and z values permits the determination of the sterilization value at temperatures other than 121 °C. Extension of this approach to heat treatment of fresh produce could allow the establishment of equivalent heating times at different temperatures to obtain a specific tissue response.

Current investigations on heat treatment of fresh produce arbitrarily select temperature-time variables, and evaluate the impact of heat treatment on targeted-responses that relate to the preservation of fresh produce. Furthermore, there is little information available with respect to temperature that may cause irreversible damages to fresh fruits and vegetables. It is imperative to acquire information with respect to temperature thresholds, above which heat treatment becomes harmful to the produce rather than imparting beneficial effects.

Thus the objective of this work was to establish a methodology to define heat doses (heating temperature and time) relative to a reference heat dose (reference temperature and time) using electrolyte leakage response in broccoli. The temperature threshold for broccoli that causes

negligible physiological damage, and the hormetic heat dose (temperature-time) were also determined.

3.4 Materials and Methods

3.4.1 Vegetable material

Freshly harvested broccoli (*Brassica oleraceae* L. var. Italica 'Diplomat') heads were obtained from a commercial farm (Ile d'Orléans, Québec, Canada). Florets (300g) of uniform size (approximately, 7 cm) were separated from heads and randomly arranged in 500 mL plastic punnets. The punnets were placed in 5 L plastic containers provided with ventilation and a layer of water at the bottom to maintain high humidity (98-100%), and the containers were stored in a controlled chamber overnight at 4 °C before heat exposure.

3.4.2 Heat treatments

Heat treatment was performed on the florets stored overnight at 4 °C in a closed 2 m^3 chamber with continuous air circulation equipped with controlled air heating system and a humidity control system using steam injection for saturation of air. Seven temperatures, 37, 42, 43, 44, 45, 47 and 52 °C were chosen based on previous studies reviewed by Lurie (1998), with heating time ranging from 5 to 1,440 min. Florets were heat treated at 37 °C for 0, 40, 120, 180, 360 and 1,440 min; at 42-44 °C for 0, 30, 60, 120, 180, 360 and 1,440 min; at 45 °C; 47 °C; and 52 °C for up to 360 min, 120 min and 60 min, respectively. The temperature of the floret stem was monitored with temperature probes (5 mm in length) placed at three locations along the length of the floret stem (bottom, middle and the top location near the buds). The come-up time to the desired temperature varied between 6 min for 52 °C and 12 min for 37 °C. The heat exposure time was logged once the desired temperature was reached as monitored by the temperature probes. Five florets were used for each treatment. After heat treatment, the heated florets were submerged up to the stem immediately into 0.9 % NaCl isotonic solution (to prevent osmotic flow) for 1 min at room temperature. Florets were then immediately surface dried and cooled down to temperature below 10 °C inside a disinfected controlled chamber with constant air flow at 1 °C for 10 min, and subsequently stored at 10 °C/90 % RH for further analysis.

3.4.3 Electrolyte leakage

Electrolyte leakage was assayed from stem cubes (5 mm in length and approx. 0.7 g) obtained from six randomly chosen floret stem discs for each time-temperature combination (i.e., at 37 °C for 0, 40, 120, 180, 360 min). After surface drying and cooling the florets, 2 to 3 stem discs ca. 7 mm were sectioned from the middle of the floret stem using a scalpel. Broccoli stem cubes were placed into 50 mL falcon tubes with 25 mL of 0.4 M mannitol solution and mixed under agitation for 1 hour. Electrical conductivity of the solution was measured at 1 min (C_1) and 60 min (C_{60}) of incubation at room temperature using a conductivity instrument (Model 3100, YSI, Yellow Springs, USA). The total conductivity (C_T) of the solution was determined on samples after autoclaving them at 121 °C for 30 min (Fan and Sokorai, 2005). Electrolyte leakage (L) was calculated as:

$$L = \frac{C_{60} - C_1}{C_T \times 100} \quad \text{.....(1)}$$

3.4.4 Color measurement

The color of broccoli florets was determined as described elsewhere in the thesis in detail, in section 2.3.3.

3.4.5 Volatile analysis

Analysis of volatiles was performed on broccoli samples stored at 10 °C/90 % RH for 0 and 24 hours. Samples consisted of about 1 g of stem tissue introduced into a 10 mL vial containing 30 μL of 0.5 % 2-propanol as internal standard. A Hewlett-Packard 6890 Network GC system equipped with a Headspace Analyzer (G1888 Network Headspace Sampler, Agilent, Wilmington, DE) and coupled to a HP 5973 mass spectrometer was used to confirm the identification of volatiles present in the standards and samples. Helium was used as carrier gas at a flow rate of 1.8 mL/min. The oven temperature was programmed as follows: hold at 90 °C for 1 min, rise to 140 °C (15 °C/min), increase to 320 °C (35 °C/min), and finally, hold for 1 min at this temperature. Three replicates were made for each treatment temperature with four replicates for each analysis.

3.4.6 Computational

The physical, chemical or microbiological changes in foods with time can be expressed according to Taoukis and Labuza (1989) in a general form as:

$$\frac{d[A]}{dt} = k[A]^n \quad \text{..... (2)}$$

where A represents a physical property, chemical entity or microbial count, n is the order of the reaction and k is the reaction rate constant. With A representing electrolyte leakage (L) of tissue which increases with the heating time, the rate equation can be expressed as:

$$\frac{dL}{dt} = kL^n \quad \text{..... (3)}$$

For a zero order reaction (i.e., electrolyte leakage increases with exposure time at a constant rate at a given temperature):

$$\frac{dL}{dt} = k \quad \text{..... (4)}$$

Up on integration on equation (4):

$$L = kt \quad \text{..... (5)}$$

The rate of electrolyte leakage (k) is expected to increase with temperature, which can be expressed by Arrhenius equation;

$$k = Be^{\frac{-E_a}{RT}} \quad \text{..... (6)}$$

where B is a pre-exponential constant; E_a is the activation energy (J mol^{-1}); R is the gas constant (8.31 J mol^{-1} $°K^{-1}$); and T is the temperature (°K). Combining equations (4) and (5), the electrolyte leakage can be expressed as:

$$L = tBe^{\frac{-E_a}{RT}} \quad \text{..... (7)}$$

Since the electrolyte leakage changes with temperature and time, the principle of time-temperature superposition can be attempted to elucidate the times at different temperatures or vice versa in order to obtain a specific response in electrolyte leakage. The time-temperature shift factor, a_T. is given by dividing the test time t_{ref} at a reference temperature, T_{ref}, by the time required, t_T, to obtain the same leakage response at a test temperature T (Ding and Wang, 2007).

$$a_T = \frac{t_{ref}}{t_T} \quad \text{..... (8)}$$

Rewriting equation (7) for electrolyte leakage at the reference temperature (L_{ref}):

$$L_{ref} = t_{ref} B e^{\frac{-E_a}{RT_{ref}}} \quad \text{..... (9)}$$

And the leakage at temperature, T (L_T):

$$L_T = t_T B e^{\frac{-E_a}{RT_T}} \quad \text{..... (10)}$$

Combining equations (9) and (10):

$$\frac{L_{ref}}{L_T} = \frac{t_{ref}}{t_T}\left[e^{\frac{Ea}{RT_{ref}} - \frac{Ea}{RT_T}} \right] \quad \text{..... (11)}$$

With the leakage response at both the temperatures being the same, equation (11) becomes:

$$\log a_T = \frac{E_a}{2.303R}\left[\frac{1}{T_{ref}} - \frac{1}{T_T} \right] \quad \text{..... (12)}$$

or

$$\log a_T = \frac{E_a}{2.303RT_{ref}T_T}\left[T_T - T_{ref}\right] \quad \text{..... (13)}$$

Also, the sensitivity of electrolyte leakage to temperature can be expressed by the activation energy (E_a):

$$E_a = \frac{2.303RT_{ref}T_T}{(T_T - T_{ref})} \log a_T \quad \text{..... (14)}$$

3.5 Results and discussion

3.5.1 Time-temperature relationships: superposition method

Because of lack of a defined heat doses for treatment of commodities, we attempted to determine time equivalence for different treatment temperatures for a specific tissue response as electrolyte leakage based on time-temperature superposition concept. The establishment of time equivalence at different temperatures to obtain 5 % electrolyte leakage response to heat allows the

application of heat at higher temperatures for short times and *vice versa.* This approach is much like the commercial sterilization of low-acids using lower temperatures for longer times and higher temperatures for shorter times, where the response is the inactivation of *Clostridium botulinum* spores (Ramesh, 2003).

Electrolyte leakage of broccoli tissue increased with both heating temperature and time (Figure 3.1), and the leakage rate increased with increase in temperature, where the increase in leakage with exposure time at a specified temperature was of zero order, i.e., long times at lower temperatures and short times at higher temperatures to bring about a certain level of leakage, suggesting that the concept of time-temperature superposition is well applicable.

The threshold electrolyte leakage that does not affect the preservation of broccoli florets was evaluated. Broccoli florets were exposed to heat at an arbitrary temperature of 43 °C based on the leakage pattern (Figure 3.1) for 0, 3, 130, 257 and 386 min to achieve five levels of electrolyte leakage (0.0, 2.5, 5.0, 7.5 and 10.0 %), stored for up to 10 days at 10 °C, and the color and visual appearance were monitored daily. It was observed that leakage level (2.5 %) was not significantly different from that of non-heat treated florets (2.3 %), whereas at the leakage level of 10 %, the florets exhibited fungal infection and off-odors at the end of storage. The presence of infection was not visually detected in florets at either 5 % or 7.5 % leakage level, but the color retention was superior at the leakage level of 5 % at the end of storage, and therefore, the heat dose required to cause a leakage level of 5 % was considered appropriate for heat treatment of broccoli florets without causing any irreversible damage to the tissue (data not shown). Time-temperature superposition was carried out to determine the equivalent heating times at various temperatures to obtain the heat dose for 5 % level of electrolyte leakage.

The time-temperature superimposed master curve seen in Figure 3.2 clearly shows that electrolyte leakage response to heat dose using different time-temperature combinations with the reference temperature of 37 °C is effectively superimposable and unified. Effectively, the electrolyte leakage-heat exposure time curves in Figure 3.1 are shifted horizontally along the time axis to the right. The amount of shifting, the shift factor, to superpose on the master curve depended on the heating temperature as seen in the inset of Figure 3.3. The electrolyte leakage rate or the shift factor was not linear with temperature, but was exponential (Figure 3.3). From the master curve, the equivalent heating times can be derived for any temperature in the range of 37-52 °C for broccoli florets can be estimated by basically reversing the procedure (Equation 11). Furthermore, it would be

interesting to see whether the temperature shift factors to electrolyte leakage in broccoli could be applicable to time-temperature behavior of other physiological responses such as respiration rate.

The non-linearity of leakage rate response or shift factor with temperature suggests the existence of a threshold temperature; below which the leakage rate is linear with temperature, and above which the rate of leakage increases exponentially (Figure 3.3). A plot of Equation 13 (Figure 3.4) and Arrhenius plot (Figure 3.5) show that the leakage rate or shift factor relation the temperature is broken linear and that there is a critical zone between 42-45 °C, and the mid-point in the critical zone being the critical temperature (Tc = 43 °C)

Interestingly, after reviewing the literature on the beneficial and adverse effects of heat treatment on ripening fruits, Paull and Chen (2000), identified two types of tissue responses to heat: a normal cellular response when the produce are heat treated at temperatures below 42 °C with beneficial effects such as delayed ripening; but cellular damage when produce are heat treated at temperatures above 45 °C. However, the critical temperature zone may not be universal for all types of fresh fruits and vegetables and this aspect may need further attention.

The existence of a critical temperature or critical temperature zone for membrane leakage rate suggests that the membrane itself undergoes thermal transition, presumably due to the melting of membrane lipids, and that the leakage behavior of the membrane is altered as a consequence. In the light of the presence of critical zone for broccoli tissue, we may class the temperatures below 42 °C as sub-critical zone, the temperature range from 42-45 °C as critical zone, and the temperatures above 45 °C as supercritical zone (Figure 3.5). The supercritical temperatures must be avoided as they can harm the tissue; and the temperatures below the critical temperature could be considered for heat treatment. It also follows that the time equivalence concept cannot be appropriate for the entire temperature range of 37-52 °C for broccoli tissue, but can well be useful for the selection of time-temperature for temperatures below the critical temperature, recognizing that the fundamental prerequisite in the selection of heat treatment is the identification of the critical temperature. The experimental evidence presented here for the presence of critical zone temperature is largely in agreement with the literature assessment of Paull and Chen (2000).

The activation energy for membrane leakage from Arrhenius plot for the three temperature zones are shown in Table 3.1. The critical zone exhibited the highest E_a (234.9 kJ mol^{-1}), followed by the super-critical zone (151.0 kJ mol^{-1}) and the sub-critical zone (27.9 kJ mol^{-1}). The high E_a (the threshold energy) for electrolyte leakage in the critical temperature zone suggests that membrane

integrity is improved by some mechanism in response to heat treatment in this range of temperatures, while it also becomes sensitive to heat that membrane could be easily perturbed by small changes in temperature, as seen by the coefficient of thermal leakage rate (α), i.e., the increase in leakage rate with an increase in temperature of 1 °C (Table 3.1). The α values were 1.92, 1.50 and 1.08 for the critical zone, upper-critical zone and sub-critical zone, respectively.

The improved membrane integrity in the critical zone in response to heat may be attributable to heat induced factors acting in tandem such as heat shock proteins (Civello et al., 1997; Lurie and Pedreschi, 2014; Pavoncello et al., 2001; Woolf and Ferguson, 2000), and metabolites such as ethanol, a primary metabolite of anaerobic respiration, at low concentrations, but can be detrimental at higher concentrations (Pesis, 2005). The above-mentioned induced factors do not appear to be highly manifest at temperatures below 42 °C (sub-critical zone). The intermediate E_a value in the supercritical zone suggests that the protective effect of the heat induced factors is mitigated, likely by the counteraction of above two factors.

3.5.2 Ethanol production in response to high temperatures

Depletion of oxygen from the plant tissue at a high treatment temperature can be expected because of reduction in the solubility of gases with increases in temperature (degasing), and also due to faster depletion of oxygen in the tissue because of high respiration rates at the elevated temperatures. Consequently, anaerobic conditions may prevail leading to increased anaerobic respiration and the conversion of pyruvate to acetaldehyde, ethanol and other volatiles (Forney and Jordan, 1998). It would seem then that the ethanol present in the heated florets at temperatures in the critical zone is optimal in contributing to the integrity of the membrane; whereas the higher levels of ethanol are produced at temperatures in the super-critical range, counteract the effect of HSPs produced in response to heat, leading to weakened membrane integrity.

Accumulation of ethanol in florets was evident after 24 h of storage with increasing exposure temperature (Figure 3.6), where the presence of ethanol was not detectable in zero-time samples. The increase of ethanol followed an exponential trend similar to the electrolyte leakage rate and a_T (Figure 3.3). Increase of ethanol in vegetable tissues is a sign of anoxia caused by depletion of oxygen from the tissue by the applied heat since oxygen solubility in water decreases with increases in temperature leading to increased anaerobic respiration. In addition to ethanol production in florets,

off-odor was perceived on florets treated at 47 and 52 °C. The latter has also been previously reported in florets treated with hot water at 52 °C by Forney and Jordan (1998). They attributed the development of off-odor to cis-3-hexen-1-ol, dimethyl trisulfide (DMTS) and dimethyl sulfide (DMS). The biosynthesis of off-odor compounds, particularly the sulfur compounds, has been related to the increased levels of free sulfur amino acids under anaerobic conditions (Derbali et al., 1998). Furthermore, protein degradation in plant cells increases with temperature (Ferguson et al., 1994), leading to the production of free sulfur containing amino acids which can act as substrate for the generation of off-odor volatiles.

3.5.3 Hormetic heat dose determination

The yellowing of broccoli florets was delayed with heat treatment above 39 °C for equivalent times (Figure 3.7). At this temperature, the yellowing was similar to the non-heated broccoli. Color change was the lowest at temperatures between 41 °C and 43 °C. Although the color retention at 45 °C and 47 °C was not significantly different from that of the florets treated at either 41 °C or 43 °C for equivalent exposure times, off–odor was evident in florets treated at 45 °C, and more so, at 47 °C. In addition, the incidence of diseases was evident in florets treated at 47 °C at end of storage of 10 days at 10 °C (data not shown), indicating that the florets had cellular damage. Thus, the heat treatment at temperatures below 43 °C for equivalent times can be considered for heat treatment of broccoli florets from the stand point of color retention. However, the attendant increased anaerobic respiration associated with critical zone temperatures would prescribe temperatures below 42 °C for equivalent times for treatment, which provide optimal color retention. In addition, heat treatment of florets below this temperature showed normal physiology, i.e., color change with the progress of senescence without causing either excessive anaerobic respiration or heat damage. Hence, a treatment temperature of 41 °C for equivalent time of 180 min is put forth as hormetic heat dose for broccoli florets not only from the point of color retention and the associated delay in senescence, but also because this temperature does not cause excessive anaerobic condition in the tissue nor cellular damage.

Figure 3.8 illustrates the evolution of color retention of heat-treated broccoli florets at 37 °C (reference temperature) for equivalent time of 1,440 min; 41 °C for 180 min (hormetic heat dose); and 47 °C (super-critical zone temperature) for 12 min during storage at 10 °C for 10 days. The color change was acceptable ($\Delta E \approx 5.0$) in broccoli florets treated at 37 °C for 5 days, but it remained

nearly unchanged in the florets treated either at 41 °C or 47 °C, but the latter also exhibited off-odors and incidence of disease.

Reduction of yellowing of broccoli has been reported to be a consequence of significant reduction in the activities of chlorophyll catabolic enzymes such as chlorophyll peroxidase, chlorophyll oxidase and ACC oxidase by heat (Funamoto et al., 2002; Funamoto et al., 2003; Terai et al., 1999). The retention of color with increasing temperature may be also related to the alteration of light reflecting characteristics of vegetable surface due to air removal from the tissue (Tijskens et al., 2001b).

Excessively heated green vegetables turn olive green and then brown due, in part, to the formation of pheophytin by exchange of Mg^{2+} and H^{+}, in part, by the liberation of plant acids during heating and the lowering of tissue pH (Tijskens et al., 2001a). However, if the heating is performed in an adiabatic system as was in this study, the plant acids such as acetic acid would escape, favoring alkalization of the cells and reducing the formation of pheophytin (Gross, 1991; Lowe, 1937).

The transition temperature zone from 42° to 45 °C demarcates the beneficial and detrimental temperature zones for heat treatment of broccoli florets. Even though the temperatures in the critical zone (42-45 °C) or above 45 °C preserve the color of broccoli (Figure 3.7), they present either moderate or severe harmful effects, respectively. Excessive anaerobic respiration and moderate production of ethanol are the marks of the critical zone, while elevated levels of ethanol production and tissue damage are the marks of the super-critical temperature zone. Thus, the temperature of 41 °C for 180 min can be considered hormetic heat dose for treating broccoli florets.

3.6 Conclusions

This study establishes a methodology to determine the application of hormetic dose of heat to broccoli florets to improve their shelf life and maintain their quality during storage, and it can be extended to other fresh crops. It also allows the establishment of equivalent heat exposure time at various treatment temperatures to obtain a specific level of electrolyte leakage response, which is 5 % for broccoli. The heat treatment at 41 °C for 180 min is considered hormetic heat dose for broccoli florets from the stand point of color retention without creating excessive anaerobic conditions as observed in the critical temperature zone, nor creating severe anaerobic conditions along with cellular damage as observed in the super-critical temperature zone. This work also shows the importance of suitable temperature selection for heat treatment designed to maintain quality of fresh produce.

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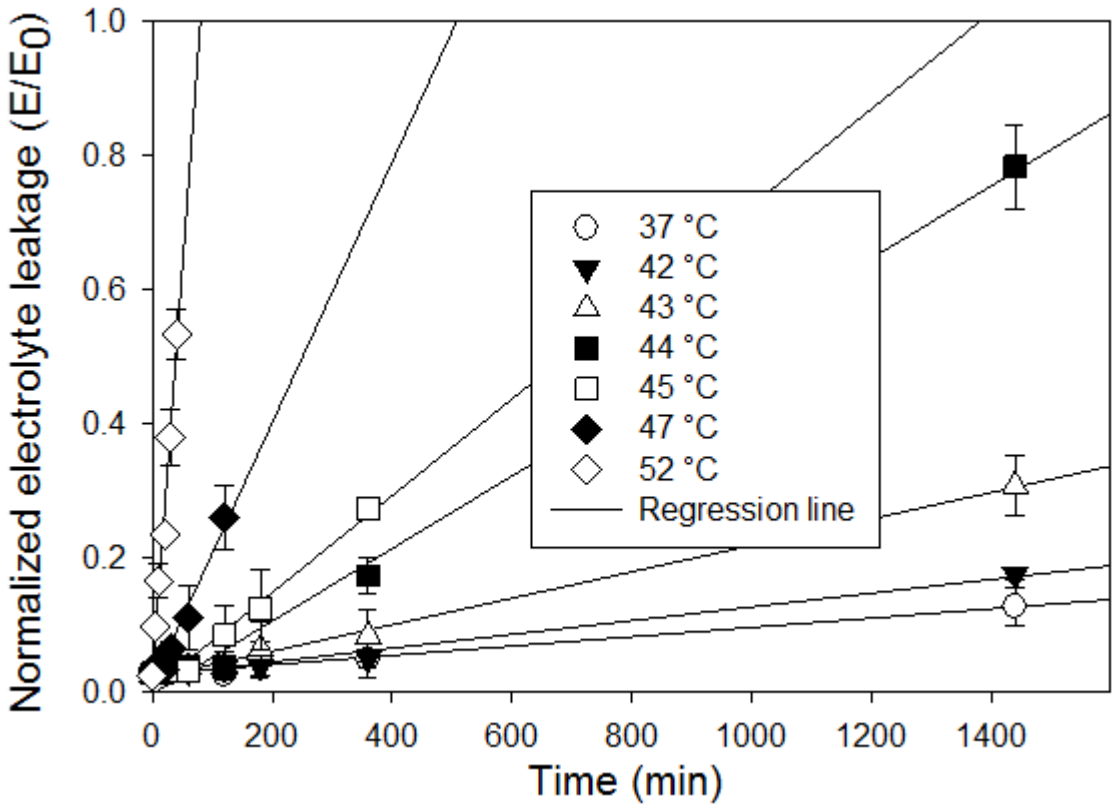

Figure 3.1 Normalized electrolyte leakage of broccoli stem tissue as a function of heating time at different temperatures. E, is the electrolyte leakage of tissue after exposure to heat (time-temperature) treatment; $E_{0,}$ is the electrolyte leakage of a completely disintegrated tissue after heating at 121 °C for 30 min. Data point is mean of 6 measurements, and the vertical bar represents standard deviation of the mean.

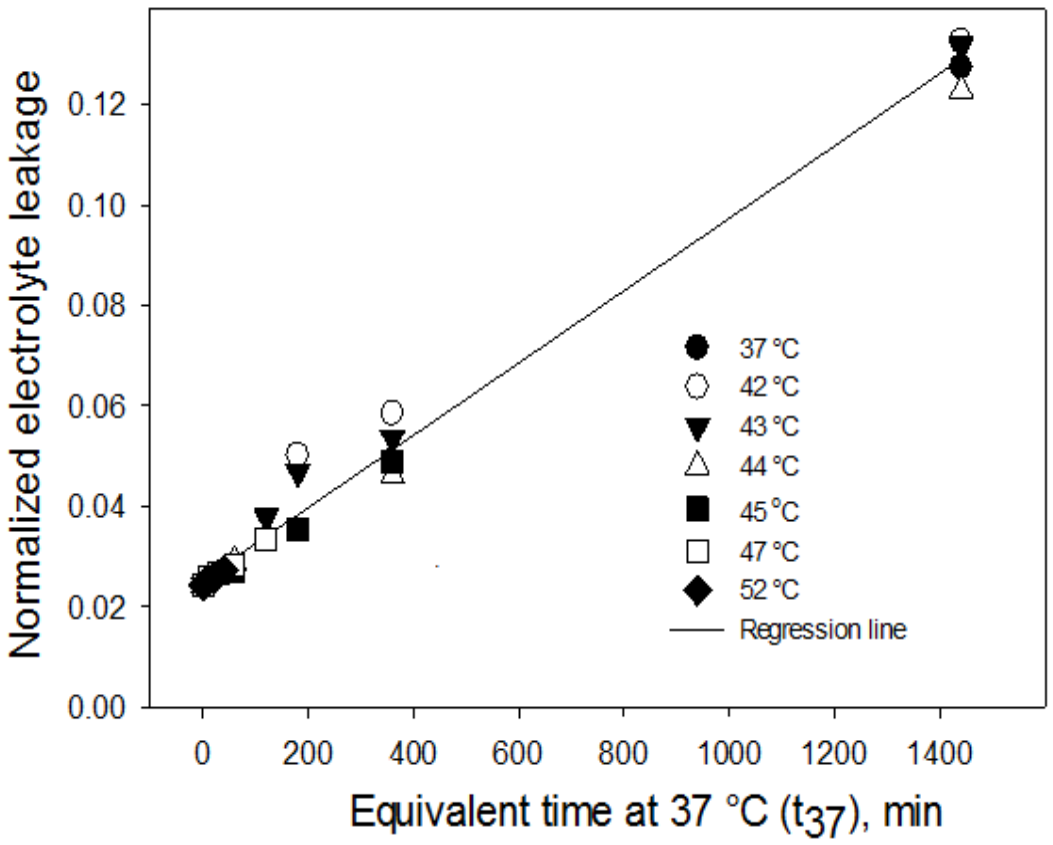

Figure 3.2 Time-temperature master curve for electrolyte leakage in broccoli tissue at reference temperature of 37 °C.

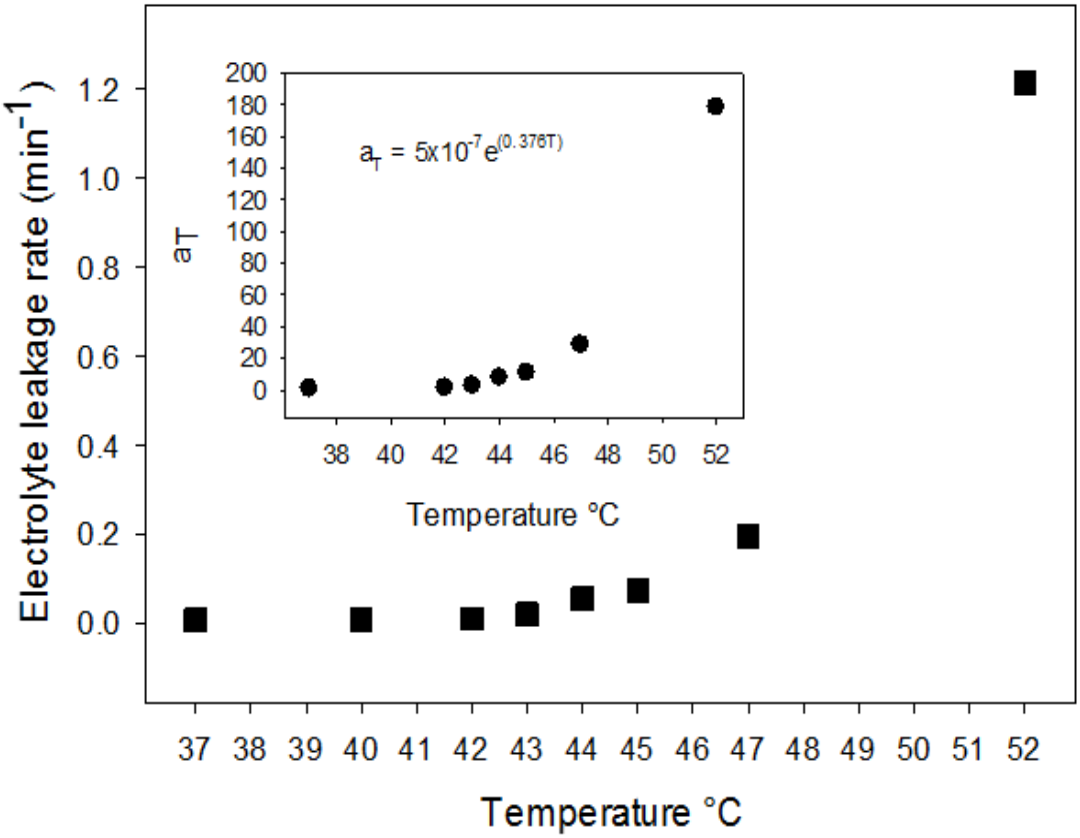

Figure 3.3 Relationship between electrolyte leakage rate and treatment temperature for broccoli florets. The electrolyte leakage rate (min^{-1}) (■) was obtained from the time course of normalized electrolyte leakage. The shift factor (a_T) (●) is the ratio of electrolyte leakage rates at different temperatures against electrolyte leakage rate at reference temperature, 37 °C.

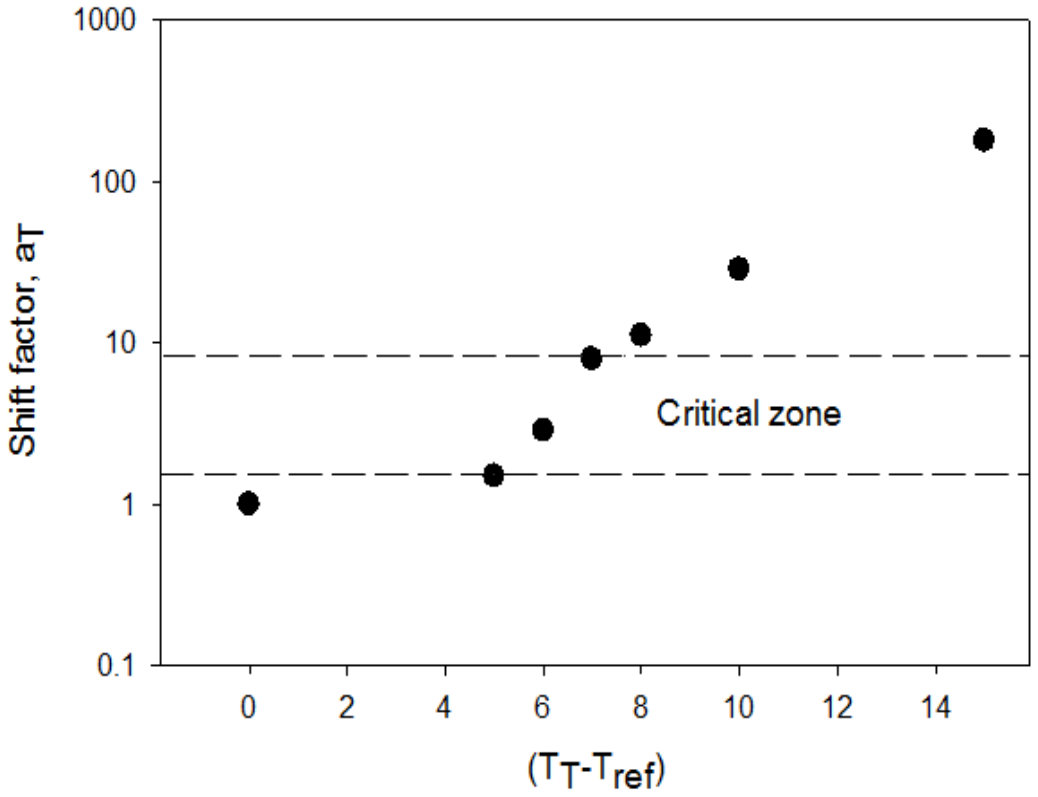

Figure 3.4 The determination of critical temperature zone for broccoli florets from the relationship between shift factor (a_T) and temperature difference ($T_T - T_{ref}$) (Equation 13).

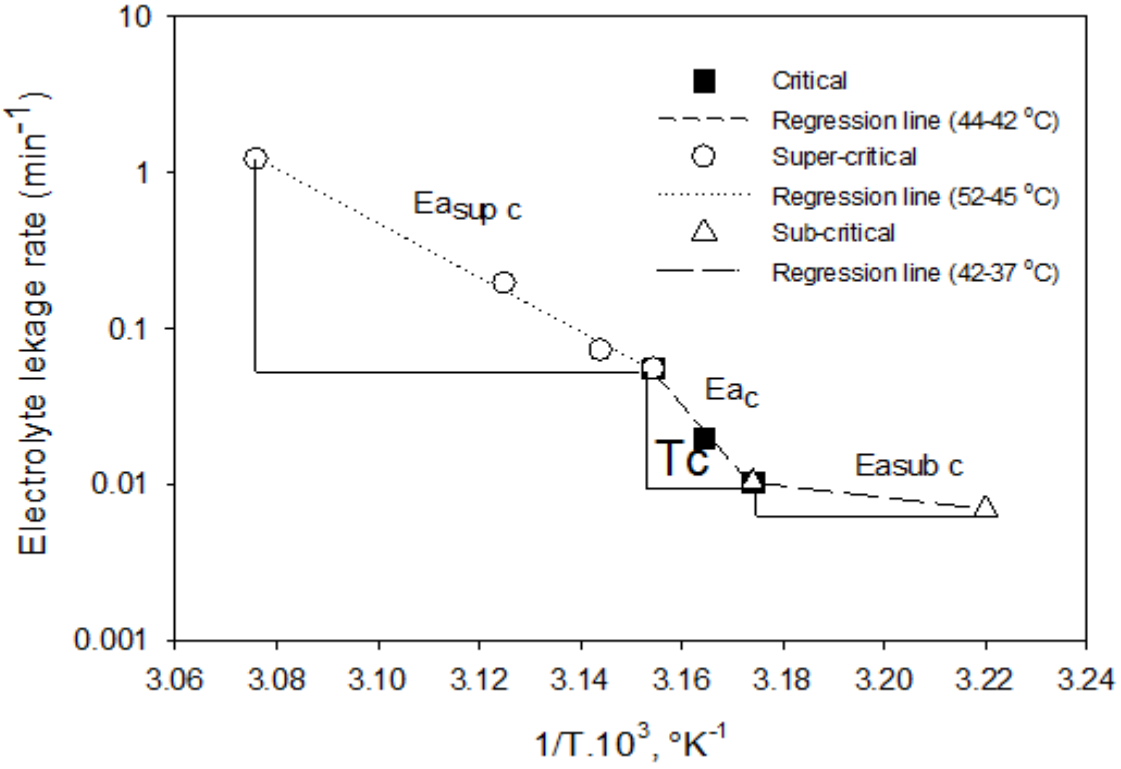

Figure 3.5 Arrhenius plot of electrolyte leakage rate against inverse treatment temperature. The activation energy ($-E_a/R$) was calculated from the slope of each temperature zone: super-critical (52-45 °C), critical (43-45 °C) and sub-critical (42-37 °C).

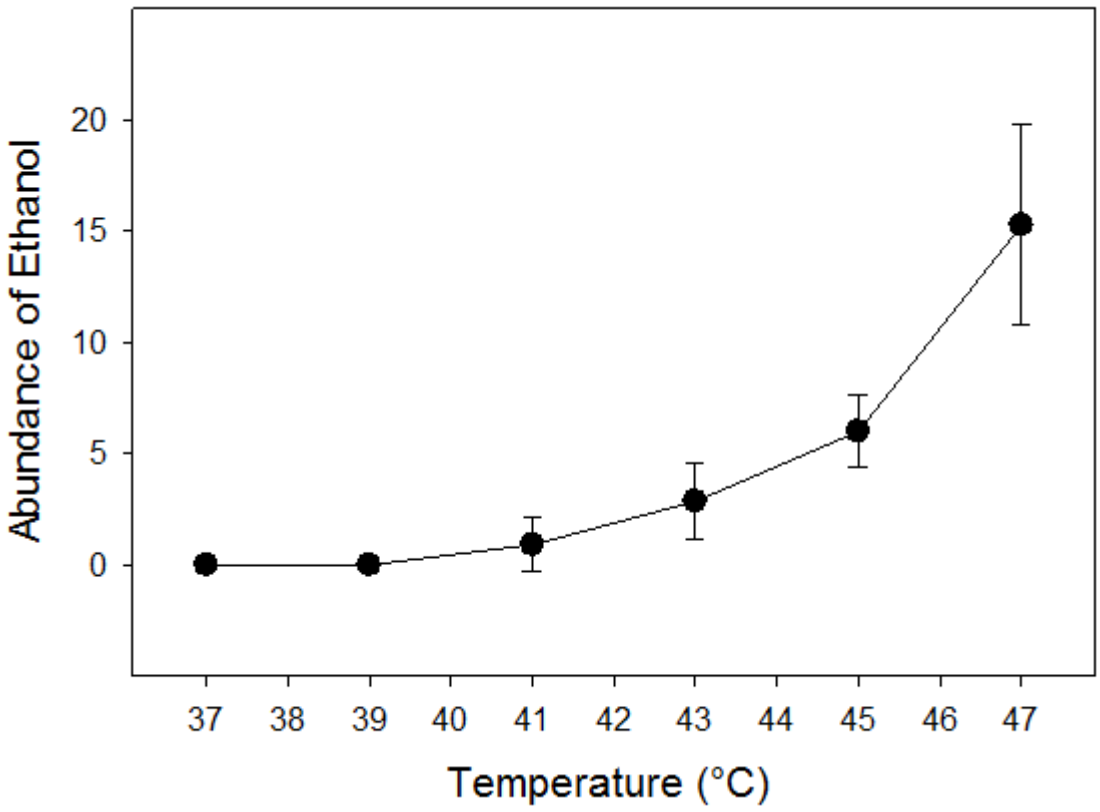

Figure 3.6 Ethanol production in broccoli florets after heating at different temperatures for equivalent times. Abundance of ethanol was detected in heat treated broccoli florets at different temperatures after 24 hours of storage at 10 °C. Data point is the mean of 3 observations, and the vertical bar represents standard deviation of the mean.

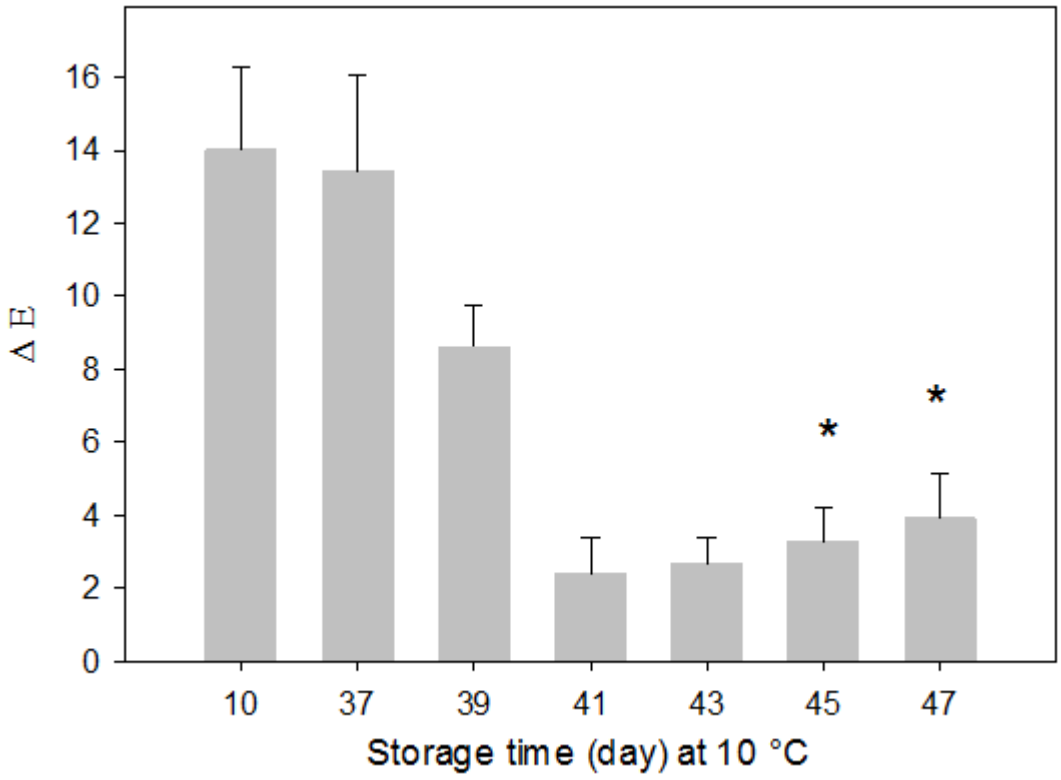

Figure 3.7 Determination of hormetic heat dose (Temperature-Equivalent time) for color retention of broccoli florets. Color changes expressed as Total color difference (ΔE) after10 days of storage at 10 °C. Data point is the mean of 6 observations, and the vertical bar represents standard deviation of the mean. (*) denotes heat damage.

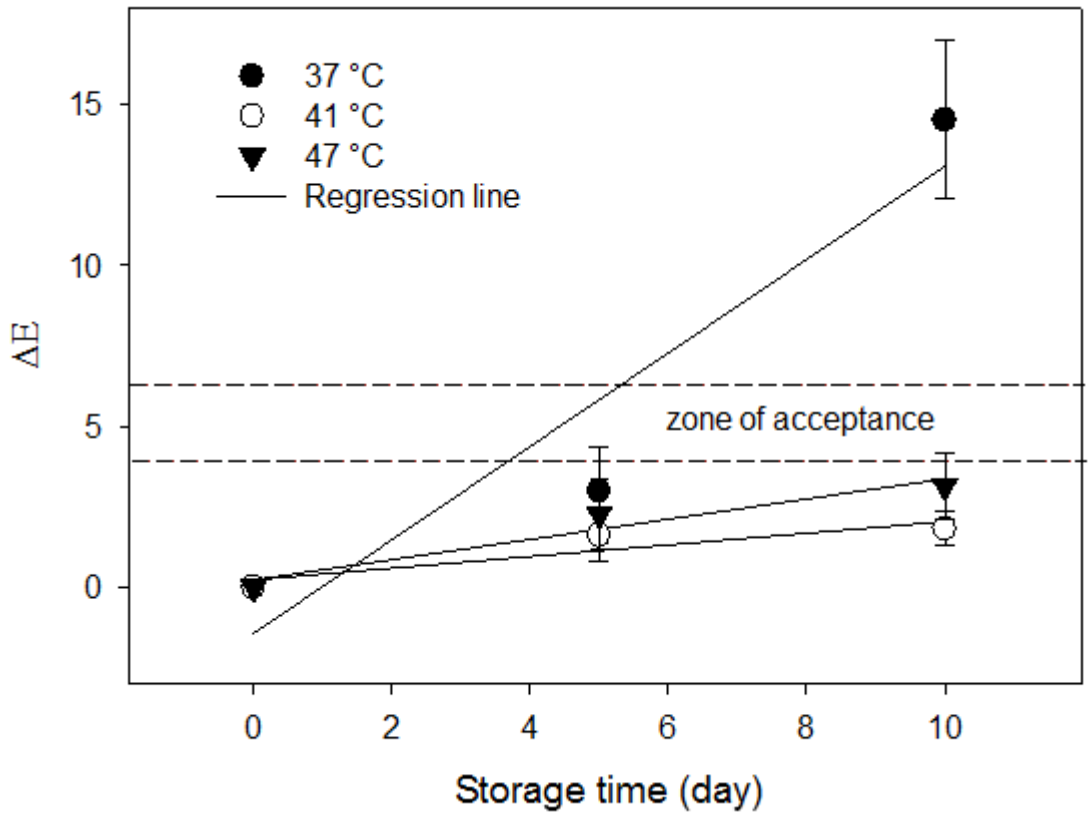

Figure 3.8 Color change in heat heated broccoli florets during storage at 10 °C for 10 days. Broccoli florets were hated at 37 °C (equivalent time, 1440 min); at 41 °C (equivalent time, 180 min) and at 47 °C (equivalent time, 12 min). The heat treatment at 41 °C/180 min represents hormetic heat dose. Data point is the mean of 6 observations, and the vertical bar represents standard deviation of the mean.

Table 3.1 Temperature activation of electrolyte leakage in broccoli tissue

Temperature (°C)	Class	Shift factor (a_T)	Activation energy, E_a (kJ.mol^{-1})	Coefficient of thermal leakage rate (α)
37	Sub-	1.00	27.9	1.50
42	critical	1.49		
43		2.85		
44	Critical	7.94	234.9	1.92
45		11.11		
47	Super-	28.57	151.0	1.08
52	critical	178.57		

a_T *= Shift factor

Chapter IV: Influence of Hormetic Heat Treatment on Quality and Health-related Secondary Metabolites of Broccoli Florets during Postharvest Storage

4.1 Résumé

L'effet du traitement de l'air chaud et humide appliqué aux fleurons de brocoli a été étudié afin de maintenir la qualité et les propriétés nutraceutiques pendant 21 jours à 4 °C. L'exposition à une dose de chaleur hormétique de 41 °C / 180 min et à la même dose de chaleur, mais livrée à une température supérieure de 47 °C pendant 12 min (dose hormétique à haute température, DHHT) ont retardé le jaunissement des fleurons par rapport aux fleurons non-chauffées pour 21 jours. La teneur en chlorophylle était également plus élevée dans les fleurons traités avec la dose de chaleur hormétique et la dose de chaleur DHHT. Il a été observé que le taux de respiration de brocoli traité à la chaleur était significativement plus élevé après exposition à la chaleur; il était 10 fois supérieur dans les fleurons exposés à la dose hormétique à celle du témoin, et 15 fois supérieure dans la DHHT par rapport au témoin au jour 0. Des différences toutefois non significatives ont été observées après 7 jours d'entreposage, même si les valeurs de CO_2 ont été inférieures dans les brocolis traités après 21 jours d'entreposage par rapport aux fleurons non traités. Des odeurs non désirables ont également été détectées dans le brocoli exposé à 47 °C / 12 min. Le contenu en glucosinolates de type indole a été significativement amélioré par les deux modes d'application des doses de chaleur, tandis que la teneur en glucoraphanine dans les fleurons a seulement augmenté avec DHHT. Une tendance similaire a été observée sur l'expression génique, où la surexpression de tryptophane N-hydroxylase (CYP79B3) était supérieure par rapport à l'expression de dihomomethionine N-hydroxylase (CYP79F1). La concentration d'acides hydroxy cinnamiques dans les fleurons a été améliorée par les deux doses de chaleur. La capacité antioxydante totale a été significativement renforcée par DHHT. De même, la surexpression de la 4-coumarate-CoA ligase (CL), chalcone synthase (CHS) et la phénylalanine N-hydroxylase (CYP79A2) a été déclenchée par la forte dose de chaleur. Les résultats indiquent que le traitement avec la dose de chaleur hormétique peut améliorer le contenu de composés phytochimiques dans les fleurons de brocoli pendant l'entreposage; l'application de chaleur à 41 °C est nécessaire pour maintenir leur qualité, bien que l'amélioration de composés phytochimiques a été quelque peu limité.

4.2 Abstract

The effect of moist hot air treatment applied to broccoli florets was studied in order to maintain quality and nutraceutical properties during postharvest storage at 4 °C. Exposure to a hormetic heat dose of 41°C/180 min and to the same heat dose but delivered at higher temperature of 47 °C for 12 min (Hormetic dose at high temperature, HDHT) delayed yellowing compared with non-heated florets for 21 days. Chlorophyll content was also higher in florets treated with hormetic heat dose and HDHT heat dose. It was observed that respiration rate of heat treated broccoli was significantly higher after exposure to treatment; with hormetic dose it was 10 times greater than that of the control, and it was 15 times greater compared with the control on day 0. However non-significant differences were observed after 7 days of storage, even though CO_2 values were lower in the treated broccoli after 21 days of storage compared with non-heated florets. Off-odors were also detected on broccoli exposed to 47 °C/12 min. Titers of indole-type glucosinolates were significantly enhanced by both modes of application of the heat doses, while the glucoraphanin content of florets only increased with the HDHT dose. A similar pattern was observed on gene expression, where overexpression of tryptophan N-hydroxylase (CYP79B3) was superior compared with the expression of dihomomethionine N-hydroxylase (CYP79F1). Titers of hydroxy-cinnamic acids of florets were improved by both doses of heat. However, the total antioxidant capacity was significantly enhanced by the HDHT. Similarly, overexpression of coumarate ligase (CoL), chalcone synthase (CHS) and phenylalanine N-hydroxylase (CYP79A2) was triggered by the high dose of heat. The results indicate treatment with the hormetic heat dose can enhance the content of phytochemicals in broccoli florets during the storage; the application of heat at 41 °C is required to maintain their quality, although the enhancement of phytochemicals was somewhat subdued.

Keywords: Broccoi, heat treatment, glucosinolates, hydroxy-cinnamic acids, respiration

4.3 Introduction

Broccoli (*Brassica oleracea* var. Italica) is a source of several vitamins (A, C, E), calcium, carotenoids, terpenes, indoles, isothyocianates, flavonoids and glucosinolates (Nestle, 1998). In fact, it is often identified as the vegetable consumed most frequently for health reasons, including cancer prevention (Schouten et al., 2009b). The most important phytochemicals in broccoli, are flavonoids and glucosinolates (Moreno et al., 2006).. Flavonoids are derived from phenylalanine through the phenylpropanoid or acetate-malonate pathway catalyzed by three important enzymes, phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) and flavonoid monooxygenase (F3H1) (Weston and Mathesius, 2013). Whereas the synthesis of glucosinolates includes the conversion of amino acids to aldoximes which is catalyzed by cytochromes P450 from CYP79 family (Mikkelsen et al., 2002).

Broccoli deteriorates rapidly due to its high respiration rate that contributes to its senescence (Finger et al., 1999; Page et al., 2001). The most evident indicator of senescence in broccoli, and also an important quality factor, is yellowing of the florets (King and Morris, 1994b; Tian et al., 1997; Toivonen and Forney, 2004). Nutrient and phytochemical contents are also affected with the progress of senescence. Quality changes such as weight loss and chlorophyll degradation occur with corresponding reduction of ascorbic acid and total phenolic compounds in cold-stored broccoli (Serrano et al., 2006). Similarly, the loss of glucoraphanin, the precursor of 4-methylsulfinylbutyl isothiocyanate (sulforaphane) has been observed during the first 3 days at 20 °C of storage of broccoli (Rangkadilok et al., 2002).

In order to delay the senescence of broccoli, low temperature 0 °C (32 °F) along with high relative humidity (98 to 100 %), and modified atmospheres (1 to 2 % O_2 + 5 to 10 % CO_2), are used during the postharvest storage of broccoli florets. (Toivonen and Forney, 2004). They are satisfactory conditions to maintain quality of broccoli florets for up to 3 weeks of storage.

In addition to the above preservation strategies, other postharvest treatments have been applied to broccoli in order to delay senescence and maintain its quality. The most effective have been heat treatment with temperatures between 41-52 °C (Duarte-Sierra et al., 2012a; Forney, 1995; Funamoto et al., 2002); ethanol fumigation at 2500 ppm (Corcuff et al., 1996); UV-C light with doses in the range of 1.2-10 $kJ.m^{-2}$ (Costa et al., 2006; Duarte-Sierra et al., 2012b; Lemoine et al., 2007) and UV-B light, ranging from 4.4 to 19.0 $kJ.m^{-2}$ (Aiamla-or et al., 2010; Aiamla-or et al., 2012).

The potential mechanisms attributed the beneficial effect of heat treatment in the delay of ripening and/or senescence and the associated changes in tissue softening of fresh produce may

involve induction of different mechanisms. They include the induction of heat shock protein (HSP) (Paull and Chen, 2000); the inhibition of ethylene production as the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene becomes vulnerable is also vulnerable to higher ambient temperatures (30 to 40 °C) (Yu et al., 1980); the partial inactivation of hydrolytic cell wall enzymes as polygalacturonase, β-galactosidase, endo-1,4-β-d-glucanase (EGase) and β-xylosidase at higher ambient temperature (45 °C) (Martínez and Civello, 2008). The cross protection of heat treatment against chilling injury is attributed to the synthesis and accumulation of HSP proteins (Paull and Chen, 2000).

