Stress response and health affecting compounds in Brassicaceae

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List of abbreviations

BRs	Brassinosteroids
CB1	Cannabinoid receptor type 1
COSY	Correlated spectroscopy
D.W.	Dry weight
ET	Ethylene
GABA	γ-amino butyric acid
GSTs	Glutathione *S*-transferases
HDL – Cholesterol	High density lipoprotein – Cholesterol
HMBC	Heteronuclear multiple bonds coherence
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum coherence
I-3-C	Indole-3-carbinol
IAA	Indole acetic acid
ISR	Induced systemic resistance
JA	Jasmonic acid
LDL – C	Low density lipoprotein – Cholesterol concentration
LDL – Cholesterol	Low density lipoprotein – Cholesterol
MeJA	Methyl jasmonate
MS	Mass spectrometry
M & S media	Murashige and Skoog media
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
PLS-DA	Partial least square-discriminant analysis
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
TMSP	Trimethyl silyl propionic acid sodium salt

Chapter 1

General introduction and outline of the thesis

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General Introduction

The nutritional health and well-being of humans are entirely dependent on plant based food, as plants are critical components of the dietary food chain. Plants are a good source of health-promoting chemicals, providing almost all essential mineral and organic nutrients to humans either directly, or indirectly.[1]

Brassica vegetables including *Raphanus sativus* (radish)[2] and *Brassica rapa*[3] are consumed all over the world and are an important part of human diet.[4] As *Brassica* vegetables are rich sources of phytochemicals including amino acids,[5] glucosinolates, flavonoids, vitamins and mineral nutrients.[6] The variability in these bioactive food components depends on preharvest growth conditions and post harvest storage and/or processing conditions.[7] The effects on health obviously also depends on the amount of consumption, and the food processing culture of the community.[8]

Brassica secondary metabolites play an important role in its food quality. Besides the minerals and vitamins, plants can provide an array of interesting phytochemicals in the diet.[1] A part from appearance and flavour of vegetables, these metabolites are also known for their biological activities[9-11] including antioxidant,[12-14] anticancer, anti-inflammatory[15] and antiviral activities.[16] Some of these phytochemicals may function solely as aforementioned activities, but many of these metabolites may activate adaptive cellular stress-response pathways, including neuron hormesis.[17] On the other hand these compounds can also act as pro-oxidants under certain conditions.[13]

During the growth period *Brassica* plants are frequently subjected to various biotic and abiotic stress factors.[18] In this case secondary metabolites are useful products for plants, as stress response.[19] The contamination of soils and water with metals has created a major environmental problem, resulting in major production losses and hazardous health problems.[20, 21] At the same time plants have also been used for remediation of metals contaminated soil.[22] In this case the degree of accumulation of toxic metals (Cd, Cu, Zn, Mn, and Fe etc.) depends on the type of metal and its concentration.[23, 24] This results in the intensification of reactive oxygen species (ROS) generation, leading to the oxidative stress in plant cells.[20, 21]

In the mean time environmental microorganisms play a critical role in the interaction of the plant with the soil. These microorganisms are also responsible for diverse metabolomic functions that affect soil

and plant health.[25] In this scenario the non-pathogenic bacteria can induce a similar systemic resistance in plants like pathogenic bacteria,[26] causing an alteration in plant metabolome, depending on the type of bacteria interacting with the plant.[27]

Preharvest growth stage and post harvest low temperature storage are also critical for the plant metabolome. Differences in plant metabolome at different developmental stages might be due to changes in channelling of precursors.[28] While at low temperatures by the disruption of cell membranes and cell walls, phytochemicals are released that may lead to changes in the metabolites pool.[29] Understanding the plant tolerance to 0^{o}C or below 0^{o}C temperatures and the relation to plant metabolite production is extremely complex.[30] But the most important postharvest condition necessary for maintaining vegetable quality is low temperature, as it maintains cellular integrity,[31] while extending the shelf life, as low temperature reduces the post harvest quality deterioration.[32]

In response to these stress factors plants produce a diverse array of primary and secondary metabolites.[19, 21] Especially amino acids, phenolics and glucosinolates play an important role in plant defence, either directly or indirectly.[13, 33]