Heat treatment either by hot water or hot air was shown to delay yellowing of broccoli (Forney, 1995; Lemoine et al., 2009; Tian et al., 1996). The immersion of broccoli at 50 °C in water for two minutes was found to be the most effective treatment to slow-down yellowing and decay (Forney, 1995). The former may be in part, due to reduced chlorophyllase activity (Funamoto et al., 2002). However, negative effects of heat treatment can occur when high temperatures and longer exposure times are used. Off-odors development and increased production of ethanol were found in treated broccoli using hot water treatment at 52 °C for 3 min (Forney and Jordan, 1998). In addition to quality maintenance, treatment of broccoli with UV-B light and the signal molecules, methyl jasmonate and salicylic acid, have improved the phytochemical composition of broccoli (Mewis et al., 2012; Pérez-Balibrea et al., 2011). Nonetheless, the elicitation and accumulation of phytochemicals by heat has not been completely explored, especially, on health-related secondary metabolites such as glucosinolates and flavonoids in broccoli.

The existence of the biological phenomenon, known as hormesis in living organisms, where low doses of harmful agents or stressors elicit beneficial or adaptive responses is generally appreciated (Chapman, 2002; Luckey, 1980). The occurrence of this phenomenon has been shown in postharvest crops in response to UV-C, mainly regarding disease resistance and delayed ripening. In this work, we have examined the effect of hormetic heat dose applied at two temperatures (41 °C/180 min and 47 °C/12 min) on quality of broccoli as well as on health-related phytochemical composition of broccoli florets stored at 4 °C.

4.4 Materials and methods

4.4.1 Broccoli

Freshly harvested broccoli (*Brassica oleraceae* L. var. Italica 'Diplomat') heads were obtained from a commercial farm (Ile d'Orléans, Québec, Canada). Florets (300g) of uniform size (approximately, 7 cm) were separated from heads and randomly arranged in small plastic punnets of 500 mL. The punnets were placed in plastic containers of 5 L with perforations for ventilation, and containing a layer of water at the bottom to maintain high humidity (98-100%), and the containers were stored inside a controlled chamber overnight at 4 °C.

4.4.2 Treatments

Heat treatments were performed in a closed chamber of 2 m^3 with continuous air circulation equipped with controlled air heating system and a humidy control system using steam injection for saturation of air. Broccoli florets were exposed to 41 °C for 180 min, the hormetic dose; and to 47 °C for 12 min, hormetic heat dose at high temperature (HDHT). Following heat treatment, the treated and control florets were cooled rapidly in a laminar flow cabinet at 0 °C for 10 min, and were subsequently stored in a controlled chamber at 4 °C/90% RH for 21 days.

4.4.3 Respiration rate of broccoli florets

The respiration rate of broccoli florets was determined as described elsewhere in the thesis in detail, in section 2.3.4.

4.4.4 Color measurement of broccoli florets

Color readings of twelve florets per dose were performed with a Chroma meter (Minolta CR200, Osaka, Japan) equipped with an 8 mm measuring head and a D 65 illuminant. The meter was calibrated using the manufacturer´s Standard white plate. Color changes were quantified in the L*, a* and b* color space. The hue angle (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 $b > 0$.

4.4.4 Chemical assays

The total phenolic content, total flavonoid content, reduced and total ascorbic acid contents, and the total antioxidant capacity were determined by the methods described elsewhere in detail in section 2.3.5.1, 2.3.5.2, 2.3.5.3, 2.3.5.4, respectively.

4.4.4.1 Chlorophyll content

The extraction of chlorophyll was performed following the method reported by Warren (2008) on lyophilized broccoli samples (0.01g) with 1 mL of methanol and shaking for 30 sec by means of a vortex (Genie 2, Vernon Hills, IL, USA). Samples were centrifuged 2 min at 11,270 g (Microcentrifuge 5418, Hamburg, Germany), and the supernatant was transferred to another microtube. Pellet was re-extracted by adding 1 mL of ethanol to the pellet under agitation, and centrifuged as previously described. The two supernatants were pooled together and used for spectrophotometric assay. Measurements were done by adding 200 μL of samples or blanks into a 96-well flat bottom polystyrene plate. The absorbance of samples was measured on a spectrophotometer (Benchmark Plus, Bio-Rad, Philadelphia, USA). The absorbance at 652 and 665 nm was converted into 1 cm path length using the measured path length of 0.58 cm by:

$$A_{652,1cm} = \frac{(A_{652,microplate} - blank)}{0.58cm} \quad (1)$$

$$A_{665,1cm} = \frac{(A_{665,microplate} - blank)}{0.58cm} \quad (2)$$

Chlorophyll concentration was calculated from 1-cm corrected path length with the following formulae:

$$Chla(\mu g/\,mL) = -8.09A_{652,1cm} + 16.51A_{665,1cm} \quad (3)$$

$$Chlb(\mu g\,/\,mL) = 27.44A_{652,1cm} - 12.16A_{665,1cm} \quad (4)$$

Total chlorophyll was the sum of Chl a and Chl b. Each measurement was performed in triplicates within four storage intervals at 0, 7, 14 and 21 days at 4 °C.

4.4.5 Glucosinolates and hydroxycinnamic acid analysis

The glucosinolates and hydroxycinnamic acids were assayed by the methods described elsewhere in detail (section 2.3.6).

4.4.6 Gene expression

The gene expression analysis section was determined by methods described elsewhere in detail in section 2.3.8.

4.4.7 Statistical analysis

The experiment was set as a complete randomized design and the data were analyzed by one-way analysis of variance (one-way ANOVA) using a significant level of 0.05. Least significant difference test at the same significant level was done when the analysis of variance found significant differences. The statistical analysis was executed using the statistical analysis system version 9.3 (SAS Institute Inc. 2011. Base SAS® 9.3 Procedures Guide. Cary, NC, USA). For chemical analysis, an average of equidistant time periods (0, 7, 14 and 21 days) was made for total phenols, flavonoids, ascorbic acid, and ORAC assay.

4.5 Results and Discussion

Based on membrane integrity of broccoli florets from previous results (Chapter III), hormetic heat dose was applied at two temperatures: 1) 41 °C applied for 180 min and 2) 47 °C for 12 min (HDTH). Both doses were equivalent, 41 °C being within the sub-critical zone (<42 °C), and 47 °C in the upper-critical zone (> 45 °C) as described in the previous chapter as well as elsewhere (Duarte-Sierra et al., 2012a).

The aim of this work was to characterize the qualitative, physiological and nutraceutical properties of broccoli florets exposed florets to heat. It was found that the two heat treatments delayed yellowing, modified respiration rates as well as titers of secondary metabolites and the gene expression pattern of broccoli florets during the storage.

4.5.1 Physiological and Biochemical Characteristics

4.5.1.1 Respiration rate and weight loss

After exposure to heat, the respiration rate of florets increased substantially and high respiration rate was observed in both hormetic and HDHT treatments doses on day 0, about six hours after treatment (Figure 4.1a). On this day, significant differences were observed among the

treatments, the respiration rate of the control florets was the lowest at 22.4 mL of $CO_2.kg^{-1}h^{-1}$, followed by hormetic dose at 169.5 and HDHT at 284.7 mL of $CO_2.kg^{-1}h^{-1}$. On day seven, respiration rates of the florets reached steady levels, but the respiration rate of the hormetic dose group remained lower compared with the control and the HDHT treatment (47 °C/12min). At this point, respiration rates of florets were 17.5, 10.9 and 16.1 mL of $CO_2.kg^{-1}h^{-1}$ for the control, the hormetic and the HDHT, respectively. Respiration rates of heat treated florets remained lower compared with the control from day 14 until the end of storage of 21 days, when respiration rates of the florets were 21, 8.3 and 9.5 mL of $CO_2.kg^{-1}h^{-1}$ for the control, the hormetic and the HDHT groups, respectively.

The rapid increase of respiration of florets in response to heat treatment is a consequence of a general adaptation process, which requires large amounts of energy in form of ATP for synthesis of protective or defense compounds. In plants exposed to heat, a general response is the production of heat-shock proteins (HSP). On the other hand, the high respiration rate can also reduce shelf-life of the heated produce (Saltveit, 2000). Despite the elevated production of CO_2 in florets immediately after heat treatment, respiration rate values of florets remained lower compared with control from day 7 until the end of storage. Similar observations have been previously reported in apples (Lurie and Klein, 1990) and mangoes (Mitcham and McDonald, 1993), where respiration rates of heat treated tissue were lower compared with non-exposed plant tissue. These authors attribute lower rates of respiration as a consequence of the heating process resulting in certain damage to the respiratory apparatus of the tissue. The generation and accumulation of ethanol previously described (Chapter III) as a result of hypoxic conditions in the heat treated tissue (decreased oxygen solubility in water increase with temperature) could play a role in the damage to the respiratory system.

Even though respiration rate of non-treated florets was lower at the beginning of storage, at the end it was higher compared with exposed-heat treatments after 21 days of storage. The latter is probably due to the development of fungal infection during the storage, since florets had not been disinfected to avoid changes on secondary metabolism that could interfere the effect of heat.

On the other hand, respiration rate may affect ethylene production. An increase in CO_2 production is generally followed by a decrease of ethylene synthesis (Klein and Lurie, 1992). Hot air was shown to inhibit the ripening of climacteric fruits by the inactivation of 1-aminocyclopropane-1-carboxylic acid (ACC) (Lurie, 1998). Broccoli is not climacteric, but is sensitive to ethylene action (Toivonen and Forney, 2004), and hot water dips have delayed yellowing of florets during the postharvest storage (Forney, 1995; Tian et al., 1996). Although, ethylene measurements were not

carried out in this study, evidence of high initial CO_2 production and delay of yellowing by the hormetic dose and HDHT were observed, giving credence to the hypothesis that heat may inhibit ethylene production.

In addition to the increase of respiration rate, broccoli florets exposed to heat, and particularly those exposed to the HDHT, suffered from significant weight loss (Figure 4.1b). In contrast, broccoli florets exposed to the hormetic dose of heat exhibited a lower level of weight loss compared with the control. An analogous observation made by Williams et al. (1994) in oranges, showed that fruit immersed in hot water (53 °C) had greatest water loss compared with fruit immersed at 45 °C. More interestingly, oranges treated at 45 °C retained more moisture compared with the non-exposed fruit, presumably due to smoother spreading of softened or molten cuticular waxes contributing to reduced loss of water from the tissue. Waxes are also present on the surface of broccoli, and their melting may have protected the tissue from desiccation, as observed with oranges. However, the high respiration rate of florets treated at 47 °C/12min could have contributed to a higher transpiration, overriding the effect of molten wax protection. The heat produced by respiration can increase WVPD contributing to increased evaporation (Ben-Yehoshua and Rodov, 2002). In addition, the released of volatile plant acids and other volatiles generated at this temperature could also aggravate weight loss (Grote et al., 2013).

4.5.1.2 Color and chlorophyll content in florets

Delay of yellowing of florets by heat treatments was evident after 21 days of storage. Likewise, the amounts of chlorophyll on both treatments were different from the control. No significant differences were observed between the non-exposed and heat-treated broccoli florets during the first 14 days of storage at 4 °C. Nevertheless, it was clear that after 21 days of storage, heat-treated florets at 47 °C/12 min or at 41 °C/180 min showed higher H° values compared with the control (Figure 4.2a). At this point, H° values were 114.7 for non-exposed florets, 128.1 for florets exposed to 41 °C/180 min and 130.5 for broccoli exposed to 47 °C/12 min, demonstrating that delay in yellowing of the florets by heat treatments compared with non-exposed florets. Similarly, at the end of the storage, of 21 days, the chlorophyll content of heat exposed broccoli was significantly higher compared with the non-heated florets (Figure 4.2b). Values were 1.8, 3.0 and 3.1 mg/g DW for control, hormetic dose and HDHT, respectively.

A closer analysis of color revealed significant differences between 41 °C/180 and 47 °C/12 min. In fact, by visual examination, broccoli florets treated at 47 °C were greener compared with

those treated at 41 °C. The improved of green color due to heat treatment has been reported for vegetables. For instance, the green color of asparagus increased with heat treatment between 70 and 98 °C (Lau et al., 2000). The index (-a*/b*), which express the green color, on heated green beans in the range of 40-90 °C was constantly superior immediately after heat treatment, however it decreased by accumulative exposure time. Also, the increase of color has been found to be proportional to the increase of temperature, (-a*/b*) was higher at 90 °C compared with 80, 70, 60, 50 or 40 °C (Tijskens et al., 2001b). The effect of the HDHT on broccoli florets also improved the green-blue color of florets. The enhancement of color with treatment temperature may be a result of the alteration of surface reflecting properties of vegetables due to air removal between cells.

In spite of the enhancement of greenness by the exposure of broccoli to heat at 47° for 12 min, a deleterious effect was evident by the perception of off-odors after treatment; which was not present in florets treated with 41 °C/180 min. A comparable observation was made when broccoli was dipped in hot water at > 50 °C resulting in off-odors generation and ethanol production (Forney, 1995; Forney and Jordan, 1998). These authors suggested that ethanol production was due to the conversion of pyruvate to acetaldehyde by prevailing anaerobic conditions caused by low oxygen solubility at high temperatures, coupled with high respiration depleting oxygen in the cells. They also suggested that even if the visual quality of florets was adequate when exposed to the high dose of heat, cellular damage may have occurred by the creation of severe anaerobic conditions. Anaerobic conditions can accelerate senescence due to the low amount of energy as a consequence of reduced ATP production and protein turnover, as well as off-odors in broccoli (De Vries, 1975; Forney et al., 1991a)

4.5.1.3 Phenylpropanoid compounds, ascorbic acid and ORAC

Florets exposed to heat showed no significant differences over the storage at 4 °C in the content of, total phenols, total flavonoids or total ascorbic acid (Table 4.1). However, ORAC (oxygen radical absorbance capacity) of florets was affected by HDHT compared with non-exposed broccoli florets. Moreover, ORAC of broccoli exposed to the high dose was 37 % superior to the control and 19 % superior compared with the hormetic dose of heat (Table 4.1). With heat application at 47 °C/12 min, the ORAC value increased during storage along with an increase in the level of oxidized ascorbic acid (Table 4.1), presumably by the increased activity of ascorbate oxidases and ROS generation in response to heat stress (Mittler et al., 2012)

4.5.2 Secondary metabolites analysis

Glucosinolates and hydroxycinnamic acids are secondary metabolites characteristic of *Brassicas* which participate in defense responses to pathogens, insects and wounding. Heat, as well as other biotic and abiotic stresses affects the stability of various cellular structures including membranes and proteins causing a state of imbalance. This imbalance leads to the generation and accumulation of undesirable products such as the reactive oxygen species (ROS), a response consequent of heat and other stresses (Mittler, 2002). One of the most important protective responses of the plants being the enhancement of secondary metabolites.

Total glucobrassicins (sum of glucobrassicin, neoglucobrassicin, 4-methoxyglucobrassicin and 4-hydroxyglucobrassicin) were significantly ($p<0.05$) enhanced by both types of heat application. After 14 days of storage, the concentration of all glucobrassicins in florets treated with HDHT was 21 % superior compared with untreated florets, and 11 % superior compared with florets treated at 41 °C/180 min (Figure 4.3a). The glucoraphanin content of florets was only affected by HDHT during the first four days of storage, but no difference was observed between treatments (Figure 4.3b). In general, tryptophan derived indole-glucosinolates are more enhanced by biotic stresses than aliphatic glucosinolates (Brader et al., 2001). The difference in gene expression between tryptophan N-hydroxylase (CYP79B3) and dihomomethionine N-hydroxylase (CYP79F1) also supports this observation with heat.

Titer of glucobrassicin was relatively constant during the storage of florets compared with the titer of the control which started to decrease from day 4 on (Figure 4.3c). A similar pattern was observed with neoglucobrassicin. Its concentration in heat-exposed (47 °C/12 min and 41 °C/180 min) was significantly higher ($p < 0.05$) compared with the untreated florets throughout the storage period (Figure 4.3d). However, other derivatives of glucobrassicin including 4-methoxyglucobrassicin (Figure 4.3e) and 4-hydroxyglucobrassicin (Figure 4.3f) showed an accumulation pattern different from the other glucobrassicins, although these two were relatively minor glucobrassicins. The concentration of the two glucobrassicin derivatives, 4-hydroxy and 4-methoxy glucobrassicins, considerably increased in the untreated florets during the storage, where 4-hydroxyglucobrassicin showed significant change during the storage. The accumulation of 4-hydroxyglucobrassicin was seen in *B. rapa* upon the challenge with *F. oxysporum* (Abdel-Farid et al., 2010). Likewise, 4-methoxyglucobrassicin appears to be necessary for the resistance to pathogens and callose formation in *Arabidopsis* (Clay et al., 2009). Therefore, the gradual induction of these compounds

over the storage in untreated florets raises the question whether there was any development of fungal disease in untreated florets , although the infection, if any, was not apparent.

The steady increase of both 4-methoxyglucobrassicin and 4-hydroxyglucobrassicin in the untreated florets during storage, as opposed to the evolution in the heat treated florets, may support the hypothesis that the untreated florets might have been susceptible to infection. It may also suggest that these two derivatives of glucobrassicin may be more effective compounds against infection than the glucosinolates, glucobrassicin, neoglucobrassicin and glucoraphanin, which tend to decrease towards the end of storage in all treatment groups.

Similar to glucosinolates, it was observed that the HDHT significantly enhanced the level of hydroxycinnamic acids in broccoli. The total HCA increased over the storage including the untreated florets, but greater increases were observed with HDHT dose followed by the hormetic heat dose (Figure 4.4), although the increase in the untreated florets was sharper after 7 days of storage. Sharp increases of 1,2-disynapoyl gentiobiose, 1,2,2'-trisinapoyl gentiobiose and 1,2-diferuloyl gentiobiose were also observed in the untreated florets after 7 days of storage similar to the total HCA and their levels reached close to levels observed for in florets treated with HDTD dose, whereas the other HCA, 1-sinapoyl-2-feruloyl gentibiose (the major HCA), 1,2-diferuloyl gentiobiose and sinapoyl-diferuloyl gentibiose, showed small increase during storage, presumably with progress in senescence of the florets. However, the sharp increases in the former HCA may not be attributable solely to senescence but may be in response to another event occurring simultaneously, such as the infection of the untreated florets, as mentioned before; and by extension, they accumulate in response to infection, and they may be more effective HCA exhibiting elevated antimicrobial activity against diseases. The induction of HCA was concomitant with the over expression of phenylpropanoid-related enzymes coumarate ligase (CoL); chalcone synthase (CHS) and phenylalanine N-hydroxylase (CYP79A2) (Figure 4.5). Usually, the induction of chalcone synthase along with phenylalanine ammonia-lyase precedes increases in the levels of constitutive phenols, which provide adequate substrates for the synthesis of fungitoxic compounds such as quinones (Lattanzio et al., 2006).

The initial contents of glucosinolates or HCA in heat treated florets either with hormetic heat dose or HDHT dose were consistently superior to those in the untreated florets. It is not clear as to the contributing factors for this observation. The enhanced synthetic enzyme activities and mobility of the substrates at higher ambient temperatures during heat treatment may play a part in higher intial

values of the compounds. In addition, the improved extractability of the compounds from heat treated tissue could contribute in part. This possibility has been observed on water-blanched vegetables (Kaiser et al., 2013).

4.6 Conclusions

Hormetic heat dose delivered at 41 °C and 47 °C showed different effects on quality and phytochemical contents of broccoli florets during the postharvest storage at 4 °C. The enhanced level of glucosinolates and HCA by heat treatment is likely the response to the accumulation of ROS in the tissue. The accumulation of 4-methoxyglucobrassicin, 4-hydroxyglucobrassicin, 1,2-disynalpoyl-2-feruloyl gentiobiose and 1,2,2'–trisinapoyl gentiobiose in the untreated florets may be in response to infection, and these compounds may likely be effective antimicrobials. The higher temperature treatment showed higher levels of glucosinolates and HCA during storage, the storability factors such as weight loss and generation of off-odors can be limiting, although the greenness of the florets were quite acceptable. The treatment at 41 °C maintained the quality of the florets, although the elevation of the phytochemicals was moderate, and at the least maintained at their initial levels throughout the storage period. Thus, the application of hormetic heat dose at 41 °C may be beneficial in terms of both quality attributes as well as phytochemical content.

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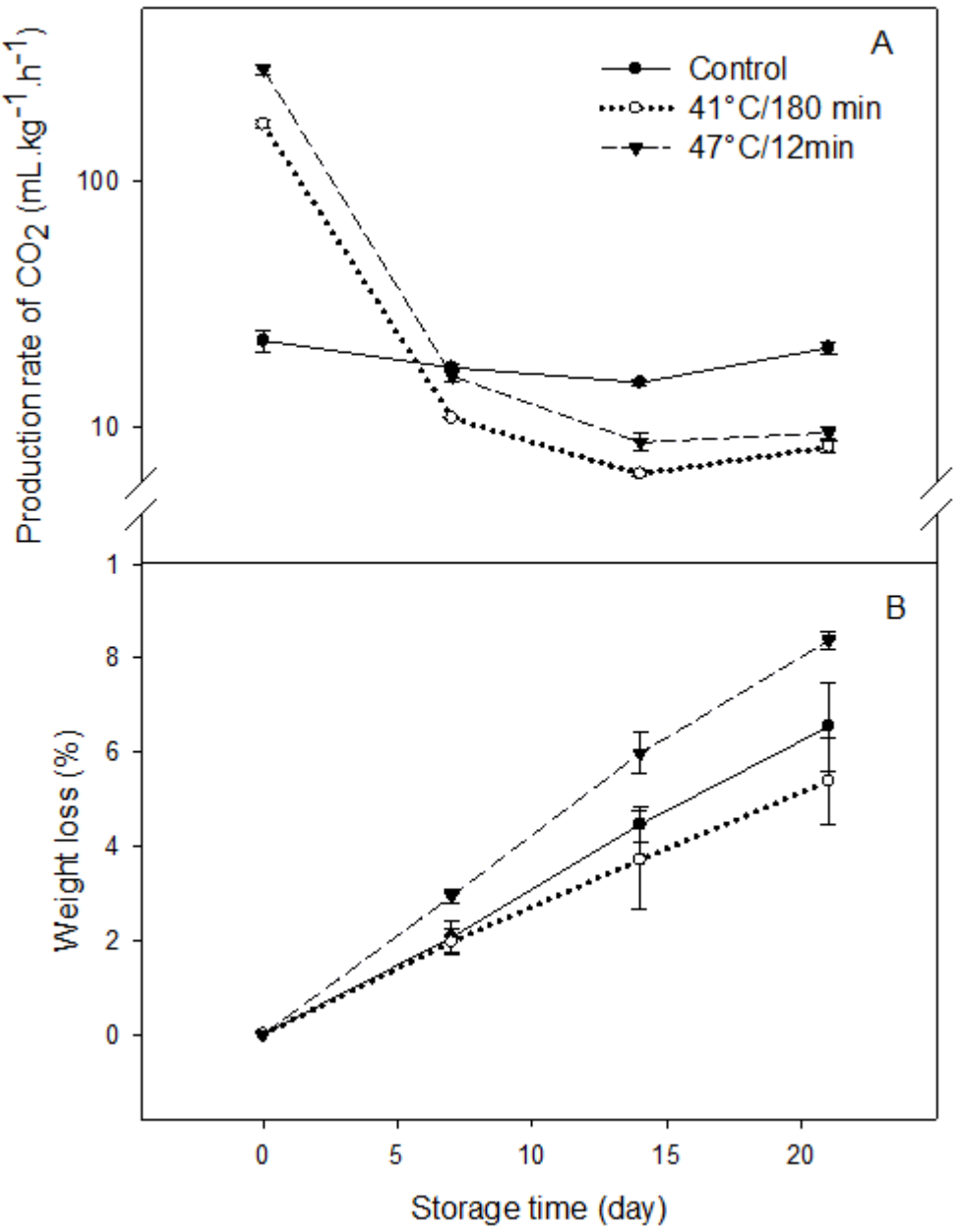

Figure 4.1 Evolution of respiration rate and weight loss of heat treated broccoli florets during the storage at 4 °C. Production of carbon dioxide (A) and weight loss (B) of florets were determined in florets exposed to three different heat doses: (●), control; (○), 41 °C/180 min (hormetic dose); and (▼), 47 °C/12min (HDHT) during 21 days of storage in darkness at 4 °C. Vertical bars represent standard deviation of the mean, n = 4.

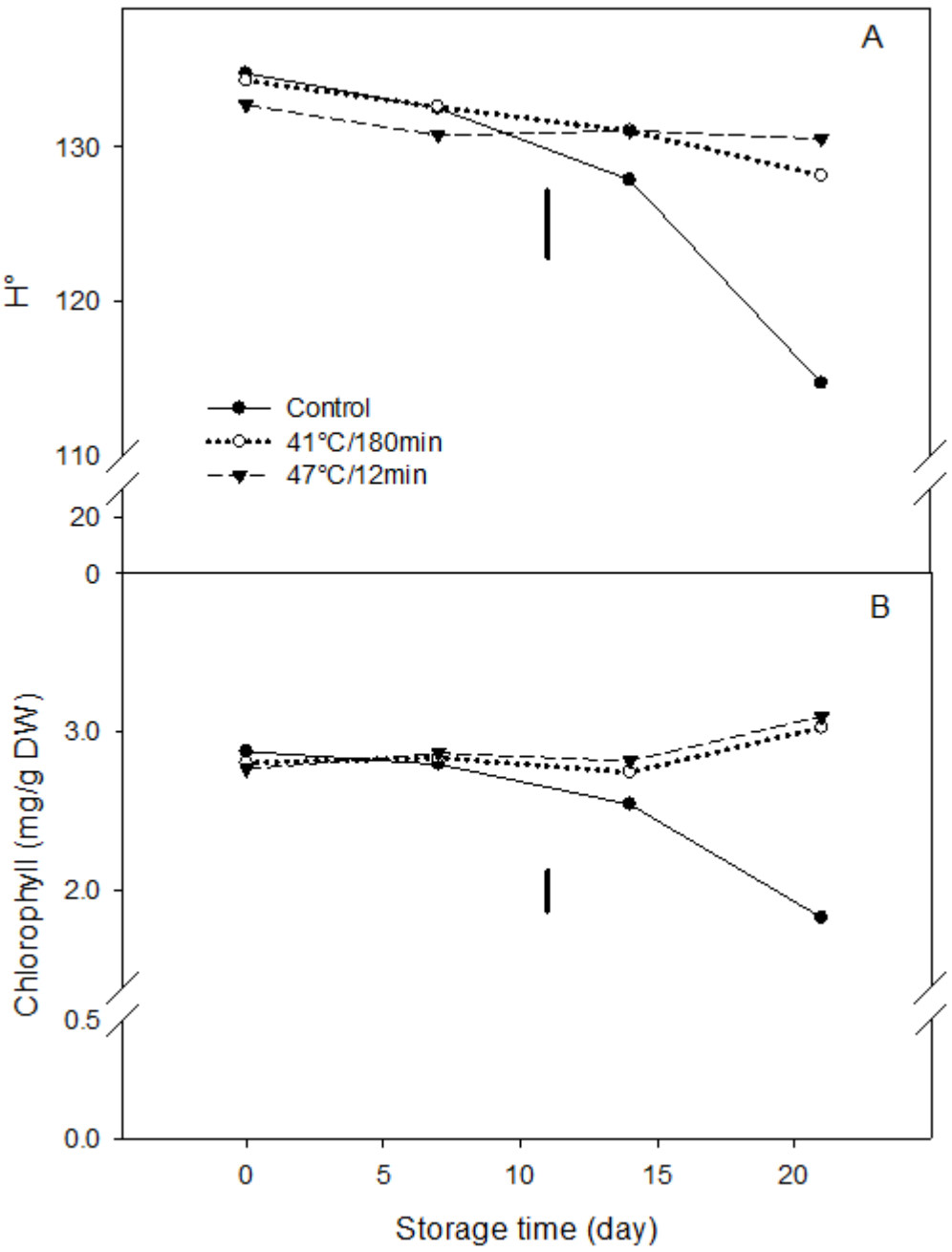

Figure 4.2 Evolution of color and chlorophyll content of heat treated florets during storage at 4 °C. Color (A) and chlorophyll content (B) of florets were measured in florets exposed to three different heat doses: (●), control; (○), 41 °C/180 min (hormetic dose); and (▼), 47 °C/12min (HDHT). Color coordinates were expressed as hue angle (H°). n=9 (color), n=4 (chlorophyll); differences between the treatments were found from LSD (0.05). Hue LSD = 4.28 and chlorophyll LSD = 0.25.

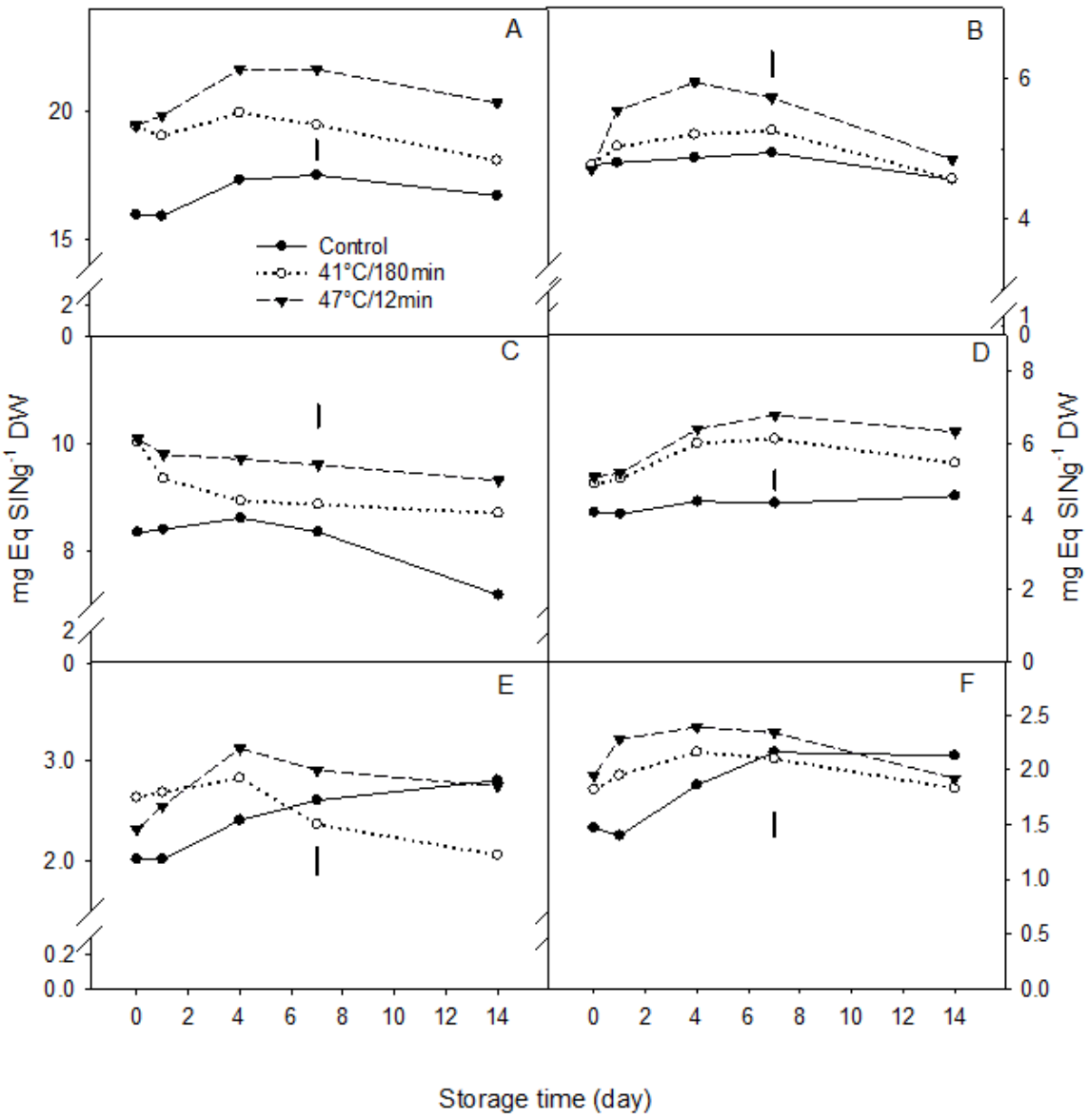

Figure 4.3 Glucosinolate content of heat-treated broccoli florets during the storage at 4 °C. Glucosinolate content was measured in florets exposed to three different heat doses: (●), control; (○), 41 °C/180 min (hormetic dose); and (▼), 47 °C/12min (HDHT). Total Glucobrassicins (A) LSD (0.05) =0.69; Glucorapharin (B) LSD (0.05) = 0.35; Glucobrassicin (C), LSD (0.05) = 0.40; Neoglucobrassicin (D), LSD (0.05) =0.51; 4-Methoxyglucobrassicin (E), LSD (0.05) =0.27; 4-Hydroxyglucobrassicin (F), LSD=0.21; n=3.

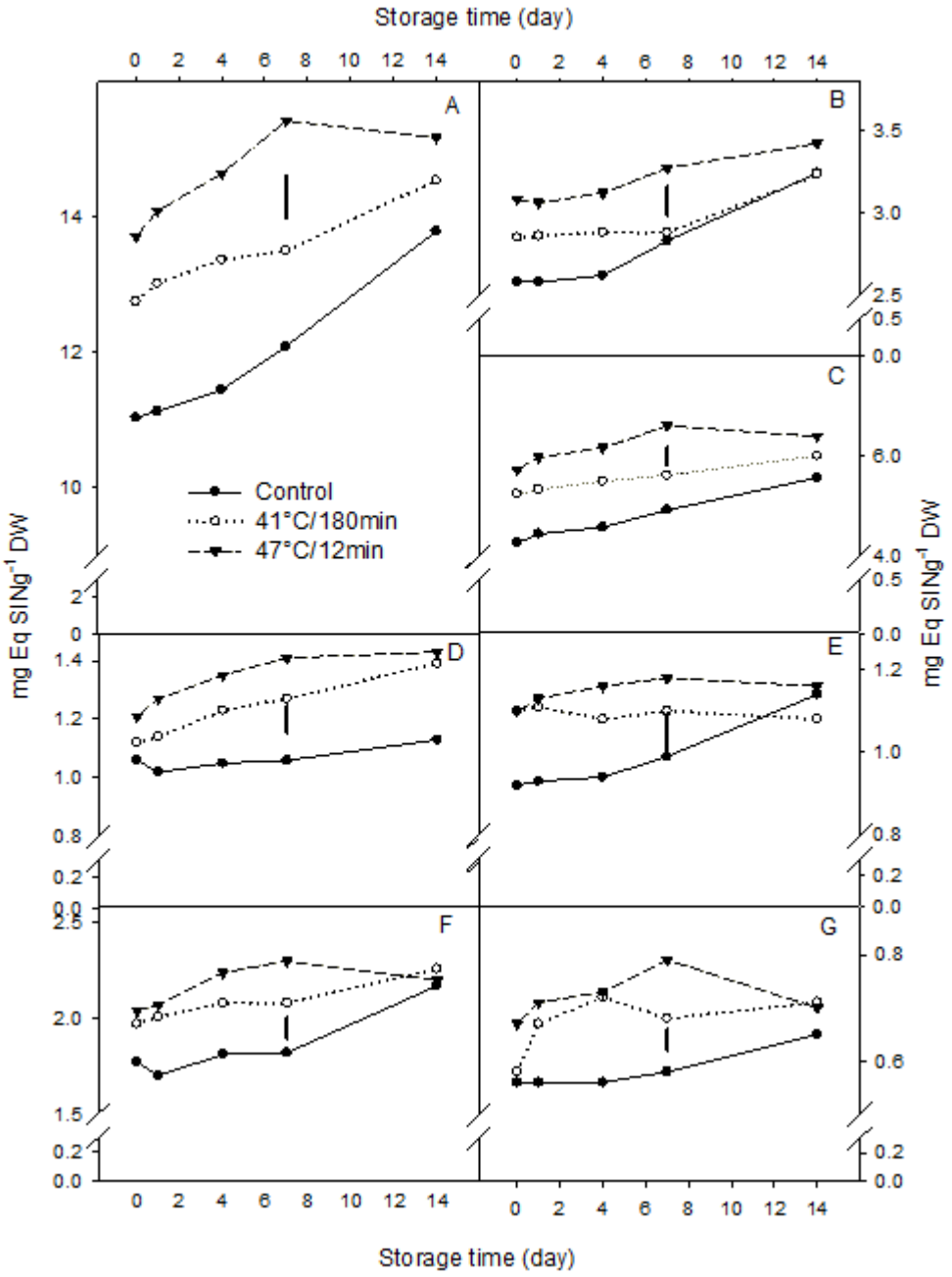

Figure 4.4 Hydroxy-cinnamic acids (HCAs) content in heat-treated broccoli florets during storage at 4 °C. HCA content was measured in florets exposed to three different heat doses: (●), control; (○), 41 °C/180 min (hormetic dose); and (▼), 47 °C/12min (HDHT). Total HCA (A); LSD (0.05) =0.63; 1,2-Disynalpoyl-2-feruloyl gentiobiose (B), LSD (0.05) = 0.17; 1-Sinapoyl-2-feruloyl gentiobiose (C), LSD (0.05) =0.37; 1,2-Diferuloyl gentiobiose (D), LSD (0.05)=0.09; 1,2,2'–Trisinapoyl gentiobiose (E), LSD (0.05) = 0.08; 1,2-Disinapoyl gentiobiose (F), LSD (0.05) = 0.11; Sinapolyl-diferuloyl gentiobiose (G), LSD(0.05) = 0.04; n=3.

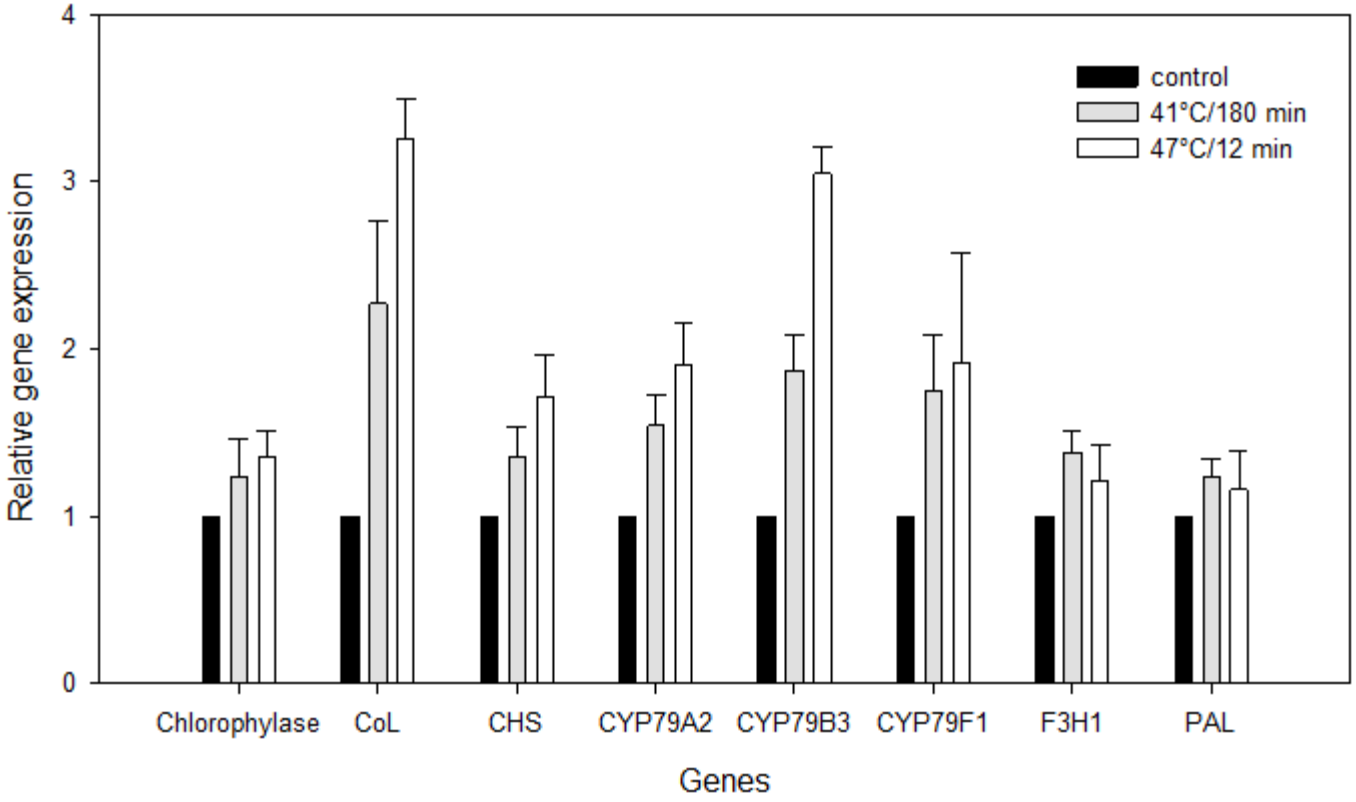

Figure 4.5 Gene expression analysis of heat-treated broccoli florets on day 0. Florets were exposed to three different heat doses: control; 41 °C/180 min (hormetic dose); and 47 °C/12min (HDHT). Gene expression was measured immediately after treatments on chlorophyllase (Chlase); coumarate ligase (CoL); chalcone synthase (CHS); phenylalanine N-hydroxylase (CYP79A2); tryptophan N-hydroxylase 2 (CYP79B3); dihomomethionine N-hydroxylase (CYP79F1) and flavanone 3-hydroxylase (F3H1). Standard deviation is presented with bars (n = 3).

Table 4.1 Concentration of ORAC (oxygen radical absorbance capacity), ascorbic acid, total phenols, total flavonoids and rutin of treated broccoli florets. Florets were exposed to heat: control; 41 °C/180 min (hormetic dose); and 47 °C/12min (HDHT) during 21 days of storage in darkness at 4 °C. The obteined values were time averaged (0, 7, 14 and 21 days).

ORAC (mg eq trolox g^{-1} DW)			
Control		152.0±14.6	
41 °C/12 min		174.1±17.0	
47 °C/180 min		208.9±16.8*	
Ascorbic acid (mg eq AA. g^{-1} DW)			
	Oxidized	**Reduced**	**Total**
Control	3.8±0.2	13.1±6.0	16.9±5.7
41 °C/12 min	3.8±0.4	12.8±5.0	16.6±5.0
47 °C/180 min	4.2±0.2	11.9±5.6	16.1±5.6
Total phenols (mg eq GA.g^{-1} DW)			
Control		16.2±3.6	
41 °C/12 min		17.4±2.8	
47 °C/180 min		18.2±2.7	
Total flavonoids(mg eq QE.g^{-1} DW)			
Control		5.4±0.7	
41 °C/12 min		5.6±0.9	
47 °C/180 min		5.8±0.7	
Rutin(mg eq SIN.g^{-1} DW)			
Control		0.6±0.1	
41 °C/12 min		0.6±0.0	
47 °C/180 min		0.7±0.01	

The asterisk indicates that the value is significantly different from the corresponding control at $p < 0.05$.

Chapter V: Glucosinolates are enhanced by oxidative stresses in broccoli florets during postharvest storage

5.1 Résumé

Les stress abiotiques sont oxydatifs de nature et provoquent la production d'espèces réactives de l'oxygène (ERO) dans les organes de la plante, où les stress sévères peuvent être nocifs pour le tissu végétal, et des doses subaiguës de stress peuvent améliorer ou induire des mécanismes de protection. L'objectif de ce travail était d'examiner l'effet hormétique ainsi que de fortes doses d'UV-B, d'ozone (O_3) et de peroxyde d'hydrogène (H_2O_2) sur la qualité et le contenu en glucosinolates dans les fleurons de brocoli pendant l'entreposage. Les doses hormétiques ont été déterminées à partir de la réponse de rétention de la couleur; et elles étaient: 1,5 kJ.m^{-2} d'UV-B; 5 ppm de O_3 pendant 60 min; et 1,25 mM de H_2O_2 pendant 180 min. Le développement de couleur, la perte de poids et le taux de respiration ont été suivis pendant 21 jours d'entreposage à 4 °C. L'expression de la dihomomethionine N-hydroxylase (CYP79F1), la tryptophane N-hydroxylase (CYP79B3), la phénylalanine N-hydroxylase (CYP79A2) et la phénylalanine ammonia- lyase (PAL), la chalcone synthase (CHS) et la flavanone 3-hydroxylase (F3H1) dans le brocoli traité ont également été évalués. Le profil de glucosinolates a été déterminé pour un maximum de 14 jours dans les fleurons de brocoli entreposés à 4 °C par LC-MS. Parmi les trois stress hormétiques, l'UV-B a été le plus efficace pour retarder le jaunissement des fleurons de brocoli. Le taux de respiration initiale des fleurons traités avec les stress était significativement élevé, surtout après l'exposition à l'O_3, qui a également abouti à la perte sévère de poids en conséquence. Dans l'ensemble, la capacité antioxydante des fleurons de brocoli traités avec l'O_3 et le H_2O_2 a diminué par rapport au témoin, mais pas dans les fleurons traités à l'UV-B. La surexpression de gènes de la voie de glucosinolates dérivés du tryptophane a été observée immédiatement (6 heures) après tous les traitements. L'expression de CYP79B3 dans le brocoli était significativement plus élevée avec les deux doses d'UV-B, ainsi qu'avec la dose élevée d'O_3 et la dose hormétique de H_2O_2. La quantité de glucobrassicines totales a augmenté de façon plus significative avec l'ozone et le H_2O_2 par rapport à l'UV-B, mais celui-ci a été plus efficace dans l'amélioration de hydroxycinnamates. L'UV-B semble être le stress le plus efficace en matière de préservation de la qualité et l'amélioration de la phyto-composés dans les fleurons de brocoli. Les résultats ont montré une bonne corrélation entre l'expression du gène CYP79B3, et les titres de glucosinolates de type indoles dans les bouquets de brocoli traités, ce qui suggère que la cible d'UV-B, O_3 et spécialement H_2O_2 est susceptible d'être la voie de la branche de glucosinolates de type indole.

5.2 Abstract

Abiotic stresses are oxidative in nature and cause production of reactive oxygen species (ROS) in plant bodies, where severe stresses can be harmful to the plant tissue, and sub-acute doses of stresses can enhance or induce protective mechanisms. The objective of this work was to examine the effect of hormetic as well as high doses of UV-B, ozone (O_3) and H_2O_2 on the quality and glucosinolate content in broccoli florets during storage. Hormetic doses were determined from color retention response; and they were: 1.5 $kJ.m^{-2}$ of UV-B; 5 ppm of O_3 for 60 min; and 1.25 mM of H_2O_2 for 180 min. Color development, weight loss and respiration rate were monitored during 21 days of storage at 4 °C. The expression of dihomomethionine N-hydroxylase (CYP79F1), tryptophan N-hydroxylase 2 (CYP79B3), phenylalanine N-hydroxylase (CYP79A2) phenylalanine ammonia lyase (PAL), chalcone synthase (CHS) and flavanone 3-hydroxylase (F3H1) in the treated broccoli were also evaluated. The profile of glucosinolates was determined for up to 14 days in broccoli florets stored at 4 °C by LC-MS. Among the three hormetic stresses, UV-B was the most effective in delaying the yellowing of broccoli florets. The initial respiration rate of the florets treated with the stresses was significantly high, especially after exposure to O_3, which also resulted in consequent severe weight loss. Overall, the antioxidant capacity of florets decreased in O_3 and H_2O_2 treated broccoli with respect to the control, but not in UV-B treated florets. The up-regulation of genes of the tryptophan-derived glucosinolate pathway was observed immediately (6 hours) after all treatments. Gene expression of CYP79B3 in broccoli was significantly higher with both doses of UV-B, as well as with the high dose of O_3 and the hormetic dose of H_2O_2. The amount of total glucobrassicins increased higher with ozone and H_2O_2 than with UV-B, but the latter was more effective in enhancing hydroxycinnamates. UV-B appears to be the stress exhibiting balanced effects with respect to quality preservation and enhancement of phyto-compounds in broccoli florets. Results showed a good correlation between gene expression of CYP79B3, and the titers of indole glucosinolates in the treated broccoli florets, suggesting that the target of UV-B, O_3 and specially H_2O_2 is likely to be the branch pathway of indole glucosinolates.

Keywords: Broccoli, postharvest, oxidative stress, glucosinolates, hydroxy-cinnamic acids, gene expression.

5.3 Introduction

Broccoli is an excellent source of nutrients including vitamin C, folic acid, vitamin K, and essential minerals. It also contains powerful health-promoting phytochemicals and antioxidants, including phenolic acid derivatives, flavonols, and organo-sulphur compounds, the glucosinolates (Duarte-Sierra et al., 2012b). It is increasingly appreciated that a significant correlation exists between the intake of these phyto-compounds and protection against cancer and cardiovascular diseases (Fahey et al., 1997). Broccoli has become an important vegetable in the human diet worldwide due to its health-promoting properties and its high nutritional value. Nevertheless, the levels of glucosinolates (GLS) tends to decrease in the edible parts of the vegetable after it is detached from the source plant, and more importantly, during its postharvest storage (Schouten et al., 2009a). GLS are also an integral part of the defense mechanisms against diseases in *Brassicas* and may be triggered by biotic as well as abiotic stresses (Doughty et al., 1995; Pereira et al., 2002; Textor and Gershenzon, 2009).

Abiotic stresses are non-living environmental factors that are of particular importance because of their negative impact on the yields of industrial crops worldwide (Gong et al., 2013). The most common abiotic stresses encountered during the production of fruits and vegetables are drought, nutrient deficiencies, extreme temperatures, salinity and light (Toivonen and Hodges, 2011). In the presence of one or more of these stresses, plants respond at the molecular, tissue, anatomical and morphological levels to cope with the unfavorable conditions (Fraire-Velázquez et al., 2011). At the molecular level, a cascade of events take place after the perception of a stress, which comprises cellular calcium spiking, cytoplasmic acidification, generation of reactive oxygen species (ROS), generation of signals and signal transduction, kinase cascades, activation of transcription factors and gene expression leading to defense responses, including secondary metabolites, heat shock factors and PR proteins (Atkinson and Urwin, 2012; Zhao et al., 2005). Abiotic stresses are oxidative stresses as the generation of ROS is a common feature when plants are exposed to them (Gill and Tuteja, 2010; Toivonen, 2003). Oxidative stress is likely to be the initial trigger, leading to the activation of the expression of genes and eventually the production and accumulation of several defense secondary metabolites in plants (Grene, 2002).

Generation of ROS generally occurs in mitochondria, chloroplast, glyoxysomes, peroxysomes and nuclei (Hodges, 2003). Chloroplasts are one of the most important target organelles with respect to plant senescence, since they operate at high oxygen concentration in the presence of light (Munné-Bosch and Alegre, 2002), and are also the location for photosynthesis, ATP

and NADPH generation. Chlorophyll degradation is also an important indicator of the quality of green vegetables during postharvest storage. In broccoli, yellowing is one of the most important marker in quality deterioration (Funamoto et al., 2002).

Abiotic stresses can be harmful to plants when they are exposed to severe levels. For instance, UV-B light (280-320 nm) can target nucleic acids, proteins, lipids, membranes, light harvesting complexes, Rubisco, ATP-ase, cell wall and pigments of plants including chlorophyll (Hollósy, 2002). Thylakoid membranes are particularly susceptible to UV-B radiation, and chlorophyll as well as carotenoids can be also affected by UV-B (Pfündel et al., 1992). The perception and signaling of UV-B is controlled by two specific signaling pathways. Low dose of UV-B are perceived by dimers of UV-B specific UV RESPONSE LOCUS 8 (UVR8), and the transduction signal is completed through CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) and a transcriptor factor ELONGATED HYPOCOTYL 5 (HY5) (Jenkins, 2009). On the other hand, responses to high UV-B intensities in plants are controlled by non-specific signaling pathways mediated by ROS and phytohormones including jasmonic acid, salicylic acid and ethylene (Schreiner et al., 2012). Plants synthetize flavonoids, hydroxycinnamic acids and sinaptate esters to protect themselves against UV-B, which also function as free radical scavengers (Ueda and Nakamura, 2011). Elicitation of glucosinolates has been also observed as a defense response to doses of UV-B applied on herbal plants (Schreiner et al., 2009).

Ozone is an air pollutant and a strong oxidizing agent that produces free radicals; and harmful effects. Ozone can react with cell structures, cell membrane lipids, proteins, nucleic acids, olefinic compounds of the cuticle, phenolic compounds (Pell et al., 1997; Roshchina and Roshchina, 2003b). The most evident visual negative effects of ozone in plants are the decline in growth, necrosis and chlorosis (Roshchina and Roshchina, 2003c). Chlorosis in green vegetables is one of the most important detrimental factors on the quality of produce induced by ozone. Ozone induces defense responses such as phytoalexins, PR proteins, lignification and antioxidant systems and pathway (signals) are very similar to those induced by pathogens (Baier et al., 2005; Sandermann, 1998). Ozone also increases the activity of catalase and peroxidases as well as the content of α-tocopherol and sinigrin in cabbage (Rozpądek et al., 2013).

Hydrogen peroxide is produced by the plants when they are exposed to biotic and abiotic stresses and plays important roles as a signal in eliciting defense responses, and in the generation of other signal molecules (Desikan et al., 2001; Foyer et al., 1997). At low concentrations hydrogen

peroxide, is related with downstream signaling events including calcium mobilization, protein phosphorylation and gene expression (Neill et al., 2002). However, high concentrations can be toxic to the cells leading to programmed cell death (Gechev and Hille, 2005), in part because H_2O_2 can be converted into more reactive ROS such as hydroxy radicals ($^{\cdot}OH$) by Fenton reaction in the presence of metals (Bienert et al., 2006).

The effects of abiotic stresses have been largely explored in plant bodies and little information is available on the effects of abiotic stresses on postharvest crops (Hodges and Toivonen, 2008). However, since commodities are living organisms with identical cell structure compared with plants, much of the knowledge of plant physiology and understanding can be expected to hold true for postharvest crops.

It has been found that quality of broccoli is affected by UV-B light. Florets remained greener after a relative high dose of 8.8 $kJ.m^{-2}$ compared with non-exposed florets (Aiamla-or et al., 2009). Furthermore, UV-B light at 0.3-1 $kJ.m^{-2}.d^{-1}$ mediated the induction of 4-methylsulfinylbutyl and 4-methoxy-indol-3ylmethyl glucosinolates in broccoli sprouts (Mewis et al., 2012). Continuous exposition of broccoli to 0.04 $\mu L.L^{-1}$ of O_3 for 21 days at 4 °C resulted in a significantly less pronounced color change compared with control stored under the same conditions (Skog and Chu, 2001), and total phenolic content of table grapes was achieved by continuous exposure to 0.1 $\mu L.L^{-1}$ of O_3 for 60 min at 0 °C (Artés-Hernández et al., 2007). Similarly, improvement of the appearance of white pepper fruits was done by the application of 15mM of H_2O_2 (Bayoumi, 2008).

Harmful stressors at low doses may elicit adaptive or beneficial processes in biological systems, known as hormesis (Calabrese and Baldwin, 2002; Luckey, 1982). UV-C hormesis has been shown in many postharvest crops and is known to induce disease resistance and delayed senescence responses (Arul et al., 2001b; Charles and Arul, 2007; Stevens et al., 2006). However, it is not known whether such a hormetic phenomenon exists with other oxidative stresses such as UV-B, ozone and H_2O_2 in postharvest systems. Plants accumulate secondary metabolites when exposed to abiotic stresses to protect themselves; hence, modification in the level of the constitutively secondary metabolites in postharvest produce occurs when abiotic stresses are applied in a controlled manner.

Thus the objective of this work was to determine hormetic doses of three stresses, UV-B, O_3 and H_2O_2 for color retention response in broccoli florets; and to study the effect of hormetic and high doses on the quality and the evolution of phytochemicals, glucosinolates and hydroxycinnamic acids

in broccoli florets during storage. The gene expression of some of the key enzymes in glucosinolate and phenylpropanoid pathways in broccoli exposed to theses stresses was also monitored.

5.4 Materials and Methods

5.4.1 Broccoli

Freshly harvested broccoli (*Brassica oleraceae* L. var. Italica 'Diplomat') heads were obtained from a commercial farm (Ile d'Orléans, Québec, Canada). Florets (300g) of uniform size (approximately, 7 cm) were separated from heads and randomly arranged in small plastic punnets of 500 mL. The punnets were placed in plastic containers of 5 L with perforations for ventilation, and containing a layer of water at the bottom to maintain high humidity (98-100%), and the containers were stored inside a controlled chamber overnight at 4 °C.

5.4.2 Treatments: selection of optimal dose

UV-B radiation was produced by 48-inch lamps (TL-40W, Phillips) with peak emission at 310 nm. The florets were treated with specified UV-B doses: 0 $kJ.m^{-2}$ (control), to 7.5 $kJ.m^{-2}$. Hydrogen peroxide treatment was carried out by immersing the stems into solutions of different concentrations to a depth of 2.0 cm: 0 mM (control) to 5mM at different times. Ozone treatment was carried out in an air tight humidified chamber at 10 °C. Ozone gas was generated by corona discharge (SF300, Burlignton, ON), the concentration was measured by an ozone detector (IN-2000, InUSA Inc., MA) and controlled with a computer (21X Micrologger, Campbell Scientific, UT). Treatments were performed using 5 ppm of the gas at different times (0-720min). Exposure times using 5 ppm of O_3 were 0, 7.5, 15, 30, 60, 120, 240 480 and 720 min. Storage conditions for treatments consisted of 30 days at 4 °C for UV-B, 10 days at 10 °C for ozone and 5 days of storage at 15 °C for hydrogen peroxide treated florets.

5.4.3 Color and respiration rate

The color and respiration rate of broccoli florets were determined as described elsewhere in the thesis in detail, in section 2.3.3 and 2.3.4, respectively.

5.4.4 Chemical assays

The total phenolic content, total flavonoid content, reduced and total ascorbic acid contents, and the total antioxidant capacity were determined by methods described elsewhere in detail in section 2.3.5.1, 2.3.5.2, 2.3.5.3, 2.3.5.4, respectively.

5.4.5 Glucosinolates and hydroxycinnamic acid analysis

The glucosinolates and hydroxycinnamic acids were assayed by the methods described elsewhere in detail (section 2.3.6).

5.4.6 Gene expression analysis

The gene expression analysis section was determined by methods described elsewhere in detail in section 2.3.8.

5.4.7 Statistical analysis

The experiment was set as a complete randomized design and the data were analyzed by one-way analysis of variance (one-way ANOVA) using a significant level of 0.05. Least significant difference test at the same significant level was done when the analysis of variance found significant differences. The statistical analysis was executed using the statistical analysis system version 9.3 (SAS Institute Inc. 2011. Base SAS® 9.3 Procedures Guide. Cary, NC, USA). For chemical analysis, an average of equidistant time periods (0, 7, 14 and 21 days) was made for total phenols, flavonoids, ascorbic acid, and ORAC assay.

5.5 Results and Discussion

5.5.1 Hormetic dose of UV-B, O_3 and H_2O_2

The susceptibility of the plant organs to the dose or severity may change with their physiological stage, and the effect of severity or dose of a stress on plants appears to depend on various factors, such as the morphological structure and specific surface area of the plant organ, the physiological age of the tissue and the dynamics of metabolite accumulation may vary with dose (Schreiner et al., 2012).

The total color difference (ΔE) was monitored during storage to determine the hormetic dose for each stress. Among the UV-B doses, 1.5 $kJ.m^{-2}$ exhibited the lowest value of ΔE, although ΔE values of florets exposed to the highest dose of 12.5 $kJ.m^{-2}$ were also low (Figure 5.1a), showing a second minimum in ΔE value. Among the exposure times to ozone at 5 ppm, the exposure of broccoli florets for 60 min was optimal for color retention (Figure 5.1b). Interestingly, the effect of ozone on color retention of broccoli was bimodal (Figure 5.1b). Total color change (ΔE) was minimum after 60 min, ΔE of 4.6 and a second minimum was registered after 720 min exposure ΔE of 5.5 (Figure 5.1b). The hormetic dose for H_2O_2 treatment was found to be 1.25 mM for 180 min (Figure 5.1c), although no significant differences were found among the other doses. Yet, a second minimum in ΔE value was apparent with 5 mM concentration for 180 min.

UV-B doses have been applied to produce to delay color change. In lime, the suppression of chlorophyll-degrading enzymes was successfully done by 19 kJm^{-2} (Kaewsuksaeng et al., 2011). In broccoli florets, with a relative large surface to volume ratio in comparison with lime, a lower dose of 8.8 kJm^{-2} (in a range of 4.4-13.1 kJm^{-2}) was found to delay the decrease in the hue angle (H°) and the content of chlorophyll (Aiamla-or et al., 2010). The dose of 8.8 $kJ.m^{-2}$ appears to be in the range of the second minimum in ΔE value observed in our study. Since these authors examined a dose range above 4.4 kJm^{-2}, they apparently to have skirted the true hormetic dose for broccoli florets.

It is worth noting that the hormetic doses of UV-B at 1.5 kJm^{-2} (Figure 5.1a) and UV-C at 1.2 kJm^{-2} (Figure 2.3) for color retention in broccoli were equivalent in photon energies. The photon energy of UV-B at peak emission of 310 nm is 3.99 eV and that of UV-C at 254 nm is 4.88 eV (Shipway, 2008). The photon energy of UV-B is 12 % lower compared with UV-C, thus a higher UV-B dose of 1.5 kJm^{-2} was comparable to the UV-C dose of 1.2 kJm^{-2} which corresponded to the photon energy difference between these two UV radiations. Further work was therefore carried out with 1.5 $kJ.m^{-2}$ as hormetic dose and a high dose of 7.2 $kJ.m^{-2}$ (5 folds greater than the hormetic dose) was used to evaluate the effects of UV-B light on the physiological characteristics and selected secondary metabolites of broccoli florets.

Fumigation of produce with ozone is normally longer compared with ozonized water treatment, and hence, it is often carried out during the storage in the storage space, mainly to inhibit bacteria and fungi (Forney, 2003). Nonetheless, ozone can also affect the physiology and the quality parameters of fruits and vegetables. For instance, floret opening and visually yellowing of broccoli exposed to continuous 0.04 $\mu L.L^{-1}$ of ozone for 21 days at 4 °C, were significantly lower compared

with the control stored under the same conditions (Skog and Chu, 2001). Color retention in celery was attributed to the inhibitory effect of ozonized water (0.03-0.18 ppm) on polyphenol oxidase activity (PPO) (Zhang et al., 2005). However, high doses of O_3 can also cause phytotoxicity that are often characterized by discoloration and browning of the tissue (Forney, 2003).

The fact that a bimodal behavior in dose – color retention relationship for the three stresses was present may be related with some mechanism operating to protect chlorophyll at high doses or its accumulation functioning as antioxidant. It is also possible that the degradation of carotenoids (e.g., lutein), renders chlorophyll more visible.

5.5.2 Physiological characteristics

5.5.2.1 Color evolution

Color evolution of broccoli florets was evaluated during storage in the dark 4 °C (Figure 5.2). Not surprisingly, the three stresses at hormetic doses delayed yellowing of broccoli florets during the storage compared with the control and stresses at high doses. Color retention of florets was not different between the hormetic and high doses of UV-B over the storage period, but was significantly different ($p<0.05$) from that of the control (Figure 5.2a). UV-B light has been reported to effectively delay the yellowing of broccoli florets by the suppression of chlorophyll-degrading enzymes, such as chlorophyllase and pheophytinase (Aiamla-or et al., 2010; Aiamla-or et al., 2012). UV-C light at 1.2 kJm^{-2} also delayed the yellowing on broccoli florets (Duarte-Sierra et al., 2012b). The effect of the latter was also attributed to reduced activities of the enzymes involved in chlorophyll catabolism such as chlorophyllase, and chlorophyll peroxidase (Costa et al., 2006). It is interesting to note that both UV-B and UV-C with different action spectra had similar effects on color retention of chlorophyll in broccoli.

Color retention of florets exposed to the high dose of ozone was comparable to that of unexposed florets, but a significant ($p<0.05$) better color retention was observed in florets exposed to the hormetic dose of O_3 (Figure 5.2b). This was probably due in part, to altered light reflectance characteristics either because of ozone reaction with cuticular waxes or because of severe weight loss of ozone treated florets described below in section 5.4.2.2 (Figure 5.3b).

It is well known that xanthophyll cycle, involving epoxidation of zeaxanthin and de-epoxidation of violaxanthin with reductants NADPH and ascorbic acid, respectively, protects the photosynthetic apparatus (thylakoids and chlrophyll) from oxidative stresses caused by drought,

chilling, heat, senescence and other abiotic stresses (Latowski et al., 2011). This cycle appears to be promoted in response to ozone exposure in tobacco leaves, where violaxanthin pool was reduced in tobacco, and that of zeaxanthin was slightly increased (Pasqualini et al., 1999). Immediately after exposure, violaxanthin de-epoxidation of zeaxanthin should be high, a substrate for ABA (Pasqualini et al., 1999). It would seem from the observation, where color change was more intense in the florets that were exposed to ozone for 240 min than in those exposed to 720 min, that the xanthophyll cycle was more operational in the latter, and that there was a build-up of reductive equivalents. These reductive equivalents can be transported to chloroplasts where they can be used to reduce plastoquinone then to plastoquinols (Wright et al., 2011). Long exposure to ozone may not only affect chlorophyll, but also carotenoids, especially lutein which is responsible of yellowing of florets, and thus reducing yellowing of florets.

Yellowing of broccoli florets was also successfully delayed by 1.25 mM/180 min of H_2O_2, but not by higher doses (Figure 5.2c). The literature regarding the application of hydrogen peroxide treatments on postharvest commodities is sparse and opposing effects on the quality of produce in response to this molecule have been reported. The application of glycolic acid (1µM) serving as substrate for hydrogen peroxide formation by the action of glycolate oxidase in tissue of pear fruit resulted in the acceleration of ripening as indicated by increased softening and ethylene evolution (Brennan and Frenkel, 1977). On the other hand, the application of vapor phase hydrogen peroxide to grapes at 1.1 $mg.L^{-1}$ did not affect their visual appearance (Forney et al., 1991b). Similarly, the application of 15 mM of H_2O_2 improved the general visual appearance, ascorbic acid content and increased the activity of antioxidant enzymes in white pepper fruits (Bayoumi, 2008).

When comparing the three hormetic doses of the applied stresses, it can be appreciated that the initial color difference for UV-B and H_2O_2 was low, and reached values of about 8 and 11, respectively. However, the initial color difference of florets treated with ozone was even lower compared with either UV-B or H2O2, reaching a value of 6.9 at the end of the storage, which points to the possibility of chlorophyll regeneration or loss of lutein (as indicated before).

5.5.2.2 Respiration and weight loss

Respiration rate increased immediately after exposure of florets to UV-B, O_3 and H_2O_2 (Figure 5.4). Significant differences in the initial respiration rate of UV-B treated floret groups were observed between the high dose (7.2 $kJ.m^{-2}$) group and the untreated group (103.1 and 22.2 $mL.kg^{-}$

$^{1}.h^{-1}$, respectively), and also between the hormetic dose (1.5 kJ.m^{-2}) group and the untreated group (44.7 and 22.2 mL.kg^{-1}.h^{-1}, respectively). By day seven, all three treatment groups showed nearly equivalent respiration rate of about 15 mL.kg^{-1}.h^{-1}, and remained steady thereafter throughout the storage period at 4 °C (Figure 5.4a). In comparison, the respiration rate of florets after the exposure to O_3 was sharply higher (Figure 5.4b). The carbon dioxide production of the florets was of 300 mL.kg^{-1}.h^{-1} following their exposure to 5 ppm of ozone for 720 min, and 170 mL.kg^{-1}.h^{-1} of CO_2 production by the florets exposed to 5 ppm of ozone for 60 min, and 14 mL.kg^{-1}.h^{-1} of CO_2 production by the untreated florets. By day seven, the high dose of ozone group exhibited a relatively high respiration rate of 40 mL.kg^{-1}.h^{-1} compared with the rate of 18 mL.kg^{-1}.h^{-1} by the hormetic and untreated groups. After 14 days of storage, similar values were observed for the three treatments groups (20-24 mL.kg^{-1}.h^{-1}) without any significant difference ($p>0.05$) between them. Hydrogen peroxide treatment also affected the respiration rate of the florets. The highest CO_2 production was reported with both hormetic and high dose groups, with 92 mL.kg^{-1}.h^{-1} on day 0. This value was significantly different ($p<0.05$) from the untreated florets, where the CO_2 production was of 27 mL.kg^{-1}.h^{-1} (Figure 5.4c). After 7 days of storage, similar values were observed for the three treatments groups (18-20 mL.kg^{-1}.h^{-1}) without any significant difference ($p>0.05$) between them.

Fruits and vegetables generally show increased respiration rate immediately after exposure to abiotic stresses (Kader, 2002), and this was observed after exposure of florets to UV-B, O_3 and H_2O_2 (Figure 5.4). The rise in the initial respiration rate with increasing dose of the stress is suggestive of the stress severity perceived by the plant tissue. The rise in the initial high respiration is also generally accompanied by stress ethylene production (Haard and Cody, 1978).

The rise in the respiration may involve alternate pathway, such as alternative oxidase (AOX) in order to eliminate excessive ROS generated by the stresses that are oxidative in nature (Vanlerberghe, 2013). It is also indicative of the occurrence of biochemical events triggered by the stresses leading to the synthesis of various defense elements, including secondary metabolites. The transient high respiration may impact the tissue with respect to energy (ATP) production from storage carbohydrates, organic acids, lipids and amino acids; and the intensification of ROS and consequent changes in the redox status of the tissue. However, the respiration rates reach steady state values for the florets treated with stresses comparable to those of the control florets without excessive depletion of carbon reserves. The additional depletion of carbon reserves during the high respiration phase may suggest that the storability and quality of the produce subjected to stress could be compromised.

But the application of hormetic doses improved the shelf life of the florets, meaning that the altered biochemistry of the treated florets prevailed, while the application of high dose compromised the shelf life

The weight loss of the florets treated with different stresses and doses during storage showed different patterns (Figure 5.3b). The weight loss of the untreated florets increased steadily in a rather linear fashion from 0 to 11.6 % after 21 days. This weight loss is likely due to loss of water by transpiration. Compared with ozone, the effect of UV-B and H_2O_2 treatments on the weight loss of florets was less evident from that of the untreated florets ((Figure 5.3ac), albeit with different outcomes with doses, suggesting that the weight loss is mainly related to the loss of water with these two stresses. The weight loss of florets treated with hormetic dose of UV-B (7.7 %) was lower compared with the unexposed florets (9.2 %), and higher when florets were exposed to the high dose (11.5 %). ((Figure 5.3a), a pattern similar to UV-C treatment described elsewhere in this thesis (Chapter II). The changes in the transpiration may be attributed to surface morphological changes in response to UV-B treatment, leading to either lower or higher transpiration rates. It is also known that the production of osmolytes, such as proline, glycine and betaine is a conserved response of plants to abiotic stresses including UV radiation (Ashraf and Foolad, 2007).

On the other hand, higher weight loss was recorded with the hormetic dose of H_2O_2 (14.9 %) than the control florets (11.6 %), and lower weight loss with high dose of H_2O_2 (9.1 %) (Figure 5.4c), although the contact time to H_2O_2 solution was the same for both doses, but the solution concentrations were different; with 1.25 mM and 5.0 mM for hormetic dose and high dose, respectively. This suggests the higher concentration of H_2O_2 probably elicits osmotic stress tolerance. Recently, H_2O_2 was found to alleviate water loss by increasing levels of oligosaccharides, proline and absicic acid (ABA) (Ishibashi et al., 2011).

Ozone was the treatment which exhibited severe weight loss during ozone exposure and during storage (Figure 5.4b). The weight loss of the florets during exposure to ozone for 720 min (high dose) was about 24 compared with 4 % with hormetic dose (exposure time of 60 min). However, the weight loss of high dose ozone treated florets during storage was small (24 to 28 %), but water loss of florets treated with hormetic dose continued to increase during storage at a higher rate until it reached the weight loss of the high dose group after 21 days od storage (27.5 %). First, the weight loss during treatment cannot be solely attributed to moisture loss. Some other event, specific to ozone, is occurring that causes weight loss in addition to moisture loss, if any. The weight

loss during storage is likely due, for most part, to moisture loss, where the small or large increases in moisture loss can be attributed to the induction of osmolytes such as proline and surface morphological changes caused by ozone. Ozone is known to modify cuticular lipids, and as a result, the produce may develop thinner cuticles, leading to elevated moisture loss (Forney, 2003). The event during exposure to ozone that causes severe weight loss during exposure to ozone, other than moisture loss, might be the emission of volatiles. The observation that the weight loss of the florets during exposure to ozone (in high humidity chamber) was very significant supports this possibility. The generation of volatiles in plants, ethylene and isoprenes, is well recognized (Fiscus, 2005; Loreto and Velikova, 2001; Roshchina and Roshchina, 2003a), and may function as a detoxification mechanism.

5.5.3 Antioxidant capacity of florets

The antioxidant activity of plants is determined not only by phenylpropanoids compounds, but also by ascorbate-glutathione and the induction of enzymes catalyzing reactions involving the scavenging of free radicals (Schreiner et al., 2012). The production of ROS during biotic and abiotic stresses is controlled, in part, by phenols and flavonoids which are also UV-screening compounds. This dual capacity was recognized when florets were exposed to UV-B. The total content of phenols was 7 % greater in the exposed florets to both doses of UV-B compared with un-exposed broccoli (Table 5.1). Furthermore, a significant enhancement of 18 % on the total antioxidant capacity of florets was observed among broccoli treated with 7.2 $kJ.m^{-2}$ of UV-B light compared with un-exposed florets. The antioxidant capacity, determined by ORAC (oxygen radical absorbance capacity) assay, of plant polyphenols such as anthocyanin cyaniding-3-glucoside and quercetin was more than four times superior to the antioxidant molecules such as α-tocopherol and ascorbic acid (Gould, 2003).

The ORAC of florets was generally reduced by O_3 and H_2O_2 treatments (Table 5.1). The ORAC values of florets were reduced by 18 % with the hormetic dose of ozone and by 24 % when florets were exposed to 5 ppm/720 min, compared with the control florets. Moreover, the titers of total ascorbic acid content in the treated broccoli were considerably reduced by 16 % after exposure of florets to 5 ppm/720 min of O_3. The effect of H_2O_2 on the total antioxidant capacity of florets was similar to ozone, 12 % and 15 % decreases into the hormetic and high dose groups, respectively, compared with the control group. Nonetheless, the amount of total phenols increased by 8 % in the high dose (5mM/180 min) group relative to the control group.

The reduction in ORAC value suggests that the tissue is under oxidative stress overall. It is increasingly recognized that the generation of ROS is a clear indication of systemic signaling and elicitation of defenses in plants (Mittler, 2002). Although it is not clear which of the ROS is specifically involved in signaling and elicitation in response to stresses, superoxide appears to be implicated in UV-B signaling and regulation of defense genes (Jenkins, 2009). Ozone, on the other hand, is soluble in water leading to the formation of multiple ROS, $O^{\cdot -}_2$, H_2O_2, peroxyl radical and other active O_2 species (Chernikova et al., 2000), in addition to its reactivity with many cell components including fatty acids, glutathione and many others possessing nucleophilic sites (Forney, 2003). Hydrogen peroxide is a reactive oxygen species *per se*, it is a stable ROS, and it can potentially generate free radicals in the presence of metal ions such as Fe^{2+} or molecules containing metal ions by the Fenton reaction (Cheeseman, 2007). Overall, the reduction in the level of antioxidant compounds or ORAC values was in line with the level of ROS generation.

5.5.4 Glucosinolates and hydroxycinnamic acids

Enhancement of glucosinolate in broccoli, especially of the indole-type, was observed in the florets exposed to all three oxidative stresses (Figure 5.5). As with the total content of phenylpropanoid described in the earlier section, there were significant differences between groups for all the stresses. Titers of total glucobrassicins of broccoli improved with both doses of UV-B and H_2O_2; in contrast, the enhancement of total glucobrassicins by O_3 was observed only in the florets exposed to hormetic dose, but with a significant decrease at the higher dose.

On the average over the storage period, the hormetic and high dose of UV-B increased the total glucobrassicins by 18 %, and 22 %, respectively, relative to the control (Figure 5.5a). Glucobrassicin was the most abundant of indole-type glucosinolate quantified in this study, followed by neoglucobrassicin, 4-methoxy-glucobrassicin and 4-hydroxy-glucobrassicin (Table 5.2). Changes in the content of individual glucosinolates in the florets exposed to UV-B was also observed. Glucobrassicin was enhanced by 18 % in both UV-B doses groups, and its conversion to neoglucobrassicin was evident by the increase to more than 30 % in both dose-groups of UV-B (Table 5.2). The titers of 4-methoxy-glucobrassicin and 4-hydroxy-glucobrassicin were also superior compared with the un-exposed florets, but less substantial compared with the increase in neoglucobrassicin.

The exposure of florets to 5 ppm of ozone for 60 min, also increased the titers of total glucobrassicins by 13 %; but the exposure of florets to the same concentration for 720 min decreased the titers by 12 % (Figure 5.5b). The same trend was perceived with titers of glucobrassicin where hormetic dose of ozone elevated level by 11 % in comparison to the high dose, which drastically reduced the titer by 58 % as compared with the control (Table 5.2). Further conversion of glucobrassicin to 4-methoxy-glucobrassicin in broccoli treated with ozone appeared to occur with the high dose, since its titer was 40 % more elevated compared with the un-exposed florets (Table 5.2).

An augmentation of 19 % on total glucobrassicins content was noted in florets exposed to 1.25 mM of H_2O_2 for 180 min, in comparison to the control florets. Also, an augmentation of 14 % was observed on florets exposed to a higher concentration of 5.0 mM of H_2O_2 for the same duration (Figure 5.5c). As observed with UV-B and O_3, the individual indole-type glucosinolates were also affected. The titers of glucobrassicin, 4-methoxy-glucobrassicin and 4-hydroxy-glucobrassicin were all enhanced compared with the un-treated florets. However, the most significant enhancement was observed on neoglucobrassicin, where the hormetic dose of 1.25 mM/180 min of H_2O_2 enhanced its titer by 34 % (Table 5.2).

Aliphatic-type glucosinolates were less susceptible to induction compared with indole-type glucosinolates, and they were less abundant as well (Figure 5.6). Glucoraphanin in florets exposed to 1.5 $kJ.m^{-2}$ was enhanced by 11 %, while UV-B dose of 7.2 $kJ.m^{-2}$ elevated the titers by 16 % (Figure 5.6a). The exposure of florets to the hormetic dose of O_3 elevated the titers by 19 % compared with the control, while the high dose produced the opposite effect, by reducing the overall concentration by 4 % (Figure 5.6b). Likewise, glucoraphanin in broccoli was enhanced by 17 % and 16 % with the hormetic and high dose of hydrogen peroxide, respectively (Figure 5.6c).

The expression of genes related to the jasmonic acid and salicylic acid pathways, accompanied by the accumulation of indole-type glucosinolates in broccoli sprouts was reported by Mewis et al. (2012). This study also shows the accumulation of indole-type glucosinolates, especially either, either 1-methoxy glucobrassicin or neoglucobrassicin. After UV-B light treatment, the glucosinolate-gene related pathway was overexpressed (Figure 5.8). Overexpression of tryptophan N-hydroxylase (CYP79B3) was the most relevant among the genes analyzed, where the expression was 6 folds higher in florets exposed to the hormetic dose, and 10 folds superior with the high dose of UV-B (Figure 5.8a). The extreme dose of UV-B light also affected the expression pattern of dihomomethionine N-hydroxylase (CYP79F1) by three folds.

Similarly to UV-B, the high dose of O_3 at 5 ppm for 720 min amplified the expression of CYP79B3 by 8 folds and also the expression of CYP79F1 by 5 folds (Figure 5.8b). The hormetic dose of 5 ppm of O_3 applied for 60 min significantly increased ($p<0.05$) the expression of phenylalanine N-hydroxylase (CYP79A2). However, the relative expression of CYP79A2 in the florets exposed to H_2O_2 was smaller in comparison to UV-B and O_3 treatments (Figure 5.8c); but the genes coding for indole glucosinolates were relatively highly expressed. The overexpression of CYP79B3 was 3 folds higher than the control in the florets exposed to 1.25 mM of H_2O_2 for 180 min (the hormetic dose of ozone).

On the other hand, the expression of the phenylpropanoid pathway enzymes was not significantly affected by the three stresses, as seen from the low relative gene expression in the treated florets (Figure 5.8). In particular, this was a surprising observation with respect to UV-B, given that phenylpropanoids, especially the flavonoids, are known to accumulate in response to UV-B and function as UV-B shields or filters as well as antioxidants (Hagen et al., 2007; Liu et al., 2011). It is possible that this did not occur because the application of UV-B in this study was acute and not chronic. Another possibility is that the synthesis of secondary metabolites, is dose-dependent (Schreiner et al., 2012), while carotenoids and glucosinolates in nasturtium are induced under low doses of UV-B (Schreiner et al., 2009), the levels of flavonols in apples (Hagen et al., 2007) increased with high doses of UV-B. Furthermore, phenylalanine and tryptophan are produced by different branches of the shikimate pathway, the biosynthesis of indole-glucosinolates would be favored if phenylalanine is limiting and not tryptophan, even when there is no enzyme limitation.

The phenolic compounds and hydroxycinnamic acids (HCA) content of florets exposed to the oxidative stresses, over the storage period, were superior to unexposed florets despite the low levels of expression of enzymes in the phenolic pathway. The pattern of changes in the total hydroxycinnamic acids (HCA) content in broccoli florets was similar to those of glucobrassicins after exposure to the three oxidative stresses, where the augmentation of HCA was observed with the application of hormetic and high doses of the three stresses, except for the high dose of O_3. The florets exposed to 1.5 and 7.2 kJm^{-2} of UV-B enhanced the titer of total HCA by 12 % (Figure 5.7a), consistent with the increase in total phenolic compounds. Moreover, significant differences between both doses of UV-B were observed in the content of 1-sinapoyl-2-feruloyl gentiobiose (Table 5.3). A decrease of 15 % in the levels of 1-sinapoyl-2-feruloyl gentiobiose was observed in florets exposed to 5 ppm of O_3 for 720 min, compared with the un-treated florets (Table 5.3). The total HCA of broccoli

was also reduced by 9 % with the high dose of O_3. However, a small increase in the total HCA of 5 % was observed when florets were exposed to the hormetic dose of O_3 (Figure 5.7b). The exposure of broccoli to H_2O_2 also increased the titer of total HCA with both doses; nonetheless, this increase was smaller in comparison with UV-B (Figure 5.7c).

Both H_2O_2 and O_3 were found to be the most effective oxidative stresses for the enhancement of glucoraphanin in florets, nonetheless, UV-B and H_2O_2 were found the most effective stresses to enhance the titers of glucobrassicins. Similarly, UV-B was the most effective stress for the enhancement of HCA and phenolic compounds, while O_3 decreased the content of phenolic-derivatives compounds in florets. Thus, exposure of florets to UV-B resulted in an optimal approach for enhancing the phytochemical content of florets. Nonetheless, the phytochemical contents were expressed on dry weight basis. With increase in moisture loss during storage, it can be expected that the dry weight of the tissue would increase as well. This would somewhat underestimate the phytochemical content of the tissue samples drawn towards the end of storage. This holds true for all the three stresses and should not affect relative comparison of the stresses with respect to their impact on phytochemical contents, except for the high dose of ozone. In this case, the weight loss may not be attributed only to moisture loss and some mass loss is possible, where some overestimation of the phytochemical content could be possible.

Effectively, the results show that the abiotic stresses influence the secondary metabolism of broccoli florets. The accumulation of secondary metabolites in plant bodies in response to stresses is well documented (Frohnmeyer and Staiger, 2003; Kangasjarvi et al., 1994; Rosemann et al., 1991). This potential is increasingly recognized in postharvest fruits and vegetables as well (Artés-Hernández et al., 2007; Mewis et al., 2012; Pérez-Balibrea et al., 2011). In plant bodies subject to drought stress, it is suggested that the stress generates a heavy oxidative stress and a surplus of reduction equivalents, leading to shifts in metabolic processes towards synthetic activities, typically those of reduced compounds (Selmar and Kleinwächter, 2013). Under drought conditions, CO_2 fixation through the Calvin cycle decreases, resulting in the reduction of the consumption of reduction equivalents ($NADPH+H^+$).

The reduction equivalents may also accrue in stressed plants by stress-induced accumulation of free proline (Hare and Cress, 1997; Liang et al., 2013). Much of the efforts towards the understanding the role of proline were focused on its function in osmotic adjustment, as buffer against stress-induced cytoplasmic acidification, as free-radical scavenger, and as stabilizer of

cellular structures. The recent developments point out that the biosynthesis of proline depletes NADP pool, and also it is coupled to pentose phosphate pathway that generates NADPH and sugar phosphates for synthetic pathways.

With the synthetic power available and the cellular environment being oxidative, it is possible that the synthetic routes for reduced compounds including antioxidants such as phenolic compounds are favored. Such reduced compounds may have disease defense function as well. The enhancement of glucosinolates by the three stresses is a case in point (Figure 5.9). Methionine and tryptophan are the two amino acid precursors of the aliphatic glucosinolate, glucoraphanin, and the indole glucosinolate, glucobrassicin, respectively, with average carbon oxidation state or number (ACON) of -0.60 and -0.36, respectively. It would seem that tryptophan at the higher oxidative state is preferentially metabolized with an end product of glucobrassicin (ACON of -0.56), whereas methionine metabolism leads to glucoraphanin with ACON of -0.50. Again the preferential metabolism of tryptophan (ACON, -0.36) compared with phenylalanine (ACON, -0.56) can be sensed on this basis. The hydroxycinnamic acids derived from phenyl alanine such as cinnamic acid, ferulic acid and sinapic acid are characterized by their ACON of -0.44, -0.20 and -0.18, respectively. The ACON values of the flavonoids, kaempherol and rutin, are -0.14, -0.08, respectively. The preferential enhancement of indole glucosinolates than hydoxy cinnamates or flavonoids seen in broccoli in response to the stresses may be because of the biosynthetic trend towards more reduced compounds. The downstream modification of glucobrassicin to 4-methoxyglucobrassacin in response to ozone leads to a reduction of ACON from -0.56 to -0.65 (4-methoxyglucobrassacin). The latter also appears to play a part in disease resistance in *Brassicas.* Clay et al. (2009) showed that ethylene and 4-methoxyglucobrassicin were necessary for resistance to pathogens and callose formation in *Arabidopsis.*

UV-B, ozone and hydrogen peroxide treatments mostly influence overexpression of glucosinolate-related genes. Compared with UV-B and O_3 treatments, the relative expression of glucosinolate-related genes in florets exposed to hydrogen peroxide were smaller, but the pattern of expression was similar for the three stresses (Figure 5.8). The correlation between the gene expression of CYP79B3, and the titers of indole glucosinolates in treated broccoli florets, suggests that the target of UV-B, O_3 and specially H_2O_2 is likely to be the pathway for indole glucosinolates.

5.6 Conclusions

The hormetic effect of the abiotic stresses, UV-B, ozone and hydrogen peroxide, was apparent for color retention in broccoli. However, the degree of color retention in broccoli in response to hormetic doses of the stresses was variable; it was superior with UV-B and was poorer with H_2O_2. The oxidative stress intensity as manifest in the stress respiration was significantly higher with O_3, even at the hormetic dose, followed by H_2O_2 and UV-B. Ozone also caused a significant weight loss during exposure of the florets even with the hormetic dose, and it was drastic at the high ozone dose. Hormetic doses of O_3 and H_2O_2 were effective in elevating the levels of glucoraphanin and glucobrassicins compared with UV-B; whereas UV-B was more effective in the enhancement of hydroxycinnamic acids than ozone and H_2O_2. Furthermore, high ozone dose depressed the levels of both glucosinolates and hydroxycinnamic acids. Thus UV-B appears to be the stress exhibiting balanced effects with respect to quality preservation and enhancement of phyto-compounds in broccoli florets. Results showed a good correlation between gene expression of CYP79B3, and the titers of indole glucosinolates in the treated broccoli florets, suggesting that the target of UV-B, O_3 and specially H_2O_2 is likely to be the branch pathway of indole glucosinolates. It appears that the accumulation of indole glucosinolates may be preferential among the glucosinolates or among the other secondary metabolites such as phenolic compounds because of their most reduced state.

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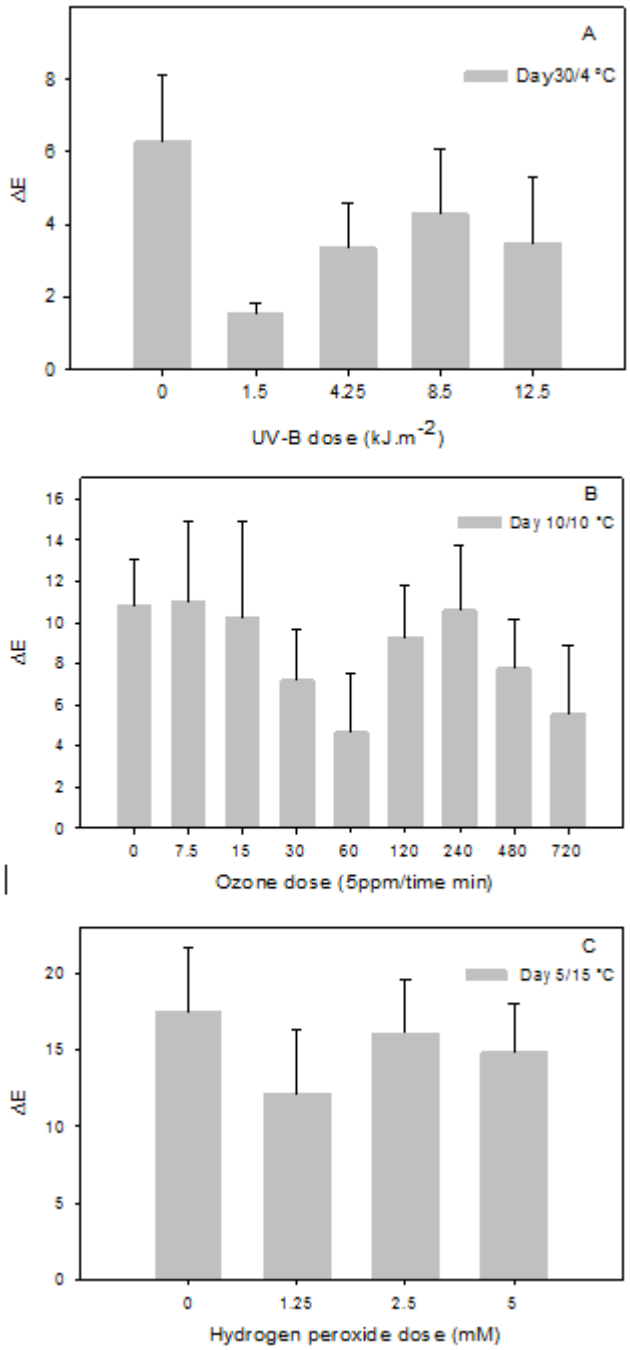

Figure 5.1 Hormetic dose determination for UV-B, Ozone (O_3) and Hydrogen peroxide (H_2O_2). The hormetic dose of the stresses UV-B (A), O_3 (B) and H_2O_2 (C) was determined from color retention response using Total Color Difference (ΔE) at the end of the storage period; UV-B, 4 °C/30 day; O_3, 10 °C/10day; and H_2O_2, 15 °C/5day. Data points are means ± standard deviation for n=9.

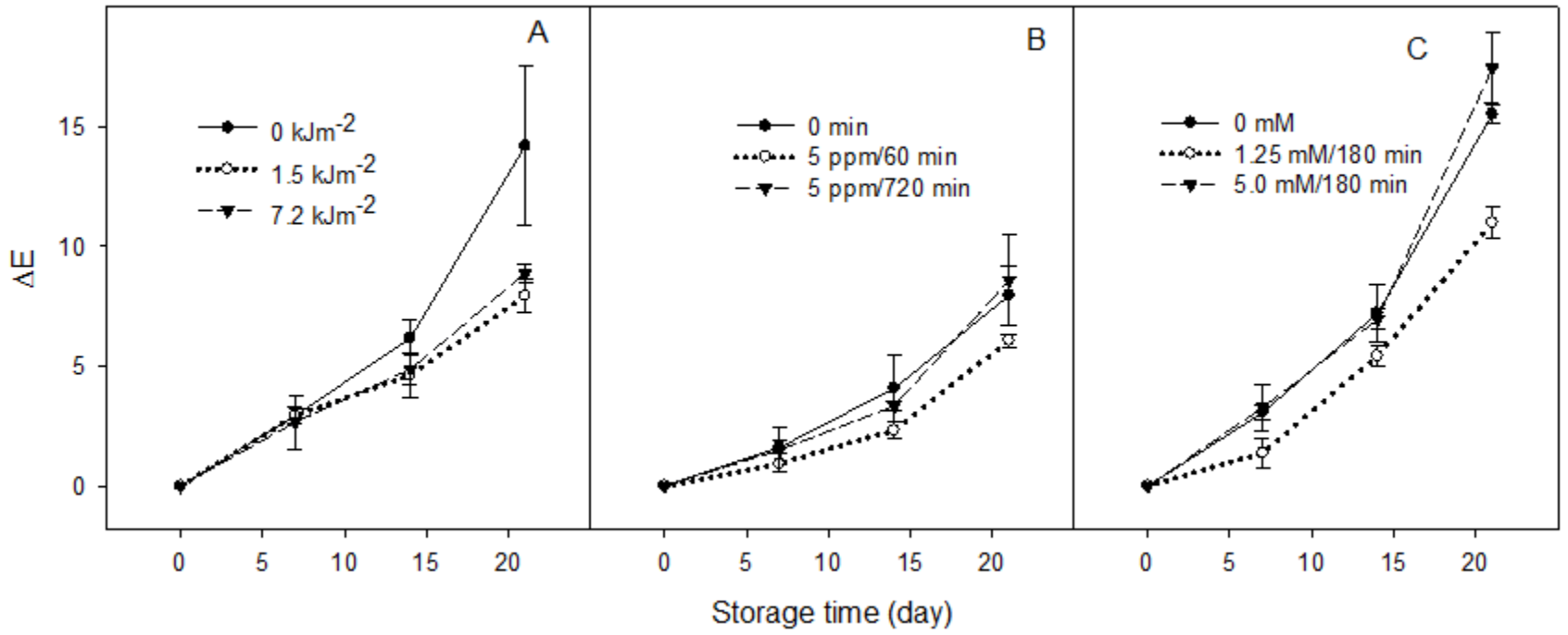

Figure 5.2 Evolution of total color change (ΔE) of broccoli florets exposed to oxidative stresses during storage for 21 days at 4 °C. Broccoli florets were treated with three different doses: UV-B (A), (●), control; (○), 1.5 kJm^{-2} (hormetic dose), and (▼), 7.2 kJm^{-2} (high dose). O_3 (B) (●), control; (○), 5ppm/60min (hormetic dose), and (▼), 5ppm/720min (high dose). H_2O_2 (C) (●), control; (○), 1.25mM/180 min (hormetic dose), and (▼), 5.0 mM/180 min (high dose). Color change (ΔE) was followed during 21. Vertical bars represent standard deviation (n=9).

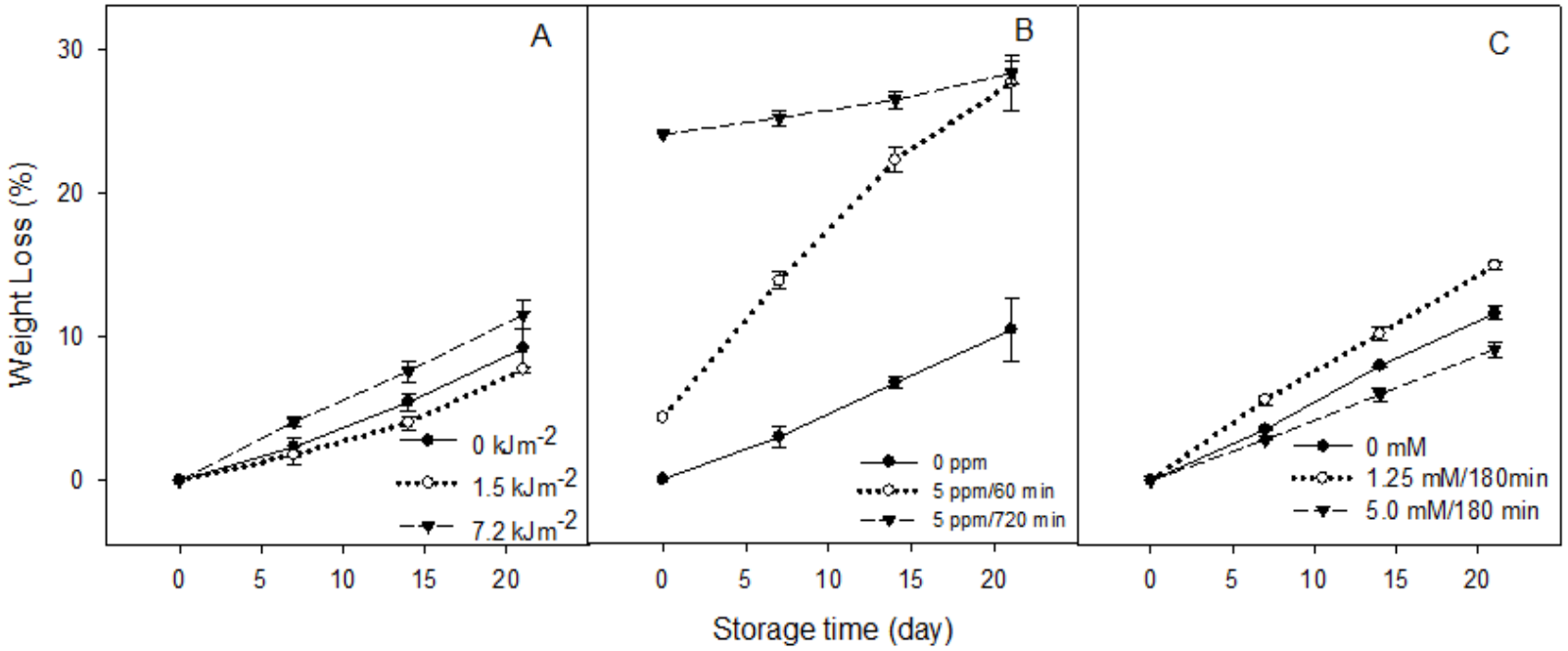

Figure 5.3 Weight loss of broccoli florets exposed to oxidative stresses during storage for 21 days at 4 °C. Broccoli florets were treated with three different doses: UV-B (A), (●), control; (○), 1.5 kJm^{-2} (hormetic dose), and (▼), 7.2 kJm^{-2} (high dose). O_3 (B) (●), control; (○), 5ppm/60min (hormetic dose), and (▼), 5ppm/720min (high dose). H_2O_2 (C) (●), control; (○), 1.25mM/180 min (hormetic dose), and (▼), 5.0 mM/180 min (high dose). Weight loss was followed during 21 days. Vertical bars represent standard deviation (n=4).

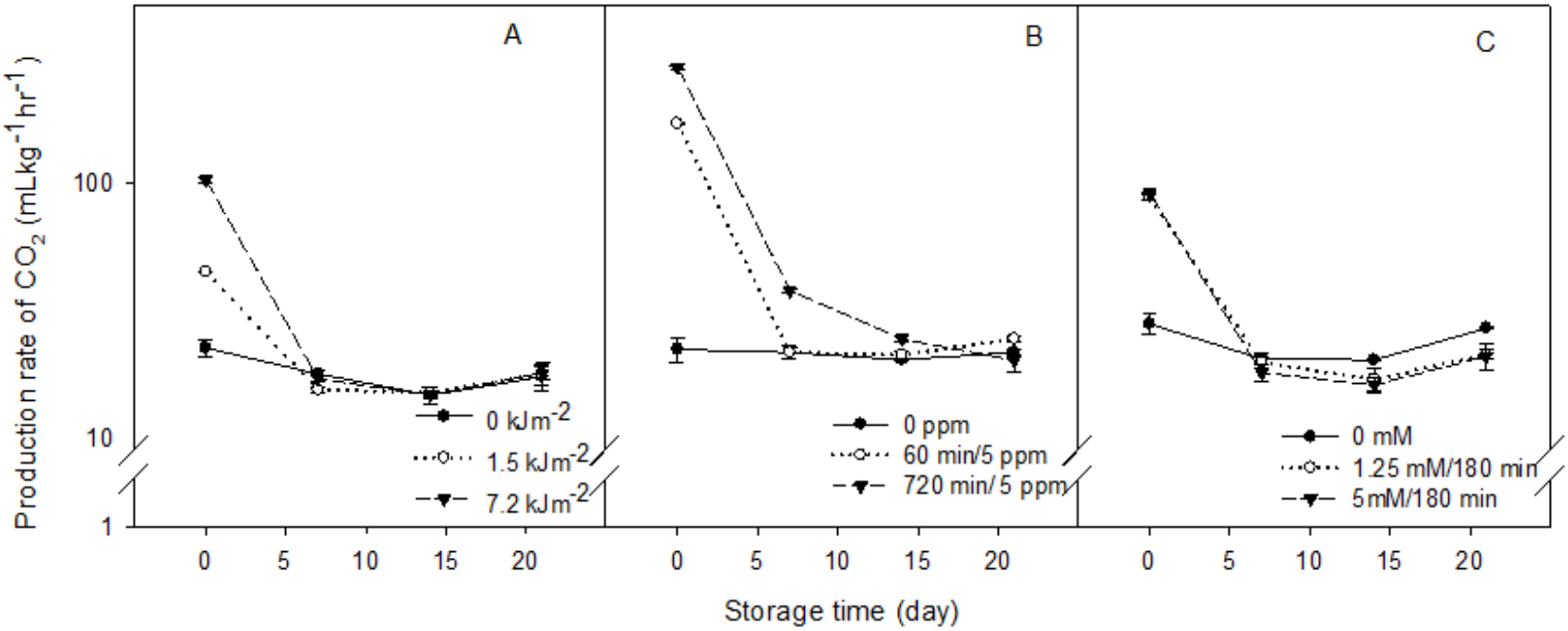

Figure 5.4 Evolution of respiration rate of broccoli florets exposed to oxidative stresses during storage for 21 days at 4 °C. Broccoli florets were treated with three different doses: UV-B (A), (●), control; (○), 1.5 kJm^{-2} (hormetic dose), and (▼), 7.2 kJm^{-2} (high dose). O_3 (B) (●), control; (○), 5ppm/60min (hormetic dose), and (▼), 5ppm/720min (high dose). H_2O_2 (C) (●), control; (○), 1.25mM/180 min (hormetic dose), and (▼), 5.0 mM/180 min (high dose). Respiration was followed during 21 days. Vertical bars represent standard deviation (n=4).