Generation of reliable metabolite profiling data for the investigation of the role of metabolites is necessary.[34] As it is impossible to measure all metabolomic changes simultaneously,[30] systems biology as a holistic approach can be used to examine different biological processes, operating as an integrated system and visualize how individual metabolomic pathways are interconnected to each other.[35] Depending on selectivity and sensitivity different analytical approaches can be used. It includes nuclear magnetic resonance (NMR), thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC/MS).[35] Among all aforementioned techniques, NMR spectroscopy is considered as, non-destructive, highly reproducible, and easy for sample handling. It allows the analysis of a large group of compounds in a single run, and is the most suited quantitative and qualitative metabolomic technique.[36, 37] It is used for high-throughput screening, metabolite fingerprinting, metabolite profiling and can also be used to investigate the operation of plant metabolomic networks.[38, 39] Multivariate data analysis techniques coupled with aforementioned analytical techniques can be used to exploit a large data set for understanding and interpreting the information in an integrative manner[40] as plotting the data in the space defined by the two

or three principal components, provides a rapid mean of visualizing similarities or differences in the data set.[35] In this case Principal Component Analysis (PCA) is one of the oldest and most widely used multivariate techniques.[35, 41] To make a clear discrimination in a data set partial least square-discriminant analysis (PLS-DA) as a supervised data analysis tool can also be applied[27] to study the plant response to various stress factors.

Aim of the thesis

The aim of the present study was to investigate the effect of preharvest and postharvest factors on the plant's primary and secondary metabolite production by using a systems biology approach.

Outline of the thesis

The thesis begins with a review of literature describing the phytochemicals in Brassicaceae and their role as health affecting compounds (**Chapter 2**) showing the importance of Brassicaceae vegetables for human diet. The next chapter is a literature survey focusing on plant metabolome changes during the various growth stages and by different stimuli. Ultimately this leads to the changes in health affecting compounds in Brassicaceae (**Chapter 3**). Nutritional value of plants at different developmental stages was also evaluated in this project. Quantitative analysis was conducted for amino acids, organic acids and sugars by NMR, and for vitamins and glucosinolates by HPLC. NMR metabolomic analysis as a non-targeted approach coupled with multivariate data analysis was also used (**Chapter 4**). The effect of non plant pathogenic bacteria (**Chapter 5**) and different metal ions (**Chapter 6**) as preharvest stress factors was evaluated, showing the change in primary and secondary metabolite production. Metabolomic changes under low temperature storage conditions are also reported in this thesis (**Chapter 7**). Extracts of several *Brassica* varieties were assayed for different biological activities including, acetylcholine esterase inhibition, antimicrobial activity, as well as CB1 and adenosine receptor binding activity (**Chapter 8**). Finally the future perspectives for further research work in related areas are discussed (**Chapter 9**).

Chapter 2

Health-affecting compounds in Brassicaceae

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Abstract

Brassicaceae vegetables are considered to be a food in many areas all over the world. *Brassica* species are not only known for their high fat and protein contents for human and animal consumption, but Brassicaceae vegetables are also recognized as a rich source of nutrients such as vitamins (carotenoids, tocopherol, ascorbic acid, folic acid), minerals (Cu, Zn, P, Mg among others), carbohydrates (sucrose and glucose), amino acids (for example, L-alanine, L-aspartic acid, L-glutamic acid, L-glutamine, L-histidine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, and L-valine), and different groups of phytochemicals such as indole phytoalexins (brassinin, spirobrassinin, brassilexin, camalexin, 1-methoxyspirobrassinin, 1-methoxyspirobrassinol, and methoxyspirobrassinol methyl ether), phenolics (such as feruloyl and isoferuloylcholine, and hydroxybenzoic, neochlorogenic, chlorogenic, caffeic, *p*-coumaric, ferulic, and sinapic acids, anthocyanins, quercetin and kaempferol), and glucosinolates mainly including glucoiberin, glucoraphanin, glucoalyssin, gluconapin, glucobrassicanapin, glucobrassicin, gluconasturtiin, and neoglucobrassicin. All these phytochemicals contribute to the reported antioxidant, anticarcinogenic, and cardiovascular protective activities of *Brassica* vegetables. However, not all members of this family are equal from a nutritional viewpoint, since significant qualitative variations in the phytochemical profiles of *Brassica* species and varieties suggest differences in the health promoting properties among these vegetables. In this review, *Brassica* phytochemicals with their nutritional value and health promoting activities are discussed to give an overview of the literature for *Brassica* as a food crop.