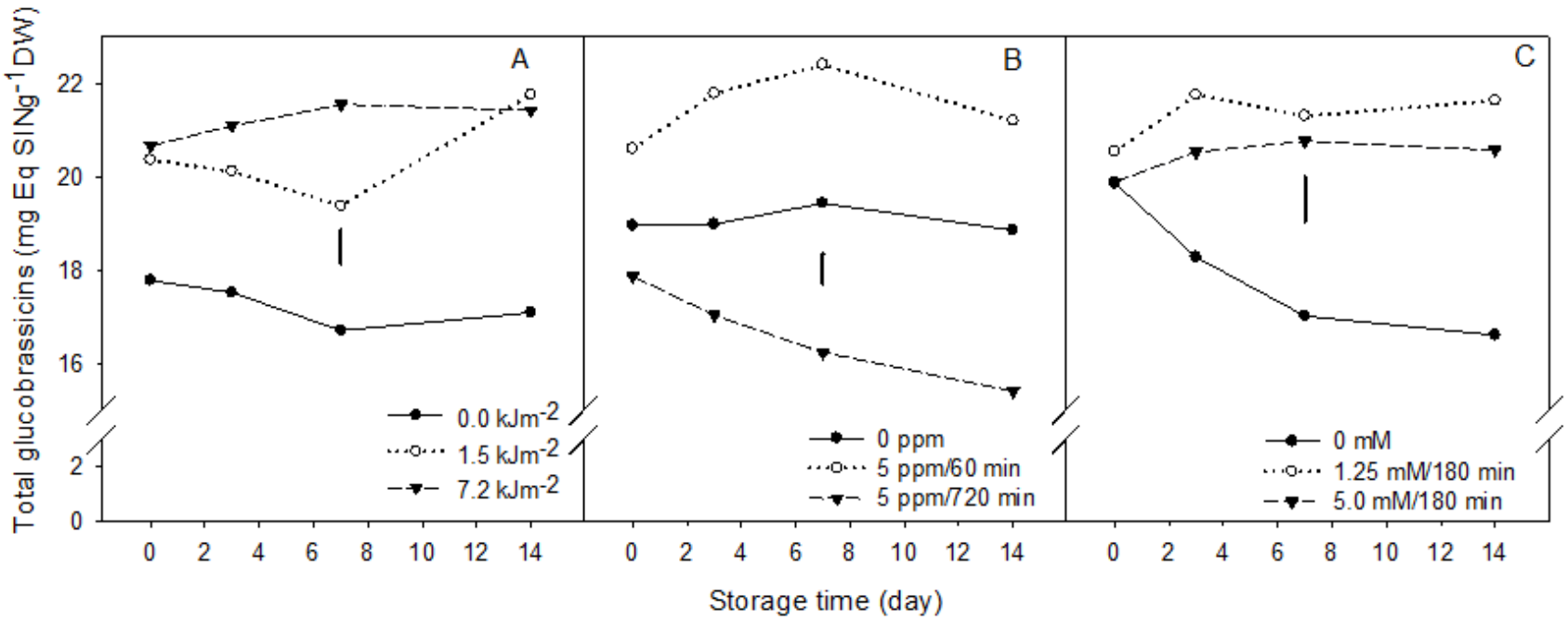

Figure 5.5 Total glucobrassicins content of florets exposed to oxidative stresses during storage for 14 days at 4 °C. Broccoli florets were treated with three different doses: UV-B (A), (●), control; (○), 1.5 kJm^{-2} (hormetic dose), and (▼), 7.2 kJm^{-2} (high dose). O_3 (B) (●), control; (○), 5ppm/60min (hormetic dose), and (▼), 5ppm/720min (high dose). H_2O_2 (C) (●), control; (○), 1.25mM/180 min (hormetic dose), and (▼), 5.0 mM/180 min (high dose). The content of total glucobrassicins (glucobrassicin + neoglucobrassicin + 4-hydroxyglucobrassicin + 4-methoxyglucobrassicin) was followed during 14 days. The vertical bar indicates differences (LSD 0.05) among the treatments. UV-B; LSD = 0.76; O_3, LSD = 0.67 and H_2O_2, LSD = 0.98.

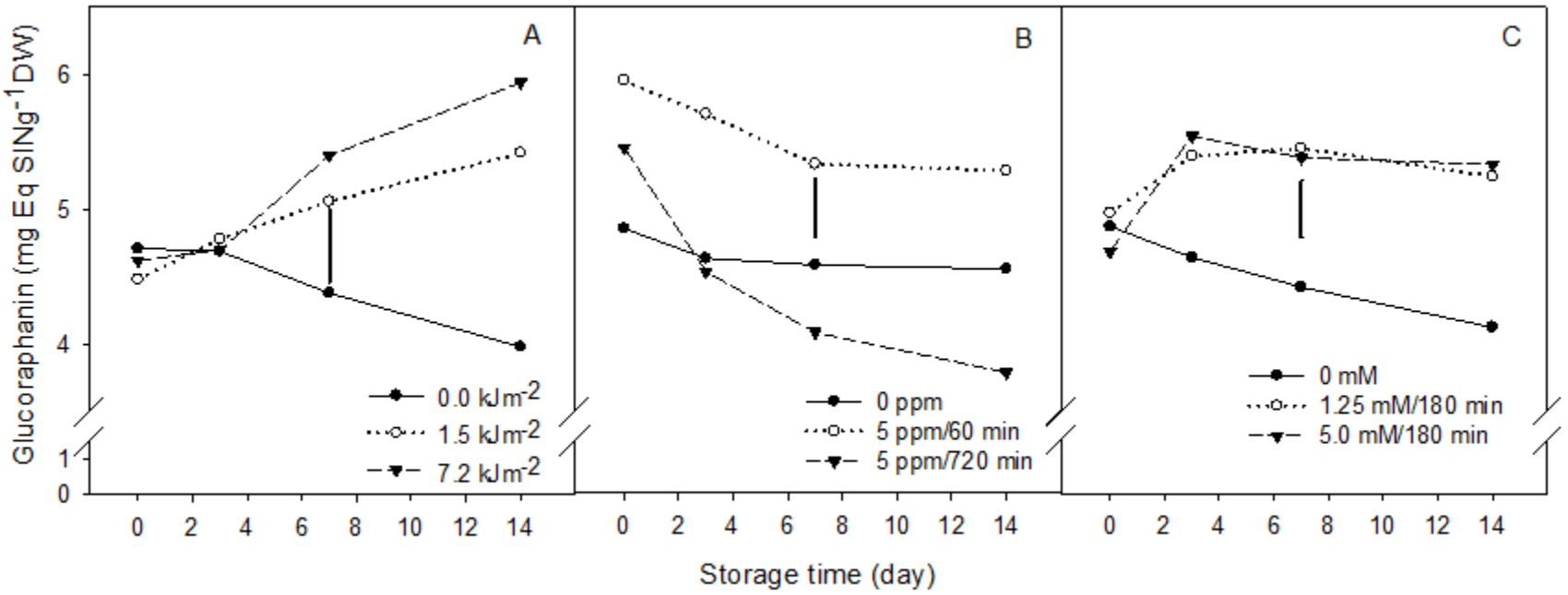

Figure 5.6 Glucoraphanin content of florets exposed to oxidative stresses during storage for 14 days at 4 °C. Broccoli florets were treated with three different doses: UV-B (A), (●), control; (○), 1.5 kJm^{-2} (hormetic dose), and (▼), 7.2 kJm^{-2} (high dose). O_3 (B) (●), control; (○), 5ppm/60min (hormetic dose), and (▼), 5ppm/720min (high dose). H_2O_2 (C) (●), control; (○), 1.25mM/180 min (hormetic dose), and (▼), 5.0 mM/180 min (high dose). The content of glucoraphanin was followed during 14 days. The vertical bar indicates differences (LSD 0.05) among the treatments. UV-B; LSD = 0.54; O_3, LSD = 0.43 and H_2O_2, LSD = 0.42.

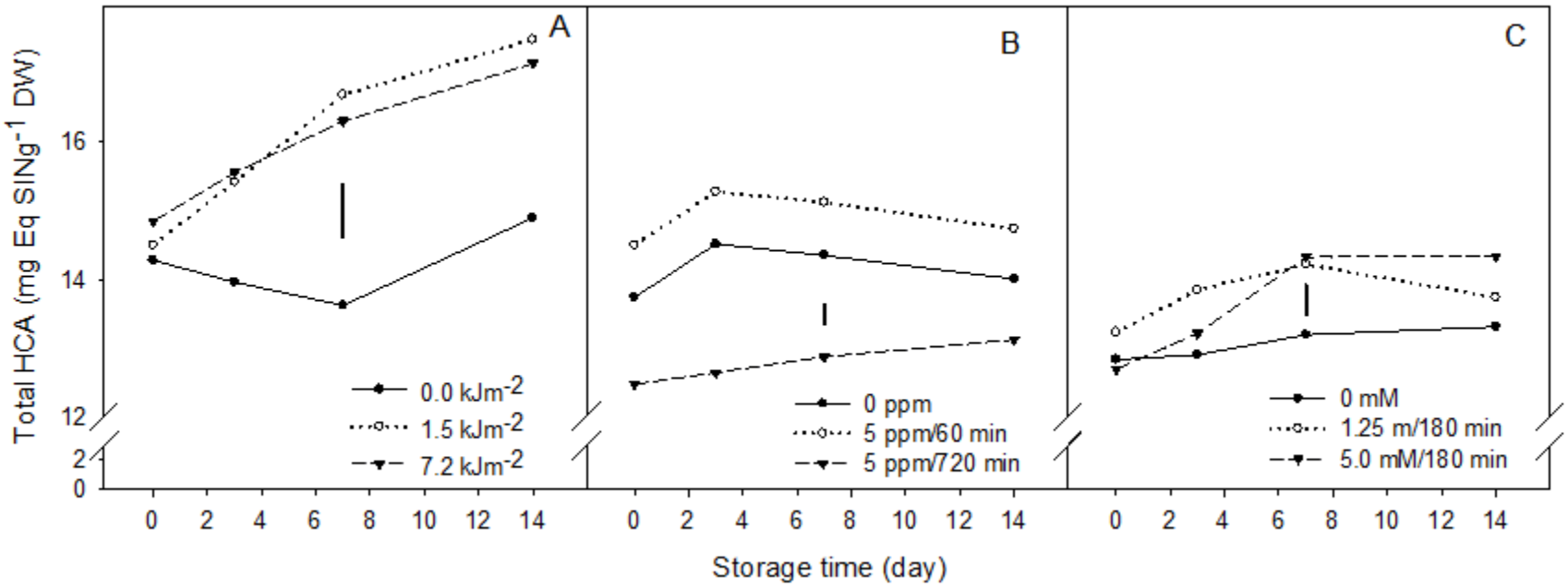

Figure 5.7 Total hydroxy-cinnamic acid (HCA) content of florets exposed to oxidative stresses during storage for 14 days at 4 °C. Broccoli florets were treated with three different doses: UV-B (A), (●), control; (○), 1.5 kJm^{-2} (hormetic dose), and (▼), 7.2 kJm^{-2} (high dose). O_3 (B) (●), control; (○), 5ppm/60min (hormetic dose), and (▼), 5ppm/720min (high dose). H_2O_2 (C) (●), control; (○), 1.25mM/180 min (hormetic dose), and (▼), 5.0 mM/180 min (high dose). The content of total HCA (1,2-disinapoyl gentibiose + 1-sinapoyl-2-feruloyl gentibiose + 1,2-diferuloyl gentibiose + 1,2,2-trisinalpoyl-gentibiose + 1,2-disynalpoyl-2-feruloyl gentibiose) was followed during 14 days. The vertical bar indicates differences (LSD 0.05) among the treatments. UV-B; LSD = 0.78; O_3, LSD = 0.28 and H_2O_2, LSD = 0.43.

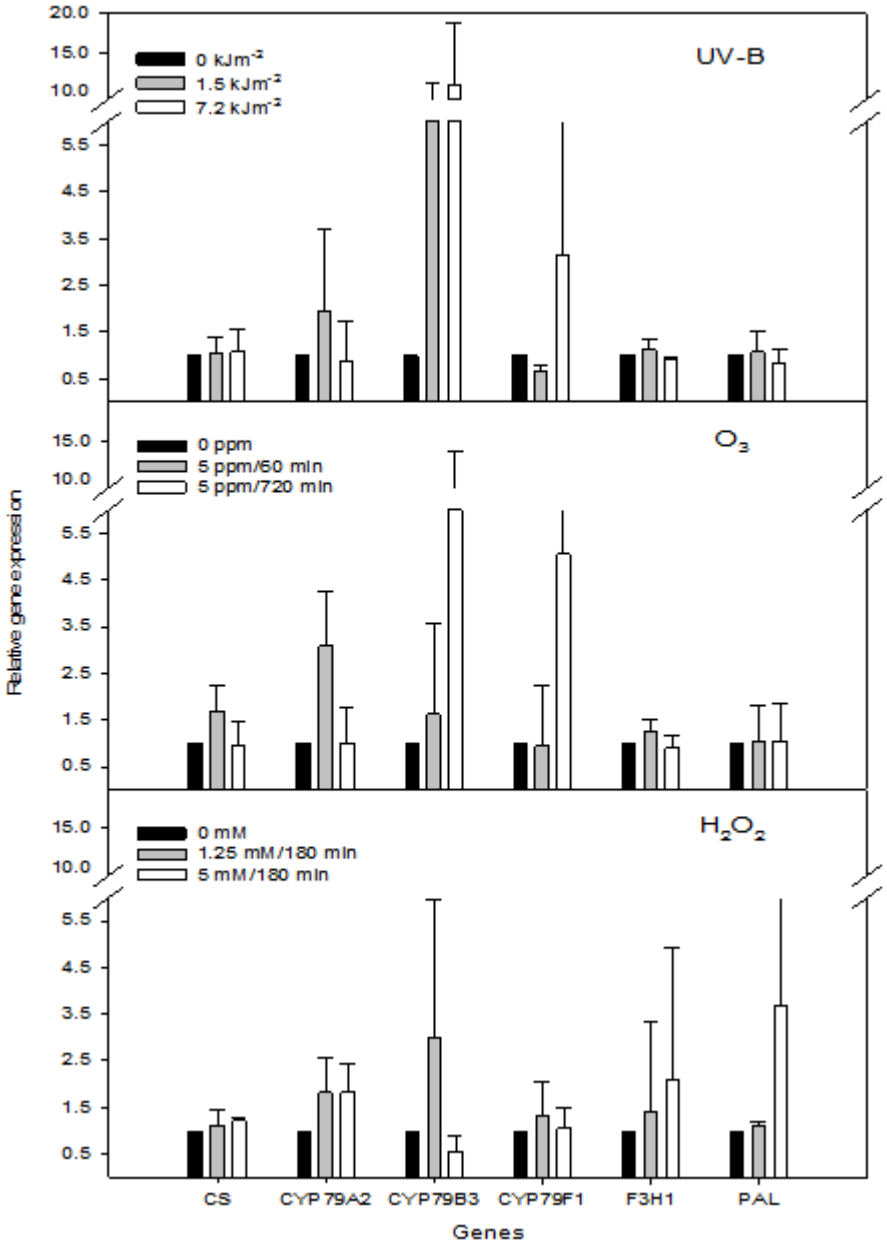

Figure 5.8 Gene expression analysis of broccoli florets exposed to oxidative stresses. Gene expression in broccoli florets exposed to UV-B, O_3 and H_2O_2 was measured after each treatment on chalcone synthase (CHS); phenylalanine N-hydroxylase (CYP79A2); tryptophan N-hydroxylase 2 (CYP79B3); dihomomethionine N-hydroxylase (CYP79F1); flavanone 3-hydroxylase (F3H1) and phenyl alanineamoniolyase (PAL). Standard deviation of the mean is presented with vertical bars (n = 3).

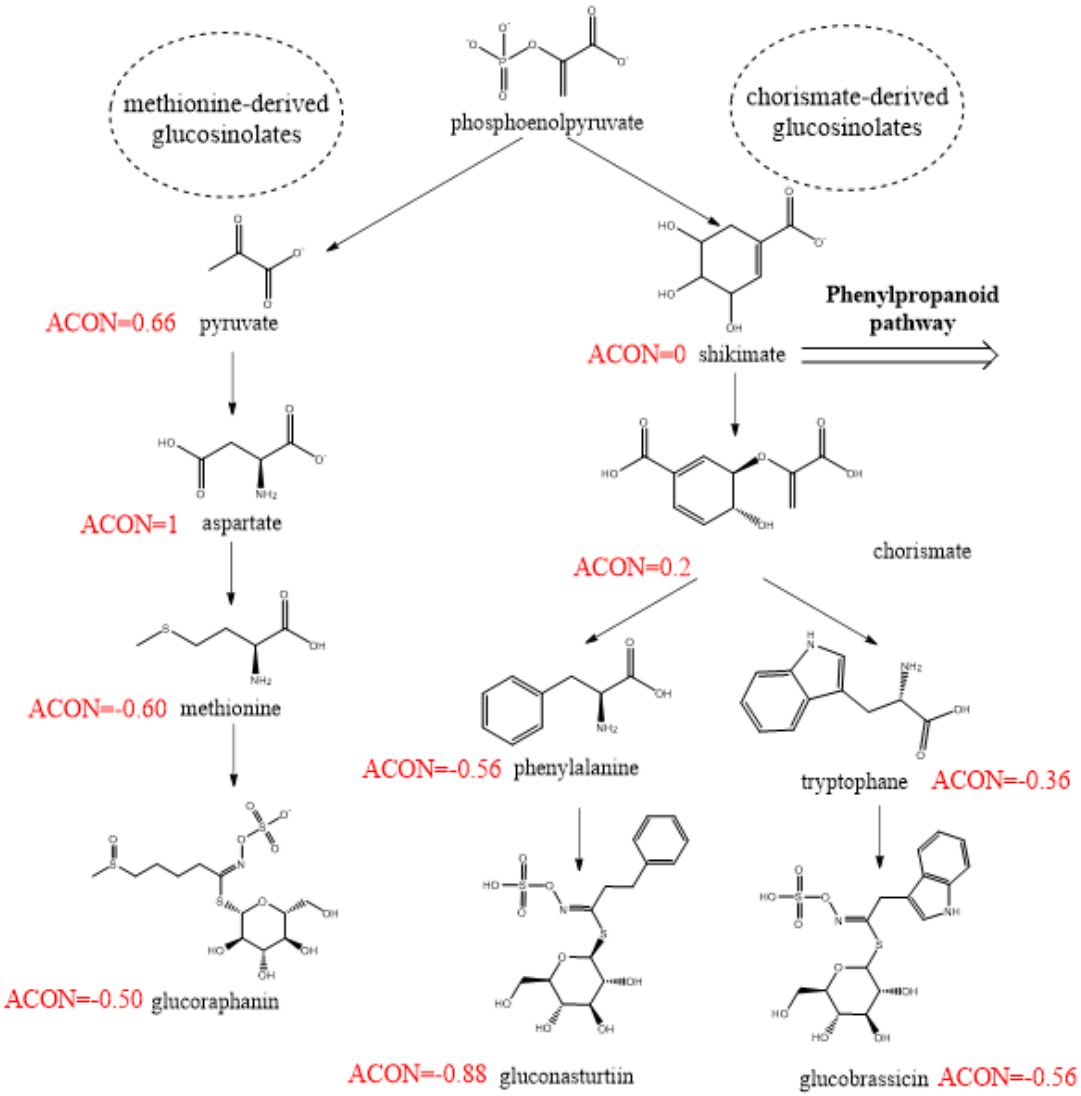

Figure 5.9 Average carbon oxidation number (ACON) in the glucosinolate pathway.

Table 5.1 Concentration values of ORAC, ascorbic acid (oxidized, reduced and total), total phenols, total flavonoids, rutin and chlorogenic acid in broccoli florets exposed to oxidative stresses. Florets were exposed to three doses of UV-B, O_3 and H_2O_2 and stored in darkness at 4 °C for 21 days. The values were time-averaged (0, 7, 14 and 21 days).

	UV-B	Ozone	H_2O_2
ORAC (mg eq trolox g^{-1} DW)			
Control	164.8±13	206.7±7.1	153.8±6.1
Hormetic	176.2±10.7	169.46±15.1	135.0±7.8
High	194.9±14.3	156.11±9.5	129.8±8.4
Total ascorbic acid (mg eq AA. g^{-1} DW)			
Control	7.5±0.4	11.2±0.5	12.8±0.6
Hormetic	7.2±0.3	11.0±0.5	12.5±0.8
High	7.1±0.2	9.4±0.6*	12.4±0.4
Reduced ascorbic acid (mg eq AA. g^{-1} DW)			
Control	4.7±0.3	7.8±0.2	9.5±0.5
Hormetic	4.4±0.2	7.6±0.2	9.2±0.5
High	4.2±0.2	5.4±0.3	8.8±0.3
Oxidized ascorbic acid (mg eq AA. g^{-1} DW)			
Control	2.8±0.1	3.3±0.2	3.3±0.1
Hormetic	2.8±0.2	3.4±0.3	3.4±0.2
High	2.9±0.1	3.9±0.3	3.6±0.1
Total Phenols (mg eq GA.g^{-1} DW)			
Control	13.5±0.6	15.2±0.9	14.0±0.4
Hormetic	14.5±0.3	16.2±1	14.7±0.4
High	14.5±0.6	14.2±0.9	15.2±0.3
Total Flavonoids(mg eq QE.g^{-1} DW)			
Control	4.3±0.7	5.9±0.5	5.4±0.3
Hormetic	5.0±0.5	7.8±0.4	5.9±0.2
High	5.1±0.4	7.1±0.6	5.8±0.5
Rutin (mg eq SIN.g^{-1} DW)			
Control	0.6±0.1	0.4±0.1	0.4±0.1
Hormetic	0.7±0.1	0.4±0.1	0.7±0.1
High	0.8±0.1	0.4±0.1	0.8±0.1
Chlorogenic acid (mg eq SIN.g^{-1} DW)			
Control	2.5±0.2	2.1±0.1	2.0±0.1
Hormetic	2.8±0.2	2.3±0.1	2.1±0.1
High	2.8±0.2	1.9±0.1	2.0±0.1

UV-B: control, 0.0 $kJ.m^{-2}$; hormetic, 1.5 $kJ.m^{-2}$; high, 7.2 $kJ.m^{-2}$. O_3: control, 0 ppm; hormetic, 5ppm/60min; high, 5ppm/720min. H_2O_2: control 0 mM; hormetic, 1.25mM/180 min; high, 5mM/180 min.The asterisk indicates that the value is significantly different from the corresponding control at $p < 0.05$.

Table 5.2 Glucosinolate content of UV-B, O_3 and H_2O_2 treated broccoli florets. Florets were exposed to three doses of UV-B, O_3 and H_2O_2 and stored in darkness at 4 °C for 21 days. The values were time-averaged (0, 7, 14 days).

	UV-B	Ozone	H_2O_2
Glucobrassicin (mg eq SIN.g^{-1} (DW))			
Control	9.2±1.0	10.4±0.9	8.6±0.3
Hormetic	10.6±1.1	11.6±0.5	9.8±0.4
High	10.9±0.6	6.1±0.4	9.6±0.4
Neoglucobrassicin(mg eq SIN.g^{1}(DW))			
Control	4.1±0.7	4.7±0.2	4.3±0.8
Hormetic	5.5±0.7	5.1±0.3	5.8±0.8
High	5.6±1.4	5.1±0.3	5.3±0.9
4-hydroxy-glucobrassicin (mg eq SIN. g^{1}(DW))			
Control	1.5±0.3	2.0±0.3	2.2±0.1
Hormetic	1.7±0.2	2.2±0.2	2.4±0.2
High	1.6±0.1	2.6±0.3	2.2±0.1
4-methoxy-glucobrassicin (mg eq SIN. g^{1}(DW)			
Control	2.4±0.3	2.0±0.2	2.9±0.2
Hormetic	2.6±0.1	2.6±0.3	3.4±0.2
High	3.0±0.3	2.8±0.1	3.3±0.2

UV-B: control, 0.0 kJ.m^{-2}; hormetic, 1.5 kJ.m^{-2}; high, 7.2 kJ.m^{-2}. O_3: control, 0 ppm; hormetic, 5ppm/60min; high, 5ppm/720min. H_2O_2: control 0 mM; hormetic, 1.25mM/180 min; high, 5mM/180 min. The asterisk indicates that the value is significantly different from the corresponding control at $p < 0.05$.

Table 5.3 Hydroxycinnamic-acid content (HCA) of UV-B, O_3 and H_2O_2 treated broccoli florets. Florets were exposed to three doses of UV-B, O_3 and H_2O_2 and stored in darkness at 4 °C for 21 days. The values were time-averaged (0, 7, 14 d).

	UV-B	Ozone	H_2O_2
1,2-disinapoyl gentibiose (mg eq SIN.g^{-1} (DW))			
Control	3.5±0.4	2.5±0.1	2.5±0.1
Hormetic	3.8±0.3	2.6±0.1	2.5±0.1
High	4.0±0.1	2.4±0.2	2.5±0.1
1-sinapoyl-2-feruloyl gentibiose (mg eq SIN.g^{-1} (DW))			
Control	4.7±0.4	6.0±0.3	4.8±0.2
Hormetic	5.7±0.6	6.3±0.3	5.1±0.4
High	5.7±0.2	5.1±0.4	4.9±0.3
1,2-diferuloyl gentibiose (mg eq SIN.g^{-1} (DW))			
Control	1.2±0.1	1.3±0.1	1.3±0.1
Hormetic	1.3±0.1	1.4±0.1	1.5±0.1
High	1.4±0.1	1.1±0.1	1.4±0.1
1,2,2-trisinalpoyl-gentibiose (mg eq SIN.g^{-1} (DW))			
Control	2.2±0.1	2.0±0.1	1.9±0.2
Hormetic	2.3±0.1	2.1±0.1	2.0±0.1
High	2.3±0.1	1.9±0.1	2.2±0.2
1,2-disynalpoyl-2-feruloyl gentibiose (mg eq SIN.g^{-1} (DW))			
Control	2.6±0.1	2.4±0.1	2.5±0.2
Hormetic	2.9±0.3	2.5±0.1	2.7±0.1
High	2.6±0.2	2.2±0.2	2.6±0.1

UV-B: control, 0.0 kJ.m^{-2}; hormetic, 1.5 kJ.m^{-2}; high, 7.2 kJ.m^{-2}. O_3: control, 0 ppm; hormetic, 5ppm/60min; high, 5ppm/720min. H_2O_2: control 0 mM; hormetic, 1.25mM/180 min; high, 5mM/180 min. The asterisk indicates that the value is significantly different from the corresponding control at $p < 0.05$.

Chapter VI: Effects of Fumigation with Ethanol and Methyl Jasmonate on Quality and Phyto-Compounds contents in Broccoli Florets during Storage

6.1 Résumé

Le brocoli est une bonne source alimentaire de glucosinolates, de flavonoïdes et d'acides hydroxycinnamiques. Ces substances ont été associées à la prévention du cancer et des maladies cardiovasculaires. Cependant les fleurons de brocoli sont également très périssables en raison de leur haut taux de respiration et une sensibilité élevée à l'éthylène. Différents traitements post-récolte incluant des stress abiotiques ont été évalués pour retarder la sénescence dans le brocoli, toutefois, l'effet de ces traitements sur des composés phytochimiques n'ont pas été complètement explorés. Des fleurons de brocoli (*Brassica oleracea*) ont été exposés à une atmosphère contenant 10000 ppm d'éthanol à la température pièce pendant 30 et 120 min. L'exposition de brocoli au jasmonate de méthyle (MeJA) a été effectuée aussi à température pièce en utilisant 1 ppm pendant 45 et 180 min. Le jaunissement des fleurons a été retardé en utilisant les deux doses d'éthanol par rapport aux fleurons non traités. Les titres de chlorophylle étaient également supérieurs dans les fleurons exposés aux deux doses d'éthanol par rapport au témoin. Les phénols totaux de fleurons ont augmenté de 15% et 18% avec l'application respectivement de la dose hormétique et la dose élevée d'éthanol suite à l'entreposage par rapport aux fleurons non exposées. Les titres de glucosinolates et acides hydroxycinnamiques ont été également renforcés par les deux doses d'éthanol. L'exposition des fleurons à 1 ppm de MeJA pour 45 min a retardé le jaunissement des fleurons, cependant des expositions plus longues ont produit le jaunissement au bout de 21 jours et le taux respiratoire des fleurons traités au MeJA a augmenté de façon significative ($p < 0.05$) par rapport aux fleurons non traités. La capacité antioxydante totale des fleurons a été considérablement réduite par les deux doses de MeJA. Pourtant, les titres d'acides hydroxycinnamiques ont augmenté avec les deux doses. La quantité de glucobrassicines totales dans le brocoli a augmenté après l'exposition des fleurons à deux doses, mais aucune différence significative n'a été observée dans le contenu de glucoraphanine. En conclusion, les traitements à l'éthanol peuvent effectivement retarder la sénescence et induire des composés phytochimiques. En revanche, l'effet sur la qualité de MeJA n'est pas considérable, mais il peut être utilisé pour améliorer le contenu phytochimique des fleurons, notamment de type indole.

6.2 Abstract

Broccoli is a good dietary source of glucosinolates, flavonoids and hydroxycinnamic acids. These substances have been associated with prevention of cancer and cardiovascular diseases. But broccoli florets are also very perishable due to their high respiration rate and ethylene sensitivity. Postharvest treatments involving abiotic stresses have been evaluated to delay senescence in broccoli, however, the effect of these treatments on phytochemicals have not been completely explored. Broccoli (*Brassica oleracea*) florets were exposed to an atmosphere containing 10,000 ppm of ethanol at room temperature for 30 and 120 min. Exposure to methyl jasmonate (MeJA) treatments was done at room temperature using 1 ppm for 45 and 180 min. Yellowing of the florets was delayed using 10,000 ppm of ethanol on both exposure times compared with untreated florets, and chlorophyll titers were also superior with both doses compared with the control. Total phenols of florets increased by 15 % and 18 % with the application of the hormetic and high dose, respectively over the storage compared with unexposed broccoli. The titers of glucosinolates and hydroxycinnamic acids were enhanced by both doses of ethanol as well. Exposure of florets to 1 ppm of MeJA for 45 min delayed the yellowing of florets, however longer exposures produced yellowing after 21 days and significantly ($p<0.05$) increased respiration rate compared with non-treated florets. The total antioxidant capacity of florets was considerably reduced by both doses of methyl jasmonate; however, titers of hydroxycinnamic acids increased with both doses. The amount of total glucobrassicins in broccoli increased after the exposure of florets to both doses, but not significant differences were observed in glucoraphanin content. In conclusion, ethanol treatments can effectively delay senescence and induce phytochemicals. In contrast, the effect of MeJA on quality is not substantial, however, it can be used to improve the phytochemical content of florets, especially of indole type.

Keywords: Broccoli, ethanol, methyl jasmonate, glucosinolates, hydroxy-cinnamic acids

6.3 Introduction

Broccoli (*Brassica oleracea* var. Italica) is a perishable vegetable susceptible to chlorophyll loss, fungal growth and off-odor generation during storage (Forney, 1995; Fukasawa et al., 2010). In order to prolong storage life, low temperature 0 °C (32 °F) along with high relative humidity, 98 to 100 %, are used during postharvest storage of broccoli florets (Toivonen and Forney, 2004). They are adequate conditions to maintain quality of broccoli florets up to 3 weeks of storage. Recently, consumers have been giving more attention not only to visual quality of produce but also to their nutritional and health-promoting properties. Broccoli florets are an excellent source of phytochemicals, including glucosinolates and flavonoids. Glucobrassicin and glucoraphanin are the most important glucosinolates in broccoli, and quercetin and kaempferol are the most abundant flavonoids (Moreno et al., 2006). Both glucosinolates and flavonoids have been recognized for the prevention of degenerative diseases such as cancer and cardiovascular diseases (Traka et al., 2008). These compounds are relatively stable during the storage at low temperatures. However, during storage at 20 °C, the amount of glucoraphanin, a precursor of a cancer preventive isothiocyanate, sulforaphane (4-methylsul-finylbutyl isothiocyanate) is lost by 55 % during the first 3 days (Rangkadilok et al., 2002).

In addition to their phytochemical role in human health, glucosinolates are the main defense compounds of *Brassicaceae* family, including broccoli (Troyer et al., 2001). Generally, the induction of these compounds is triggered by herbivore attack during the development of the plant in the field (Textor and Gershenzon, 2009). Abiotic stresses such as UV-B light are known to induce the production of glucosinolates and phenylpropanoids in broccoli sprouts (Mewis et al., 2012). Induction of defense mechanisms in postharvest commodities by physical agents has been used as an adjunct to cold storage to reduce phytochemical losses (Charles et al., 2009; Cisneros-Zevallos, 2003; Terry and Joyce, 2004). Abiotic stresses and plant signal modulators could have a potential commercial application, especially those which has been already Generally Recognized As Safe (GRAS) by the U.S. Food and Drug Administration (FDA). One example is the combination of methyl jasmonate (MeJA) and ethanol (EtOH) that reduced decay, maintained quality and enhanced antioxidant activity in Chinese bayberries (Wang et al., 2010).

Ethanol is commonly used as disinfectant in food industry and it has also been used to improve the shelf-life of postharvest commodities. The most common applications are dips and vapor treatment, which are utilized to inhibit discoloration, and reduce decay and ethylene biosynthesis (Jin

et al., 2013; Wang et al., 2014; Zhang et al., 2007). The suppression of ethylene synthesis is one of the implied effects of ethanol in stored produce (Jin et al., 2013), its suppression is mainly due to the loss of the activities of enzymes: 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase (Asoda et al., 2009). The delay in chlorophyll loss and the prevention of off-odors generation in broccoli may also be attributable to the reduction of ethylene production (Corcuff et al., 1996). Ethanol is also associated with the protection of reduced compounds, such as those associated with the ascorbate-glutathione cycle in broccoli florets (Mori et al., 2009). On the other hand, the generation of reactive oxygen species (ROS) (Albano, 2006) and oxidative damage to membrane lipids, proteins and DNA after ethanol intoxication in animals is known (Sergent et al., 2005). Even though ROS are highly toxic substances in the cell, they can also participate as signal molecules to control various processes including biosynthesis of defense compounds and programmed cell death (Apel and Hirt, 2004).

Jasmonic acid and methyl jasmonate (MeJA) are collectively referred to as jasmonates which are cellular signals implicated in fruit ripening and senescence (Cheong and Choi, 2003; Fan and Mattheis, 1999; Hung et al., 2006). Jasmonates are plant signals derived from α-linolenic acid, a polyunsaturated fatty acid (Cheong and Choi, 2003). Jasmonates are considered as hormones since they elicit cellular responses at low concentrations and far-remote locations (Rohwer and Erwin, 2008). The use of MeJA in crops has been focused on plant protection against microbial development, and enhancement of phytochemical compounds (Rohwer and Erwin, 2008). The defense-related responses of jasmonates in plants include synthesis of toxic secondary metabolites, formation of morphological barriers and changes in the rate of vegetative growth (Koo and Howe, 2009). Enhancement of secondary metabolites by jasmonates in crops includes resveratrol in grapevine (Vezzulli et al., 2007), and glucosinolates accumulation in pak choi (Wiesner et al., 2013). Similar to ethanol treatment, the generation of ROS in MeJA treated plants was observed in chloroplasts (Zhang and Xing, 2008).

This work reports on the effect of pre-storage application of ethanol and MeJA on senescence, quality and secondary metabolites (glucosinolates and hydroxycinnamic acids) in broccoli florets during storage at 4 °C.

6.4 Materials and Methods

6.4.1 Broccoli

Freshly harvested broccoli (*Brassica oleraceae* L. var. Italica 'Diplomat') heads were obtained from a commercial farm (Ile d'Orléans, Québec, Canada). Florets (300g) of uniform size (approximately, 7 cm) were separated from heads and randomly arranged in small plastic punnets of 500 mL. The punnets were placed in plastic containers of 5 L with perforations for ventilation, and containing a layer of water at the bottom to maintain high humidity (98-100%), and the containers were stored inside a controlled chamber overnight at 4 °C.

6.4.2 Treatment: Selection of optimal dose of ethanol and MeJA

Fumigation of ethanol and MeJA was carried out in a hermetic chamber made of Plexiglas with sampling ports and $59 \times 59 \times 60 cm$ size with a total volume of 0.2 m^3. A solution of 2 L of 25 % ethanol-water (v/v) was place in the chamber for 2 h in order to saturate desired concentration of ethanol in the atmosphere, prior to exposure of broccoli florets. A 10,000 ppm (v/v) of ethanol was selected based from preliminary experiments (data not shown). The ethanol vapor concentration in the atmosphere of the chamber was analyzed by gas chromatography. Standard curve for ethanol was prepared for a concentration range of 1 to 100 ppm and measurements were repeated four times for each concentration using an injection volume of 300 µL with a split ratio of 200 by GC-MS (Hewlett-Packard (HP) 6890 Network GC system (Wilmington, DE) coupled to a HP 5973 mass spectrometer). Broccoli florets were exposed to 10,000 ppm of ethanol was for 0, 30, 60, 120, 240, 300, 420 and 540 min. Saturation of the chamber with MeJA (Sigma Aldrich, 392707) was done using 10 mL of the substance which was introduced inside the chamber 24 h prior to the treatment. Methyl jasmonate concentration was of 1ppm based on vapor pressure of MeJA of $1.28 \times 10^{-04} mmHg$ at 23 °C (Acevedo et al., 2003) and the exposure times were from 0 to 720 min. The concentration of MeJA in the atmosphere of the chamber was analyzed by gas chromatography. Standard curve for MeJA was prepared for a concentration range of 1 to 10 ppm repeated 4 times using an injection volume of 200µL. MeJA concentrations were measured by GC-MS (Hewlett-Packard (HP) 6890 Network GC system (Wilmington, DE) coupled to a HP 5973 mass spectrometer).

6.4.3 Color and respiration rate of broccoli florets

The color and respiration rate of broccoli florets were determined as described elsewhere in the thesis in detail, in section 2.3.3 and 2.3.4, respectively.

6.4.4 Glucosinolates and hydroxycinnamic acid analysis

The glucosinolates and hydroxycinnamic acids were assayed by the methods described elsewhere in detail (section 2.3.6).

6.4.5 Chemical assays

The chlorophyll content, total phenolic content, total flavonoid content, reduced and total ascorbic acid contents, and the total antioxidant capacity were determined by methods described elsewhere in detail in section 4.3.4.1, 2.3.5.1, 2.3.5.2, 2.3.5.3, 2.3.5.4, respectively.

6.4.6 Statistical analysis

The experiment was set as a complete randomized design and the data were analyzed by one-way analysis of variance (one-way ANOVA) using a significant level of 0.05. Least significant difference test at the same significant level was done when the analysis of variance found significant differences. The statistical analysis was executed using the statistical analysis system version 9.3 (SAS Institute Inc. 2011. Base SAS® 9.3 Procedures Guide. Cary, NC, USA). For chemical analysis, an average of equidistant time periods (0, 7, 14 and 21 days) was made for total phenols, flavonoids, ascorbic acid, and ORAC assay.

6.5 Results and Discussion

6.5.1 Hormetic Dose

Broccoli florets were fumigated with atmosphere containing ethanol and MeJA vapor because lower consumption of ethanol or MeJA are required in vapor phase than their application in liquid phase (Dao and Dantigny, 2011).

Very few investigations have focused on the preservation of the quality with emphasis on the enhancement or maintenance of phytochemicals contents of vegetables during storage. Since

broccoli is an excellent dietary source of health-promoting glucosinolates and hydroxycinnamic acids, the effect of ethanol and MeJA on florets was examined.

Hormetic dose of ethanol was determined by exposing broccoli florets to an atmosphere containing 10,000 ppm for various durations ranging from 0 to 540 min, and monitoring their color retention by Total color difference (ΔE) during 10 days of storage at 10 °C (Figure 6.1). An exposure time of 30 min was beneficial for color retention compared with the control or longer exposure times. By increasing the exposure time of florets to ethanol, the ΔE value increased as a consequence of yellowing in those florets up to 240 min. However, ΔE value decreased to values comparable to that of the control above the exposure time of 240 min for up to 420 min, and increased once again with further increase in the exposure time (Figure 6.1a). Such bimodal pattern in color retention in broccoli florets was also observed with UV-B, ozone and hydrogen peroxide. The exposure of broccoli florets to an atmosphere containing 1 ppm of MeJA showed a minimum in ΔE value with exposure time of 45 min (Figure 6.1b), and it increased steadily with longer exposure time, unlike with ethanol. Thus the exposure of the florets to an atmosphere containing 1 ppm of MeJA ppm for 45 min was considered hormetic.

6.5.2 Color evolution and respiration rate during storage

Exposition of florets to 10,000 ppm of ethanol for 30 min exhibited a better color retention compared with the untreated florets during storage at 4 °C (Figure 6.2a). The chlorophyll degrading enzyme activities, magnesium dechelatase and chlorophyll-degrading peroxidases remained nearly unchanged in ethanol-treated broccoli (Fukasawa et al., 2010). In addition, ethylene biosynthesis and its action in broccoli appears to be inhibited by ethanol vapor treatment (Asoda et al., 2009).

It has been shown that the increase in the storability of carnation cut flower with ethanol is reversed when the oxidation of ethanol is blocked, suggesting that the increased storage life of the cut-flower is likely due to the oxidation of ethanol to acetaldehyde, which is naturally present in plants at a ratio of 1 part of acetaldehyde to 100 parts of ethanol (Podd and Van Staden, 1998). Such a mechanism can be expected to operate in broccoli floret, also a flower body. The accumulative intake of ethanol in broccoli florets and its possible conversion to acetaldehyde has also been discussed previously (Corcuff et al., 1996). The conversion of ethanol to acetaldehyde generates an imbalance in NAD^+/NADH ratio, with the equilibrium shifting towards the NADH production. In yeast, this shift has a number of consequences such as the stimulated conversion of pyruvate to lactate, the

inhibition of fatty acid oxidation and respiration rate as well as the decrease in the ATP/ADP ratio (Jones, 1989). The bactericidal effect of ethanol is well known and its action include the disturbance of cell membranes, the denaturation of proteins as well as the decrease of water activity (A_w) of the medium. The bactericidal effect may also be accentuated by the oxidation of ethanol into acetaldehyde. Indeed, the aldehydes exhibit bactericidal effect, which increases with decreasing chain length, formaldehyde and acetaldehyde being the most toxic (Jones, 1989).

The effect of acetaldehyde on ethylene action has been proven to be more pronounced compared with ethanol. For instance, the inhibition of ACC-treated grapes and mango disks was more effective with acetaldehyde compared with ethanol, at equal concentration (Burdon et al., 1996; Pesis and Marinansky, 1992). But this is not the only response of fruits and vegetables to the exposition of ethanol and acetaldehyde.

While high doses of ethanol and the corresponding high levels of acetaldehyde delay ripening and ethylene production, low concentration of ethanol can induce the synthesis of ethylene. Also at high concentrations these compounds can be phytotoxic and induce off-flavors and enhanced respiration by the breakdown of organic acids (Pesis, 2005). However, at concentration of ethanol used in this study, the respiration rates observed in florets treated with both doses of ethanol were very similar to the unexposed florets and were not significantly different immediately after the exposure to ethanol (Figure 6.3a). Moreover, no off-odors and higher titers of chlorophyll (Figure 6.4a) were detected at the end of the storage, suggesting that the applied concentrations didn't affect the tissue irreversibly.

Treatment with methyl jasmonate was less effective for color retention in broccoli florets compared with ethanol, even though hormetic dose of MeJA showed better color retention compared with untreated florets (2 folds), but longer exposure times of florets to MeJA displayed poorer color retention compared with the control (Figure 6.1b). Increasing the exposure time of florets to MeJA was in proportion to color change (Figure 6.1b). Methyl jasmonate and jasmonic acid are collectively identified as jasmonates. They are cyclopentanone compounds which function in signal transduction and elicitation of defense responses against pathogens in plants (Nimitkeatkai et al., 2011). Interaction between jasmonate and ethylene signalling pathways is one of the most significant in defense responses, including plant-microbe interactions, plant-insect interactions and wounding, also, jasmonates can lead to ethylene synthesis in plants (Zhao et al., 2005).

Exposure of broccoli florets to ethylene causes a dose-dependent decline of green color (Tian et al., 1994). The steady increase in color change in broccoli florets with increase in duration of exposure to MeJA suggests that some agent such as ethylene that increases with exposure to MeJA is at play. Contrary to ethanol, MeJA concentration was very low, yet the effect on the yellowing of florets with increasing exposure times might be associated with ethylene production and its action on senescence development. Nonetheless, the color retention of florets was slightly superior in florets treated with the hormetic dose of MeJA compared with the control or the high dose (Figure 6.2b).

In addition, the high initial respiration rate (and the high metabolic activity) of the florets exposed to MeJA may be attributable to ethylene production (Figure 6.3b). Also, chlorophyll titers of treated florets with both doses were significantly lower ($p<0.05$) compared with the unexposed florets (Figure 6.4b). This is similar to the effect of MeJA in apples. Fan and Mattheis (1999) fou nd that treatment with MeJA stimulated respiration. These authors proposed two mechanisms by which MeJA may modulate color change: (1) by promoting ethylene biosynthesis, or (2) MeJA itself promote, regardless of ethylene action as the apples were previously treated with 1-MCP and also underwent degreening.

6.5.3 Secondary metabolism modification by ethanol and methyl jasmonate

The influence of MeJA, a signal molecule, on plant secondary metabolism is known, and it has been shown to induce the biosynthesis of phenolic compounds (Wang et al., 2010), anthocyanins (Shan et al., 2009), resveratrol (Vezzulli et al., 2007), and indole glucosinolates (Bodnaryk, 1994). Ethanol, on the other hand, can cause perturbation of the membrane by increasing its fluidity (Gibson Wood and Schroeder, 1988; Goldstein, 1986), and it can affect enzyme activities through changes in the conformation of proteins (Dufour and Haertl´, 1990). Furthermore, ethanol can be further metabolized into CO_2, organic acids and amino acids in different tissues including storage organs, fruits, stems and leaves in relatively short periods of time (Cossins and Beevers, 1963).

Analysis of both, individual and total glucosinolates and HCA, was performed on florets after exposure to methyl jasmonate and ethanol. Total glucobrassicin content was the sum of glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin and 4-hydroxyglucobrassicin contents, and the total HCA content was the sum of 1,2-Disinapoylgentiobiose (DSG), 1-sinapoyl-2-feruloylgentiobiose (SFG), 1,2-Diferuoylgentiobiose (DFG), 1,2,2-Trisinapoylgentiobiose (TSG) and 1,2-Disynalpoyl-2-feruloylgentiobiose (DSFG) contents.

The total amount of glucobrassicins was significantly (p<0.05) enhanced by treatments of ethanol throughout storage (Figure 6.5a). Overall, titers of total glucobrassicins were 10 and 11 % higher in broccoli exposed to 10,000 ppm for 30 (hormetic dose) and 120 min (high dose), respectively, compared with unexposed broccoli florets. However, no significant differences were observed among the content of individual glucobrassicins in ethanol-exposed and unexposed florets (Table 6.2). Only glucoraphanin was enhanced by ethanol treatments (Figure 6.6a). Overall, titer of glucoraphanin was 20% higher in broccoli exposed to the hormetic dose and 24% higher in broccoli exposed to high dose of ethanol, compared with untreated florets. In addition, the total content of HCA in broccoli was generally enhanced by 17 and 15 % (Figure 6.7a) with hormetic and high doses of ethanol, respectively, compared with control, and the content of SFG of florets was increased by 20% with both doses of ethanol compared with unexposed florets (Table 6.3).

The content of total glucobrassicins in broccoli florets exposed to MeJA was also enhanced by 14 and 15 % with 1 ppm of the gas applied for 45 and 180 min, respectively, compared with unexposed florets (Figure 6.5b). Moreover, substantial enhancement was observed in the titers of neoglucobrassicin and 4-hydroxyglucobrassicin exposed to MeJA doses. Titer of neoglucobrassicin was 26 and 20 % higher on exposed florets to the hormetic and high doses of MeJA, respectively, compared with unexposed florets, and that of 4-hydroxyglucobrassicin was 38 and 44 % superior in exposed florets to the hormetic and high doses of the gas, compared with unexposed florets (Table 6.2). However, no significant differences were observed in glucoraphanin content when florets were exposed to methyl jasmonate at both doses compared with the unexposed florets (Figure 6.6). On the other hand, the overall concentration of HCA, significantly (p<0.05) increased with the dosage of 1 ppm of MeJA for 45 min by 5 % and by 11 % with 1 ppm of MeJA applied for 180 min (Figure 6.7b). Similar to ethanol exposure, titer of SFG in broccoli florets treated with the high dose of MeJA increased by 10 % (Table 6.3).

Enhancement of secondary metabolites (SM) in broccoli florets exposed to ethanol may be attributed to the augmented reductive power (NADH) generated by the oxidation of ethanol into acetaldehyde. In addition, ethanol is metabolized in the tissue, producing acetate, organic acids, and amino acids (Fletcher and Beevers, 1970) that are substrates for the production of glucosinolates and HCA. On the other hand, the exposure of plants to methyl jasmonate appears to be a response similar to insect or mechanical wounding, leading to large systemic increases in the concentration of

indole-type glucosinolates in *Brassicas* (Bodnaryk, 1994; Brader et al., 2001; Doughty et al., 1995; Pérez-Balibrea et al., 2011).

In *Brassicas*, the main defense mechanism is the glucosinolate-myrosinase system, in which glucosinolates are converted to isothyocianates to protect the tissue against herbivores (Textor and Gershenzon, 2009). While sulforaphane or 4-methylsulfinylbutyl isothiocyanates of broccoli is a very potent inducer of phase 2 enzymes for achieving protection against carcinogenesis in humans (Fahey et al., 1997), tryptophan-derived indole glucosinolates are more responsive to wounding, insect feeding, treatments with phytohormones as well as fungal infection compared with the aliphatic glucosinolates (Brader et al., 2001; Ku et al., 2013). Indeed, Ku et al. (2013) reported the enhancement of glucoraphanin, glucobrassicin and neoglucobrassicin content in cauliflower by 1.5, 2.4 and 4.6-folds over the controls, in response to methyl jasmonate, respectively. In comparison, an enhancement of indole-type glucosinolates by 1.25 fold was noted in MeJA treated florets in this study, which was significant, albeit to a lesser extent (Figure 6.5b).

MeJA and ethylene are two phytohormones that normally act in conjunction in the elicitation of defense responses. However, the enhancement of the aliphatic glucoraphanin in the florets exposed to ethanol, and the lack of such enhancement by MeJA treatment raises the question regarding the role of ethylene in glucosinolate biosynthesis. As mentioned before, the inhibition of yellowing of florets by ethanol could be attributable to reduced ethylene production and/or action by possible denaturation of the proteins involved in ethylene production (ACC oxidase) and its action (ethylene receptor). Thus it would seem that reduced ethylene in the tissue by ethanol leads to the enhancement of glucoraphanin, but not in response to the exposure of florets to MeJA. In this case, higher concentration of ethylene can be expected (Hudgins and Franceschi, 2004; Xu et al., 1994) and it is likely the consequence of increased yellowing of florets exposed to MeJA. It would seem likely that the presence of both MeJA and ethylene interact leading to the synthesis of indole glucosinolates, which are effective defense compounds. Or it may also suggest that MeJA alone transduces the synthesis of indole-glucosinolates. In this respect, Brader et al. (2001) observed that the elicitors of *E. carotovora* triggered specific induction of indole glucosinolates in *Arabidopsis*, which was mainly jasmonic acid-dependent, and the role of either ethylene or salicylic acid was of minor importance.