Keywords: Brassicaceae, Health effects, Metabolites

1 Introduction

The Brassicaceae (Cruciferae) family is composed of 350 genera and about 3500 species,[42] including some crops of great economical importance such as *Brassica napus* L., *B. rapa* L., and *Sinapis alba* L.[43, 44] These species are used as food, spices, and as a source of vegetable oils.[45] The Brassicaceae vegetables represent a major part of the human diet[4] being consumed by people all over the world[46-48] and are considered important food crops in China, Japan, India, and European countries.[42, 49, 50] Over the past 3 decades *Brassica* production has grown steadily becoming an important source of oil and protein of plant origin for animal and human nutrition, respectively. Rapeseed (canola) ranks currently as the third source of vegetable oil (after soy and palm) and the third leading source of oil meal (after soy and cotton).[51] *Brassica* is an inexpensive though very nutritive source of food, providing nutrients and health-promoting phytochemicals such as phenolic compounds, vitamins,[52-55] phytic acid, fibre, soluble sugars,[56] glucosinolates,[57] minerals, polyphenols,[58] fat, and carotenoids (**Figure 1**).[59] There is currently much interest in identifying phytochemicals with useful biological activity in food[60] and any significant finding related to the presence of valuable compounds in *Brassica* species will be welcomed by the food industry.[51]

There is ever-increasing evidence that a higher consumption of *Brassica* vegetables, for example, broccoli, cabbage, kale, mustard greens, Brussels sprouts, and cauliflower, reduces the risk of several types of cancer.[61, 62] The anticarcinogenic effect of these vegetables has been attributed to decomposition products of glucosinolates, indoles, and iso-thiocyanates,[63] phytoalexins, and other antioxidants.[64, 65] Indole-3-carbinol, a natural component of *Brassica* vegetables[66] has an interesting anticarcinogenic potential, acting via different metabolomic and hormonal pathways.[65] It reduces the incidence of tumours in reproductive organs[66] and the growth of human breast cancer cells.[67]

Overall, to date, the most promising anticarcinogenic dietary compounds have been detected in cruciferous vegetables and further elucidation of their protective mechanisms and the identification of other active constituents may contribute to the development of highly health supporting *Brassica* varieties.[68]

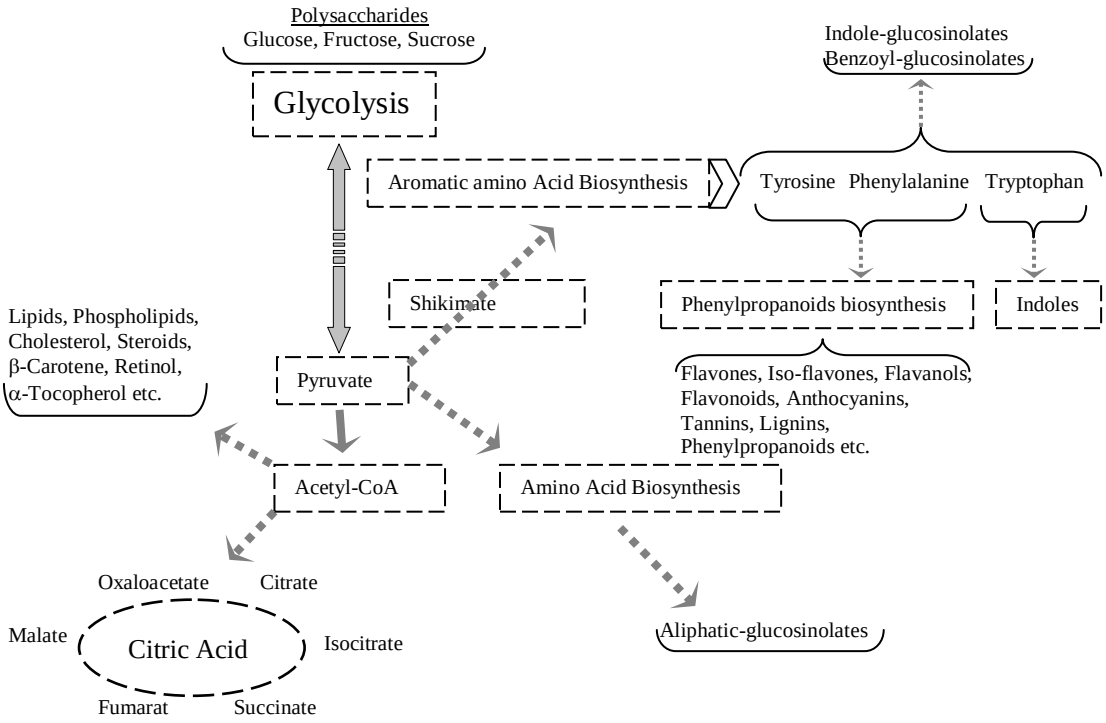

Figure 1 – General biosynthesis pathway for Brassicacae metabolites.[69-73]

Extracts of the different species of the Brassicaceae family show antioxidant effects[74] and decrease oxidative damage,[75] while the juice of some *Brassica* species has been proved to protect human hepatoma cells from the genotoxic effects of carcinogens.[68] However, compounds such as glucosinolates and phytates may also have a negative effect on human and animal health. For example, glucosinolates and glucosinolate by-products can be toxic and are responsible for the bitter, hot, and pungent flavours of Brassicaceae vegetables.[76] Also, thiocyanates, isothiocyanates, and oxazolidine-2-thiones have been shown to be goitrogenic.[77] As Brassicaceae vegetables can be a good source of minerals, antinutrients such as phytates can decrease their bioavailability.[78]

The purpose of this review is to provide an overview of health-affecting compounds identified in the *Brassica* genus.

2 Vitamins

Brassica vegetables contain high levels of vitamins[58] including carotenes, tocopherols,[79] vitamin C, and folic acid[80] (**Table 1**). It is a well-known fact that the first 3 vitamins have the potential to prevent and treat malignant and degenerative diseases.[79] Broccoli (*Brassica oleracea*) extracts are protective against reactive oxygen species (ROS) presumably due to the presence of vitamin C, quercetin, kaempferol, lutein, zeaxanthin,[81] α-tocopherol, γ-tocopherol, and β-carotene.[82] Bioavailabilty is a critical feature in the assessment of the role of these compounds in human health. When 200 g of broccoli was consumed by healthy volunteers, significant changes in serum of both men and women were observed for lutein, whereas for γ-tocopherol a significant change was detected in women only, whereas no changes were observed for α-tocopherol, β-carotene, and retinol.[83]

- **Carotenoids**

In some *Brassica* species, carotenoid content is two-fold higher than in spinach.[84] Sixteen carotenoids were identified in *B. chinensis*, *B. parachinensis*, and *B. pekinensis*, out of which lutein and β-carotene were the most abundant.[85, 86] Lutein has also been isolated from extracts of fresh raw kale (*Brassica oleracea* var. *acephala*)[87] and high levels of other carotenoids, mainly β-carotene, were also detected.[76, 79, 88] Two other vegetables, Brussels sprouts and green cabbage, have been reported to contain significant amounts of *trans*-β-carotene and *cis*-β-carotene.[89] Carotenoids present in dark green leafy vegetables might be involved in the prevention of several diseases related to oxidative stress.[86]

- **Tocopherols**

The predominant tocopherol in all *Brassica* vegetables is α-tocopherol with the exception of cauliflower, which predominantly contains γ-tocopherol.[90] The tocopherol content of rapeseed oil consists of 64% γ-tocopherol, 35% α-tocopherol, and less than 1% is the mixture of δ-tocopherol and plastochromanol-8.[91]

- **Vitamin C**

High levels of vitamin C have been reported in Chinese cabbage, broccoli, cauliflower and cabbage[92] (**Table 1**). The content of this vitamin in different cultivars of cabbage (*Brassica oleraceae* L.) ranges from 12.0 to 112.5 mg/100 g.[93]

- **Folic acid**

Raw broccoli,[94] cauliflower[95] and cabbage contain folic acid[96] (**Table 1**), a scarce and important vitamin which acts as a coenzyme in many single carbon transfer reactions in the synthesis of DNA, RNA, and protein components.[97] Folic acid reduces the risk of neural tube defects and may be associated with the reduced risk of vascular disease and cancer,[98, 99] while low folate intake has been identified as a main cause of anaemia.[100]

Table 1 – Variation of vitamins (μg/g) among different Brassicaceae vegetables on fresh weight basis.