Improvement of HCAs was also observed in ethanol and MeJA treated florets compared with the controls (Figure 6.7). Ethanol can inhibit the activity of peroxidase (POD) and the accumulation of

phenolic compounds is one of the consequences (Xu et al., 2012a). In this work, enhancement of HCA and total phenols in florets was observed by the application of both doses of ethanol (Table 6.1). Even if total phenolic compounds were accumulated after exposure to ethanol, no differences were observed neither on ascorbic acid or total flavonoid content of broccoli. Only the amount of kaempferol was slightly enhanced by both doses of ethanol (Table 6.1). The accumulation of total phenols and HCA can be also attributed to the possible utilization of ethanol as a carbon source for the synthesis of sugars and amino acids (Cossins and Beevers, 1963). Ethanol may be converted into amino acids such as phenylalanine which can be transformed into phenolic compounds by enzymes including phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) and others. Another possibility for the increase concentration of phenols in tissue after ethanol exposure could be associated to weight loss. When comparing various hormetic stresses, ethanol caused significant weight loss (Figure 7.1c). Weight loss due to ethanol can be explained by the disruption of the osmotic balance of the cell as well as the disturbance of water structure which can affect the structure of membranes. Thus, dehydration of tissue could have concentrate HCAs, which could have been also possible for the observed augmentation of GLS in the florets exposed to ethanol.

On the other hand, the induction of PAL, which is the key enzyme for the synthesis of phenylpropanoids compounds can be mediated by jasmonates (Sharan et al., 1998). Results of this investigation revealed a significant enhancement of HCAs with the hormetic and high dose of MeJA. The augmentation of HCAs was directly related to the exposure time of florets to MeJA. However, a substantial decrease on ORAC was not related with the exposure time of broccoli to the phytohormone (Table 6.1). This observation differs from results of studies where MeJA enhanced antioxidant activity in blackberries and raspberries (Wang et al., 2008; Wang and Zheng, 2005).

Since total phenols, flavonoids and ascorbic acid of exposed-MeJA florets were not very different from those of the control (Table 6.1), it should be expected that the antioxidant capacity should also remain the same as that of the control. Besides, oxidized ascorbic acid content of the MeJA treated florets was also nearly the same as that of the control, suggestive of a low ROS generation. It is also different from the observations in response to oxidative stresses in broccoli florets. However, MeJA also caused yellowing of the florets in a dose-dependent manner. It raises the question whether redox reactions to protect chlorophyll occur mainly in the chloroplast, involving the antioxidants protecting the photosystem such as glutathione or α-tocopherol.

6.6 Conclusion

Results from this work exhibit the influence of ethanol and methyl jasmonate treatments on broccoli florets during the postharvest storage. Ethanol increased visual quality, delayed yellowing and increased levels of glucosinolates, possibly by the lack of ethylene endogenous production and action. On the other hand, methyl jasmonate increased titers of indole glucosinolates and phenolic compounds a response similar to mechanical wounding or pathogenic attack, but quality was not enhanced. It seems that commercial application for these molecules is possible, since they are considered as safe for human health. However, their application may not improve both the quality characteristics and the nutraceutical value of the florets.

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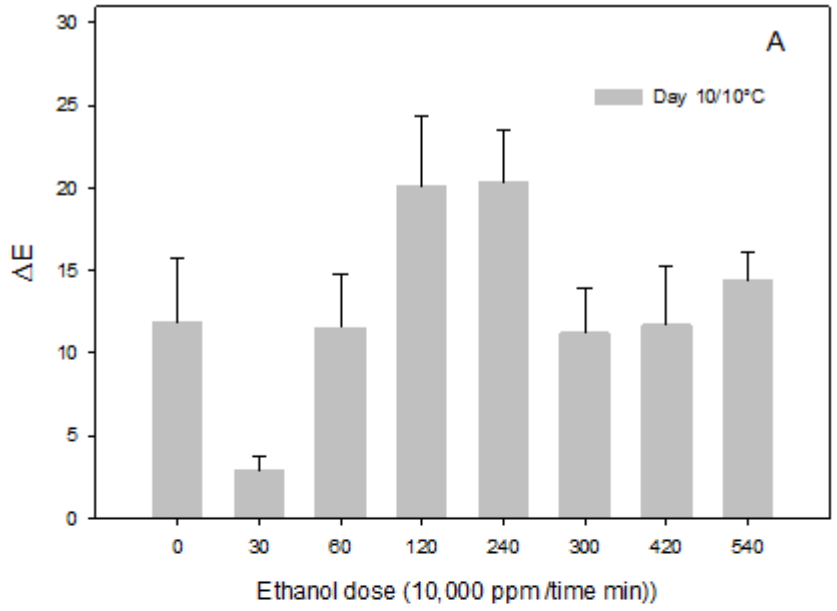

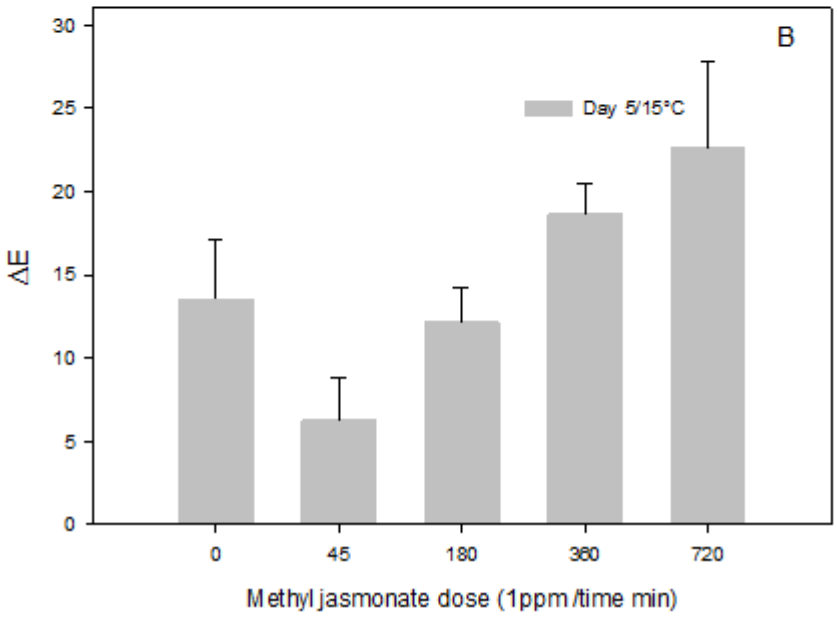

Figure 6.1 Hormetic dose determination for ethanol and methyl jasmonate. The determination of hormetic dose of the stress ethanol (A) and methyl jasmonate (B) was based on Total Color Difference (ΔE) at the end of the storage period. Data points are means ± standard deviation of the mean for n=9.

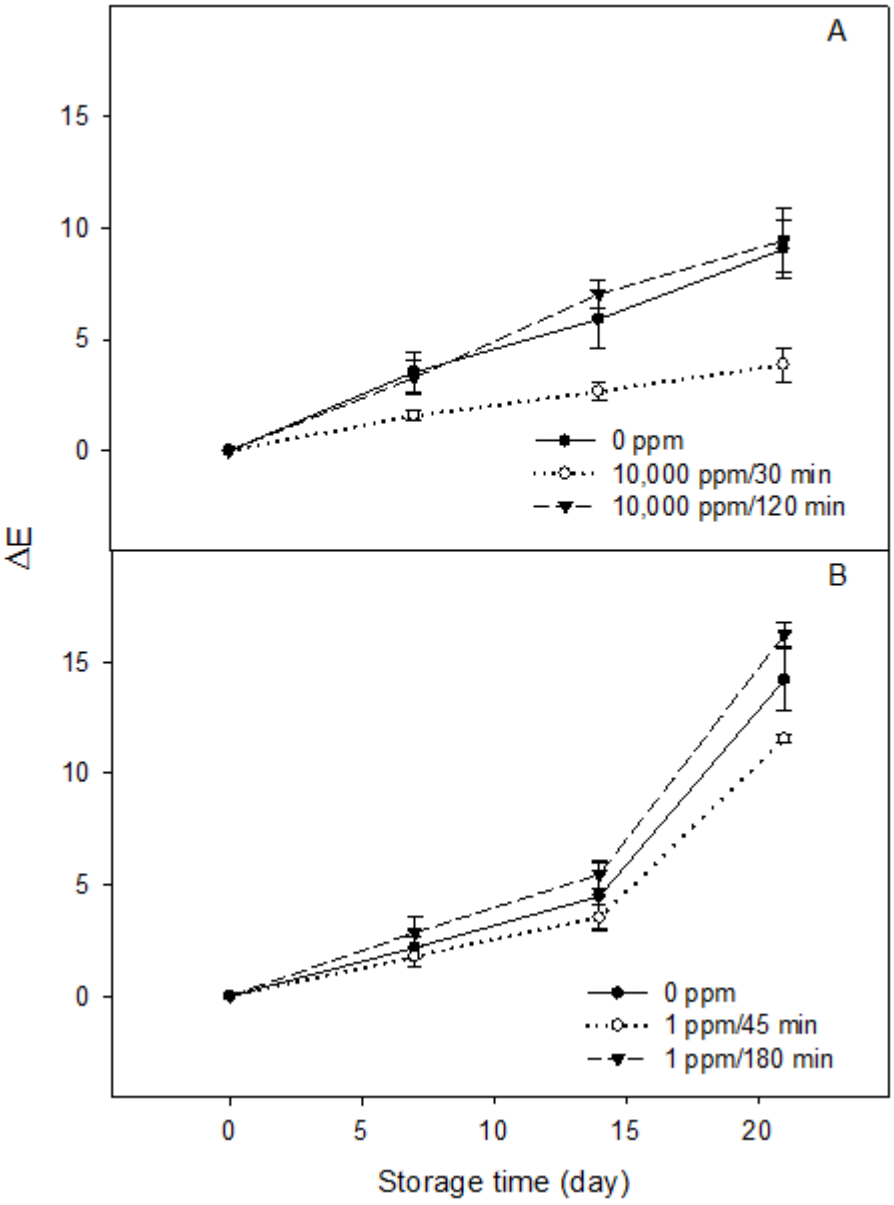

Figure 6.2 Evolution of total color change of broccoli florets exposed to ethanol and methyl jasmonate. Broccoli florets were treated with ethanol (A) at three different doses: (●), control; (○), 10,000 ppm/30 min (hormetic dose); and (▼), 10,000 ppm/120 min (high dose). Methyl jasmonate (B) (●), control; (○), 1ppm/45min (hormetic dose); and (▼), 1ppm/180min (high dose). Color change (ΔE) was followed during 21 days of storage in darkness at 4 °C. Vertical bars represent standard deviation of the mean (n=9).

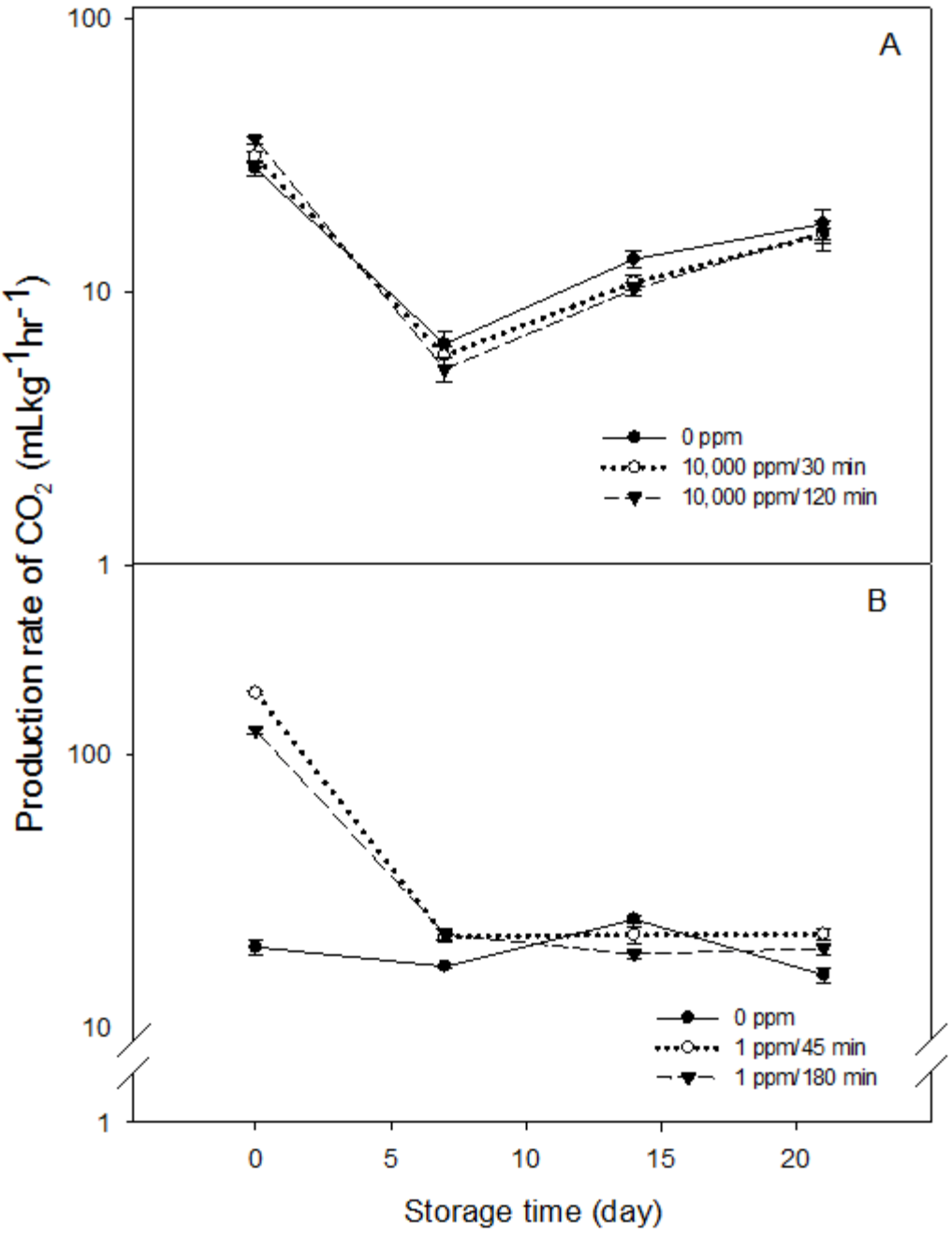

Figure 6.3 Evolution of respiration rate of broccoli florets exposed to ethanol and methyl jasmonate. Broccoli florets were treated with ethanol (A) at three different doses: (●), control; (○), 10,000 ppm/30 min (hormetic dose); and (▼), 10,000 ppm/120 min (high dose). Methyl jasmonate (B) (●), control; (○), 1ppm/45min (hormetic dose); and (▼), 1ppm/180min (high dose). Color change (ΔE) was followed during 21 days of storage in darkness at 4 °C. Vertical bars represent standard deviation of the mean (n=4).

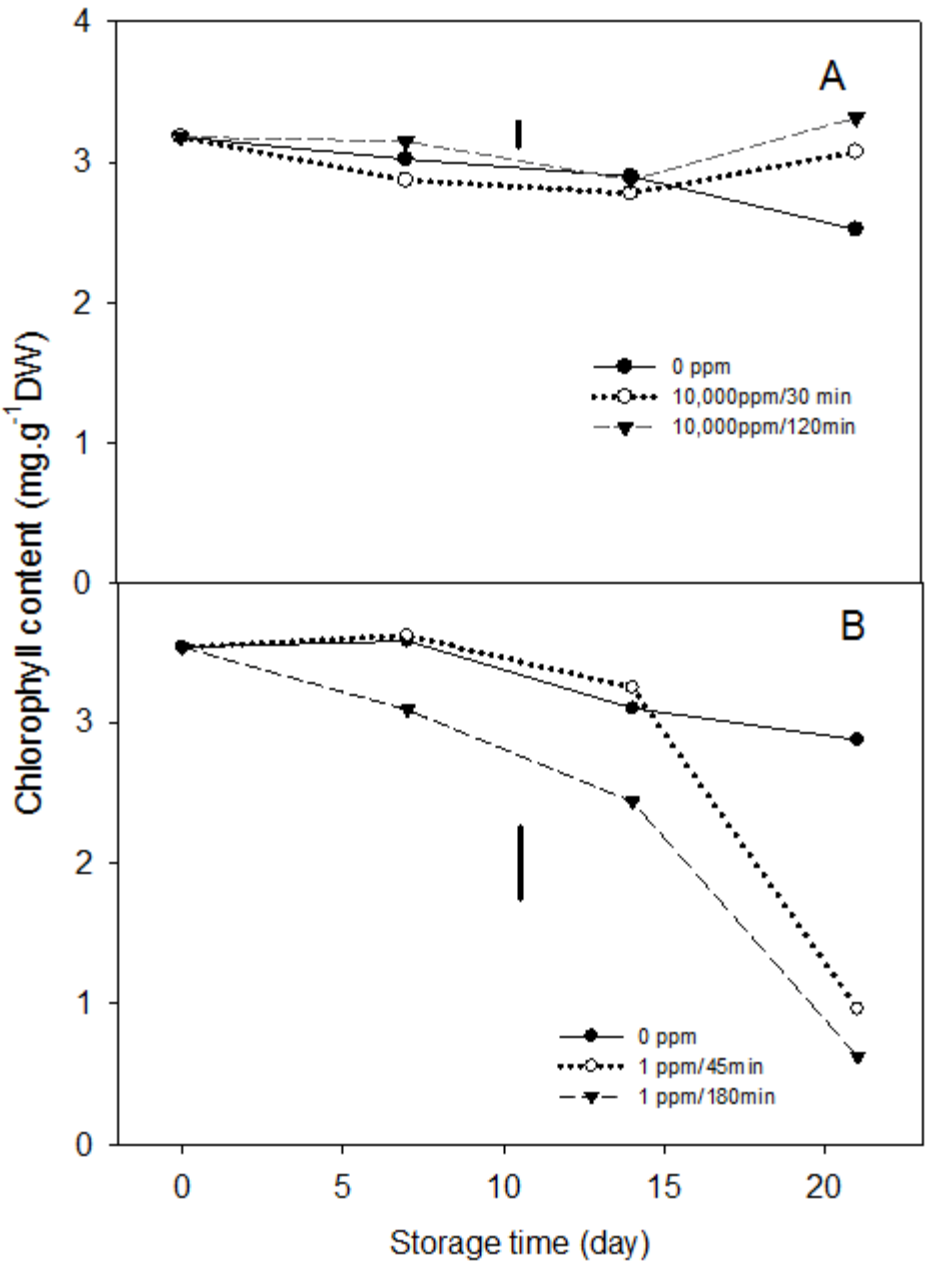

Figure 6.4 Evolution of chlorophyll content of broccoli florets exposed to ethanol and methyl jasmonate. Broccoli florets were treated with ethanol (A) at three different doses: (●), control; (○), 10,000 ppm/30 min (hormetic dose); and (▼), 10,000 ppm/120 min (high dose). Methyl jasmonate (B) (●), control; (○), 1ppm/45min (hormetic dose); and (▼), 1ppm/180min (high dose). Color change (ΔE) was followed during 21 days of storage in darkness at 4 °C. Vertical bars represent standard deviation (n=4).

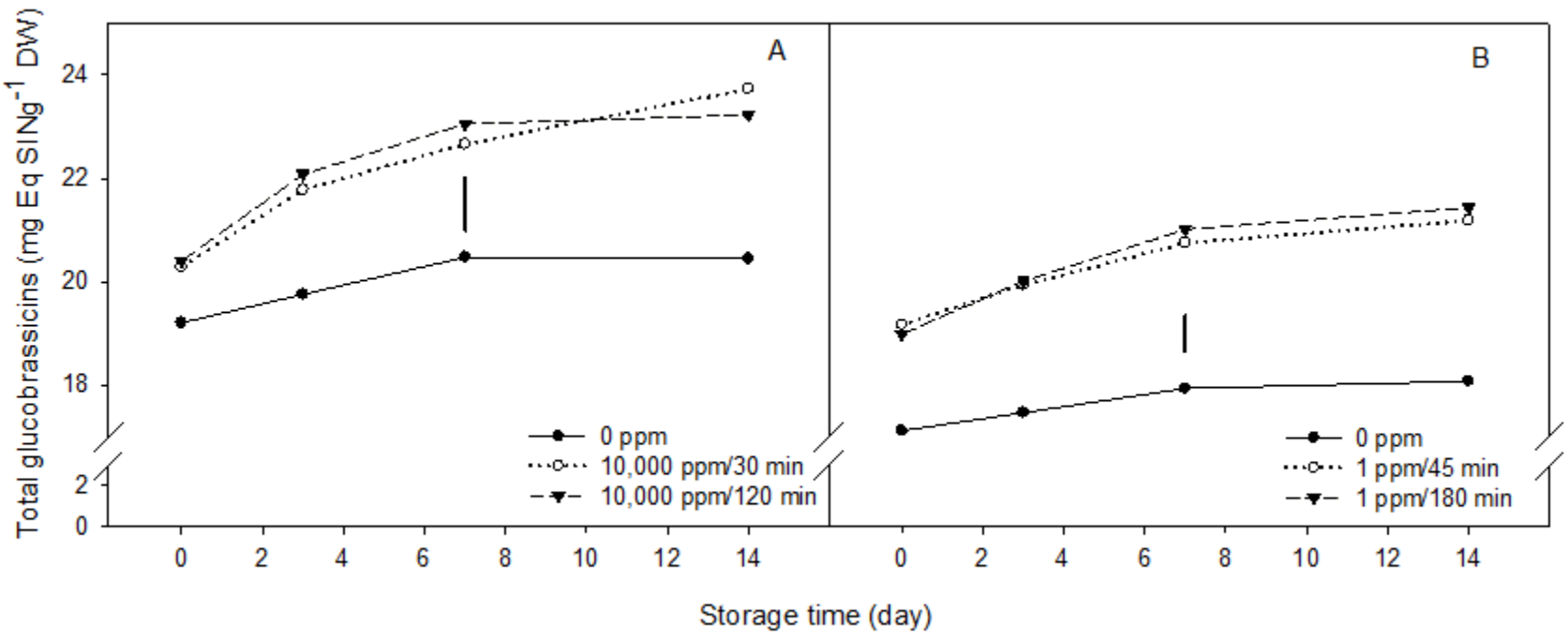

Figure 6.5 Total glucobrassicins content of florets exposed to ethanol and methyl jasmonate. Broccoli florets were treated with ethanol (A) at three different doses: (●), control; (○), 10,000 ppm/30 min (hormetic dose); and (▼), 10,000 ppm/120 min (high dose). Methyl jasmonate (B) (●), control; (○), 1ppm/45min (hormetic dose); and (▼), 1ppm/180min (high dose). The content of total glucobrassicins (glucobrassicin + neoglucobrassicin + 4-hydroxyglucobrassicin + 4-methoxyglucobrassicin) was followed during 14 days of storage in darkness at 4 °C The vertical bar indicates differences (LSD 0.05) among the treatments. Ethanol; LSD = 1.05 and MeJA, LSD = 0.70.

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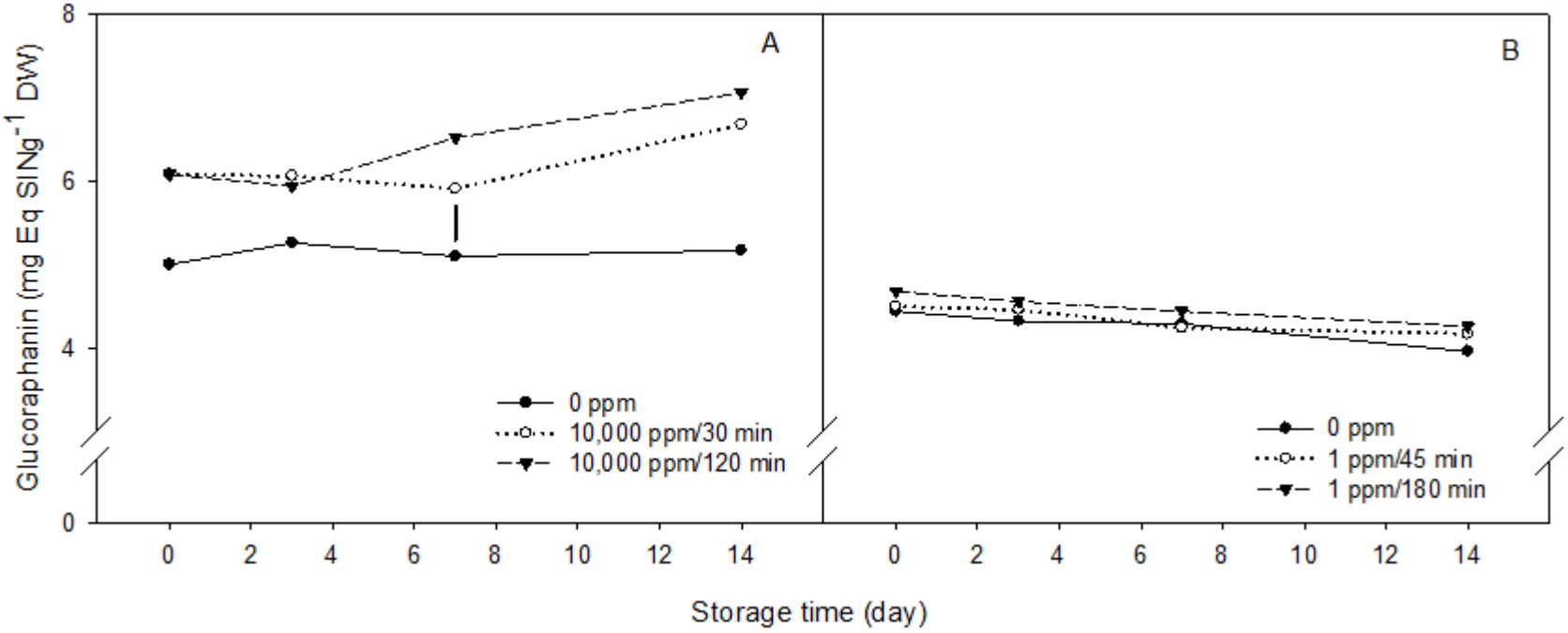

Figure 6.6 Total glucoraphanin content of florets exposed to ethanol and methyl jasmonate. Broccoli florets were treated with ethanol (A) at three different doses: (●), control; (○), 10,000 ppm/30 min (hormetic dose); and (▼), 10,000 ppm/120 min (high dose). Methyl jasmonate (B) (●), control; (○), 1ppm/45min (hormetic dose); and (▼), 1ppm/180min (high dose). The content of glucoraphanin was followed during 14 days of storage in darkness at 4 °C The vertical bar indicates differences (LSD 0.05) among the treatments. Ethanol; LSD = 0.40.

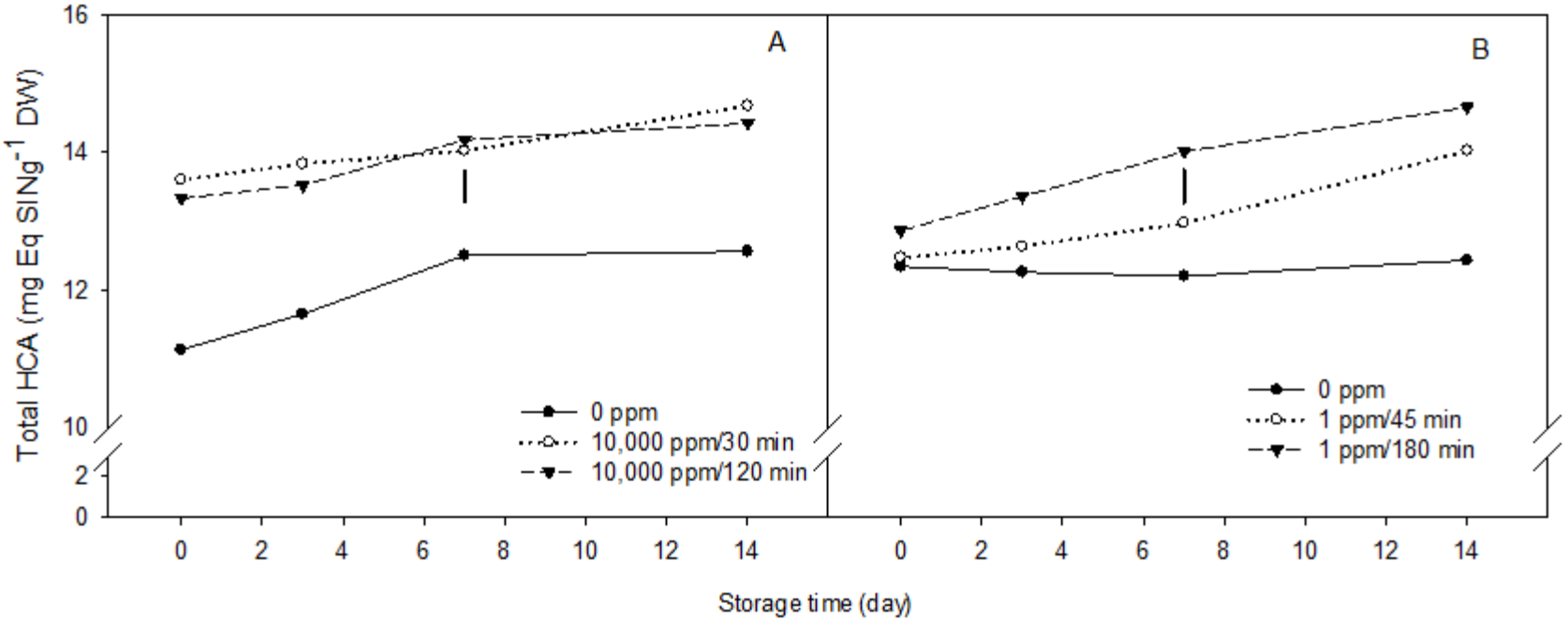

Figure 6.7 Total hydroxy-cinnamic acid (HCA) content of florets exposed to ethanol and methyl jasmonate. Broccoli florets were treated with ethanol (A) at three different doses: (●), control; (○), 10,000 ppm/30 min (hormetic dose); and (▼), 10,000 ppm/120 min (high dose). Methyl jasmonate (B) (●), control; (○), 1ppm/45min (hormetic dose); and (▼), 1ppm/180min (high dose). The content of total HCA (1,2-disinapoyl gentibiose + 1-sinapoyl-2-feruloyl gentibiose + 1,2-diferuloyl gentibiose + 1,2,2-trisinalpoyl-gentibiose + 1,2-disynalpoyl-2-feruloyl gentibiose) was followed during 14 days of storage in darkness at 4 °C. The vertical bar indicates differences (LSD 0.05) among the treatments. Ethanol; LSD = 0.45 and MeJA, LSD = 0.47.

Table 6.1 Concentration values of ORAC, ascorbic acid (oxidized, reduced, total), total phenols, total flavonoids, chlorogenic acid and kaempferol in broccoli florets exposed to ethanol and methyl jasmonate. Florets were exposed to three different doses of ethanol and methyl jasmonate and stored during 21 days in darkness at 4 °C. The obteined values were time averaged (0, 7, 14 and 21 days).

	Ethanol	MeJA
ORAC (mg Eq trolox g^{-1} DW)		
Control	149.5±5.7	180.6±11.8
Hormetic	150.1±3.9	137.3±12.8*
High	147.7±4.1	134.6±7.7*
Total ascorbic acid (mg Eq AA. G^{-1} DW)		
Control	14.1±0.4	8.9±0.5
Hormetic	13.7±0.5	8.7±0.5
High	14.1±0.7	8.7±0.6
Reduced ascorbic acid (mg Eq AA. G^{-1} DW)		
Control	10.1±0.3	5.7±0.3
Hormetic	9.9±0.3	5.5±0.4
High	9.9±0.3	5.4±0.3
Oxidized ascorbic acid (mg Eq AA. G^{-1} DW)		
Control	4.0±0.1	3.2±0.2
Hormetic	3.8±0.2	3.3±0.1
High	4.2±0.4	3.3±0.3
Total phenols (mg Eq GA.g^{-1} DW)		
Control	12.8±0.3	14.5±0.6
Hormetic	14.8±0.4*	14.5±0.5
High	15.1±0.2*	14.7±0.8
Total flavonoids(mg Eq QE.g^{-1} (DW))		
Control	3.7±0.4	4.0±0.4
Hormetic	3.7±0.1	3.9±0.2
High	4.0±0.2	4.5±0.4
Chlorogenic acid (mg Eq SIN.g^{-1} DW)		
Control	2.2±0.1	1.9±0.1
Hormetic	2.5±0.2	2.0±0.1
High	2.5±0.2	2.0±0.1
Kaempferol (mg Eq SIN.g^{-1} DW)		
Control	1.6±0.1	1.4±0.1
Hormetic	2.0±0.2*	1.5±0.1
High	2.0±0.1*	1.5±0.1

Ethanol hormetic: 10,000 ppm/30 min, high: 10,000 ppm/120 min; MeJA hormetic: 1 ppm/45min, high 1ppm/180 min.The asterisk indicates that the value is significantly different from the corresponding control at $p < 0.05$.

Table 6.2 Glucosinolate content of ethanol and methyl jasmonate treated broccoli florets. Broccoli florets were treated with ethanol and methyl jasmonate, stored for 14 days in darkness at 4 °C, and the obteined values were time averaged (0, 7, 14 days).

	Ethanol	**MeJA**
Glucobrassicin (mg Eq SIN.g^{-1} DW)		
Control	10.9±0.8	8.4±0.8
Hormetic dose	11.9±1.5	8.9±0.2
High dose	11.8±0.9	8.9±0.5
Neoglucobrassicin (mg eq SIN.g^{-1} DW)		
Control	4.3±0.3	4.9±0.3
Hormetic dose	4.8±0.6	6.2±0.4*
High dose	4.9±0.4	5.9±0.4*
4-Hydroxyglucobrassicin (mg eq SIN.g^{-1} DW)		
Control	1.7±0.1	1.8±0.0
Hormetic dose	1.9±0.3	2.5±0.3*
High dose	2.0±0.2	2.6±0.3*
4-Methoxyglucobrassicin (mg eq SIN.g^{-1} DW)		
Control	3.1±0.3	2.6±0.1
Hormetic dose	3.5±0.3	2.8±0.2
High dose	3.4±0.2	2.9±0.2

Ethanol hormetic: 10,000 ppm/30 min, high: 10,000 ppm/120 min; MeJA hormetic: 1ppm/45min, high: 1ppm/180min. The asterisk indicates that the value is significantly different from the corresponding control at $p < 0.05$.

Table 6.3 Hydroxycinnamic-acid content (HCA) of ethanol and methyl jasmonate treated broccoli florets. Broccoli florets were treated with ethanol and methyl jasmonate, stored for 14 days in darkness at 4 °C, and the obteined values were time averaged (0, 7, 14 days).

	Ethanol	MeJA
1,2-Disinapoylgentibiose (mg eq SIN.g^{-1} DW)		
Control	2.2±0.1	2.3±0.2
Hormetic dose	2.5±0.1	2.4±0.1
High dose	2.5±0.3	2.5±0.1
1-sinapoyl-2-feruloylgentibiose (mg eq SIN.g^{-1} DW)		
Control	4.9±0.2	4.6±0.1
Hormetic dose	5.9±0.4*	4.8±0.2
High dose	5.9±0.4*	5.1±0.3*
1,2-Diferuoylgentiobiose (mg eq SIN.g^{-1} DW)		
Control	1.3±0.1	1.1±0.1
Hormetic dose	1.5±0.1	1.1±0.1
High dose	1.5±0.1	1.2±0.2
1,2,2-Trisinapoylgentibiose (mg eq SIN.g^{-1} DW)		
Control	1.5±0.1	1.8±0.1
Hormetic dose	1.7±0.1	1.9±0.0
High dose	1.7±0.1	2.0±0.1
1,2-Disynalpoyl-2-feruloylgentiobiose (mg eq SIN.g^{-1} DW)		
Control	2.0±0.2	2.6±0.1
Hormetic dose	2.4±0.2	2.7±0.1
High dose	2.3±0.1	2.9±0.2

Ethanol hormetic: 10,000 ppm/30 min, high: 10,000 ppm/120 min; MeJA hormetic: 1ppm/45min, high: 1ppm/180min. The asterisk indicates that the value is significantly different from the corresponding control at $p < 0.05$.

Chapter VII: Comparative Evaluation of the Effect of Several Abiotic Stresses on Postharvest Storage of Broccoli Florets

7.1 Résumé

Le brocoli est un végétal ayant des caractéristiques nutritionnelles importantes, y compris la vitamine C, les composés phénoliques et les glucosinolates. Cependant, il est très périssable dans la phase post-récolte en raison de son taux de respiration élevé, résultant en une perte rapide de la qualité nutritionnelle et commercialisable. Des traitements de post-récolte peuvent être utilisés en conséquence comme un outil pour améliorer la qualité et les propriétés nutraceutiques de fruits et légumes au cours de l'entreposage. L'objectif de ce travail était de comparer les effets de doses subaiguës ou hormétiques de stress abiotiques sur la qualité et le contenu phytochimique des fleurons de brocoli-traitées pendant l'entreposage après récolte. Les fleurons de brocoli ont été exposés à sept stress abiotiques hormétiques et entreposés pendant 21 jours à 4 °C / 90% RH. Les stress abiotiques incluaient: UV-B, 1,5 $kJ.m^{-2}$; UV-C, 1,2 $kJ.m^{-2}$; chaleur, 41 ° C / 180 min; ozone (O_3) 5 ppm / 60 min; peroxyde d'hydrogène (H_2O_2), 1,25 mM / 180 min; éthanol, 10,000 ppm / 30; et jasmonate de méthyle (MeJA) à 1 ppm / 45 min. Le développement de la couleur, la teneur en chlorophylle, la perte de poids, le contenu en acide ascorbique, ORAC, et en glucosinolates, ainsi que la teneur en acide hydroxycinnamique ont été suivis pendant l'entreposage. Les valeurs de développement de la couleur, de la teneur en chlorophylle et de la perte de poids ont été calculés comme une moyenne au cours des 21 jours de la période d'entreposage. Les valeurs moyennes pour l'acide ascorbique, ainsi que les valeurs ORAC et des métabolites secondaires ont été calculés sur une période d'entreposage de 14 jours et normalisés par rapport au témoin. La chaleur et l'éthanol ont été les meilleurs stress hormétiques pour la rétention de la couleur, la teneur en chlorophylle a été mieux conservé dans fleurons traités avec de l'éthanol, la chaleur et l'UV-B. La perte de poids de fleurons était considérablement élevée par la dose hormétique d'O3. Tous les stress hormétiques ont réduit la teneur en acide ascorbique totale, cependant ORAC a été renforcée dans les brocolis exposés à la chaleur et à l'UV-B. Les métabolites secondaires, glucosinolates et hydroxycinnamates (HCA), ont été légèrement améliorés par les doses hormétiques de stress abiotiques à des degrés divers. Bien que le contenu de glucoraphanine, de type aliphatique, a été augmenté par l'éthanol et l'ozone, la plupart des glucosinolates de type indole ont été améliorés par le peroxyde d'hydrogène et l'UV-B. Néanmoins, la spécificité de MeJA et de l'O_3 a été montrée par l'augmentation respectivement de hydroxy et méthoxy glucobrassicines. La chaleur et l'éthanol sont les stress les plus efficaces pour l'amélioration de HCA. Ainsi, les stress abiotiques peuvent être utilisés soit comme un moyen pour maintenir la qualité des fleurons ou comme un outil pour l'amélioration de métabolites secondaires.

7.2 Abstract

Broccoli is vegetable with significant nutritional characteristics, including vitamin C, phenolic compounds and glucosinolates. However, it is highly perishable in the postharvest phase due to its high respiration rate, resulting in a rapid loss of nutritional and marketable quality. Postharvest treatments can be used, as a tool to enhanced quality and nutraceutical value of fruits and vegetables during the storage. The objective of this work was to compare the effects of sub-acute or hormetic doses of abiotic stresses on the quality and the phytochemical content of treated-broccoli florets during postharvest storage. Broccoli florets were exposed to seven hormetic abiotic stresses and stored over 21 days at 4 °C/90 %RH. The abiotic stresses included: UV-B, 1.5 $kJ.m^{-2}$; UV-C, 1.2 $kJ.m^{-2}$; heat, 41 °C/180 min; ozone (O_3) 5ppm/60 min; hydrogen peroxide (H_2O_2), 1.25mM/180 min; ethanol, 10,000 ppm/30; and methyl jasmonate (MeJA) at 1 ppm/45 min. Color development, chlorophyll content, weight loss, ascorbic acid, ORAC, and the glucosinolate as well as hydroxycinnamic acid content were monitored during storage. Color development, chlorophyll content and weight loss were averaged over the 21 days of storage period. Ascorbic acid, as well as ORAC values and secondary metabolites were averaged over 14-day storage period and normalized relative to the control. Heat and ethanol were the best hormetic stresses for color retention, and chlorophyll content was better retained in florets treated with ethanol, UV-B ant heat. Weight loss of florets was considerably high by the hormetic dose of O_3. All the hormetic stresses reduced the content of total ascorbic acid, however ORAC was enhanced in heat and UV-B treated broccoli. The secondary metabolites, glucosinolates and hydroxycinnamates (HCA), were modestly enhanced by hormetic doses of abiotic stresses to varying degrees. While the content of glucoraphanin, of aliphatic type was increased by ethanol and ozone, most of the indole-type glucosinolates were enhanced by hydrogen peroxide and UV-B hormetic stresses. Nevertheless, the enhancement of hydroxy- and methoxy-glucobrassicins showed the specificity to methyl jasmonate and O_3, respectively. Heat and ethanol were the most effective stresses for enhancement of HCA. Thus, abiotic stresses can be use either as a mean to maintain the quality of florets or as a tool for secondary metabolites enhancement.

Keywords: Broccoli, abiotic stress, glucosinolates, hydroxycinnamic acids.

7.3 Introduction

Abiotic stresses are environmental factors that can disturb plant growth and crop production. Drought, flooding, salinity, extreme temperatures and high light intensities are the most important due to their impact on industrial crops (Gong et al., 2013). Abiotic stresses produce morphological, physiological and biochemical changes that reduce plant's stress exposure and/or limit damage (Patakas, 2012). The responses of plants towards abiotic stresses are complex and dynamic and they are also elastic (reversible) or plastic (irreversible) (Cramer et al., 2011). Among these responses, the earliest events involve generation of reactive oxygen species (ROS) (Gill and Tuteja, 2010; Miller et al., 2008) and the interaction with hormones such as abscisic acid (ABA) (Tuteja, 2007), ethylene (Wang et al., 2002b) and methyl jasmonate (MeJA) (Turner et al., 2002). Additionally, a common response of plants in relation to abiotic stresses is the phenomenon of priming, where primed plants are more resistant to future exposures of stresses (Bruce et al., 2007; Capanoglu, 2010).

The intensification of natural disease resistance by physical, biological or chemical elicitors is relatively a new approach in postharvest (Dann, 2003; Terry and Joyce, 2004). This approach is based on the concept of hormesis, that low levels of normally harmful agents or stressors can stimulate beneficial responses in living organisms (Luckey, 1980; Stevens et al., 1998; Stevens et al., 2006). One of the most studied elicitors for disease resistance of vegetables during the postharvest storage is UV-C light, which has been widely used in Canada by Arul group (Ait-Barka et al., 2000a; Arul et al., 2001b; Baka et al., 1999; Charles et al., 2008a; Charles et al., 2008b; Charles et al., 2008c; Charles et al., 2008d; Charles et al., 2009; Maharaj et al., 2010; Mercier et al., 1993a). Abiotic stresses have also been used for quality preservation of produce, where a part of the research has been focused on color and chlorophyll retention of broccoli. UV-B light, for instance, has been extensively studied in Japan by Yamauchi group in order to inhibit enzymes related with chlorophyll loss (Aiamla-or et al., 2010; Aiamla-or et al., 2012; Aiamla-or et al., 2009). Similarly, Martinez group in Argentina has broadly studied the effect of UV-C, heat and visible light on broccoli preservation during the postharvest storage (Büchert et al., 2011a; Büchert et al., 2011b; Costa et al., 2006; Lemoine et al., 2009; Lemoine et al., 2008; Lemoine et al., 2007; Martínez and Civello, 2008).

The use of abiotic stresses is not limited to induced resistance and preservation of fruits and vegetables. Recently two independent laboratories in Germany have shown the potential of UV-B light to enhanced secondary metabolites in brassica plants, especially phenolic compounds and

glucosinolates compounds (Harbaum-Piayda et al., 2010; Mewis et al., 2012). This has opened the door to find other alternatives to induced important health-related compounds as well as maintaining quality properties of produce during postharvest storage. For example, ozone is known to induce signal molecules such as ethylene and salicylic acid as well as secondary metabolites such as phytoalexins related with oxidative defense in crop plants (Sandermann Jr et al., 1998). Although positive effects of ozone on postharvest preservation of produce is debatable (Forney et al., 2003; Skog and Chu, 2001), it has been recently found that this gas can increase the aliphatic/indole glucosinolate ratio in broccoli (Vandermeiren et al., 2012). Similarly, exogenous application of MeJA has improved the content of flavonoid and indole glucosinolates in broccoli sprouts (Pérez-Balibrea et al., 2011). Nonetheless, MeJA can induce ethylene, leading to yellowing and senescence in broccoli (Rohwer and Erwin, 2008).

In contrast, hot water treatments have successfully reduced yellowing in broccoli florets (Forney, 1995; Tian et al., 1996). However, the effect of heat on secondary metabolites has not been explored. Likewise, ethanol which has shown its potential by reducing yellowing of broccoli florets during the postharvest storage (Asoda et al., 2009; Corcuff et al., 1996; Fukasawa et al., 2010), has not been exploited as potential secondary metabolite inducer. Less explored alternatives have also been used for preservation during the postharvest storage of commodities. For instance, hydrogen peroxide is mainly used as disinfectant and suppressor of surface discoloration of cut-produce (Forney et al., 1991b; Kim et al., 2007; Peng et al., 2008). Yet, H_2O_2 is involved in many physiological functions in plants and it is also a potent signal molecule (Petrov and Van Breusegem, 2012) and is responsible to mediate responses to biotic and abiotic stresses as a second messenger (Neill et al., 2002).

Thus, in this research the effect of various abiotic stresses on preservation and enhancement of metabolites, more specifically glucosinolates, were investigated in broccoli florets during postharvest storage.

7.4 Materials and Methods

7.4.1 Broccoli

Freshly harvested broccoli (*Brassica oleraceae* L. var. Italica 'Diplomat') heads were obtained from a commercial farm (Ile d'Orléans, Québec, Canada). Florets (300g) of uniform size

(approximately, 7 cm) were separated from heads and randomly arranged in small plastic punnets of 500 mL. The punnets were placed in plastic containers of 5 L with perforations for ventilation, and containing a layer of water at the bottom to maintain high humidity (98-100%), and the containers were stored inside a controlled chamber overnight at 4 °C.

7.4.2 Color and respiration rate of broccoli florets

The color and respiration rate of broccoli florets were determined as described elsewhere in the thesis in detail, in section 2.3.3 and 2.3.4, respectively.

7.4.3 Chemical assays

The chlorophyll content, reduced and total ascorbic acid contents, and the total antioxidant capacity were determined by methods described elsewhere in detail in section 4.3.4.1, 2.3.5.3, 2.3.5.4, respectively.

7.4.5 Glucosinolates and hydroxycinnamic acid analysis

The glucosinolates and hydroxycinnamic acids were assayed by the methods described elsewhere in detail (section 2.3.6).

7.4.6 Comparison of effects of abiotic stresses

The effects of various stresses at the hormetic dose were compared. The data presented in this section was obtained from other previous chapters. Preservation indicators, color development, chlorophyll content and weight loss were averaged on a 21-day storage time. Total and reduced ascorbic acid, ORAC values, and secondary metabolites were averaged on 14-day storage period. Each attribute for the seven stresses was normalized with respect to their controls, since treatments were performed using broccoli harvested at different seasons, the responses were represented as the percentage of change.

7.5 Results and Discussion

The objective of this chapter was to compare the results from all the quality, physiological, biochemical and phytochemical parameters obtained from the different hormetic stresses applied to florets.

7.5.1 Preservation and quality indicators of broccoli florets exposed to abiotic stresses

Color retention is one of the most important quality parameter in broccoli since green color is related to its high quality, and the presence of yellow florets terminates the marketability of this produce, as the consumer associates yellowing of florets with loss of freshness (Toivonen and Forney, 2004). From the physiological standpoint, chlorophyll loss is associated with senescence. This process along with gradual destruction of chlorophyll is accompanied by other symptoms such as softening of tissue, membrane disintegration and the loss of proteins (Page et al., 2001).

Evolution of color during the storage was the first parameter evaluated in florets. Hormetic doses of heat and ethanol contributed to color retention of broccoli florets considerably more that that of the untreated florets (Figure 7.1). Color retention was enhanced by 3 folds with the application of heat, and 128 % with ethanol. Retention in color was also enhanced by 62 % with UV-C, 50 % with UV-B, 46 % with ozone, 44 % with hydrogen peroxide, and 24 % with methyl jasmonate (Figure 7.1). The effect of hormetic stresses on chlorophyll retention remained quite similar to color retention with ethanol and heat (Figure 7.1). The most elevated chlorophyll contents were measured in florets exposed to ethanol with 21 %, followed by UV-B (18 %), heat (13 %), hydrogen peroxide (10 %), UV-C (9 %), and ozone as well as methyl jasmonate (7 %) (Figure 7.1).

The retention of color and chlorophyll with heat and ethanol can be explained in part by the partial inhibition of ethylene-biosynthetic enzymes as well as of the ethylene membrane-protein receptors. Heat treatment has been shown to inhibit ripening by preventing ethylene biosynthesis, and also by reducing the effect of exogenous ethylene in apples and tomatoes (Lurie, 1998). Although these effects are frequently reported in the literature, heat treatment can also alter the surface reflecting properties of vegetables due to air removal between cells. Volatilization of organic acids such as acetic acid, also favors the alkalization of cell contributing to chlorophyll retention (Chapter IV). Similarly, ethanol vapor treatment can prolong the shell-life of broccoli florets by suppressing the activities of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase (Asoda et al., 2009). In addition alcohols can perturb cellular membranes and ethylene

receptor sites (Saltveit, 1989). Thus, the color preservation of broccoli florets by heat and ethanol exposure may be related to the partial suppression of enzymes as well as changes in visual properties.

Weight loss was also affected on treated broccoli florets. Exposure of broccoli to the hormetic dose of ozone resulted in the highest weight loss (about 2.4 folds) compared with the untreated florets, registered among all the applied hormetic stresses (Figure 7.1). After heat, florets exposed to the hormetic dose of ethanol showed the second most important weight loss, reaching 92 %, followed by hydrogen peroxide with 32 %. For the other stresses, weight loss was smaller compared with the untreated florets: UV-C (-51 %), followed by UV-B and methyl jasmonate (-20 %) and heat (-15 %) (Figure 7.1).

The weight loss of produce during treatment and storage represent both moisture loss as well as of volatiles generated by stresses. Although we did not account for each component of weight loss, the generation of volatiles in response to stresses in plant bodies is increasingly receiving attention (Blande et al., 2014; Niinemets et al., 2013), but this aspect is investigated in postharvest fruits in the context of fruit aroma and flavor than as stress response (Kader, 2008; Klee, 2010; Mattheis and Fellman, 2000). The weight loss was significantly higher than the untreated florets exposed to ozone (2.3 fold greater), ethanol (92 % higher) and to a smaller extent after exposure to H_2O_2 (about 32 % greater). This extent of weight loss cannot be attributed to moisture loss alone, otherwise the florets would have wilted and be subject to severe water stress that contributes to loss of turgidity and senescence (Ben-Yehoshua et al., 1983) with yellowing of the florets. Since this did not seem to be the case, the loss of volatiles might have been a significant contributor to weight loss along with moisture loss. The observation that the weight loss of the florets during exposure to ozone (in high humidity chamber) was very significant supports this possibility.