	Ascorbic acid	α-Carotene	β-Carotene	α-Tocopherol	Folate
Broccoli	748 ± 62 [a]	0.3 [b]	8.9 [b]	16.2 [b]	1.771 [d]
Kale	186 [e]	0.6 [b]	48.6 [b]	19.2 [b]	–
Cauliflower	499 ± 53 [a]	–	72 ± 0.5 [g]	1.7 [b]	0.53 [e]
Chinese cabbage	253 [a]	–	0.1 [c]	0.8 [c]	0.81 [f]
White cabbage	188 ± 13 [a]	0.02 [b]	0.8 [b]	1.7 [b]	–
Brussels sprouts	158 [c]	–	1.4 [c]	1.5 [c]	–

a = [92]; b = [79]; c = [101]; d = [94]; e = [95]; f = [97]; g = [102].

3 Minerals

Brassica plants have been found to be rich in many minerals including calcium and iron.[84] Among the green leafy vegetables, *B. oleracea* L. *acephala* (kale) is an excellent source of minerals,[76] accumulating high levels of P, S, Cl, Ca, Fe, Sr, and K (**Table 2**).[103] Broccoli accumulates Se to concentrations many times above that found in soil, which may greatly enhance its health-promoting properties.[104] Different *Brassica* vegetables such as cauliflower, bok choy (*B. rapa*) stems and leaves, broccoli (*B. oleracea v. botrytis*), and kale (*B. oleracea v. acephala*) are reported to have high mineral contents[96] (**Table 2**). Interestingly, all these *Brassica* vegetables exhibit excellent calcium bioavailability.[105] Cabbage leaf (*B. oleracea* var. *capitata*) also contains potentially useful amounts of copper, zinc, iron, and a number of other essential minerals and trace elements.[106]

Brassica can be cultivated under hydroponic conditions that lead to high levels of nutritionally important minerals such as Cr, Fe, Mn, Se, and Zn. Owing to reproducible and high concentration of minerals in the

edible plant tissue small quantities of this enriched plant can be processed to make capsules or tablets that supply 100% of the recommended daily intake of these elements, with the advantage of using a natural plant source.[107] However, the bioavailability of some of these minerals might be reduced by the presence of glucosinolates, phytates, and phenolics.[78]

Table 2 – Variation of minerals (μg/g) among different Brassicaceae vegetables on fresh weight basis

	Broccoli	Kale	Cauliflower	Chinese cabbage	White cabbage	Brussels sprouts
Ca	272 ± 20 [a]	2860 ± 430 [b]	175 ± 17 [a]	470 ± 60 [b]	440 ± 60 [b]	356 ± 13 [a]
Fe	8.7 ± 0.5 [a]	4 ± 2 [b]	5.0 ± 0.3 [a]	2 ± 0.3 [b]	1.4 ± 0.3 [b]	7.6 ± 0.2 [a]
Cu	0.94 ± 0.07 [a]	0.4 ± 0.2 [b]	0.56 ± 0.07 [a]	0.4 ± 0.2 [b]	0.5 ± 0.5 [b]	0.9 ± 0.09 [a]
Mg	181 ± 8 [a]	510 ± 40 [b]	145 ± 22 [a]	130 ± 30 [b]	140 ± 20 [b]	207 ± 12 [a]
K	2890 ± 70 [a]	7120 ± 5170 [b]	2210 ± 140 [a]	2280 ± 1120 [b]	2660 ± 870 [b]	4250 ± 250 [a]
Zn	9.5 ± 0.3 [a]	2.9 ± 0.5 [b]	6.4 ± 0.3 [a]	2.3 ± 0.4 [b]	2 ± 1 [b]	5.8 ± 0.4 [a]
Na	180 ± 6 [a]	120 ± 40 [b]	192 ± 27 [a]	50 ± 20 [b]	30 ± 10 [b]	107 ± 7 [a]
Mn	1.92 ± 0.09 [a]	3 ± 1 [b]	1.31 ± 0.07 [a]	0.5 ± 1.4 [b]	2 ± 1 [b]	2.31 ± 0.13 [a]

a = [108]; b = [109].

Heavy metals (for example, Mo, B, Co, Se, Cd, Pb, Cr, Ni, Hg, and As) and others such as Cu, Zn, Mn, Fe may be found in high concentration in contaminated soils and have toxic effects on plants, animals, and human beings.[110] The use of metal-accumulating plants to remove toxic metals from soil is known as phytoremediation[22] and *Brassica* species such as *B. oleracea* and *B. napus*, known for their metal accumulator properties, are used for this purpose.[111] However, this characteristic, which constitutes an advantage for the former use entails, an important toxicological risk if these fruits and vegetables grown in contaminated soils are ingested.[112]

4 Lipids

Rapeseed oil is one of the most common edible oils in the world. Its nutritive value is excellent due to its unsaturated fatty acid content.[113] Mustard oil is also a significant source of unsaturated fatty acids

containing about 94.2%, and only 5.4% saturated fatty acids. These are recognized as essential dietary elements with important effects on human health.[114] Mustard oil contains linolenic acid 21.4% (omega – 3), palmitic acid 2.9%, palmitoleic acid 0.2%, stearic acid 1.0%, oleic acid 19.4%, linoleic acid (omega – 6) 9.7%, and erucic acid 44.4% [115], showing an inhibition of mutagenicity.[114] Oil content in seeds of different *B. campestris* genotypes varies from 38.9% to 44.6% and major fatty acids found are oleic, linoleic, linolenic, eicosaenoic, erucic acid ranging from 10.1% to 17.3%, 5.9% to 14.5%, 5.2% to 15.0%, 7.7% to 13.7% and 39.6% to 59.9%, respectively.[116] Canola seed oil is one of the richer sources of omega-3-unsaturated fatty acids[65] and in particular of α-linolenic acid.[117] The oil of commercial *B. napus* L. is rich in oleic acid and contains moderate levels of linoleic and linolenic acid.[118]

Cauliflower is considered to be a food of high nutritional value and some authors relate its quality to the stability of its fatty acids. Environmental stress may enhance the fatty matter content (linolenic acid) and polyphenols.[119]

The essential oil of *B. rapa* var. *perviridis* leaves was found to contain 48 volatile components, representing 94.0 – 96.6% of the oil. The main constituents were found to be 3-butenyl-isothiocyanate (1.4 – 29.2%), 4-pentenyl isothiocyanate (8.2–23.5%), 2-methyl 5-hexenenitrile (1.3–16.8%), 2-phenylethyl isothiocyanate (7.0– 13.7%), and phytol (6.1–23.5%).[84] Volatile chemicals emitted by rape seed oil also contain monoterpenes (limonene, sabinene, β-myrcene, and *cis*-3-hexen-1-ol acetate), sesquiterpenes, short-chain aldehydes and ketones, other green leaf volatiles and organic sulfides including the respiratory irritant, dimethyl disulfide.[120] The emission of volatiles from cabbage, consisted mainly of monoterpenes (sabinene, limonene, α-thujene, 1,8-cineole, β-pinene, myrcene, α-pinene, and γ-terpinene). (Z)-3-Hexenyl acetate, sesquiterpene (E, E)-α-farnesene, and homoterpene (E)-4, 8-dimethyl-1, 3, 7-nonatriene were emitted mainly from herbivore-damaged plants.[121]

In *Brassica* oils, triacylglycerols are the main constituents making up about 98% of the oils. The remaining nonglyceridic fraction consists of different lipophylic phytochemicals such as tocopherols, sterols, and sterol esters.[122] Similarly, in *Brassica* oils the remaining 2% consists of sterols, phospholipids, and sphingolipids. The major sterols were identified as stigmasterol,[123] sitosterol, campesterol, and cholesterol,[122] the phospholipids as phosphatidylethanolamine and phosphatidylcholine, and the sphingolipids as cerebrosides.[124]