The release of ethylene and isoprenes by ozone-exposed plants is fairly well documented (Fiscus, 2005; Loreto and Velikova, 2001; Roshchina and Roshchina, 2003a). The premise is that these emissions are likely de-toxicants of ozone, and that the reaction products of volatiles with ozone may not be as toxic as ozone could be. By extension, H_2O_2 being an oxidizing agent, but less potent than ozone, may also be expected to cause a similar reaction in the plant tissue leading to the production volatiles or small soluble molecules to detoxify ROS. However, it would be difficult to emphasize the loss of volatiles for weight loss caused by ethanol exposure, because ethanol forms hydrogen bond with water and is a dehydrating agent depending on its concentration. The

evaporation of ethanol during storage would inevitably entrain moisture in its wake, contributing to water loss as well.

In any event, moisture loss of florets exposed to ozone and ethanol compared with the untreated florets could be a factor in causing water stress in florets, as opposed to other hormetic stresses that exhibited lower weight loss compared with the controls.

Although harvested organs are susceptible to moisture loss because of trans-evaporation, and adaptational physiological mechanisms such as LEA (late embryogenesis abundance proteins) in seeds or the accumulation of sugars in ripening fruits that increases osmotic potential and reduces moisture loss and delays their senescence (Ben-Yehoshua and Rodov, 2002). Such mechanisms may not be present in broccoli florets, which are not susceptible to detachment from the plant body. In addition, abiotic stresses exacerbate water loss or contribute to reduction in moisture loss. Since ozone stimulates the production of ethylene that prevents the action of abscisic acid (ABA) action in closing stomata, and consequently, moisture loss can be significant (Wilkinson and Davies, 2010). The exposure of florets to high ambient temperatures may lead to the melting of waxy bloom present in several cultivars of *Brassica oleracea* (Denna, 1970) could lead to the closure of stomata and reduction in moisture loss, as seen in the heat treated florets.

7.5.2 Respiration rate of broccoli florets

Respiration rate, an indicator of metabolic rate of produce, is associated with its shelf-life and very often high rates are correlated with short shelf-life of fresh fruits and vegetables (Kader, 2002). Abiotic stresses appear to stimulate respiration rate as result of enhanced metabolism (Tiwari et al., 2002). Following the exposure of broccoli florets to abiotic stresses, there was a transient increase in respiration rate (the stress respiration), which varied with the type of hormetic stress. Nonetheless, the respiration rates decreased with time and reached steady-state levels (steady respiration) after certain time and remained constant for the rest of the storage period.

The effect of hormetic stresses on stress respiration was significant, in particular, the exposure of florets to ozone, where they exhibited large weight loss. The stress respiration rate of florets exposed to hormetic dose of ozone was 6.7 folds higher compared with the unexposed florets. It was also very high in heat-treated florets (6.5 folds higher) compared with the untreated florets. Methyl jasmonate treatment also increased significantly the stress respiration rate by 4.5 folds. Among the other hormetic stresses, the stress respiration was also significantly higher than the

untreated florets but at lower levels, except for ethanol exposure: 2 folds higher with H_2O_2, 1.35 folds higher with UV-C, and about 1.0 fold higher with UV-B. The increase in the stress respiration of ethanol exposed florets was about 10 % (Figure 7.2).

It would seem that O_3, heat and MeJA may have the most impact in stimulating the metabolic activity of the florets. In particular, the very elevated respiration rate values registered in ozone-treated florets may have been a consequence of the high amount of energy required to maintain cell antioxidant defenses. Initial breakdown of O_3 in apoplast is responsible for the synthesis of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Fiscus, 2005). Moreover, ozone can activate defense responses similar to pathogen infection (Baier et al., 2005; Sandermann, 1998) which requires large quantities of energy. These responses are modulated by signaling pathways involving heat shock proteins (HSPs), ethylene, jasmonic and salicylic acid, which also require energy (Kangasjarvi et al., 1994; Koch et al., 2000; Mittler et al., 2012; Rao et al., 2000). However, the high stress respiration rate following heat treatment may be a consequence of two simultaneous stresses including thermal stress and hypoxic stress occurring during heat application by the depletion of oxygen resulting from the effect of temperature on tissue respiration (Kader and Saltveit, 2002) and oxygen solubility as discussed earlier. The effect of methyl jasmonate on respiration might be also enhanced by a defense response displayed in conjunction with ethylene.

With other hormetic stresses, UV-B, UV-C, H_2O_2 and ethanol, the stress respiration rates were significantly lower compared with ozone, heat and methyl jasmonate. At first glance, this may appear that the response of the tissue against these hormetic stresses may have been somewhat subdued. However, this assumption may not be consistent with the observation that hydrogen peroxide and UV-B were the most effective, among the hormetic stresses, in elevating the levels of glucosinolates, which are defense compounds in broccoli (Section 7.4.3). Thus, the high stress respiration may not be a strict yardstick for the magnitude of defense reactions in broccoli subject to hormetic stresses. Yet, the stress respiration may be an indicator of level or virulence of the stress as perceived by the tissue and generation of ROS as well as diversity of responses potentially mounted by the tissue as documented for ozone in plant bodies (Baier et al., 2005; Forney, 2003).

7.5.3 Glucosinolates in broccoli

A stress is considered as a potentially unfavorable factor for a living organism; an enhanced mobilization of defense responses after exposure of vegetal tissue to stress is a positive adaptation

process (Imahori, 2012). Application of abiotic stresses on *Brassicas* can influence the biosynthesis of different types of primary and secondary metabolites such as carbohydrates, amino acids, phenolic compounds and glucosinolates (Jahangir et al., 2009). Moreover, induction of secondary metabolites is a common response to the production of ROS (Grene, 2002; Lehmann et al., 2009), and other signaling molecules such as jasmonic acid and ethylene (Hung et al., 2006; Jenkins, 2009; Morgan and Drew, 1997). All the abiotic stresses in this study, i.e., UV-B, UV-C, Heat, O_3, EtOH, H_2O_2 and MeJA are oxidative stresses (Mewis et al., 2012; Orozco-Cárdenas et al., 2001; Pastori and Foyer, 2002; Vandermeiren et al., 2012; Xu et al., 2012a; Yamauchi, 2013), which is typical of biotic agents.

The glucosinolates constitutively present in broccoli can be grouped as: tryptophan-derived indole-type glucosinolates, and glucoraphanin, an aliphatic glucosinolate derived from methionine. Glucoraphanin accumulated mostly in response to the oxidative stresses including ethanol, ozone, hydrogen peroxide, UV-C and UV-B light. The accumulation of glucoraphanin in florets was less evident with heat and methyl jasmonate treatments (Figure 7.3), despite these stresses are also oxidative in nature. It suggests that not all oxidative stresses have the same or similar ROS and other signal footprints. The relationship between stresses and the accumulation of secondary metabolites can be appreciated if we have some understanding of the ROS and signal profiles of the stresses. For example, in incompatible plant-pathogen interactions, the production of superoxide anions ($O_{2\bullet}^{-}$) is favored, and they are rapidly converted to hydrogen peroxide (Apel and Hirt, 2004). Hydrogen peroxide is a stable ROS having an intermediate oxidation number which can be converted into other ROS, and it is a fundamental component of signal transduction responses in biotic an abiotic stresses. The titers of total glucobrassicins in florets were mostly affected by hydrogen peroxide treatment, UV-B and methyl jasmonate, where there was an enhancement of 18 % for both H_2O_2 and UV-B, and 14 % for hydrogen peroxide (Figure 7.4). While hydrogen peroxide elevated both aliphatic and indole glucosinolates accumulation, it was more effective towards the induction of the indole glucosinolates than that of glucoraphanin (Figure 7.3, Figure 7.4).

Glucobrassicin was the most abundant glucosinolate found in this study, and the trend in the changes of its level was quite similar to that of total glucobrassicins. Nonetheless, glucobrassicin was more abundant in florets treated with the hormetic dose of UV-B, followed by that of hydrogen peroxide and heat. Interestingly, the induction of this glucosinolate in florets treated with methyl jasmonate was very low, and it seems that glucobrassicin is further converted into neoglucobrassicin and 4-hydroxyglucobrassicin with this treatment (Figure 7.4). Neoglucobrassicin was the second

most abundant glucosinolate after glucobrassicin, which was enhanced with the hormetic doses of UV-B, hydrogen peroxide and heat, and they were the three effective stresses for glucobrassicin as well (Figure 7.4). Of note was the most significant change observed with neoglucobrassicin was its sharp increase in comparison with glucobrassicin due to the hormetic dose of methyl jasmonate (Figure 7.4). While the signature of the hormetic doses was similar for glucobrassicin and neoglucobrassicin, further hydroxyation and methoxylation of glucobrassicin revealed the specificity of hormetic stresses. The treatment of florets with methyl jasmonate significantly enhanced titers of 4-hydroxyglucobrassicin compared with the other stresses, while heat in particular, showed little change in its level (Figure 7.4). Similarly, a significant enhancement of 4-methoxyglucobrassicin was detected with the hormetic dose of ozone compared with the other stresses, and its level was nearly the same as the control florets in the heat-treated florets (Figure 7.4).

Indole glucosinolates accumulate in plants in response to wounding, herbivores and infection (Mikkelsen et al., 2002). For instance, *Erwinia carotova* elicits specifically the accumulation of indolymethylglucosinolate (glucobrassicin) in *Arabidopsis* plants mediated by jasmonic acid signalling pathway (Brader et al., 2001). The deposition of the glucan polymer, callose, on the plant cell wall against pathogen attack, an innate immune response in *Arabidopsis*, is mediated by ethylene and 4-methoxyglucobrassicin (Clay et al., 2009). The accumulation of 4-hydroxyglucobrassicin has been observed also in *Brassica rapa* after the infection of *Fusarium oxysporum* (Abdel-Farid et al., 2010). Thus, the induction of indole glucosinolates by abiotic stresses appears to be a response similar to pathogenic attack. However, the modification of glucobrassicin is more characteristic of the stresses. The florets treated with MeJA exhibited higher accumulation of only 4-hydroxyglucobrassicin, which was also observed in ozone-treated florets; but 4-methoxyglucobrassicin seems to be specific for this gas. On the other hand, neoglucobrassicin appears to be more of a generalized response to the application of abiotic stresses.

In addition, indole-type glucosinolates are enhanced to a greater extent and maintained for longer periods in comparison with aliphatic or methionine-derived glucosinolates (Mithen, 2001). Of them, the indole-type accumulates in large quantities in plant tissues; and among the glucobrassicins, glucobrassicin and neoglucobrassicin are the most abundant (Butcher et al., 1974). Indole-type glucosinolates derived from tryptophan are converted to indole-3-acetaldoxime, via cytochromes P450 of the CYP79B subfamily, which is the branching point for the biosynthesis of indole glucosinolates, indole 3-acetic acid (IAA) and other indole natural products (Naur et al., 2003a). The

over-expression of CYP79B3 in response to applied abiotic stresses, in particular UV-B, suggest that the biosynthesis of indole-3-acetaldoxime was likely enhanced, leading to increased synthesis of indole glucosinolates and IAA; in particular UV-B, that enhances the expression of CYP79B3 as well as neoglucobrassicin. Interestingly, IAA and kinetin were shown to inhibit chlorophyll loss in aging wheat chloroplasts (Misra and Biswal, 1980), suggesting that IAA may have some role in the delay of yellowing in the treated broccoli florets.

7.5.4 Hydroxycinnamic acids and Antioxidant capacity of the tissue

Hydroxycinnamic acids (HCA) including ferulic and sinapic acids and their glycosides are phenolic compounds possessing antioxidant properties (Teixeira et al., 2013), antimicrobial properties (Harris et al., 2010), and health-beneficial attributes (Nardini et al., 1995). The phenolic compounds identified in this study were glycosides derived from sinapic acid and ferulic acid. Four sinapic acid derivatives 1,2-disinapoyl gentibiose (DSG), 1-sinapoyl-2-feruloyl gentiobiose (SFG), 1,2,2-trisinapoyl gentiobiose (TSG), 1,2-disynalpoyl-2-feruloyl gentiobiose (DSFG) and one ferulate derivative 1,2-diferuoyl gentiobiose (DFG) were identified (Figure 7.5). The total HCA content is the sum of individual HCA contents. The total HCA was mostly affected by ethanol and heat hormetic doses. It was observed that the enhancement of HCA decreased in the order: ethanol (17.3 %) > heat (13.8 %) > UV-B (12.9 %) > UV-C (10.6 %) > MeJA (5.8 %) > H_2O_2 and O_3 (5.3 %) (Figure 7.5). Most significant changes on individual HCA were detected in heat and ethanol treated florets, while the less changes were observed in florets treated with either O_3 or H_2O_2, with an exception that the enhancement of DSFG and DFG by O_3 was lower compared with H_2O_2 (Figure 7.5). The enhancement pattern of HCA in response to hormetic stresses was different from that of glucosinolates, and the less oxidative heat and ethanol treatments exhibited higher elevation of HCA in comparison with more oxidative O_3 and H_2O_2.

SFG was the most abundant HCA in broccoli florets as reported elsewhere in this thesis, followed by DSG, DSFG, DFG and TSG (Chapter IV). On the other hand, this hierarchy was modified by the exposure of florets to stresses. Although the pattern of HCA accumulation in response to stresses remained similar to the total HCA content, only the accumulation of ferulate derivative, DFG, followed by DSFG was more pronounced with all stresses (Figure 7.5). The radical scavenging activity of hydroxy-cinnamic acids decrease in the following order: caffeic acid > sinapic acid > ferulic acid > ferulic acid esters (Kikuzaki et al., 2002). Also, there is some indication that ferulate may

marginally have a higher antimicrobial activity than sinapate (Zabka and Pavela, 2013). While the majority of HCA in the treated broccoli were somewhat enhanced depending the stress, the most significant increases were observed was with DFG, the simple ferulate. It seems that the enhancement of HCA by hormetic stresses may be part of both antioxidant and antimicrobial defenses.

7.5.5 Antioxidant capacity of the tissue

In order to understand whether the oxidative stress in the tissue following the application of stress persists during storage, the antioxidant capacity of the tissue was monitored by the evolution of oxygen radical absorbance capacity (ORAC) and of ascorbic acid content in the treated florets (Figure 7.6). The changes in ORAC values of treated florets compared with the untreated florets indicate that the remnants of oxidative stress are present in UV-C, ozone and H_2O_2, and surprisingly, in MeJA treated florets, and oxidative stress appears to be largely resolved in the florets treated with heat and UV-B, and to a smaller extent, in the florets treated with ethanol. Although a number of antioxidant substances in the tissue influence ORAC, only ascorbic acid was monitored in this study. Generally, an increased level of oxidized ascorbic acid was observed in the tissues treated with all stresses, except with ethanol. The increase in oxidized ascorbic acid was lower in the tissues treated with heat and UV-B, which also showed increase in ORAC. The average level of reduced ascorbic acid decreased in the treated tissues relative to the untreated florets for all stresses, in particular UV-C and UV-B. The reduction in both oxidized as well as reduced ascorbic acid in ethanol treated florets is probably not a simple consequence of the redox status of the tissue and involvement of redox enzymes, but more likely due to chemical decomposition of ascorbic acid. Hsu et al. (2012) reported on the degradation of ascorbic acid in ethanol solutions and they suggested that the degradation pathway is predominantly aerobic with the formation of 3- hydroxy- 2 pyrone, but the mechanisms are not clear.

7.5.6 Summary

The marketable life of produce is terminated by the progress of senescence and by the incidence of diseases. While this work did not examine the effect of hormetic stresses on disease resistance of broccoli that can potentially be induced by abiotic stresses it examined the potential

impact of abiotic stresses on the health-beneficial compounds in broccoli in response to abiotic stresses, a new dimension towards the preservation of fresh fruits and vegetables. Because of the lack of evaluation of disease resistance, an attribute of importance in the storability of crops, the selection of abiotic stresses capable of imparting all the three attributes to broccoli is not feasible at this time, pending further investigations. Nevertheless, it is possible to identify the hormetic stresses suitable for preservation of quality attributes and/or for the enhancement of or maintenance of health-beneficial compounds in broccoli.

Generally, treatment of broccoli florets with hormetic stresses help extend not only the shelf life, but also modestly enhance glucosinolates and hydroxy cinnamic acids, both to different degrees. Although doses of stresses higher than the hormetic doses, in general, were shown to enhance the levels of secondary metabolites in broccoli, its shelf life was compromised. No one hormetic stress was uniquely effective on both of these counts. From the standpoint of color retention in broccoli, the hormetic doses of heat and ethanol were the effective stresses, while MeJA and ozone treatments were the least effective. Severe weight loss was the mark of florets treated with ozone, and to a lesser extent, ethanol treatment. UV-C, UV-B, heat and MeJA treatments caused smaller weight losses during storage. Considering these two factors, heat and UV-C are probably the most effective hormetic stresses.

Although the enhancement of glucosinolates and HCA in response to hormetic stresses was modest, ozone, ethanol and H_2O_2 were effective in the enhancement of glucoraphanin; and UV-B and H_2O_2 as well as ozone, heat and MeJA for glucobrassicins. The difference between the hormetic stresses in the enhancement of HCA was not quite significant, although ethanol, heat, UV-C and UV-B were nearly equally effective. For both marketable quality and secondary metabolites, hormetic dose of UV-B, followed by heat and ethanol, can be considered beneficial treatments for broccoli.

7.6 Conclusion

Abiotic stresses can increase the storability of broccoli florets or enhanced health related compounds but not both for most of the applied treatments (Figure 7.7). Heat and ethanol were the best treatments for color preservation of the florets; both stresses delayed the yellowing of florets due to the effect on enzyme activity. Ozone and hydrogen peroxide were found to be efficient stresses to enhance indole glucosinolates as well as glucoraphanin, but ozone, the most intense oxidative stress, may not be advantageous from the standpoint of severe weight loss during treatment as well

as during storage. The hormetic dose of UV-B was the most balanced treatment, since yellowing was delayed and neoglucobrassicin was enhanced. Overall, hormetic doses tend to improve the preservation of broccoli, whereas the higher doses tend to increase secondary metabolites compromising the storability.

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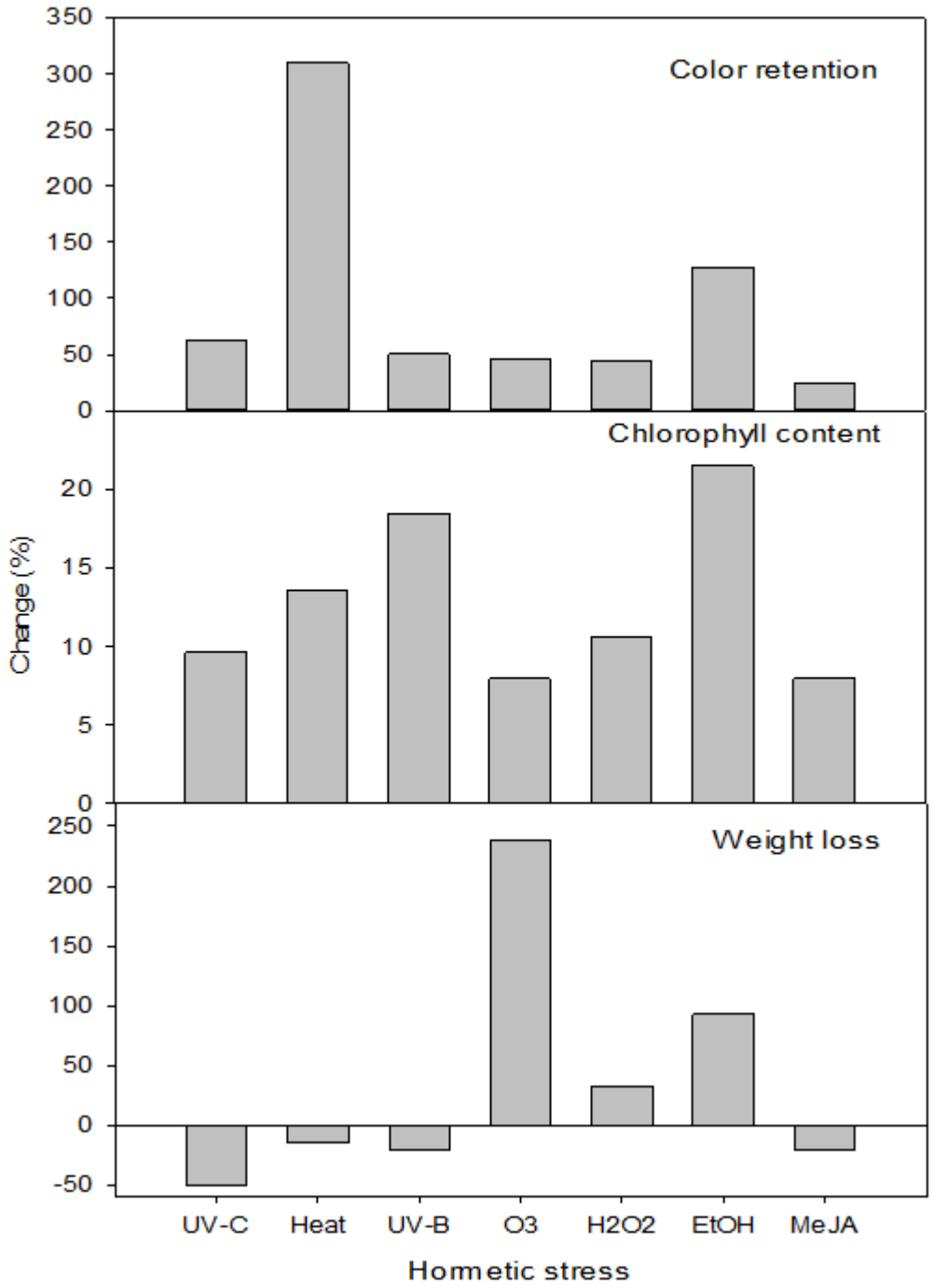

Figure 7.1 The effect of hormetic doses of abiotic stresses on the preservation of broccoli florets during storage at 4 °C for 14 days. Change of color, chlorophyll content and weight loss relative to control treated with abiotic stresses: UV-B at 1.5; UV-C at 1.2 $kJ.m^{-2}$; O_3 at 5 ppm for 60 min; H_2O_2 at 1.25 mM for 180 min; ethanol at 10,000 ppm for 30 min; heat at 41 °C/180 min and MeJA at 1 ppm for 45 min. Values were averaged over 14 days storage period and normalized with respect to the control.

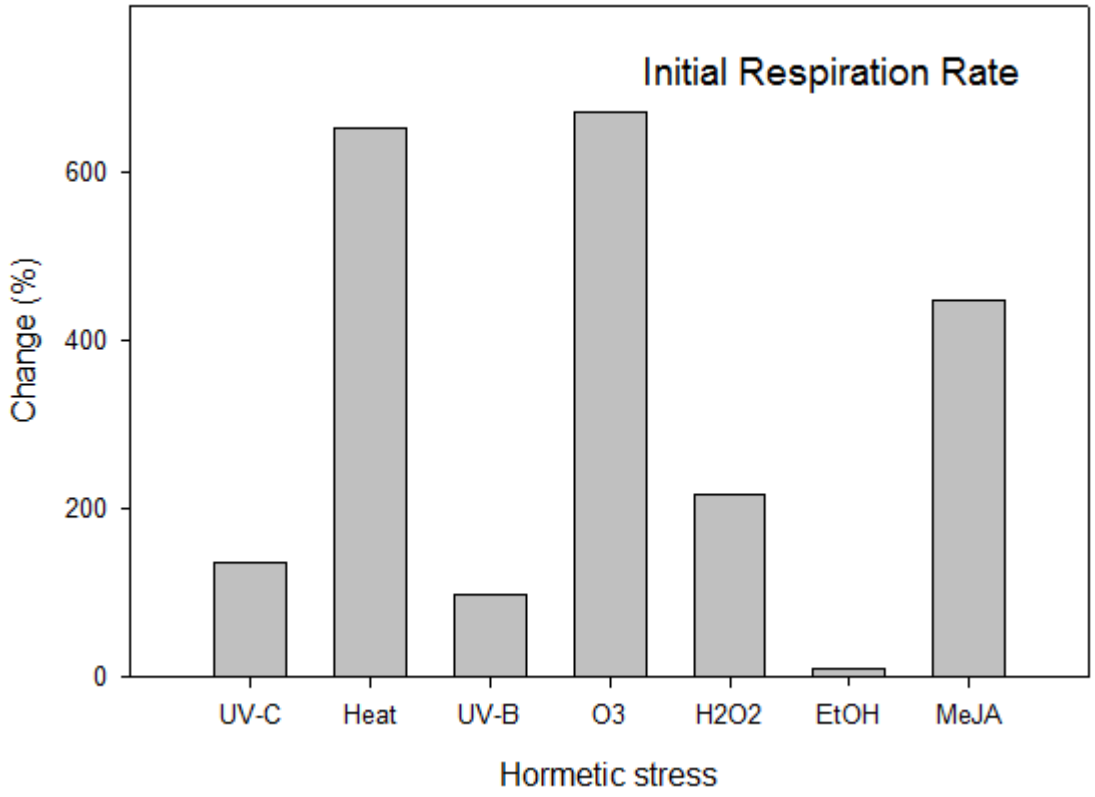

Figure 7.2 The effect of hormetic doses of abiotic stresses on the respiration rate of broccoli florets. Change in respiration rate relative to control treated with abiotic stresses: UV-B at 1.5; UV-C at 1.2 $kJ.m^{-2}$; O_3 at 5 ppm for 60 min; H_2O_2 at 1.25 mM for 180 min; ethanol at 10,000 ppm for 30 min; heat at 41 °C/180 min and MeJA at 1 ppm for 45 min. Values were averaged over 14 days storage period and normalized with respect to the control.

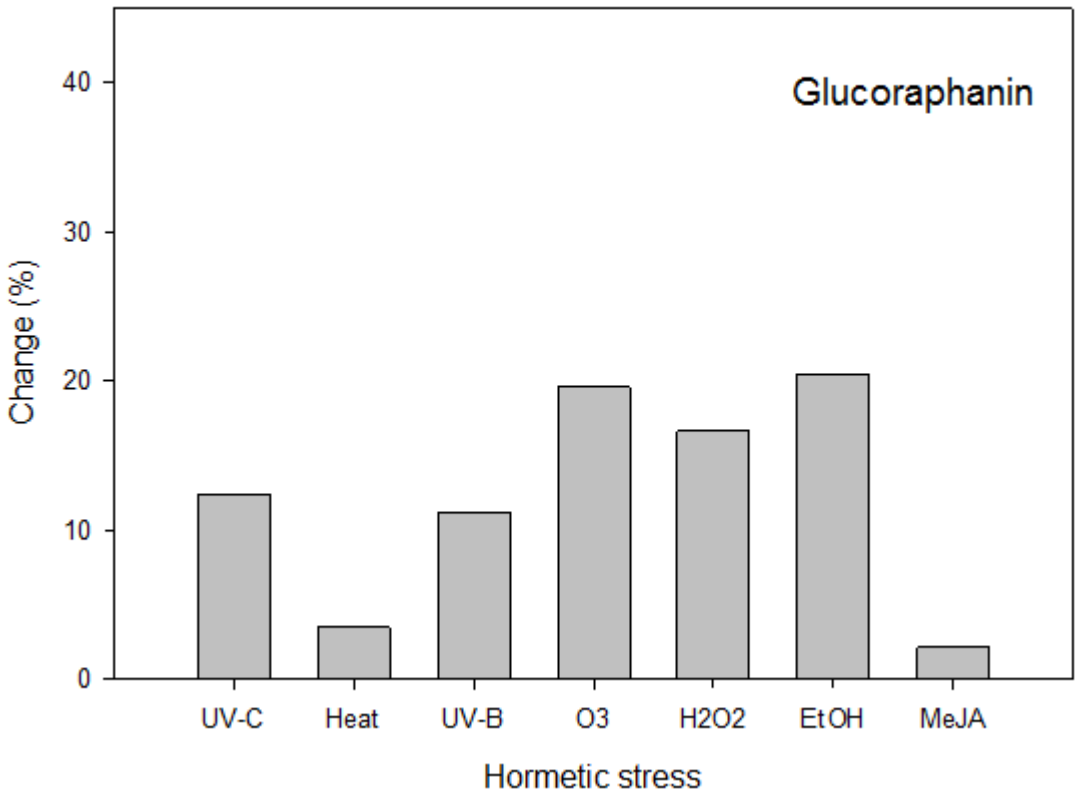

Figure 7.3 The effect of hormetic doses of abiotic stresses on the glucoraphanin content of broccoli florets. Change in glucoraphanin content relative to control treated with abiotic stresses: UV-B at 1.5; UV-C at 1.2 $kJ.m^{-2}$; O_3 at 5 ppm for 60 min; H_2O_2 at 1.25 mM for 180 min; ethanol at 10,000 ppm for 30 min; heat at 41 °C/180 min and MeJA at 1 ppm for 45 min. Values were averaged over 14 days storage period and normalized with respect to the control.

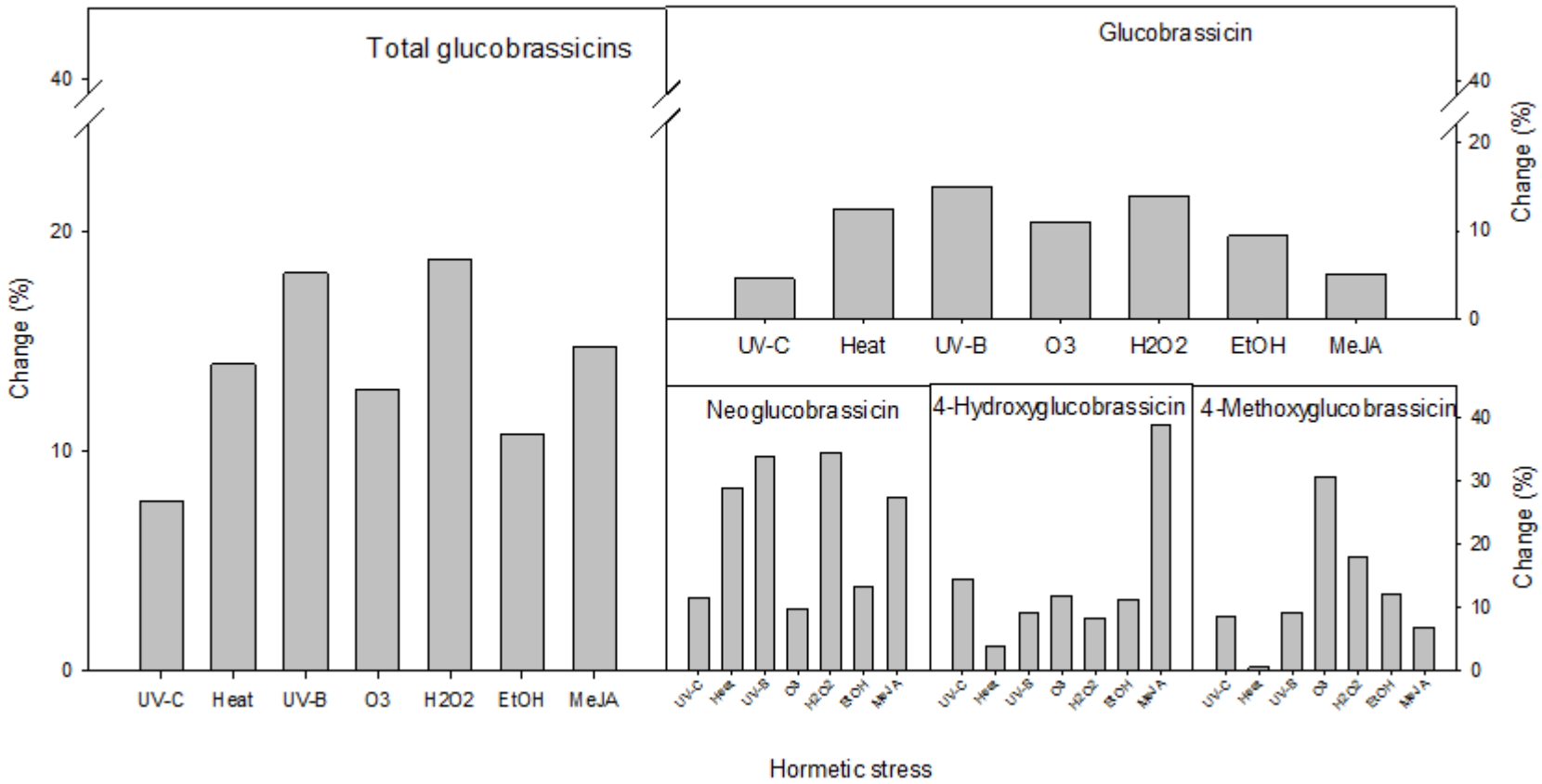

Figure 7.4 The effect of hormetic doses of abiotic stresses on the total glucobrassicins and individual indole-type glucosinolates of broccoli florets. Changes in glucobrassicin, neoglucobrassicin, 4-hydroxyglucobrassicin and 4-methoxyglucobrassicin content relative to control treated with abiotic stresses: UV-B at 1.5; UV-C at 1.2 kJ.m^{-2}; O_3 at 5 ppm for 60 min; H_2O_2 at 1.25 mM for 180 min; ethanol at 10,000 ppm for 30 min; heat at 41 °C/180 min and MeJA at 1 ppm for 45 min. Values were averaged over 14 days storage period and normalized with respect to the control.

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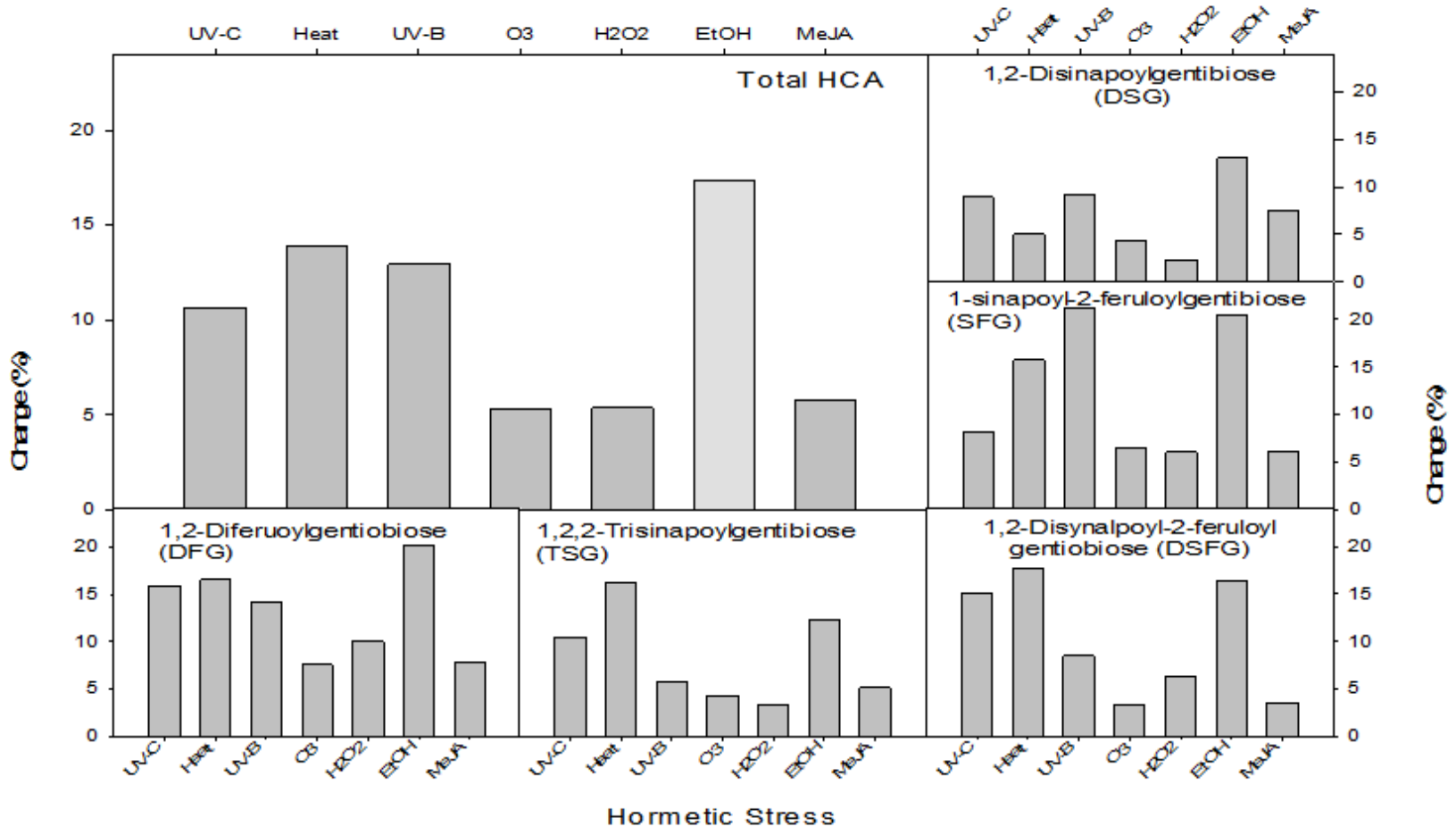

Figure 7.5 The effect of hormetic doses of abiotic stresses on hydroxy-cinnamic acids (HCA) in broccoli florets. Changes in total HCA 1,2-disinapoyl gentiobiose, 1-sinapoyl-2-feruloyl gentiobiose, 1,2-diferuoyl gentiobiose, 1,2,2-trisinapoyl gentiobiose and 1,2-disynalpoyl-2-feruloyl gentiobiose content relative to control treated with abiotic stresses: UV-B at 1.5; UV-C at 1.2 $kJ.m^{-2}$; O_3 at 5 ppm for 60 min; H_2O_2 at 1.25 mM for 180 min; ethanol at 10,000 ppm for 30 min; heat at 41 °C/180 min and MeJA at 1 ppm for 45 min. Values were averaged over 14 days storage period and normalized with respect to the control.

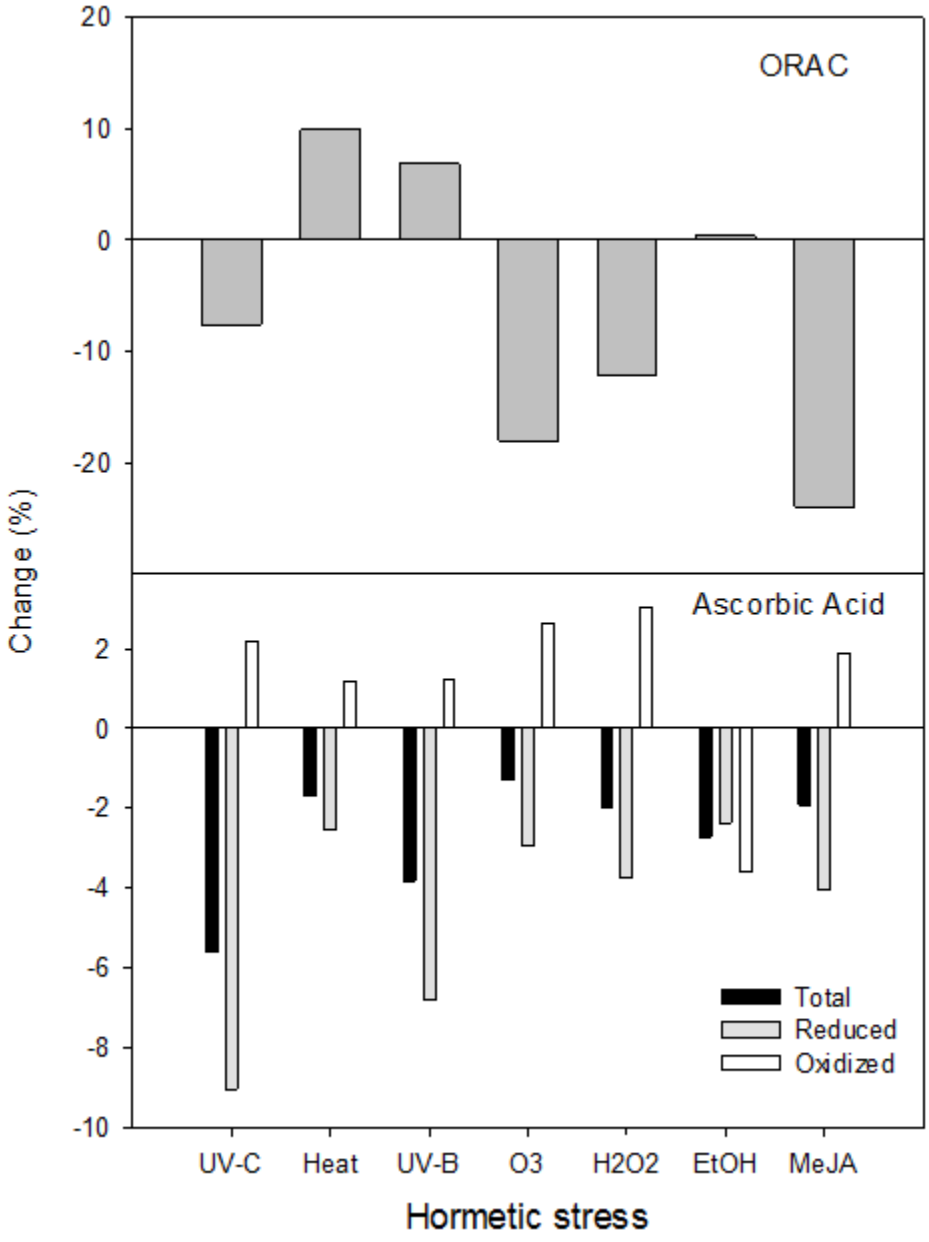

Figure 7.6 The effect of hormetic doses of abiotic stresses on the antioxidant parameters of broccoli florets. Changes in ORAC and total ascorbic acid content relative to control treated with abiotic stresses: UV-B at 1.5; UV-C at 1.2 $kJ.m^{-2}$; O_3 at 5 ppm for 60 min; H_2O_2 at 1.25 mM for 180 min; ethanol at 10,000 ppm for 30 min; heat at 41 °C/180 min and MeJA at 1 ppm for 45 min. Values were averaged over 14 days storage period and normalized with respect to the control.

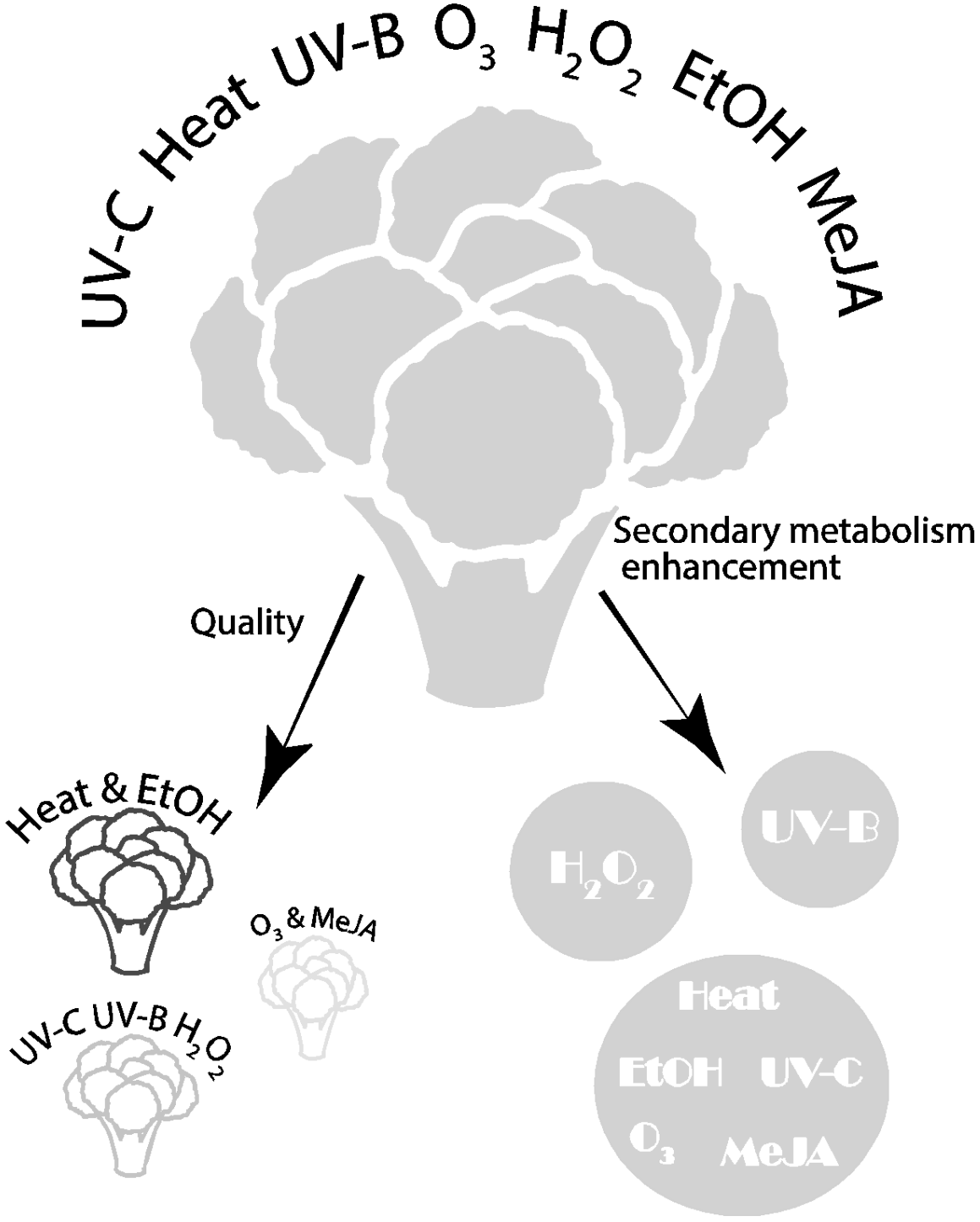

Figure 7.7 Effect of hormetic doses of abiotic stresses in broccoli quality and secondary metabolism.

Chapter VIII: Concluding Remarks

General conclusions

Broccoli is an important crop for human nutrition because of its high content in fiber, vitamins and minerals. In addition, broccoli has two important classes of phyto-compounds: glucosinolates and phenylpropanoids, which have been recognized for the prevention of degenerative diseases such as cancer. The application of abiotic stresses prior to the storage of broccoli florets can be an adjuvant to cold storage, which can be carried out on large quantities at relatively low cost. Abiotic stresses can delay yellowing of broccoli florets, the main indicator of senescence that affects its marketability. In addition, it has the potential to activate defense mechanisms that could lead to the enhancement of health-beneficial secondary metabolites.

The objective of this thesis was to explore the potential of abiotic stresses, UV-B, UV-C, heat, O_3, H_2O_2, ethanol (EtOH) and methyl jasmonate (MeJA) to enhance the levels of bioactive phyto-compounds in broccoli, especially, the glucosinolates, while also improving or maintaining its storage life.

The results of this work show that the abiotic stresses appear to be beneficial in maintaining the quality and the storability of broccoli at low doses. High doses are often required for significant enhancement of health beneficial phytochemicals such as glucosinolates, but it is at the expense of preservation of broccoli. This raises the question whether it is possible to achieve both, preservation of and enhancement of phyto-compounds in broccoli, with a single stress at a low dose.

The first objective centered on the identification of the hormetic dose for each type of abiotic stress, based on color retention response of broccoli florets during storage. In this regard, color is a convenient indicator to determine the effect of a specified stress, because it is an indicator of senescence and also of quality. The hormetic dose of various stresses for color retention were: UV-B, 1.5 $kJ.m^{-2}$; UV-C, 1.2 $kJ.m^{-2}$; heat, 41 °C for 180 min; ozone, 5 ppm for 60 min; hydrogen peroxide, 1.25 mM for 180 min; ethanol, 10,000 ppm for 30 min; and MeJA, 1 ppm for 45 min.

Among the hormetic stresses, heat was the peerless treatment to maintain color of florets during the storage. Pre-treatment of broccoli with the hormetic heat dose using humid hot air preserved the green color of broccoli florets for 4 weeks at 4 °C. Although higher temperatures could be used to deliver the hormetic heat dose and achieve color retention, 41 °C was found optimal to avoid off-odor generation and minimize anaerobic respiration. Exposure of broccoli to ethanol was also an effective treatment to delay yellowing without causing any appreciable weight loss. Like heat treatment, ethanol can suppress catabolic enzymes of chlorophyll as well as the biosynthesis of

ethylene. UV-C doses ranging from 0.9 to 1.5 kJ.m^{-2} were effective in maintaining the color of broccoli florets for almost 4 weeks of storage at 4 °C. Interestingly, the hormetic dose of UV-B and UV-C were equivalent in terms of their photon energies and color retention. Furthermore, it was possible to delay yellowing of broccoli with the powerful oxidative agent, ozone, using an atmosphere containing 5 ppm and a short exposure time. Nonetheless, the weight loss and the texture or turgidity of the florets, associated with water loss, could limit their acceptability towards the end of the storage. Hydrogen peroxide, a mild oxidative agent in comparison to ozone, delayed the yellowing of florets at the hormetic dose of 1.25 mM for 45 min. On the other hand, the signal molecule, methyl jasmonate, tended to accelerate the rate of yellowing of treated florets, presumably, by its effect on ethylene production, resulting in a decline of the green color of the florets.

The second part of the work focused on the effects of the abiotic stresses on physiological and biochemical characteristics of florets during the storage. The working hypothesis was that hormetic doses of the abiotic stresses for color retention could also enhance the levels of antioxidants including phenols, flavonoids, ascorbic acid, thereby, also increasing the ORAC (oxygen radical absorbance capacity) values; in other words, the delay in yellowing or senescence might relate to elevated levels of antioxidants. However, this was not the case for some of the studied stresses. For instance, more elevated concentrations of total phenols and flavonoids as well as ORAC were observed on the high dose of heat, compared with the hormetic dose, yet, yellowing of florets was delayed by both doses. Similarly, even if better color retention was observed in the broccoli florets exposed to the hormetic doses of UV-C and UV-B, the titer of total phenols in florets was superior in the florets exposed to a high dose of UV-C; and ORAC concentration was improved in the florets exposed to the high dose of UV-B. Therefore, the hormetic dose for quality preservation of broccoli is not always the most appropiate for the enhancement of the antioxidant capacity of florets.

Consistently, the initial respiration rate and ascorbic acid content of the florets subject to abiotic stresses were inversely related, especially at high doses. Invariably, the high intial respiration activity of the florets was accompanied by a significant decrease of ascorbic acid, suggesting that the depletion of ascorbic acid in response to the stresses is an indicator of the intensity of the oxidative stress by the generation of reactive oxygen species (ROS). In this regard, the intensity of high doses of oxidative stress followed a decreasing order: O_3, followed by UV-C, UV-B, heat, hydrogen peroxide, ethanol and methyl jasmonate. Yet, the oxidative nature of methyl jasmonate is not clear,

since only the long exposure to this molecule caused the depletion of ascorbic acid in florets. In addition to the inverse relationship between the rise of respiration rate and the depletion of ascorbic acid, the ORAC (oxygen radical absorbance capacity) value appears to be stress-specific, where only UV-B and heat enhanced ORAC value and it is likely because of the enhanced levels of total phenolic content as well as hydroxy-cinnamic acids (HCA). Moreover, a common enhanced HCA for these two stresses was 1,2 – disynalpoyl -2- feruloyl gentiobiose, which was the most abundant HCA.

The third part of the work was to study the evolution of phenolic acids and glucosinolates in broccoli florets treated with abiotic stresses and stored at 4 °C. Hydroxy-cinnamic acids were enhanced by UV-C and by hormetic heat dose at temperature above the critical zone (HDHT). The high enhancement of 1,2 – diferuoyl gentiobiose in broccoli treated with HDHT may be used as a marker of heat damage. The high heat dose (HDHT), the high dose of UV-B and both the hormetic and high doses of hydrogen peroxide enhanced the titers of total glucobrassacins. The hormetic dose of UV-B enhanced the titer of neoglucobrassin, a tryptophan-derived indole glucosinolate. However, the most effective stress to enhance glucosinolates was hydrogen peroxide, where both indole and aliphatic glucosinolates were boosted. Ozone enhanced neoglucobrassicin, and especially 4-methoxyglucobrassicin. In spite of a decreased level of the total glucosinolates with methyl jasmonate treatment, the glucobrassicin, 4-hydroxyglucobrassicin, was highly enhanced by this signal molecule.

Glucosinolates are known defense compounds in *Brassicas* and particularly, in broccoli, stresses can also be viewed from the standpoint of their elicitation of defense compounds in broccoli. In this sense, the responses to the applied stresses are similar to wounding response, i.e., ROS production and the subsequent enhancement of glucosinolates, especially the indole glucosinolates. In this respect, the application of hydrogen peroxide stands out, which is also a secondary messenger in plant defense responses, including the accumulation of glucosinolates and phenylpropanoids. Yet, there is an apparent hierarchy of defense responses observed with this stress, where the enhancement of glucosinolates was more prominent compared with the phenylpropanoids while these two classes of compounds share the same precursor, chorismate.

Thus the response of broccoli to oxidative stresses appears to be primarily the enhancement of glucosinolates rather than phenolic compounds. This response was similar with UV-B, O_3 and UV-C to a lesser extent. On the other hand, heat causes unspecific responses, as it involves thermal effect as well as anaerobic stress with impacts on protein conformation, cell membrane structure and the kinetics of reactions. The effect of ethanol and methyl jasmonate appears to be very specific, that

ethanol may affect the conformation of proteins, particularly ethylene receptors, while methyl jasmonate is a signal molecule that can enhance defense responses at physiologically effective concentrations.

The fourth part of the investigation was to have a better understanding on how stresses affect the gene expression of key enzymes involved in the biosynthesis of the above secondary metabolites (the conclusions here exclude the treatments with methyl jasmonate and ethanol, as gene expression in response to their exposure was not assayed). The initial overexpression at the start of storage of broccoli florets was examined in relation to biosynthesis of secondary metabolites. Genes coding for key enzymes were divided into two categories, phenylpropanoids and glucosinolates. The shikimic acid pathway, including phenylalanine ammoniolyase (PAL) and chalcone synthase (CS) were induced by UV-C and H_2O_2 and the expression was in proportion to the enhancement of total phenolic content. Likewise, high heat dose, HDHT, enhanced the biosynthesis of HCA in florets corresponded with the specific overexpression of coumarate ligase gene. On the other hand, doses of UV-B doses below 7.2 $kJ.m^{-2}$ did not effectively induce the expression of the key enzymes of the shikimic acid pathway. In addition, the effect of applied doses of ozone on the shikimic acid pathway enzymes was nearly inexistent.

The expression of tryptophan N-hydroxylase (CYP79B3) was significant with the oxidative stresses, UV-B, O_3 and H_2O_2. Similarly, UV-B light and high ozone dose had a strong influence on the overexpression of phenylalanine N-hydroxylase (CYP79A2) gene in broccoli florets. Both tryptophan and phenylalanine share a common precursor, the chorismate. While phenylalanine, is the main gate for the phenylpropanoid biosynthetic pathway, the indole glucosinolates are derived from tryptophan. Thus the influence of abiotic stresses appears to be specific for the biosynthesis of glucosinolates or phenylpropanoids, and the gene expression profile can be a good approach to identify this specificity. For instance, in the gene expression profile of UV-C light, there was a more predominant overexpression of phenylpropanoids compared with the indole glucosinolates. This pattern was opposite for UV-B light and ozone and to a lesser extent for hydrogen peroxide. The expression pattern with heat was more arbitrary with some trend for phenylpropanoid gene overexpression. Also, there was no clear indication of relationship between gene expression and glucosinolate titers with ozone treatment. Even when the genes are expressed, the product may not

be synthesized because of either lack of substrates or synthetic power (reductive power, NADPH). In the case of ozone, it is possible that there might have been less supply reductive equivalents.

The following presents a summary of main observations regarding the impact of various stresses on the yellowing of broccoli florets during storage at 4 °C and on the contents of glucosinolates and HCA to determine the most appropriate abiotic stress.

- UV-C light

  It is a short-time and relatively inexpensive treatment that can adequately delay yellowing of broccoli florets at low doses as well as enhance the titers of hydroxy-cinnamic acids, 1,2-disynalpoyl-2-feruloyl gentiobiose, and neoglucobrassicin in the florets. The high dose of UV-C elevates total glucosinolates, but it also increases ascorbic acid depletion as well as yellowing and browning of the florets.

- Heat

  Heat was the best among the abiotic stresses evaluated in this study to maintain the green color of broccoli florets during storage at 4 °C. The application of this treatment on florets can be easily accomplished once the equipment is started up. However, the hormetic dose of heat must be delivered at proper temperatures to be beneficial; and in the case of broccoli, the temperature must be below 42 °C. Higher temperatures can cause off-odors as well as tissue softening. Temperatures higher than 45 °C can cause severe anaerobic respiration and tissue damage rendering the tissue susceptible to spoilage.

- Ozone

  The effect of ozone in broccoli may be limited with respect to freshness of the florets, even with low exposure time, because of the weight loss of the florets. It also causes considerable decreases in ascorbic acid and ORAC value. Unfortunately, the enhancement of glucosinolates by this gas can be imbalanced by the reduced quality of florets at the end of the storage period. From a practical standpoint, the control of the ozone concentration during the treatment requires specialized equipment.

- UV-B light

  Like UV-C light, UV-B is an inexpensive treatment to delay yellowing and increase the phytochemical content of florets. Low doses of UV-B light can delay yellowing of broccoli and enhance total glucosinolate content of the florets, especially the titer of neoglucobrassicin.

Total HCA is also enhanced by low doses of UV-B light, particularly sinapic and ferulic acid derivatives, which can contribute to the antioxidant capacity of the florets. As a result, hormetic dose of UV-B light can be an ideal postharvest treatment for broccoli florets since quality is not unduly affected and the phytochemical content is reasonably boosted.

- Hydrogen peroxide

  No significant improvement in the quality of florets was observed with H_2O_2 treatment compared with other stresses such as heat or ethanol. However, glucosinolates, especially total glucobrassicins are largely enhanced compared with the other stresses, and has a potential for commercial application to improve the phytochemical content in broccoli.

- Ethanol

  Besides heat, ethanol can be an excellent treatment to preserve the green color of broccoli florets, while it can also enhance the concentration of glucosinolates and HCA. It is also an unexpansive treatment due to the low cost of the ethanol.

- Methyl jasmonate

  Contrary to ethanol, methyl jasmonate, which is also a fumigation treatment, accelerates the yellowing of broccoli florets, although it enhances glucobrassicin derivatives, neoglucobrassicin and 4-hydroxyglucobrassicin.

Thus, among the stresses evaluated in this study, hormetic doses of heat and ethanol were the best treatments for delaying yellowing florets, but UV-C and UV-B were also effective for delaying yellowing of florets over the storage. The antioxidant capacity of the florets was moderately enhanced by heat and by UV-B. Hormetic doses of ozone and hydrogen peroxide were found effective in elevating glucoraphanin and indole glucosinolates, but they can be disadvantageous because of weight loss and poor color retention, respectively. It was evident that none of the stresses was most favorable from the standpoint of both color retention as well as enhancement of phyto-compounds. However, UV-B was the most balanced stress in terms of both quality preservation and enhancement of glucosinolates and hydroxycinnamic acids.

Originality of the contribution

This thesis research has contributed to the knowledge and provided insights into the postharvest physiology of stored broccoli. The initial respiration rate of florets following treatments can be used as a rapid method to determinate the severity of abiotic stresses, and foresee potential effects on quality during storage. Likewise, changes in free amino acid pools of commodities can be used to predict the potential influence of abiotic stresses on secondary metabolites of interest for plant protection and human health. In particularly, some amino acids can be used as markers of stress severity; the branched-chain amino acid, leucine, is a possible marker of broccoli senescence. Like initial respiration rate, electrolyte leakage can be used as a rapid technique to determine the general status of florets cell membrane integrity.

Another contribution of this work was the application of the time-temperature superposition concept and methodology to establish hormetic heat dose for heat treatment of broccoli florets. As was shown for broccoli, the identification of a critical temperature or a crtitical temperature zone would help avoid physiological damages to crops subject to heat treatment. This methodology can be used in other produce and could help to perform postharvest heat treatments.

The use of gene expression on postharvest biology is not new and it has often been used to study gene expression in the phenylpropanoid pathway. However, the glucosinolate pathway has been less explored in broccoli exposed to abiotic stresses. This study has shown that oxidative stresses can induce the expression of key enzymes of the glucosinolate pathway. More specifically, the indole glucosinolate pathway appears to be affected by oxidative stresses, and the cytochrome P450 family 79 subfamily b polypeptide 3 (CYP79B3) appears to be specifically expressed in response to oxidative stresses. Its expression was particularly evident in response to hydrogen peroxide, a compound that is widely used for postharvest disinfection, but not as inductor of plant defense responses. Likewise, heat treatment is known to maintain the quality of broccoli florets during storage, but little is known about its influence on phytochemicals content of florets. Thus, this work points to the possibility of preserving broccoli florets while improving their phytochemical content with abiotic stresses, but only certain such as UV-B and H_2O_2 are able to improve the phyto-compounds without unduly compromising the storability.

Perspectives for further work

Even though the outlook to extend the shelf life of and enhance the the phytochemical content of broccoli florets is put in evidence by this work, additional research is necessary to optimize doses of abiotic stresses and treatment conditions. For instance, even if the hormetic dose was established for UV-C, and UV-B, the light fluence rate was not, and it is quite possible that the rate of delivery may have impact on the hormetic effects. Likewise, the concentrations of hydrogen peroxide or the fumigants, ozone, ethanol or MeJA need further investigations.

From the metabolomic perspective, there is a lack of information regarding the effect of abiotic stresses on glucosinolate and phenylpropanoids precursors, including amino acids and primary metabolites. For instance, influence of abiotic stresses on chorismate derived from erythrose 4-phsophate and phosphoenolpyruvate can help the understanding of the potential changes in phenylpropanoids and indole/aromatic glucosinolates. The alterations in oxaloacetate – aspartate and methionine pools may provide insights into the biosynthesis of aliphatic glucosinolate.

Phytohormones are of crucial importance in plant-abiotic stress interactions, and as recent reports show that abscisic acid (ABA) appears to be playing key roles in the interactions, including the production of ROS, and it needs to be monitored in the treated produce. In addition, monitoring key signals such as ethylene, jasmonic acid and salicylic acid, as well as stress metabolites such as proline could be use to mark indirectly the severity of abiotic stresses. In addition, the redox status of produce after an abiotic stress event could also provide some insights into the type of secondary metabolites synthesized as well as the nature of reactive oxygen species involved.

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Annexes

Annex I: Plant metabolomics

Abstract

Plant metabolomics is relatively a new science sustained by three disciplines: analytical chemistry, chemometrics and bioinformatics. Together with genome sequence and transcriptomics, metabolomics is an important part of functional genomics which aims at the understanding of the physiology of biological systems. The present review is focused on the most common analytical techniques used in plant metabolomics. Analytical techniques are outlined as well as the experimental design, plant cultivation and extraction protocols. In analytical chemistry, hyphenated techniques such as gas chromatograph – mass spectroscopy (GC-MS) and liquid chromatography – mass spectroscopy (LC-MS) are more and more emphasized. Some background is also given on chemometrics; principal components analysis (PCA) and partial least squares (PLS) which are valuable multivariate methods for data mining in metabolomics. A brief description of databases and visualization software is also discussed. Finally, some applications of metabolomics are illustrated in plant science and technology.
Key words: metabolomics; GC-MS; LC-MS; chemometrics; bioinformatics; metabolic networks

Introduction

Efforts to characterize the molecular constituents of life has allowed the development of high-throughput genome-wide experimentation also known as 'omics' (Bruggeman and Westerhoff, 2007). These technologies have been adapted from known methods and improved for large scale analysis. Functional genomics was the first approach to understand how genes works, transcriptomics, on the other hand was used to demonstrate how raw sequence data could be transformed to gene function. Similarly, proteomics has been used to study the amount of total proteins expressed at a given time or under certain condition (Holtorf et al., 2002). Plant metabolomics is a relative new science, originated from metabolomic profiling. Plants were not the first target of metabolomic profiling.

One of the first studies of metabolomics with biological molecules was on steroids using gas chromatography coupled with mass spectroscopy in 1960 (Horning et al., 1961) The concept of metabolite profiling has decades and the first measures of metabolites were done as targeted approaches with spectrometric assays or by chromatography separations in the context of medical and diagnostic purposes (Lisec et al., 2006). There are three major approaches used in metabolomics studies: (i) targeted analysis, (ii) metabolite profiling and (iii) metabolic fingerprinting. Targeted analysis is the most developed analytical approach in metabolomics and is used to measure the concentration of a limited number of known metabolites precisely. Metabolic fingerprinting does not attempt to identify or precisely quantify all the metabolites in the sample. Rather, it considers a total profile, or fingerprint, as a unique pattern characterizing a snapshot of the metabolism. Fingerprinting is usually performed with spectroscopic techniques such as nuclear magnetic resonance (NMR), Fourier transform infrared spectroscopy (FT-IR), Fourier transform ion cyclotron resonance mass spectroscopy (FTICR-MS) or mass spectrometry (MS). Metabolomics on the other hand, pretends to quantify all these molecules of a certain system at the same time; however this is impossible at the present time due to the complexity of molecules and the lack of analytical strategies that can accomplish this task. Moreover, it has been estimated that the plant kingdom possess more than 200 000 different metabolites (Fiehn, 2002), showing how difficult the task can be.

Metabolomic analysis

Due to the complexity of metabolome, there no one unique one method capable of measuring all of it and thus, many technologies have been developed in order to complete the task. Among methods that have been used in plant metabolomics are: nuclear magnetic resonance (NMR) (Abdel-Farid et al., 2007); capillary electrophoresis coupled with mass spectrometry (CE/MS) (Edwards et al., 2006); gas chromatography-mass spectrometry (GC-MS) (Forney et al., 1991a); liquid chromatography-mass spectrometry (LC-MS) (Vallejo et al., 2003b); liquid chromatography tandem mass spectrometry (LC/MS/MS) (Tian et al., 2005) and Fourier transform ion cyclotron mass spectrometry (FTMS). The potential of HPLC coupled with mass spectrometry and nuclear magnetic resonance (LC/NMR/MS) or (LC/NMR/MS/MS) for metabolomics analysis has been also reviewed (Sumner et al., 2003). These are not the only technologies, but they represent the most common techniques (Figure A.1).

Because of the availability of a variety of analytical tools, the best way to select a tool will be asking exactly the problem that one wants to resolve. This is a fundamental part for a successful strategy in the metabolomic analysis. It is also important to have some background knowledge on the analyte chemistry in order to divide analysis into subsets of the problem. Part of the strategy for metabolome analysis is to answer the following questions: what kind of information is needed? What kind of chemistry is expected? and what are the analytical facilities available? (Villas-Bôas et al.,

2007). In general, the approaches used for metabolome analysis are often divided into four different strategies: Targeted analysis; Metabolomic fingerprinting; Metabolomic profiling and Metabolomics. Although there is an overlap between these strategies, they can give not only quite different but also complementary results. These strategies share some common methodologies and analytical approaches but are typically implemented quite differently (Villas-Bôas et al., 2007).

Targeted analysis

Targeted analysis is a quantitative approach used to determine precise concentrations of limited number of known metabolites. In plants this approach is widely use to follow limited metabolites which are known to be involved in a particular environmental condition (Shulaev et al., 2008b). A major limitation of this approach is that the analysis must be done on compounds already known and in purified form which is not useful for the identification of novel metabolites (Shulaev, 2006b).

Metabolomic fingerprinting

In this approach, a total profile or fingerprint, is a unique pattern characterizing the metabolism of a particular cell or tissue (Shulaev 2006). Fingerprinting can be performed with a variety of analytical techniques, including NMR, Fourier transform ion cyclotron resonance mass spectrometry or Fourier transform infrared (FT-IR) spectroscopy (Shulaev et al., 2008a).

Metabolite profiling

The profiling involves the analysis of a class of metabolites, for example, amino acids, or some components associated with a metabolic pathway (Goodacre et al., 2004). Metabolite profiling is aimed to measure of all or a set of metabolites in a sample and quantifies them. To date, GC-MS is the most developed analytical platform for plant metabolite profiling. GC-MS is generally performed using electron impact (EI) quadrupole or time-of-flight (TOF) mass spectrometry. The major advantage of GC-MS for metabolomics is the availability of both commercially and publicly available EI spectral libraries. The limitation of the GC-MS profiling is that it can only analyze volatile compounds or compounds that can be volatilized by chemical derivatization (Shulaev et al., 2008a). For non-volatile compounds, LC-MS and CE-MS provide a better alternative. Ionization can be performed with 'soft' ionization methods, such as electrospray ionization (ESI); atmospheric pressure chemical ionization (APSI) or photoioniztion (PI) (Hall, 2006b). CE-MS provides a viable alternative for metabolite profiling due to its high resolving power, low sample volume requirements and the ability to separate cations, anions and uncharged molecules simultaneously (Shulaev et al., 2008a).

Metabolomics

Metabolomics aims at obtaining an unbiased picture of the metabolome of a particular organism, both quantitative and qualitative (Hall et al., 2002). Because of the complexity of the mixtures, major changes in metabolite levels will almost certainly include unidentified spectra. Therefore, metabolomic research should include approaches aimed at elucidating chemical structures, for example by combining liquid chromatography with nuclear magnetic resonance detection (NMR) and mass spectrometry (Fiehn, 2001). Analytical methods are very similar to those used for profiling and include GC/MS, NMR, and LC/UV/MS. All the protocols used currently in metabolomic approaches have biases and reproducibility is very hard to assess. Thus quantification

for all the metabolites present in a sample is often impossible to achieve; hence, metabolomics at the present remains a 'quick-and-dirty' method (Fiehn, 2001).

Technical elements of metabolomics

Metabolomics consists of several complicated technical elements with each step possibly giving rise to an experimental error. To establish a robust system, close collaboration among researchers in the fields of analytical chemistry, organic chemistry, chemo-metrics, informatics, and bioscience is required (Fukusaki and Kobayashi, 2005). In order to accomplish a metabolomic analysis there are basically six steps (Griffiths, 2007) that might be followed: 1) experimental design; 2) plant cultivation; 3) sample preparation; 4) analysis of metabolites 5) metabolomics data analysis and 6) data integration.

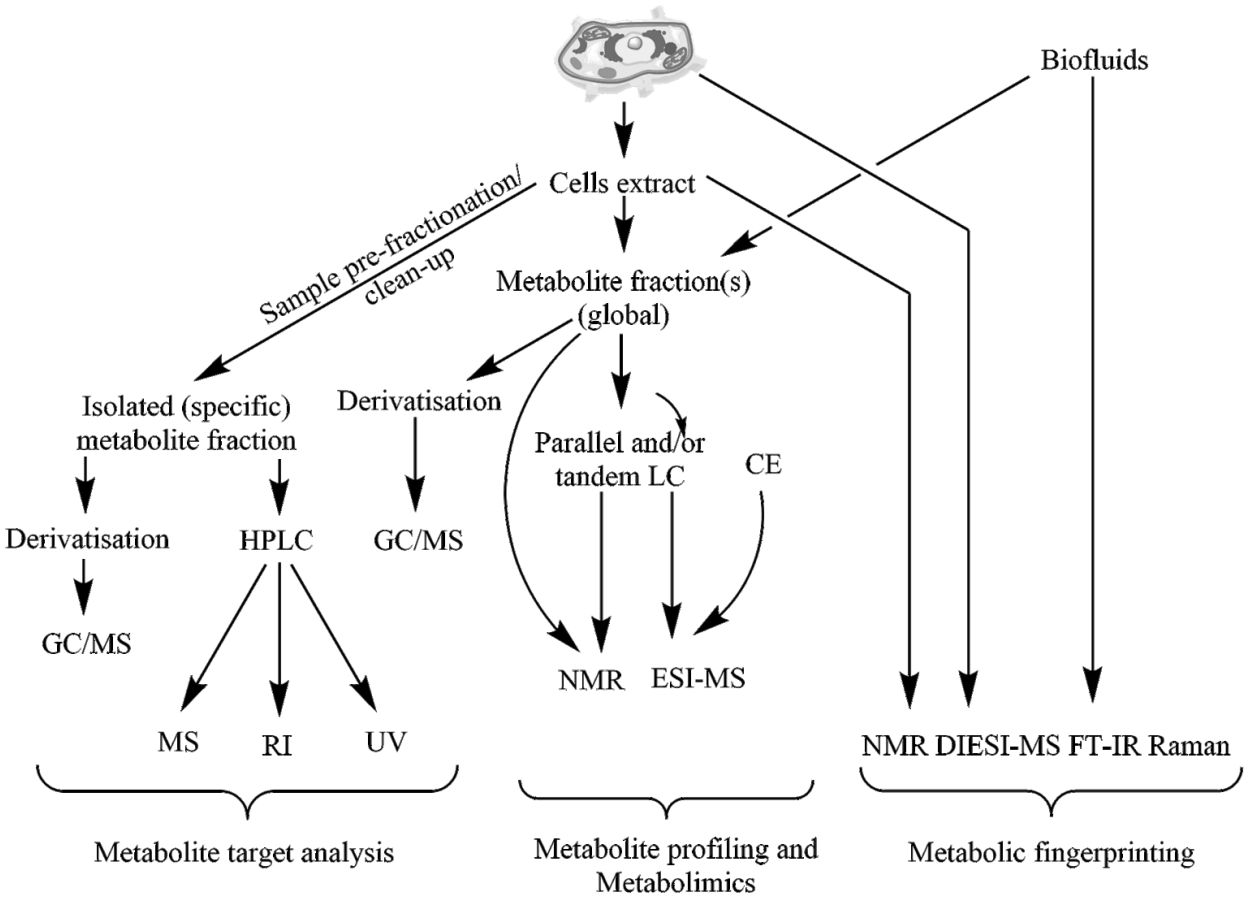
Experimental design

A well designed experiment can reduce bias and variance; help ensure that the experiment is feasible; can answer the question of interest; and can minimize errors. The basic principles of the design are the same as those used in all areas of science: randomization, replication and controls. For metabolomics studies in particular, it has been suggest following the MIAMET (Minimum Information About a Metabolomics Experiment) experimental design (Jenkins et al., 2004). Experimental design is an effectively way to visualize the experiment that may not guarantee success, but is a way through it. (Goodacre et al., 2004).

Plant cultivation

Sample procurement represent the initial and most critical step in any analytical procedure and underpins the success of the results obtained (Ryan and Robards, 2006) A typical description of the plant material must contain details of the origin of the sample; species and genotype; plant organ and tissue function; age or extent of maturation and environmental conditions. Uniformity in plant cultivation is very hard to accomplish. Small differences in light, humidity and composition of soil are expected due to the position of each one of the plant in the chamber, leading to differences in the metabolome. To avoid this problem, plants could be rotated or be cultivated without soil (hydroponic) (Fukusaki and Kobayashi, 2005). Plant preparation is important since identical conditions could exceeded the experimental error by a factor of 10 (Ryan and Robards, 2006)

Figure A.1 General strategies for metabolome analysis. (Goodacre et al., 2004)



Sampling and extraction of metabolites

If sample is crucial for metabolomic analysis, sample preparation and extraction is of vital importance as well. To achieve a low variability, not only the plant cultivation is important but also the timing of sampling must be controlled. The latter is even more important when handling postharvest produce (Fukusaki and Kobayashi, 2005). An optimal protocol may involve four steps for sampling: 1) the control of growth stage and the exact time of sampling; 2) the homogenization of the plant material; 3) a solid matrix for pre-column work; and 4) the concentration for targeted metabolites should be considered (Bamba and Fukusaki, 2006). It should be also considered that protocols for metabolomics are tissue dependent. Since the aim of metabolomics is to take a 'picture' of the metabolome under certain conditions, it's of absolute importance to stop all enzymatic activity, to avoid changes in the sample. This is done with a rapid quenching of all biochemical processes after sample harvesting (Villas-Bôas et al., 2007). After quenching, the metabolites are made accessible for analysis by extraction of the metabolites of intra or extracellular space. Most used techniques for quenching include freezing with liquid nitrogen, freeze drying, and addition of alcohol or acid treatments (Ryan and Robards, 2006). After quenching, homogenization is the next step that is the most vulnerable in terms of contamination. Homogenization is usually made with mortar and pestle, although a freezer mill under liquid nitrogen atmosphere will be better. The last step in sampling preparation is sample storage normally at -80 °C (Figure A.2).

The extraction procedure supposed to break cell structures liberating all or the maximum number of metabolites in their original state. To make an efficient extraction one must know some aspects of the tissue, such as the composition of cell walls structures, chemical nature of the metabolites and the possible losses due to processing (Villas-Bôas et al., 2007). To disturb cell wall, there are two methods: mechanical and non-mechanical. Mechanical methods are divided into: those use liquids such as ultrasonics, microwave extraction or supercritical fluid extraction; and those involve solids such as manual grinding or ball milling. Non mechanical methods are divided in enzymatic; chemical (organic solvents) and physical (osmotic shock, heat) (Villas-Bôas et al., 2007). In plant metabolomics the most common combination is mortar grinding and organic solvents, sometimes with an additional enzymatic step (Gratacós-Cubarsí et al., 2010; Song et al., 2005).

In chemical extraction there are several considerations; the solvent characteristics, ratio solvent and sample, duration of the extraction and temperature (Kim and Verpoorte, 2010). Appropriate solvent depends also on analytical approach. In the case of GC/MS the range of possible solvents is limited to volatile compounds and for polar mixtures derivatization is a must. In the case of LC/MS there can be limitations, although compatibility with mobile phase is desired. On the other hand, in NMR there is less limitation regarding solvents and a mid-polar solvent (aqueous methanol) is often used (Kim and Verpoorte, 2010). When the extract is ready the next step is preparation for analytical method. This is an easy step regarding NMR or LC, but in the case of GC, if the compound is polar, derivatization must be done to render them more volatile. The most frequently derivatized functional groups are hydroxy, carboxy, and amino groups. In the case of hydroxy compounds the most used derivatization is the transformation to silyl; with amines sylitation is done also with acylation and for carboxylic acid the most common method is the formation of methyl esters (Görög, 2005). Because chromatographic methods are time consuming a direct injection mass spectroscopy (DIMS) is usually done in metabolomics to save time (Kim and Verpoorte, 2010).

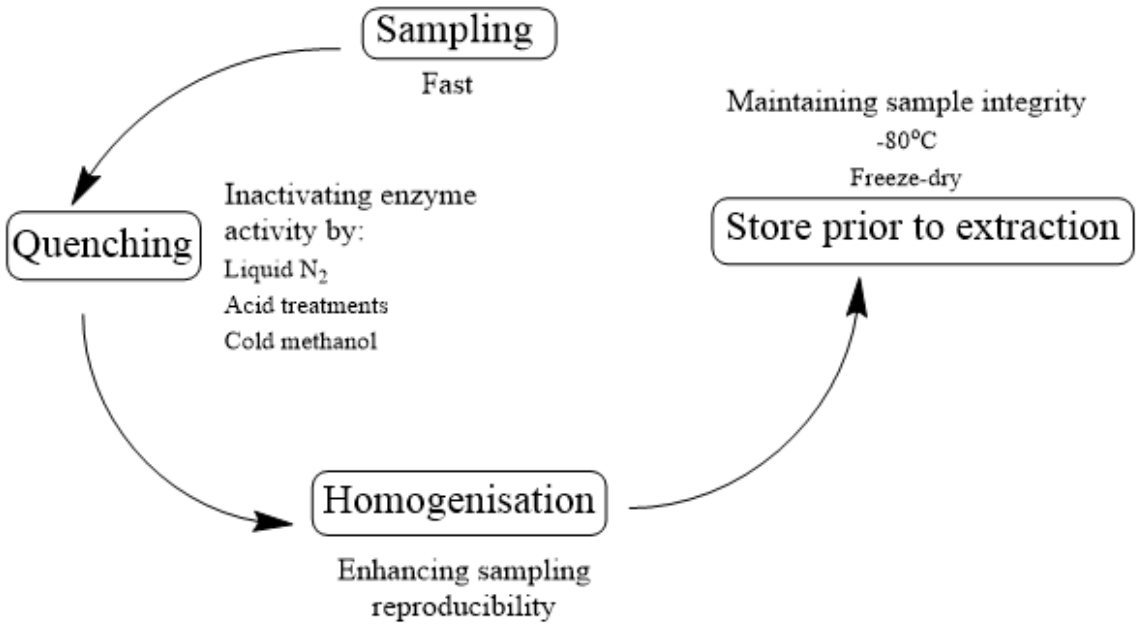


Figure A.2 Mainly steps for sample preparation. From (Villas-Bôas et al., 2007)

Analysis of metabolites

A variety of spectroscopic methods are possible for metabolite analysis. This review will not cover all those theoretical aspects behind spectroscopy, but a brief introduction is presented, for more details reader may refer to good reviews on the subject (Bainbridge, 2005; Harvey, 2005; Welham, 2005; Wright, 2005). Both mass spectroscopy (MS) and nuclear magnetic resonance (NMR) are fundamental for metabolomics and they provide complementary information. Mass spectrometry works on the principle of ionization, in a very general outlook this is caused by an energy source which impacts a molecule, breaking it and generating ions. The fragmentation pattern of a molecule provides structural information such as the mass to charge ratio (m/z) allowing its identification. A simple mass spectrometer consists in an ion source, a m/z analyzer and a detector. The most common ways to generate ions are: Electron ionization (EI); Chemical ionization (CI); Electrospray (ES); Atmospheric pressure chemical ionization (APCI); Atmospheric pressure photoionization (APPI); Desorption electrospray ionization (DESI); Liquid secondary ion mass spectrometry (LSIMS); Fast atom bombardment (FAB); Matrix-assisted laser desorption/ionization (MALDI); Desorption ionization in silicon (DIOS) (Griffiths, 2007). Mass analyzers work by separating ions according to their m/z, most common analyzers are Linear quadruple filters; Quadruple ion trap; Time-of-Flight (TOF); Fourier Transform Ion Cyclotron Resonance (FTICR) and Linear ion trap – Orbitrap mass spectrometer (Griffiths, 2007).

Nuclear magnetic resonance (NMR)

NMR has been extensively used for metabolite fingerprinting profiling and metabolic flux analysis. NMR is well-suited to metabolomics because it can 'quantify' and detect a wide range of organic compounds at the same time in the micro-molar range and sample is not destroyed. Sample preparation for NMR is straight-forward and largely automated, however its major limitation is its relatively low sensitivity, making it inappropriate for the analysis of large number of low abundance metabolites (Shulaev, 2006a). A NMR spectrum is created by radiating the sample with a short pulse of high-energy radio frequencies (100–1000 MHz) that excite all nuclei; and the energy emitted back is measured as free induction decay (FID) signal. By a Fourier transformation of this signal, the decay can be converted to a pattern of frequencies emitted representing different energy emissions from different nuclei when they return to their low-energy states. Usually, the scale is calibrated to the frequency of reference compounds and frequencies are converted to parts per million (ppm) concentration (Villas-Bôas et al., 2007). Due to the abundance of hydrogen in organic compounds, analysis with 1H-NMR generally contains more signals, and it is generally used as a reference method in plant metabolomics (Schripsema, 2010).

Nonetheless, plant metabolomics it is not restricted to 1H, and isotopes 2H, 13C and 15N, have been used regardless of their very low natural abundance and difficult detection. Stable isotope labeling leads to the enhancement of certain signals and is a powerful method to follow metabolic pathways (Ward et al., 2007). NMR has been used also with LC and MS, in a hyphenated method, LC-NMR-MS, or in a new variant with a solid phase extraction (SPE), LC-SPE-NMR. This relative new variant was used to identify some flavonoids and phenolic acids on Greek oregano, where the acquisition times were short (Exarchou et al., 2003). Two dimensional NMR is another alternative for one of the main problems related with 1D, 1H NMR, and peak congestion. This is particularly frequent in 1D NMR analyzing small metabolites, whereas 2D NMR can easily resolved. However, 2D is time-consuming that a 15 min analysis on 1D could go up to 16 hours on 2D (Griffiths, 2007). Thus,

technology needs to be further developed in order to have more powerful magnets and increase the sensitivity which is the main limitation of NMR.

Direct analysis mass spectrometry

Although NMR has shown its potential in plant metabolomics, mainly because only one internal stardard is needed, its insensitivity has led mass spectroscopy in a better position in terms of popularity and usefulness in most of plant metabolomics studies. Mass spectroscopy is generally coupled with GC or LC, both are the more used hyphenated techniques. As separation of components has to be done before analysis by MS in some large-scale studies which are time-consuming, direct analysis by mass spectrometry is an alternative method to NMR when high-throughput screening is mandatory and high sensitivity is required. Thus there are some established methods and other that are emerging for the direct analysis of complex mixtures without previous separation by GC or LC.

Direct infusion mass spectrometry (DIMS)

DIMS has been used in clinical studies for some time, is a fast and sensitive technique with an average of 5 minutes per sample. Since it is not coupled to chromatographic separation, it is susceptible to ionization suppression (Antignac et al., 2005). It is also known that DIMS by itself is not able to differentiate isomers and because of this tandem or FT-ICR have been used to differentiate isomeric compounds (Bedair and Sumner, 2008). The most powerful tool for DIMS is FT-ICR, due to its ultra-high mass resolution and mass accuracy that has been used mainly in metabolic fingerprinting studies (Bedair and Sumner, 2008). An example of the later was made in plant extracts in combination with 13C isotope label in a FT-ICR spectrometer, in order to identify compounds in *Arabidopsis thaliana* (Giavalisco et al., 2008). The employment of multivariate statistical analysis is common in DIMS to discriminate ions.

Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS)

MALDI-MS is an important tool in proteomics as well in as lipodomics and it is often used in imaging. One of the best combination for MALDI is time of flight (TOF) analyzer and its major advantages are high-throughput capacity and higher tolerance for salts compared with electrospray ionization (ESI) (Bedair and Sumner, 2008). In MALDI, the sample is mixed with a matrix, often α-cyano-4-hydroxycinnamic acid or 3,5-dimethoxy-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid (Griffiths, 2007). This matrix is then mixed in aqueous acetonitrile with 0.1 % of trifluoacetic acid on a stainless plate allowing co-crystallization. The MALDI plate is placed under high vacuum system of a mass spectrometer, and irradiated with laser light. The matrix absorbs the light energy resulting in ionization of the sample and the matrix (Griffiths, 2007). The utility of this technique was assessed for plant metabolomics studies with some plant organs, including flowers, leaves, fruits, bulbs, tuber and seeds. In atmospheric pressure infrared MALDI mass spectrometry (AP IR-MALDI) more than 50 metabolites, including lipids, amino acids oligosaccharides and flavonoids compounds were detected (Li et al., 2008b).

Desorption electrospray ionization (DESI)

DESI is a soft-ionization technique in which an electrospray emitter is used to generate micro droplets that are directed to the sample. Then the molecules are desorbed, ionized, desolvated and directed to the MS inlet (Bedair and Sumner, 2008). Its application in metabolomics is new, and is promising, since desorption ionization takes place under 'soft' conditions even without sample preparation. The method has high sensitivity, is very fast and is applicable to small-molecule organic compounds; DESI method is applicable to the analysis of proteins, oligonucleotides, as well as organic molecules (Takáts et al., 2005). Recently DESI-MS was used with success to identify signaling in plant defense process, where secondary metabolites were identify by imaging (Lane et al., 2009). On the other hand, a study involving plant alkaloids showed similar mass spectra, number of alkaloids, and signal intensities to those obtained when extraction and separation processes were performed prior to mass spectrometric analysis (Talaty et al., 2005). This study shows the potential of DESI, because it was done in-vivo, thus can be a promising technique in plant metabolomics.

Chromatography coupled to mass spectrometry

Direct MS analysis is a good alternative for relatively high concentrated samples and even if it is powerful, depth-coverage is relative low. Besides direct MS cannot discriminate between isobaric molecules such as isomers and enantiomers. For this reason chromatographic methods are needed, first to increase the coverage, and second, to significantly reduce suppression/competitive ionization (the major limitation of direct mass analysis) (Bedair and Sumner, 2008).

Gas chromatography – mass spectrometry (GC-MS)

At present the most mature technology for metabolite profiling is gas chromatography coupled with electron impact (EI) quadruple or time-of-flight (TOF) MS (GC–MS) (Shulaev, 2006a). The popularity of the couple is basically due to the robustness of both separation and the electron impact spectrometry, and also because of the availability of deconvulation software. GC-MS is principally used for separation and identification of naturally volatile metabolites at temperatures up to 250 °C (alcohols, monoterpenes, and esters). It is also used in non-volatile polar compounds (primary metabolites) such amino acids, sugars and organic acids by chemical derivatization (Hall, 2006a). Metabolic profiling with GC-MS involves 6 steps (Kopka, 2006): 1) extraction of metabolites form biological samples; 2) derivatization of metabolites; 3) separation by GC; 4) Ionization (EI is the most wildly used); 5) detection, with different detector devices, including quadruples detectors, ion trap technology and time of flight detectors. Quad is normally employed for routine analysis; ion trap allows tandem analysis MS-MS and TOF gives fast scanning rates; and acquisition and evaluation of data files.

The principal challenge for GC-MS is automatic deconvulation for all the peaks that shape a GC-MS chromatogram. There are several examples of studies regarding this technique in different plants, vegetables and fruits. GC-MS has been used for determination of primary metabolites in *Arabidopsis thaliana* (Tam and Normanly, 1998); in profiling of volatile compounds (Rohloff and Bones, 2005) and to measure jasmonic acid under heavy metal stress (Maksymiec et al., 2005). It is wildly utilized for detecting pesticide residues in vegetables (Garrido Frenich et al., 2003; Martínez Vidal et al., 2002). It has been used to identify volatiles in naranjilla fruit (Brunke et al., 1989); and from macauba fruit (Fortes and Baugh, 2004). However a majority of the secondary metabolites can be resolved by other techniques such as LC-MS.

Liquid chromatography – mass spectrometry (LC-MS)

LC-MS is a more versatile technique compared with GC-MS regarding secondary metabolites identification, because chemical derivatization is not needed. Advances in chromatographic technologies like ultraperformance liquid chromatography (UPLC) and constant advances in columns (Table A.1) are improving separation efficiency. In addition, various ionization methods are available according the chemistry of metabolites. Among them, electrostray ionization (ESI); atmospheric pressure chemical ionization (APCI); and photo ionization (PI) are the most popular (Hall, 2006a). LC-MS couple is the 'universal' separation method and HPLC is considered the universal separation technique. HPLC can perform in various scales using different columns sizes (Sumner, 2006) (Table A.1). However, the generally poorer retention time reproducibility of LC compared with GC, limits the establishment of a single optimized analytical procedure. Making difficult the comparison of LC-MS chromatograms between laboratories, and that analyses of mass signal datasets are left to manual searches (Moco et al., 2006). Unlike GC-MS, few mass spectral libraries are available for LC-MS and this is a topic to take in account for a better identification.

Capillary electrophoresis – mass spectrometry (CE-MS)

CE is an analytical separation technique capable of high-resolution separation of a diverse range of chemical compounds and is capable of separating polar and charged compounds. The amounts of sample and reagents are smaller as capillaries instead of columns are used (Ramautar et al., 2006). However, its major limitation is precisely due to the small amounts of samples, which affect the sensitivity. Capillary zone electrophoresis (CZE) is the most popular technique used for CE-MS analysis of metabolites, mainly due to its simplicity (Bedair and Sumner, 2008). The mode of separation involved is that charged molecules are separated based on the differential electrophoretic mobility and neutral molecules by electro-osmotic flow. CE will have a space in plant metabolomics if its sensitivity could be improved, perhaps with some optimized protocol to concentrate the samples.

Table A.1 General liquid chromatographic scales

Scale	Column internal diameter	Flow rate
Preparative	2.1->200 mm	10mL/min
Analytical (conventional)	2.1-4.6 mm	1.0 mL/min
Micro	1.0 mm	200 µL/min
Capillary	300 µm -1 mm	4µL/min
Nano	25 – 300 µm	200 nL/min

Multidimensional Approaches

A multidimensional system consists of coupling two or more chromatographic system in order to increase separation power or peak capacity of the system, leading to greater resolution (Giddings, 1987). Among the advantages using multidimensional separation, the principal is the minimum sample preparation. Multidimensional separation are represented with the multiplex symbol (e.g., $GC \times GC$) which is different from hyphenated techniques using the hyphen symbol (e.g., GC-MS) (Ryan and Robards, 2006). In order to have a clear idea of multidimensional separations, Ryan and Robards stated it this way: a single column of gas chromatograph is one dimension; the same column coupled with a LC system has 2 dimensions. In metabolomics it is very easy to get more than two dimensions, since every organic group need a different separation method. Thus, if one desires to identify amino acids, phenylpropanoids, terpenoids and glucosionolates the sample would have a dimension of four (Ryan and Robards, 2006). Such a system is difficult to assess and it would be even more difficult if an additional dimension is added to the system.

Metabolomics data analysis

Metabolomics is a multidisciplinary science and analytical chemistry is only a part of it (Table A.2). Metabolomic studies produce large amounts of data, which would be useless without appropriate treatment. Thus, metabolomics needs a relative new science of chemometrics. In metabolomics one can track several metabolites at the same time, which differ from only few in targeted metabolites where classical statistical methods are applied. However traditional t-student or one-way ANOVA are not recommended if the objectives are several (hundreds) of metabolites at same time, and thus multivariable analysis is the best option. There are three categories of analysis in chemometrics: exploratory analysis; classification and discriminant analysis; and regression analysis and prediction models. Exploratory analysis is used to find patterns and cluster and detect trends, a classical method is principal component analysis (PCA). In classification and discriminant analysis one can classify samples into categories (e.g., control and treatment) discriminant analysis on principal components or partial least squares scores are some examples. Regression analysis and prediction models are used for quantitative relationships between two blocks of data (e.g., partial least squares regression PLS) (Trygg et al., 2006).

Table A.2 Common analytical techniques used in metabolomics

Analytical Method	Advantage	Disadvantage
NMR	Rapid Analysis	Low sensitivity
	High resolution	Convoluted spectra
	No derivatization needed	More than one peak per component
	Non-destructive	Limited libraries
GC-MS	Sentitive	Slow
	Robust	Derivatization
	Large linear range	Thermally-unstable analytes
	Large commercial libraries	
LC-MS	No derivatization needed	Slow
	Many modes of separation	Limited commercial libraries
	Large sample capacity	
CE-MS	High separation power	Limited commercial libraries
	Small sample requirement	Poor retention time reproducibility
	Rapid analysis	
	No derivatization needed	
	Can analyzed neutrals, anions	
FTIR	and cations	Extremely convoluted spectra
	Rapid analysis	More than one peak/component
	Complete fingerprint of sample	Metabolite identification nearly impossible
	chemical composition	Requires sample drying

Principal component analysis (PCA)

PCA is a mathematical procedure in which many correlated variables are transformed into a small group of uncorrelated variables called principal components. The first two components are used to define a plane into a dimensional space , and if one projects all the sample points in the plane inside the dimensional space it is possible to visualize all the samples (Trygg et al., 2006). The coordinates of these samples onto this plane are called scores T, and they are weighted averages of all X-variables (e. g. metabolites). The score plot is very informative because it gives an overview of all samples in X, and how they relate to each other. This may reveal groups of samples (clusters) and trends. When patterns are found it is possible to understand the reason of relationship. This is because there is another plot for measured variables (e.g., metabolites) known as the loading plot which measures the influence of variables in the model. PCA is a linear additive model in which each principal component (PC) is part of the total variance of the data, however only two PC can account for over 90 % of total variance (Sumner et al., 2003). The first principal axis is the direction in which the data is primarily distributed (Figure A.3). In biological sciences, the principal component is often the most abundant or the most important (Fukusaki and Kobayashi, 2005)

Partial Least Squares (PLS)

PLS is used instead of PCA when more information of samples is on hand. For example, when the genotype of certain plant is known (wild vs. mutant) where an additional y matrix is available. PLS is normally used when correlation between a descriptor matrix x and a response matrix y is needed. In this case, matrix y can be quantitative (concentration) or qualitative (genotype). PLS differs from PCA because it only presents the variation according y matrix and not the variation among the whole system. The y matrix consists of the same number of rows as the x matrix and each column in y indicate a certain property (e. g., concentration or genotype) for each sample. When y is qualitative, the PLS method is called PLS Discriminant Analysis (PLS-DA), to distinguish it from the situation when y is quantitative (Trygg et al., 2006).

Bioinformatics for metabolomics

Like the other omics technologies there is a huge amount of information to deal with, and it is impossible in terms of time to handle manually. In this sense bioinformatics is a very appreciable tool, as it has been reviewed in the previous section, mostly with multivariate analysis. On the other hand, metabolomics is related with all kind of databases, from GC-MS libraries through metabolic pathways. Another bioinformatic tool is software visualization on molecular interaction networks. Now with bioinformatics, analytical chemistry and chemometrics, metabolomics is complete.

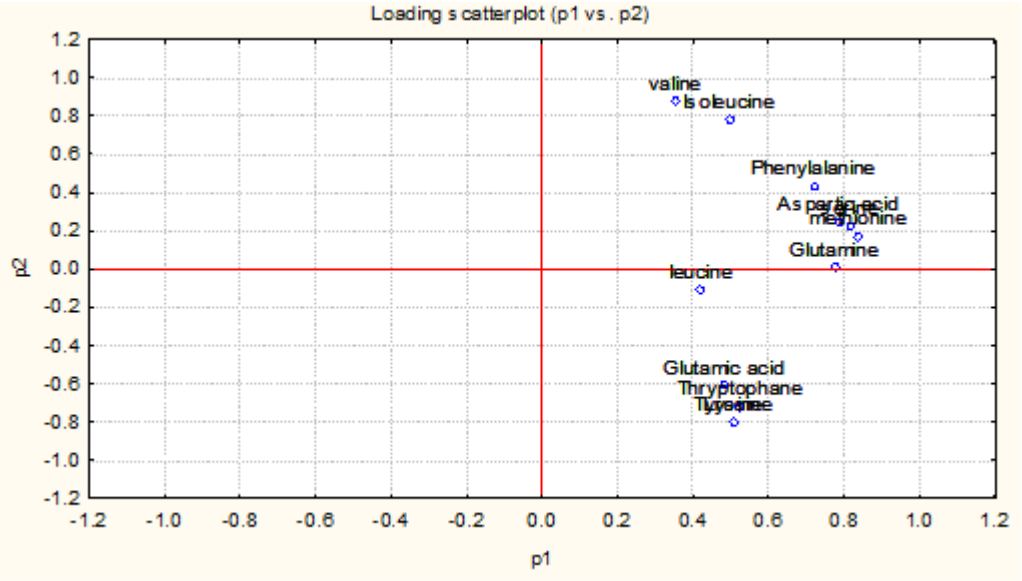


Figure A.3 Principal component analysis (PCA). Preliminary results from UV-C treated broccoli

Databases

Databases are designed for visualization and interpretation of metabolomic data in a context of network reactions in an organism. Databases might have information of enzymes and the genes that code for those enzymes; i.e., the biochemistry of a system (Sumner et al., 2003). Such data bases already exist, perhaps the most well-known is the Kyioto Encyclopedia of Genes and Genomes KEGG (Kanehisa et al., 2010) database. KEGG database is a collection of 16 main databases; of which the most important for metabolomics is the KEGG pathway database, although KEGG genome and KEGG gene are also appreciated. KEGG is based on information from GenBank/EMBL/DDJB, and information about reactions and enzymes from the Enzyme Nomenclature of the IUBMB, accessible from the ENZYME database (Chang et al., 2009) this has been reviewed by Sumner et al. (2003). One of the problems with KEGG and databases based on GenBank is that much of the sequences reported therein, were inferred by sequence similarity and may not have any experimental support. Therefore, many researchers prefer to create their own databases resulting in the lack of appropriate public databases for exchanging information. Nonetheless, KEGG is a valuable reference tool for metabolomics studies; although it is not specific for plant metabolomics.

PlantCyc is a database from Plant Metabolic Network Center containing pathways and their catalytic enzymes and genes, as well as compounds from many plant species (Karp et al., 2010). Information in this database is supported by experimental evidence and only a few by computational prediction. Plant Metabolic Center offers data bases for plant models, like Arabidopsis thaliana (AraCyc) and Populus trichocarpa (PoplarCyc). Some other plant/vegetable models also exist: CapCyc (pepper); CoffeaCyc (coffee); LycoCyc (Tomato); MedicCyc. MetaCyc is another valuable database for all organisms; its most interesting characteristic is that only experimental supported data is included for pathways and enzymes. ExPASy offers a huge digitalized map of a number metabolic pathways by Roche Applied Science. Golm metabolome database (Kopka et al., 2005) and PubChem are both compound databases. Recently Plant Metabolomics Organization. made a very interesting web portal for exploring, visualizing, and downloading plant metabolomics data (Bais et al., 2010). The reader may visit the sites listed in (Table A.3) for further information.

Metabolic networks

The amount of transcriptomics, proteomics and metabolomics data is huge and represents a challenge for those who want to make sense of them. In this context, efforts must be made towards developing new tools to confront this very real problem. This problem could be very easily resumed as the need for methods that automatically display functional genomics results on metabolic charts or maps. One could manually mapped transcriptional changes to metabolic maps. However, this is a time-consuming process, and thus visualization software has come to deal with this, in automated manner (Cavalieri and De Filippo, 2005). Among the different visualization software, three applications are of great importance: GenMapp (Salomonis et al., 2007); Cytoscape (Figure A.4) (Cline et al., 2007) and DAVID (Database for Annotation, Visualization, and Integrated Discovery) (Dennis et al., 2003).

GenMapp is an application for visualization of gene expression and other genomics data in charts representing biological pathways and grouping genes. Its features include draw pathways easily, underline genes of imported data and query data against gene ontology. Cytoscate is an open source application that allows user to visualize, analyze and model molecular and genetic interaction networks.

Table A.3 Databases for metabolomics

Database name	Web site
KEGG	http://www.genome.jp/kegg/
PMNc	http://plantcyc.org/
PlantCyc	http://www.plantcyc.org:1555/PLANT/server.html?
AraCyc	http://www.plantcyc.org:1555/ARA/server.html?
PoplarCyc	http://www.plantcyc.org:1555/POPLAR/server.html?
Others Cycs	http://solgenomics.net/index.pl
MetaCyc	http://metacyc.org/
ExPASy pathway	http://www.expasy.ch/cgi-bin/search-biochem-index
PubChem	http://pubchem.ncbi.nlm.nih.gov/
Golm metabolome	http://gmd.mpimp-golm.mpg.de/
Plant metabolomics	http://tht.vrac.iastate.edu:81/
UCDavis Genome	http://www.genomecenter.ucdavis.edu/
Fiehn Laboratory	http://fiehnlab.ucdavis.edu/
Other resources	http://www.bmrb.wisc.edu/metabolomics/external_metab_links.html

It works with several plug-ins for additional file format support, scripting, and connection with databases. Nevertheless one of its restrictions is precisely the visualization, because it is not necessarily intuitive (Cavalieri and De Filippo, 2005). Finally, DAVID provides several functional annotation tools such as discover enriched functional-related gene groups; visualize genes on BioCarta (http://www.biocarta.com/Default.aspx) and KEGG pathway maps and list of interacting proteins A common denominator of visualization software according to Cavalieri and De Filippo (2005) is that they do not show the statistical significance of the change in a pathway making difficult the selection of possible biomarkers.