For the purpose of human nutrition, a high ingestion of oleic acid, linoleic and linolenic acids is advantageous.[118] All polyunsaturated fatty acids including both linoleic and linolenic acids are essential because they cannot be synthesized by humans.[125] However, the type of fatty acids in the dietary fat is very important, being considered, for example, as one of the detrimental factors in colon cancer development. Fats containing omega 6-polyunsaturated fatty acids were found to enhance chemically induced colon cancer,[115] while omega-3-polyunsaturated fatty acids reduce it.[75, 115] Consumption of diet rich with canola fat may also alter the fatty acid composition of lipids of adipose tissue, muscle, kidney, and liver.[126] A diet high in *trans*–α–linolenic acid may increase plasma LDL/HDL cholesterol and total cholesterol/HDL-cholesterol ratios. Careful deodorization prevents the formation of *trans*-α-linolenic acid and may help to improve the diet.[117]

5 Carbohydrates

The type and concentration of free sugars influence the flavour of *Brassica* products.[127] Fructose, glucose, and sucrose are the major soluble sugars found in *Brassica*.[128] A comprehensive evaluation of the nutritive profiles of *Brassica* seed meals of yellow-seeded types (*B. napus*, *B. rapa*, *B. juncea*, and *B. carinata*) and conventional brown-seeded (canola) type showed that all contain sucrose (7.5% – 8.7%), oligosaccharides (2.3% – 2.5%), ash (6.9% – 7.0%), and non-starch polysaccharides (20.4% – 19.7%).[129] Fructose is the major sugar in the different types of *Brassica*, representing between 48.8 and 56.9% of the total sugar content in broccoli cvs. *Marathon* and *Senshi*, respectively, 48.7% (cv. *Mirandela*) and 53.8% (cv. *Murciana*) in the other cabbages. Glucose is the second major sugar, while sucrose represents a maximum of 20.5% in broccoli cv. *Shogun* and 11.1% in cv. *Murciana*.[127]

- **Dietary Fibre**

It is composed of non-starch polysaccharides[130] and is an important constituent in Brassicaceae vegetables, contributing to prevent colon cancer.[131] In white cabbage (*B. oleracea* var. *capitata*) dietary fibre represents one-third of the total carbohydrate content, the other two-third being low-molecular weight carbohydrates, including glucose (37%), uronic acid (32%), arabinose (12%), and galactose (8%).[132, 133] The dietary fibre content of 6 cultivars of white cabbage (*B. oleracea* var *capitata*) was evaluated finding that of the average total dietary fibre of

241 mg/g of dry matter, approximately 25% was soluble.[132] Dietary fibre content of other species was found to vary between 271 and 352 mg/g for the yellow-seeded *B. napus* and brown-seeded *B. napus*, respectively,[134] with intermediate values in other species, such as cauliflower (302 mg/g of D.W.), broccoli (330 mg/g of D.W.), and cabbage (226 mg/g of D.W.).[96]

6 Protein and Free Amino Acids

The defatted meal of *Brassica* oilseeds is a valuable source of protein for the livestock feed industry[135] and may constitute an important protein source for human nutrition thereby increasing the value of *Brassica* crops. However, the high temperatures and organic solvents used during the oil extraction process cause denaturation of proteins in *Brassica* meal.[56] Protein and free amino acid content in rapeseed meal have a high nutritive value, but the utilization of rapeseed/canola as a source of food-grade proteins for human consumption is still limited due to the presence of antinutrients such as glucosinolates, phytates, and phenolics.[113, 136, 137] Therefore, it is used only for animal feeding.[138] There is a variation in protein content in different groups of *Brassica*, such as *B. napus* seeds have higher protein solubilities than meals from *B. rapa* seeds. Meals with higher protein solubility values also have higher foaming capacity values.[139] Seeds of rape, *B. napus*, and related cruciferous oilseed crops, such as *B. campestris*, *B. juncea*, *B. carinata*, and *B. nigra* are rich sources of edible protein and rapeseed/canola meal, the by-product of the oil-extraction process, contains up to 42.7% – 50% protein.[129, 140]