Metabolomic studies for stresses in plants

Metabolomics has been a valuable tool for fundamental science, mostly to differentiate genotypes based on metabolite levels that may affect phenotypes. Metabolic approaches are used to explain the biochemical causes or consequences of those phenotypes (Sumner et al., 2003). Selective metabolic profiling has been used for fingerprinting of species, genotypes or ecotypes for gene discovery and metabolome quantification when chemical or physical stimuli are applied and for comparison of metabolites from mutant and wild type plants. However, more practical applications are already emerging, for example, such as crop quality characteristics (Hall et al., 2005). Metabolomics could be used in the identification of metabolites implied in defense-response; transduction or acclimatization of vegetal tissues. The same metabolites could be correlated then with transcriptomic and proteomic studies to identify new genes and metabolic pathways. However most of the studies related to stresses generally employ only or perhaps a combination of the two approaches and the combination of the three is less frequent. The problem with studies employing transcriptomics, proteomics and metabolomics is the data integration (Mehrotra and Mendes, 2006). Some of the metabolomics studies conducted on the stress physiology of plants have been with temperature; water and salt stress; deficiency in minerals; oxidative stress; heavy metal stress; and combination of stresses (Shulaev et al., 2008a).

Temperature stress; may involve low-temperature signaling and heat shock metabolism. In low temperature signaling, the interest has been finding signal transduction pathways that confer chilling or freezing resistance to plants. On the other hand, heat shock metabolism has identified very conservative amino acid and oligosaccharides related to heat (Guy et al., 2008). A work showed a combination of proteomics and metabolomics with the purpose of understand the acclimatization mechanisms of a C4 plant challenged by salt and water stress (Sobhanian et al., 2010). Another interesting work attempted at finding the differences through transcriptomics and metabolite analysis of a well-adapted salt conditions *Thellungiella halophila* and *Arabidopsis thaliana* was made. A transcriptomic profiling (microarray) and a metabolic GC-MS profiling revealed a stress-anticipatory preparedness in Thellungiella (Gong et al., 2005). Oxidative stress is least explored, and only few reports are available using metabolomics (Shulaev et al., 2008a). Oxidative stress in heterotrophic Arabidopsis cells had profound effects on central metabolic pathways analyzed by microarray and GC-MS. It was found that overall response was very similar of oxidative stress responses in bacteria (Baxter et al., 2007). Finally, it should be underlined that plants are exposed to more than one stress in nature, and recent studies have suggested that the response to a combination of stresses is unique and not be extrapolated from the responses of individual stresses (Mittler, 2006).

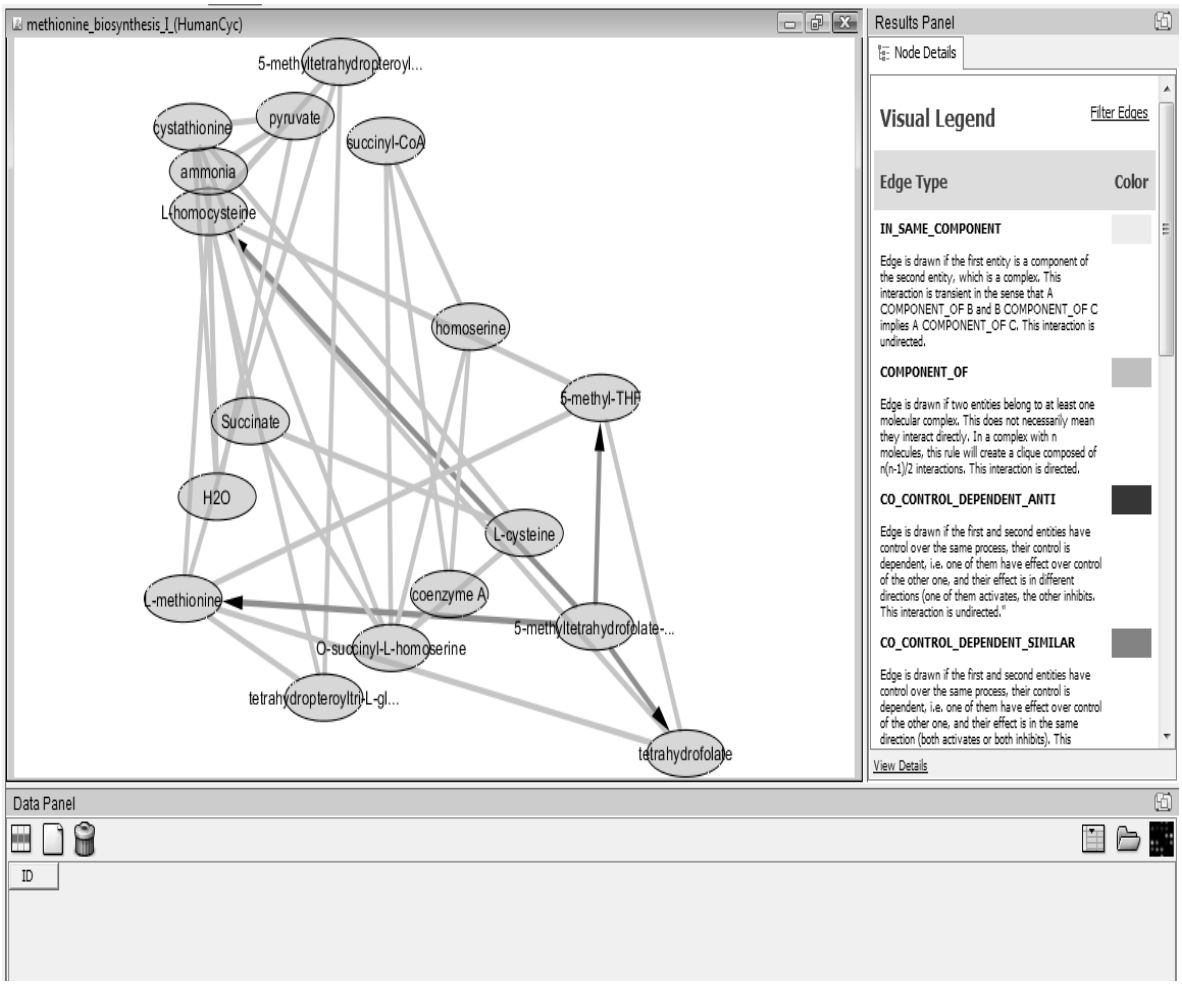


Figure A.4 Methionine biosynthesis in Human: Metabolic network made with Cytoscape software

Analysis of secondary metabolites of brassica

Liang et al. (2006a) showed that it is possible to detect differences in metabolite profiles between control and MJ treated Brassica by multivariate analysis of J-resolved 2D NMR spectra. Using this method, they were able to analyze the metabolite changes produced by MJ treatment involving a wide range of compounds such as glucosinolates, hydroxycinnamates, sugars and amino acids. In the case of flavonoids, Brassica leaves have been reported to accumulate flavonols (quercetin, kaempferol, and isorhamnetin) and flavones (apigenin and luteolin) (Onyilagha et al., 2003). Isorhamnetin, kaempferol, and quercetin glycosides were also identified in turnip tops of Brassica rapa (Romani et al., 2006). Another important group of compounds in *Brassicas* are phenylpropanoids, which show a different metabolic profile depending on the species and tissues studied. In a recent study, a new phenylpropanoid, malate conjugated 5-hydroxyferulic acid, was identified in Brassica rapa leaves (Liang et al., 2006b). One of the major Brassica metabolite groups, the glucosinolates, shows a large variation among species, up to the point that there are many species specific glucosinolates (Fahey et al., 2001). For example, it has been reported that the glucosinolate content in Brassica is dependent on several factors such as cultivar, age, seasonal, and environmental factors (temperature, soil type, and fertilizer application) (Abdel-Farid et al., 2007). Thus, a metabolomic study can be effective only if changes in the target group are superior compare with those biological variations of controls.

Glucosinolates

Successful analysis of glucosinolates depends of an appropriate collection and preparation of fresh samples and avoidance of glucosinolate hydrolysis by the endogenous myrosinase-catalyzed reaction is a critical step (Wathelet et al., 2004). Samples must be grind in liquid nitrogen and stored at a minimum of -20 °C to avoid enzymatic activity (Rosa, 1997). Extraction of glucosinolates is done with protic solvents such as water, ethanol or methanol. Ethanol-water (1:1) or methanol water (7:3) are recommended for freeze-dried tissue. The use of ultrasound and high temperatures enhance penetration of extract solvent into the tissue and accelerates mass transfer and diffusivity (Wang and Weller, 2006).

Analysis of glucosinolates can be done basically by three approaches: degradative totals colorimetric techniques, nondestructive totals or individual components by chromatographic techniques (Clarke, 2010). Colorimetric methods for total glucosinolate content are based on glucosinolate hydrolysis by myrosinase and free glucose quantification (Smith and Dacombe, 1987). Assays are also designed to detect UV absorbance of some complexes such as thiourea (Wetter and Youngs, 1976) or ferricyanide (Jezek et al., 1999). Since glucosinolates are ionic molecules, direct analysis in GC is not possible and required a pre column derivatization for their conversion into volatile desulphoglucosinolates derivatives. Thus, high performance liquid-phase separation such as HPLC and capillary electrophoresis are more used for the analysis of these molecules (Moreno et al., 2006).

Absorption spectroscopy UV/Visible and mass spectrometry (MS) are useful methods for the detection of glucosinolates. However, the most common method for glucosinolate analysis is LC-MS and octadecyl (C18) reverse phase. One of the most important features of this method is the simultaneous analysis of intact and desulfated glucosinolates. Most conventional ionization techniques and detector configurations are: fast atom bombardment (FAB), matrix assisted laser desoption ionization time of flight (MALDI-TOF), atmospheric pressure chemical ionization (APCI) and electrostray ionization (ESI) (Clarke, 2010). Depending on the compounds, ESI can be

performed in negative or positive mode. Since glucosinolates from Brassicas have a sulfate-glucose structure, they ionize extremely well in negative mode on electrospray ionization. Also, some molecules such as phenol, flavonoids and other glycosylated compounds can be detected in ESI negative mode (Moco et al., 2007).

Phenolic compounds

Most of the extraction methods for phenolic compounds are based on manual protocols that include techniques such as liquid-liquid extraction, solid-liquid extraction or soxhlet extraction which are labor intensive. To overcome this, new extraction processes that provide automation and lower solvent composition have been developed (e.g. countercurrent chromatography) (Herrero et al., 2012). Quantification has to be highly sensitive and selective for identification of total or individual polyphenols. Spectroscopy is the main technique used for the quantification of total phenols, flavonoids or anthocyanins, because of its advantage of being, fast and not very expensive. However, the main inconvenient is that it only gives an idea of the average composition of certain tissue and it does not quantify individual compounds. Quantification of total phenols is generally done by the Folin-Ciocalteu colorimetric assay, while total flavonoids can be quantified also by colorimetry using a method based on the complexation of these compounds with Aluminum (III) (Ignat et al., 2011). Even though, spectroscopy gives a good idea of the polyphenol content, HPLC is preferred because it can not only quantify but also identify different compounds in a specific matrix. Reverse phase (RV) HPLC is now the most useful technique among with diode array detector (DAD) and mass spectrometry for the analysis of polyphenols (Sakakibara et al., 2002).

Simultaneous analysis of glucosinolates and phenolic compounds

Identification and quantification of glucosinolates and phenolic compounds is laborious and time-consuming. Analysis of these compounds used to be carried out separately due to differences in their physico-chemical properties of these two compounds. Early attempts to assay glucosinolates and phenolic compounds simultaneously was done in *Moringa oleifera* L, using ion-pair chromatography with UV-vis and electrospray ionization (ESI) mass spectrometer (Bennett et al., 2003). LC-UV photodiode array detection – electrospray ionization (ESI) has also been used for the identification and quantification of glucosinolates and phenolic compounds in *Brassica rapa*. The main glucosinolate identified was gluconapin and the main flavonoids were kaempferol, quercetin and isohamnetin (Francisco et al., 2009).

Conclusions

- Plant metabolomics has growth in studies, technologies and applications.
- New strategies are necessary for compound identification in analytical chemistry.
- Bioinformatic tools are very important for identification, analysis and visualization, but databases must to be update to today's needs.
- Fundamental science is the niche for plant metabolomics in most of the studies done until now, but more practical application will find a space.
- Plant metabolomics is constrained to some model organisms, in the future, with functional genomics, studies will be done in more important commercial crops.

Annex II: Additional stresses

White light

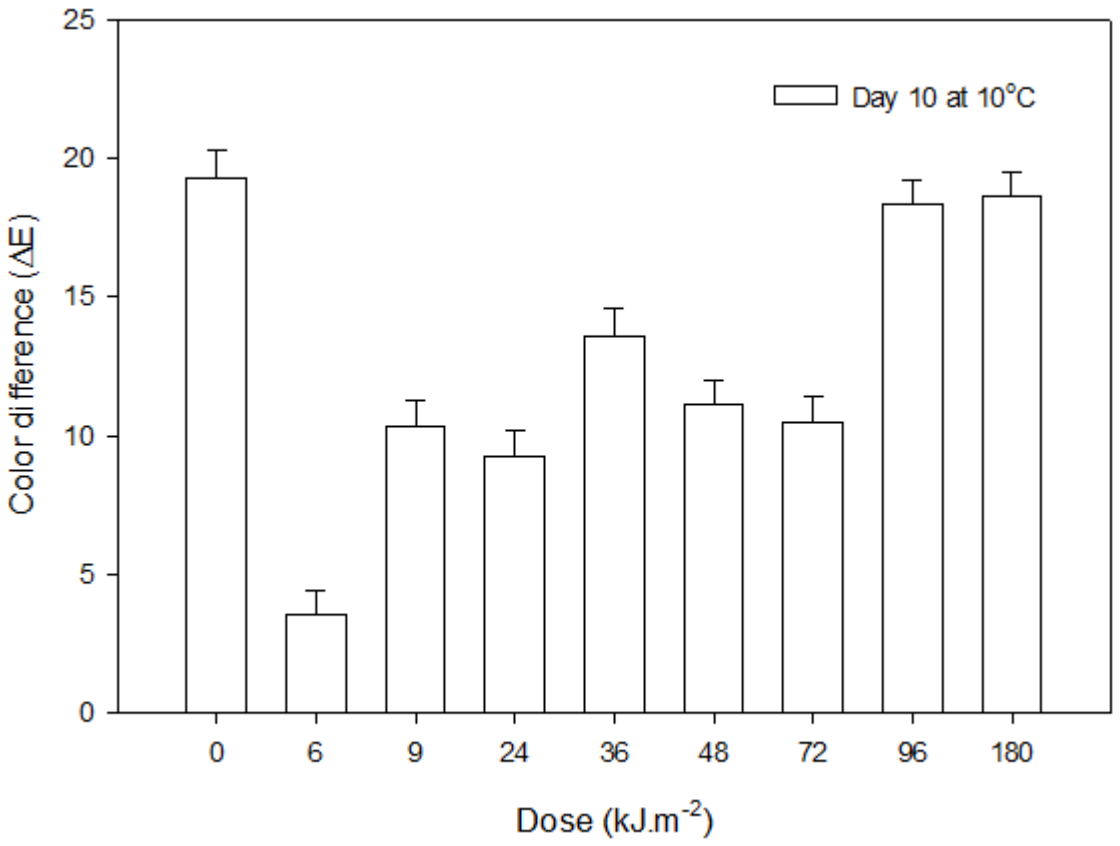


Figure A.5 Total color difference on broccoli florets exposed to white light and stored at 10 °C for 10 days.

Ethylene

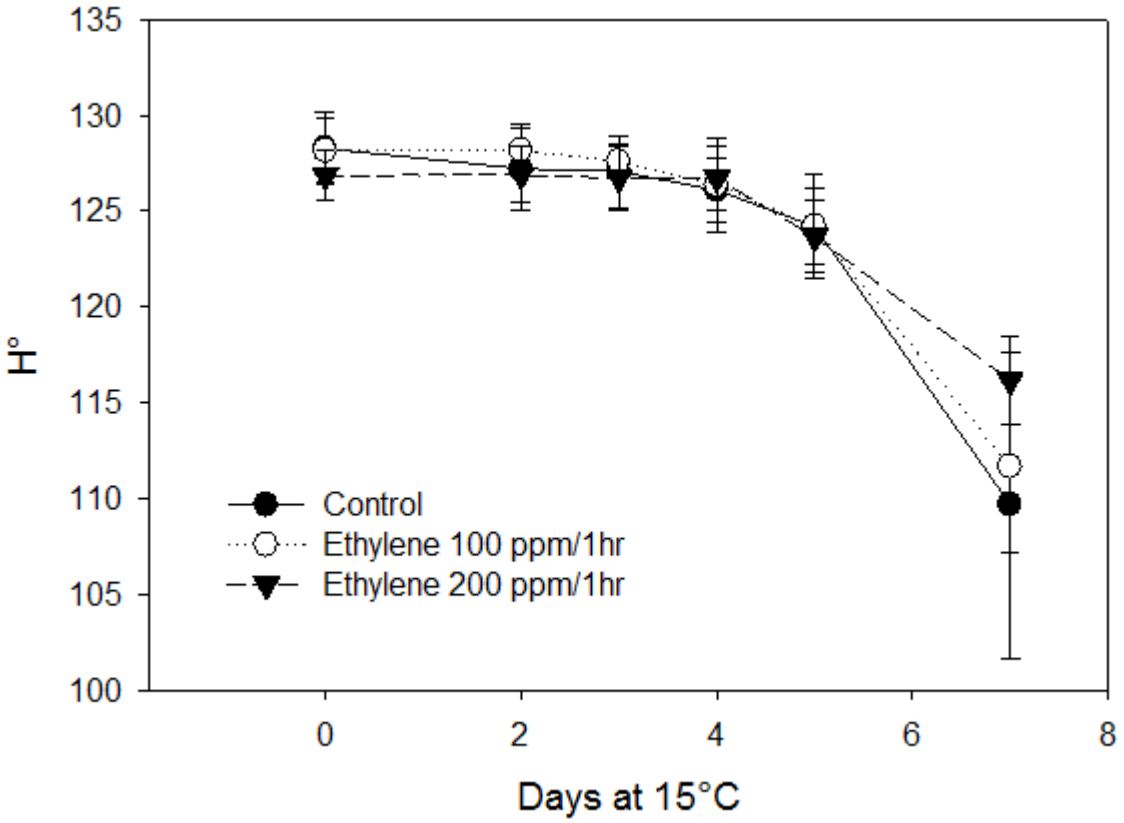


Figure A.6 Broccoli florets exposed to ethylene 100 and 200 ppm for 1 hour stored at 15 °C for 5 days.

Annex III: Sequences

Actin

LOCUS AF044573 1500 bp mRNA linear PLN 05-JAN-1999
DEFINITION Brassica oleracea actin (Act1) mRNA, complete cds.

>gi|4105261|gb|AF044573.1| Brassica oleracea actin (Act1) mRNA, complete cds
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Chlorophyllase 1

LOCUS AF337544 1115 bp mRNA linear PLN 07-DEC-2007
DEFINITION Brassica oleracea chlorophyllase 1 mRNA, complete cds.

>gi|24210532|gb|AF337544.1| Brassica oleracea chlorophyllase 1 mRNA, complete cds
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P450 CYP79F1

LOCUS GU385846 1623 bp mRNA linear PLN 17-FEB-2010

>gi|288812701|gb|GU385846.1| Brassica oleracea var. alboglabra cytochrome P450 CYP79F1 (CYP79F1) mRNA, complete cds

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P450 CYP79B3

LOCUS FJ376047 1783 bp mRNA linear HTC 03-AUG-2009

>gi|237682411|gb|FJ376047.1| Brassica rapa subsp. pekinensis cytochrome P450 79b3 (CYP79B3) mRNA, complete cds

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GTGTATAATCTAAAGTTTGGTCAATCATTAACC

P450 CYP79A2

LOCUS EU877074 2123 bp mRNA linear PLN 10-AUG-2008
DEFINITION Brassica rapa subsp. chinensis cytochrome P450 CYP79A2 mRNA,

>gi|195593047|gb|EU877074.1| Brassica rapa subsp. chinensis cytochrome P450 CYP79A2 mRNA, complete cds
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PAL

>gi|302201603|gb|HM623311.1| Brassica oleracea var. botrytis phenylanlanine ammonia-lyase (PAL) mRNA, complete cds

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GAATCTGAGACAAACGGTGAAGAACACAGTTTCTCAAGTGGCGAAGAAAGTGCTAACCACTGGAGTCAAC
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CGTACGTGGATGATCCTTGTAGCGCTACGTACCCGTTGATGCAGAAACTGAGACAAGTTATTGTTGATCA
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CHS

>gi|29423730|gb|AY228486.1| Brassica oleracea chalcone synthase (chs) mRNA, complete cds
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CoA

LOCUS AF207572 756 bp DNA linear PLN 07-MAY-2003
DEFINITION Brassica oleracea 4-coumarate:CoA ligase (4CL-A) gene, partial cds.

>gi|6683990|gb|AF207572.1| Brassica oleracea 4-coumarate:CoA ligase (4CL-A) gene, partial cds
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F3H1

LOCUS EU402420 1536 bp mRNA linear PLN 09-FEB-2008
DEFINITION Brassica rapa subsp. campestris flavonoid 3` hydroxylase 1 protein

>gi|166798282|gb|EU402420.1| Brassica rapa subsp. campestris flavonoid 3` hydroxylase 1 protein (F3`H1) mRNA, complete cds

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Annex IV : Numerical values of figures

Chapter II

Figure 2.3

Dose(kJm^{-2})	ΔE	SD
0.0	12.0	2.7
0.3	11.8	2.8
0.6	8.8	2.6
0.9	6.0	1.1
1.2	5.5	1.5
1.5	6.5	1.2
3.0	8.7	1.5
6.0	13.7	2.1

Figure 2.4

Day	Control	SD	1.2kJm^{-2}	SD
0	0.0	0.0	0.0	0.0
3	1.4	0.7	1.1	0.8
6	2.5	0.6	1.7	0.7
9	3.5	0.6	2.1	0.6
12	4.5	1.6	3.0	1.0
15	5.7	1.1	3.5	1.0
18	6.5	1.6	4.4	1.3
21	8.5	1.9	5.0	1.2
24	10.2	1.5	5.1	1.5
27	12.0	2.7	5.5	1.5

Figure 2.5

Day	0 kJm^{-2}	SD	1.2 kJm^{-2}	SD	3 kJm^{-2}	SD
0	30.4	1.5	71.6	2.4	81.0	0.8
7	13.5	1.5	17.2	0.9	20.1	1.8
14	12.2	2.0	12.3	0.9	14.2	1.5
21	12.4	1.0	12.5	1.1	14.3	1.4

Figure 2.56a

	Leucine		Valine		Isoleucine	
0.0	32.6	1.9	57.4	3.8	46.2	3.7
1.2	28.6	1.0	55.4	2.6	40.2	1.9
3.0	30.3	0.4	61.2	4.2	42.9	0.7

Figure 2.6b

	Serine		Glutamic A		Aspartic A	
0.0	208.2	45.1	136.7	5.7	138.4	1.9
1.2	139.3	11.5	83.4	0.9	88.7	8.2
3.0	130.3	28.2	73.5	11.9	96.6	3.7

Figure 2.6c

Dose (kJm-2)	Methionine	SD	Lysine	SD	Tyrosine	SD	Tryptophan	SD	Phenylalanine	SD	Histidine	SD	Glutamine	SD
0.0	3.4	0.2	17.93	0.1	8.7	0.5	3.4	0.5	21.9	1.6	6.1		12.1	4.6
1.2	1.8	0.1	4.8	0.4	3.5	0.4	1.5	0.2	15.1	0.1	3.1		9.1	6.0
3.0	1.6	0.09	4.0	0.5	3.7	0.9	1.5	0.3	15.2	0.3	1.65	0.6	7.3	1.6

Figure 2.7

Days	0 kJm^{-2}	SD	1.2 kJm^{-2}	SD	3 kJm^{-2}	SD
0	0.0	0.0	0.0	0.0	0.0	0.0
7	2.3	0.2	1.2	0.2	3.7	0.7
14	4.8	1.1	2.4	0.1	7.8	0.1
21	7.0	1.4	3.3	0.4	11.0	2.1

Figure 2.9

	Day0					
	0 kj.m^{-2}		1.2 kj.m^{-2}		3.0 kj.m^{-2}	
Chlorophyllase	1	0	2	0.7	3.5	0.9
CHS	1	0	3.4	1.4	3.7	1.7
CoL	1	0	2.7	0.5	3.3	1.4
CYP79A2	1	0	1.6	0.2	2.6	0.4
CYP79B3	1	0	3.1	0.8	3.7	0.2
CYP79F1	1	0	1.5	0.3	1.3	0.6
F3H1	1	0	2.5	0.3	2.9	1.8
PAL	1	0	2.7	0.5	1.6	0.3

	Day2					
	0 kj.m^{-2}		1.2 kj.m^{-2}		3.0 kj.m^{-2}	
Chlorophyllase	2	0.2	1.5	0.4	1.9	0.3
CHS	2.1	0.2	3.5	1.9	4	1.3
CoL	1.3	0.6	2.7	0.8	3.7	0.7
CYP79A2	0.9	0.3	1.7	1	2	0.7
CYP79B3	1.1	0.3	3.7	1.8	4.4	0.9
CYP79F1	0.9	0.2	1.6	0.7	0.8	0.4
F3H1	2.2	1.9	2.3	1	3.4	0.1
PAL	1.3	0.9	2.2	0.6	1.8	0.7

	Day 4					
	0 kj.m^{-2}		1.2 kj.m^{-2}		3.0 kj.m^{-2}	
Chlorophyllase	2.1	0.5	1.4	0.2	2.1	1.2
CHS	2.5	0.6	3.8	0.5	4.2	0.4
CoL	1.4	0.2	2.5	1.1	2.9	0.4
CYP79A2	1.2	0.2	1.9	0.6	2.3	0.4
CYP79B3	1.3	0.6	4	0.5	5.3	1.3
CYP79F1	0.9	0.3	1.8	0.9	0.9	0.2
F3H1	2.7	0.3	2.3	0.2	3.3	1.5
PAL	1.2	0.1	1.7	0.7	2	0.7

Figure 2.10

Day	Control	SD	1.2kJ.m^{-2}	SD	3.0 kJ.m^{-2}	SD
0	4.6	0.2	5.7	1.1	5.6	0.3
1	5.2	0.5	6.0	0.9	5.6	0.2
4	5.2	0.4	6.3	0.6	6.1	0.4
7	6.0	0.0	6.2	0.1	6.4	0.1
14	5.7	0.2	6.0	0.8	6.0	0.4

Figure 2.11

Day	Control	SD	1.2 kJm^{-2}	SD	3.0 kJm^{-2}	SD
0	21.2	0.4	20.7	0.5	20.3	0.3
1	20.6	0.2	21.1	0.5	20.8	0.2
3	19.7	0.3	21.4	0.3	22.2	0.2
7	19.4	0.4	21.7	0.2	22.6	0.5
14	18.4	0.2	21.1	0.4	22.4	0.6

Figure 2.12

Day	Control	SD	1.2 kJm^{-2}	SD	3.0 kJm^{-2}	SD
0	21.2	0.4	20.7	0.5	20.3	0.3
1	20.6	0.2	21.1	0.5	20.8	0.2
3	19.7	0.3	21.4	0.3	22.2	0.2
7	19.4	0.4	21.7	0.2	22.6	0.5
14	18.4	0.2	21.1	0.4	22.4	0.6

Figure 2.13

Day	Control	SD	1.2 kJm^{-2}	SD	3.0 kJm^{-2}	SD
0	11.6	0.2	12.1	0.2	12.5	0.3
1	12.1	0.1	12.6	0.1	13.0	0.2
4	12.7	0.2	14.1	0.1	14.7	0.2
7	13.2	0.3	14.8	0.2	15.2	0.3
14	13.7	0.2	15.7	0.2	15.6	0.1

Chapter III

Figure 3.1

min	32 °C	SD	37 °C	SD	42 °C	SD	47 °C	SD	52 °C	SD
0	2.8	0.4	2.8	0.6	3.0	0.2	3.4	0.3	2.4	0.3
5									9.7	0.8
10							4.3	0.5	16.5	2.5
20							3.3	2.0	23.4	0.8
30					3.4	0.3	6.5	1.1	37.9	4.1
40			3.2	0.6					53.3	3.7
60					3.2	0.0	11.0	4.7		
80	2.9	0.3								
120	2.4	0.2	2.6	0.5	3.5	0.3	26.0	21.0		
180			4.8	2.7	3.8	0.5				
360	2.2	0.3	4.9	2.7	5.0	1.6				
960	2.6	0.4								
1440	2.5	0.5	12.7	2.9	17.5	27.7				

Figure 3.2

min	37 °C	42 °C	43 °C	44 °C	45 °C	47 °C	52 °C
0	0.02	0.02	0.02	0.02	0.02	0.02	0.02
5							0.02
10						0.03	0.03
20							0.03
30						0.03	0.03
40							0.03
60				0.03	0.03	0.03	
80							
120			0.04			0.03	
180		0.05	0.05		0.04		
360	0.05	0.06	0.05	0.05	0.05		
960							
1440	0.13	0.13	0.13	0.12			

Figure 3.3

Temperature (°C)	Rate	at
52	1.22	178.57
47	0.19	28.57
45	0.07	11.11
44	0.05	7.94
43	0.02	2.85
42	0.01	1.49
40	8.76E-03	
37	6.90E-03	1

Figure 3.4

(Tt-Tref)	aT
15	178.57
10	28.57
8	11.11
7	7.94
6	2.85
5	1.49
0	1

Figure 3.5

$1/T.10^3, °K^{-1}$	Min $^{-1}$
3.08	1.22
3.13	0.19
3.14	0.07
3.15	0.05
3.16	0.02
3.17	0.01
3.22	6.90E-03
3.27	7.00E-05

Figure 3.6

Temperature °C	Ethanol	SD
37	0.0	0.0
39	0.0	0.0
41	0.9	1.2
43	2.9	1.7
45	6.0	1.6
47	15.3	4.5

Figure 3.7

Day at 10 °C	ΔE	SD
10	14.0	2.3
37	13.4	2.6
39	8.6	1.1
41	2.4	1.0
43	2.7	0.7
45	3.3	0.9
47	3.9	1.2

Figure 3.8

Days	37 °C	SD	41 °C	SD	47 °C	SD
0	0.0	0.0	0.0	0.0	0.0	0.0
5	3.0	1.4	1.6	0.8	2.3	1.1
10	14.5	2.5	1.8	0.5	3.1	1.0

Chapter IV

Figure 4.1

Respiration ($mL.kg^{-1} h^{-1}$)

Day	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
0	22.5	2.4	169.5	6.2	284.8	12.8
7	17.5	0.6	10.9	0.1	16.2	0.9
14	15.2	0.4	6.5	0.2	8.7	0.7
21	21.0	1.1	8.3	0.4	9.5	0.5

Weight loss (%)

Day	Control	SD	41°C/180 min	SD	47°C/12 min	SD
0	0.0	0.0	0.0	0.0	0.0	0.0
7	2.1	0.3	2.0	0.3	2.9	0.2
14	4.5	0.4	3.7	1.0	6.0	0.4
21	6.5	0.9	5.4	0.9	8.4	0.2

Figure 4.2

HUE

Day	Control	41 °C/180 min	47 °C/12 min
0	134.8	134.3	132.7
7	132.5	132.6	130.8
14	127.9	131.0	131.1
21	114.7	128.1	130.5

Chlorophyll

Day	Control	41 °C/180 min	47 °C/12 min
0	2.9	2.8	2.8
7	2.8	2.8	2.9
14	2.5	2.7	2.8
21	1.8	3.0	3.1

Figure 4.3

Glucoraphanin

Day	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
0	4.8	0.6	4.8	0.8	4.7	0.8
1	4.8	0.1	5.0	0.1	5.5	0.2
4	4.9	0.5	5.2	0.1	5.9	0.1
7	4.9	0.4	5.3	0.4	5.7	0.1
14	4.6	0.4	4.6	0.0	4.9	0.1

Glucobrassicin

Day	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
0	8.3	0.2	10.0	0.2	10.1	0.5
1	8.4	0.4	9.3	0.2	9.8	0.3
4	8.6	0.5	8.9	0.5	9.7	0.2
7	8.4	0.1	8.9	0.6	9.6	0.2
14	7.2	0.4	8.7	0.3	9.3	0.6

Neoglucobrassicin

Day	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
0	4.1	0.0	4.9	0.3	5.1	0.3
1	4.1	0.3	5.1	0.0	5.2	0.1
4	4.4	0.2	6.0	0.1	6.4	0.4
7	4.4	0.1	6.1	0.4	6.8	0.9
14	4.6	0.1	5.5	0.7	6.3	0.7

4-methoxyGB

Day	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
0	2.0	0.0	2.6	0.2	2.3	0.3
1	2.0	0.1	2.7	0.2	2.6	0.0
4	2.4	0.2	2.8	0.9	3.1	0.1
7	2.6	0.1	2.4	0.1	2.9	0.1
14	2.8	0.1	2.1	0.1	2.8	0.1

4-hydroxyGB

Day	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
0	1.5	0.1	1.8	0.0	2.0	0.0
1	1.4	0.0	2.0	0.2	2.3	0.1
4	1.9	0.1	2.2	0.1	2.4	0.1
7	2.2	0.2	2.1	0.1	2.3	0.1
14	2.1	0.3	1.8	0.3	1.9	0.1

Total Glucobrassicins

Day	Control	41 °C/180 min	47 °C/12 min
0	16.0	19.4	19.5
1	15.9	19.0	19.8
4	17.3	19.9	21.7
7	17.5	19.5	21.7
14	16.7	18.1	20.3

Figure 4.4

1,2-disinapoyl

Day	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
0	2.6	0.1	2.9	0.2	3.1	0.2
1	2.6	0.1	2.9	0.1	3.1	0.1
4	2.6	0.1	2.9	0.2	3.1	0.2
7	2.8	0.1	2.9	0.2	3.3	0.1
14	3.2	0.1	3.2	0.3	3.4	0.2

1, sina, 2-feru

Day	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
0	4.3	0.3	5.2	0.1	5.7	0.3
1	4.4	0.4	5.3	0.2	6.0	0.5
4	4.6	0.2	5.5	0.4	6.2	0.6
7	4.9	0.2	5.6	0.4	6.6	0.6
14	5.6	0.4	6.0	0.5	6.4	0.6

1,2-diferuoyl

Day	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
0	1.1	0.0	1.1	0.1	1.2	0.0
1	1.0	0.1	1.1	0.1	1.3	0.2
4	1.1	0.1	1.2	0.1	1.4	0.0
7	1.1	0.0	1.3	0.1	1.4	0.2
14	1.1	0.0	1.4	0.2	1.4	0.1

1,2,2 trisinalpoyl

Day	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
0	0.9	0.0	1.1	0.1	1.1	0.1
1	0.9	0.0	1.1	0.1	1.1	0.1
4	0.9	0.1	1.1	0.0	1.2	0.1
7	1.0	0.1	1.1	0.1	1.2	0.1
14	1.1	0.0	1.1	0.1	1.2	0.1

1,2-disina, 2-feru

Day	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
0	1.8	0.0	2.0	0.2	2.0	0.1
1	1.7	0.1	2.0	0.1	2.1	0.1
4	1.8	0.1	2.1	0.1	2.2	0.1
7	1.8	0.1	2.1	0.1	2.3	0.1
14	2.2	0.1	2.3	0.1	2.2	0.1

sino-diferuloyl

Day	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
0	0.6	0.0	0.6	0.0	0.7	0.1
1	0.6	0.1	0.7	0.1	0.7	0.1
4	0.6	0.0	0.7	0.0	0.7	0.1
7	0.6	0.0	0.7	0.1	0.8	0.0
14	0.7	0.1	0.7	0.1	0.7	0.0

Total HCA						
Day	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
0	11.0	0.1	12.8	0.1	13.7	0.1
1	11.1	0.1	13.0	0.0	14.1	0.1
4	11.5	0.1	13.4	0.1	14.6	0.1
7	12.1	0.0	13.5	0.1	15.4	0.1
14	13.8	0.1	14.5	0.1	15.2	0.1

Figure 4.5

Gene expression	Control	SD	41 °C/180 min	SD	47 °C/12 min	SD
Chlorophylase	1.0	0.0	1.2	0.2	1.4	0.2
CoL	1.0	0.0	2.3	0.5	3.3	0.2
CHS	1.0	0.0	1.4	0.2	1.7	0.3
CYP79A2	1.0	0.0	1.5	0.2	1.9	0.3
CYP79B3	1.0	0.0	1.9	0.2	3.1	0.2
CYP79F1	1.0	0.0	1.8	0.3	1.9	0.7
F3H1	1.0	0.0	1.4	0.1	1.2	0.2
PAL	1.0	0.0	1.2	0.1	1.2	0.2

Chapter V

Figure 5.1

UV-B			Ozone			Hydrogen Peroxide		
Dose	ΔE	SD	Dose	ΔE	SD	Dose	ΔE	SD
0.0	6.3	1.8	0.0	10.8	2.2	0.0	17.5	4.2
1.5	1.6	0.3	7.5	11.0	3.8	1.3	12.1	4.2
4.3	3.4	1.2	15.0	10.3	4.7	2.5	16.1	3.4
8.5	4.3	1.8	30.0	7.2	2.5	5.0	14.9	3.2
12.5	3.5	1.8	60.0	4.7	2.9			
			120.0	9.3	2.5			
			240.0	10.6	3.2			
			480.0	7.8	2.3			
			720.0	5.6	3.4			

Figure 5.2

UV-B						
Days	control	SD	1.5 kJm^{-2}	SD	7.2 kJm^{-2}	SD
0	0.0	0.0	0.0	0.0	0.0	0.0
7	2.9	0.3	2.9	0.4	2.7	1.1
14	6.2	0.8	4.6	0.9	4.9	0.6
21	14.2	3.3	7.9	0.7	8.9	0.4
Ozone						
Days	control	SD	5ppm/60min	SD	5ppm/720min	SD
0	0.0	0.0	0.0	0.0	0.0	0.0
7	1.6	0.3	0.9	0.1	1.5	0.9
14	4.1	1.4	2.3	0.4	3.3	0.2
21	8.0	1.2	6.1	0.3	8.6	1.9
Hydrogen peroxide						
Days	control	SD	1.25mM/180 min	SD	5.0 mM/180 min	SD
0	0.0	0.0	0.0	0.0	0.0	0.0
7	3.1	0.3	1.4	0.6	3.3	1.0
14	7.2	1.2	5.4	0.5	7.0	0.4
21	15.5	0.4	11.0	0.7	17.5	1.5

Figure 5.3

UV-B						
Days	control	SD	1.5 kJm^{-2}	SD	7.2 kJm^{-2}	SD
0	22.5	1.9	44.8	0.2	103.2	2.7
7	17.8	0.5	15.4	0.5	17.0	0.5
14	14.7	1.2	15.0	0.8	14.7	0.1
21	17.3	2.1	17.9	1.7	17.9	1.1
Ozone						
Days	control	SD	5ppm/60min	SD	5ppm/720min	SD
0	22.3	2.5	172.5	2.7	286.4	4.2
7	21.7	1.2	21.7	1.3	38.1	0.8
14	20.3	0.3	21.2	0.7	24.6	0.7
21	21.8	0.6	24.6	0.7	20.2	2.0
Hydrogen peroxide						
Days	control	SD	1.25mM/180 min	SD	5.0 mM/180 min	SD
0	28.1	2.5	88.9	2.8	91.8	0.2
7	20.5	1.2	19.8	0.9	18.1	1.5
14	20.3	0.3	17.0	1.7	16.2	1.1
21	27.1	0.3	21.3	0.9	21.0	2.4

Figure 5.4

UV-B						
Days	control	SD	1.5 kJm^{-2}	SD	7.2 kJm^{-2}	SD
0	0.0	0.0	0.0	0.0	0.0	0.0
7	2.3	0.7	1.8	0.6	4.1	0.4
14	5.5	0.6	4.0	0.5	7.6	0.7
21	9.2	1.3	7.7	0.2	11.5	1.0
Ozone						
Days	control	SD	5ppm/60min	SD	5ppm/720min	SD
0	0.0	0.0	4.3	0.0	24.0	0.0
7	2.9	0.7	13.8	0.6	25.1	0.5
14	6.7	0.3	22.2	0.8	26.4	0.5
21	10.4	2.2	27.5	1.9	28.2	0.8
Hydrogen peroxide						
Days	control	SD	1.25mM/180 min	SD	5.0 mM/180 min	SD
0	0.0	0.0	0.0	0.0	0.0	0.0
7	3.5	0.1	5.6	0.3	2.8	0.0
14	8.0	0.1	10.2	0.5	6.0	0.4
21	11.6	0.5	14.9	0.2	9.1	0.5

Figure 5.5

UV-B						
Days	control	SD	1.5 kJm^{-2}	SD	7.2 kJm^{-2}	SD
0	17.8	0.7	20.4	0.8	20.7	0.5
7	17.5	0.6	20.1	0.1	21.1	0.8
14	16.7	1.0	19.4	1.1	21.6	1.1
21	17.1	0.7	21.8	1.1	21.4	0.6
Ozone						
Days	control	SD	5ppm/60min	SD	5ppm/720min	SD
0	18.9	0.9	20.6	0.3	17.9	0.2
7	19.0	0.6	21.8	0.6	17.0	0.3
14	19.4	0.5	22.4	0.3	16.2	0.4
21	18.8	0.3	21.2	0.2	15.4	0.2
Hydrogen peroxide						
Days	control	SD	1.25mM/180 min	SD	5.0 mM/180 min	SD
0	19.9	0.4	20.5	0.4	19.9	0.8
7	18.3	0.1	21.7	0.6	20.5	2.3
14	17.0	0.5	21.3	0.3	20.8	0.7
21	16.6	0.6	21.6	0.6	20.6	1.8

Figure 5.6

UV-B						
Days	control	SD	1.5 kJm^{-2}	SD	7.2 kJm^{-2}	SD
0	4.7	0.5	4.5	0.3	4.6	0.6
7	4.7	0.3	4.8	0.7	4.7	0.7
14	4.4	0.7	5.1	0.7	5.4	0.5
21	4.0	0.6	5.4	0.8	5.9	0.3
Ozone						
Days	control	SD	5ppm/60min	SD	5ppm/720min	SD
0	4.9	0.5	6.0	0.5	5.5	0.1
7	4.6	0.2	5.7	0.5	4.5	0.3
14	4.6	0.5	5.3	0.1	4.1	0.1
21	4.6	0.3	5.3	0.6	3.8	0.1
Hydrogen peroxide						
Days	control	SD	1.25mM/180 min	SD	5.0 mM/180 min	SD
0	4.9	0.5	5.0	0.5	4.7	0.6
7	4.6	0.3	5.4	0.5	5.5	0.7
14	4.4	0.2	5.5	0.8	5.4	0.1
21	4.1	0.5	5.2	0.5	5.3	0.3

Figure 5.7

UV-B						
Days	control	SD	1.5 kJm^{-2}	SD	7.2 kJm^{-2}	SD
0	14.3	0.5	14.5	0.7	14.9	0.2
7	14.0	0.1	15.4	0.3	15.6	0.1
14	13.6	0.2	16.7	0.2	16.3	0.2
21	14.9	0.3	17.5	0.2	17.2	0.1
Ozone						
Days	control	SD	5ppm/60min	SD	5ppm/720min	SD
0	13.7	0.1	14.5	0.1	12.5	0.3
7	14.5	0.2	15.3	0.2	12.6	0.1
14	14.4	0.2	15.1	0.1	12.9	0.3
21	14.0	0.2	14.7	0.2	13.1	0.3
Hydrogen peroxide						
Days	control	SD	1.25mM/180 min	SD	5.0mM/180 min	SD
0	12.8	0.1	13.2	0.1	12.7	0.2
7	12.9	0.1	13.9	0.3	13.2	0.2
14	13.2	0.1	14.2	0.2	14.3	0.1
21	13.3	0.3	13.7	0.3	14.3	0.3

Figure 5.8

UV-B	Control		1.5kJ.m^{-2}		7.2 kJ.m^{-2}	
CS	1	0	1.1	0.3	1.1	0.5
CYP79A2	1	0	2.0	1.8	0.9	0.9
CYP79B3	1	0	6.6	4.4	10.9	7.8
CYP79F1	1	0	0.7	0.1	3.2	4.7
F3H1	1	0	1.1	0.2	0.9	0.1
PAL	1	0	1.1	0.4	0.8	0.3

Ozone	Control		5 ppm/60 min		5 ppm/720 min	
CS	1	0	1.7	0.6	1.0	0.5
CYP79A2	1	0	3.1	1.1	1.0	0.8
CYP79B3	1	0	1.6	1.9	8.1	5.7
CYP79F1	1	0	0.9	1.3	5.1	3.1
F3H1	1	0	1.2	0.3	0.9	0.3
PAL	1	0	1.0	0.8	1.0	0.8

H_2O_2	Control		1.25 mM/180 min		5.0 mM/180 min	
CS	1	0	1.1	0.3	1.2	0.1
CYP79A2	1	0	1.8	0.7	1.8	0.6
CYP79B3	1	0	3.0	3.0	0.5	0.3
CYP79F1	1	0	1.3	0.7	1.1	0.5
F3H1	1	0	1.4	1.9	2.1	2.8
PAL	1	0	1.1	0.1	3.7	4.8

Figure 6.1

Ethanol

Dose	ΔE	Sd
0	11.8	3.9
30	2.9	0.8
60	11.5	3.2
120	20.1	4.2
240	20.4	3.1
300	11.2	2.7
420	11.7	3.5
540	14.4	1.6

MeJA

Dose	ΔE	Sd
0	13.6	3.6
45	6.3	2.5
180	12.2	2.1
360	18.6	1.9
720	22.6	5.1

Figure 6.2

Ethanol

Days	control	SD	10,000 pmm/30min	SD	10,000 pmm/120min	SD
0	0.0	0.0	0.0	0.0	0.0	0.0
7	3.5	0.9	1.6	0.2	3.3	0.8
14	5.9	1.3	2.7	0.4	7.0	0.6
21	9.0	1.3	3.9	0.8	9.4	1.4

MeJA

Days	control	SD	1 ppm/45min	SD	1 ppm/180 min	SD
0	0.0	0.0	0.0	0.0	0.0	0.0
7	2.2	0.5	1.8	0.4	2.9	0.7
14	4.5	1.5	3.5	0.6	5.5	0.7
21	14.2	1.4	11.5	0.2	16.2	0.5

Figure 6.3

Ethanol

Days	control	SD	10,000 pmm/30min	SD	10,000 pmm/120min	SD
0	28.3	1.9	31.2	1.5	36.0	1.1
7	6.5	0.7	5.9	0.4	5.2	0.5
14	13.2	1.0	10.8	0.6	10.2	0.5
21	17.7	2.2	16.2	2.0	16.6	1.5

MeJA

Days	control	SD	1 ppm/45min	SD	1 ppm/180 min	SD
0	19.6	1.3	107.7	3.3	122.1	3.7
7	16.7	0.4	21.4	1.0	21.9	1.0
14	24.8	0.7	21.7	1.5	18.5	0.7
21	15.5	1.0	21.8	1.2	19.4	0.9

Figure 6.4

Ethanol

Days	control	10,000 pmm/30min	10,000 pmm/120min
0	3.2	3.2	3.2
7	3.0	2.9	3.1
14	2.9	2.8	2.9
21	2.5	3.1	3.3

MeJA

Days	control	1 ppm/45min	1 ppm/180 min
0	3.5	3.5	3.5
7	3.6	3.6	3.1
14	3.1	3.2	2.4
21	2.9	1.0	0.6

Figure 6.5

Ethanol

Days	control	10,000 pmm/30min	10,000 pmm/120min
0	19.2	20.3	20.4
7	19.8	21.8	22.1
14	20.5	22.7	23.1
21	20.4	23.7	23.2

MeJA

Days	control	1 ppm/45min	1 ppm/180 min
0	17.1	19.2	19.0
7	17.5	19.9	20.0
14	17.9	20.8	21.0
21	18.1	21.2	21.4

Figure 6.6

Ethanol

Days	control	10,000 pmm/30min	10,000 pmm/120min
0	5.0	6.1	6.1
7	5.3	6.1	5.9
14	5.1	5.9	6.5
21	5.2	6.7	7.1

MeJA

Days	control	1 ppm/45min	1 ppm/180 min
0	4.4	4.5	4.7
7	4.3	4.5	4.6
14	4.3	4.2	4.4
21	4.0	4.2	4.3

Figure 6.7

Ethanol

Days	control	10,000 pmm/30min	10,000 pmm/120min
0	11.1	13.6	13.3
7	11.7	13.8	13.5
14	12.5	14.0	14.2
21	12.6	14.7	14.4

MeJA

Days	control	1 ppm/45min	1 ppm/180 min
0	12.3	12.5	12.9
7	12.3	12.6	13.4
14	12.2	13.0	14.0
21	12.4	14.0	14.7

Chapter V11

Figure 7.1 & 7.2

	ΔE	Chlorophyll	Weight loss	Respiration
UV-C	62.3	9.6	-51.0	135.8
Heat	309.8	13.6	-15.4	653.8
UV-B	50.5	18.5	-20.5	99.1
O_3	46.0	7.9	238.5	673.0
H_2O_2	45.0	10.6	32.9	216.0
EtOH	128.2	21.5	92.5	10.4
MeJA	24.0	8.0	-20.5	449.4

Figure 7.3 & 7.4

	Glucoraphanin	Glucobrassicin	4-Methoxy	NeoGlu	4OHGLB	Total GLB
UV-C	12.4	4.6	8.5	11.4	14.4	7.8
Heat	3.4	12.4	0.5	28.9	3.8	14.0
UV-B	11.1	15.0	9.1	33.7	9.3	18.2
O_3	19.6	11.0	30.5	9.8	11.8	12.8
H_2O_2	16.6	13.9	18.0	34.4	8.3	18.8
EtOH	20.5	9.4	12.0	13.2	11.2	10.8
MeJA	2.1	5.1	6.8	27.2	38.8	14.8

Figure 7.5

	DSG	SFG	DFG	TSG	DSFG	Total
UV-C	8.9	8.1	15.8	10.5	15.1	10.7
Heat	5.1	15.8	16.5	16.3	17.8	13.9
UV-B	9.3	21.2	14.3	5.8	8.5	12.9
O_3	4.3	6.4	7.6	4.4	3.3	5.4
H_2O_2	2.3	6.0	10.0	3.5	6.3	5.4
EtOH	13.1	20.4	20.1	12.3	16.5	17.3
MeJA	7.5	6.0	7.9	5.2	3.6	5.8

Figure 7.6

	ORAC	Total AA
UV-C	-7.6	-5.6
Heat	9.9	-1.7
UV-B	6.9	-3.8
O_3	-18.0	-1.3
H_2O_2	-12.2	-2.0
EtOH	0.4	-2.7
MeJA	-24.0	-1.9