The rape seed (*B. napus*) meal contains napin and cruciferin as storage proteins and oleosin as a structural protein associated with oil bodies.[138, 140] The 2S albumins or napins in oilseed rape and turnip rape are potential food allergens.[141] Free amino acids are involved in secondary plant metabolism and in the production of compounds which directly or indirectly play an important role in plant-environment interactions and human health. A total of 17 amino acids were identified (L-alanine, L-arginine, L-asparagine, L-aspartic acid, glycine, L-glutamic acid, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine) in *B. oleracea var italica*.[5, 125] *S*-methylcysteine sulfoxide, a naturally occurring *S*-containing amino acid, is contained at high concentrations in *Brassica* vegetables such as broccoli and cabbage. Its

cholesterol-lowering effects have been demonstrated in animals, observing a significant decrease of the serum level of LDL-C (14% decrease) following the oral administration of broccoli (*B. oleracea L.* var. *botrytis L.*) and cabbage (*B. oleracea L.* var. *capitata L.*).[142]

7 Indoles

Plants may respond to pathogen attack by producing phytoalexins.[143] Phytoalexins are a group of structurally diverse molecules[144, 145] that are generally non-specific in their antimicrobial activities.[145 145, 146] A number of phytoalexins have been isolated from crucifers (**Figure 2**).[147]

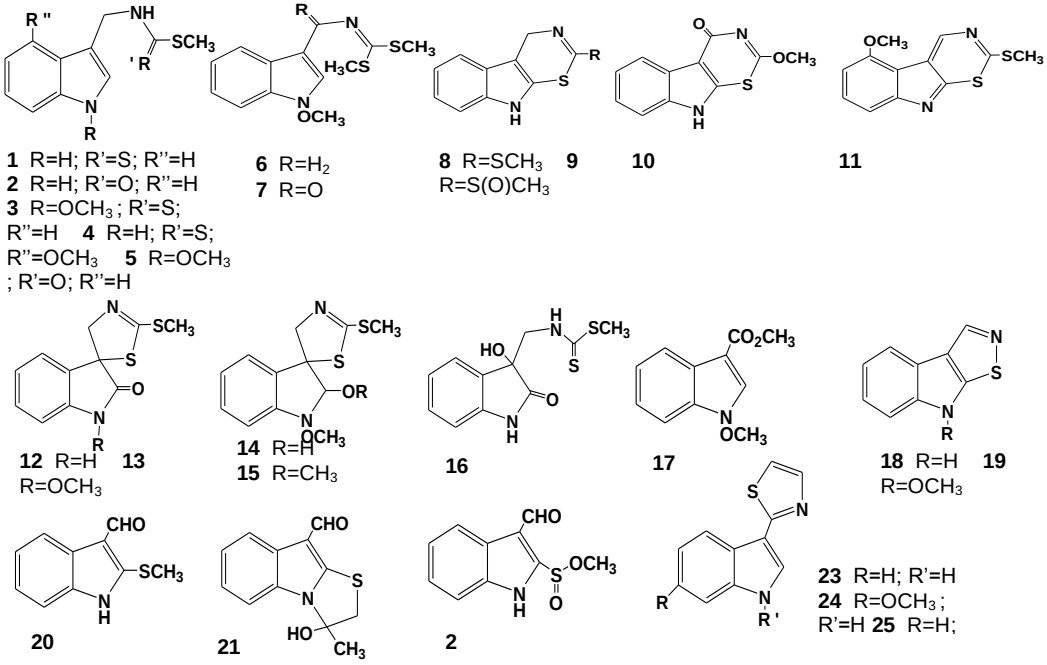

Figure 2 – Structures of cruciferous phytoalexins: **1:** brassinin, **2**: brassitin, **3:** 1-methoxybrassinin, **4:** 4-methoxybrassinin, **5:** 1-methoxybrassitin, **6:** 1-methoxybrassenin A, **7:** 1-methoxybrassenin B, **8:** cyclobrassinin, **9:** cyclobrassinin sulfoxide, **10:** cyclobrassinone, **11:** dehydro-4-methoxycyclobrassinin, **12:** spirobrassinin, **13:** 1-methoxyspirobrassinin, **14:** 1-methoxyspirobrassinol, **15:** 1-methoxyspirobrassinol methyl ether, **16:** dioxibrassinin, **17:** methyl 1-methoxyinodole-3-carboxylate, **18:** brassilexin, **19:** sinalexin, **20:** brassicanal A, **21:** brassicanal B, **22:** brassicanal C, **23:** camalexin, **24:** 6-methoxycamalexin, **25:** 1-methylcamalexin [147].

In *Brassica* indole phytoalexin (camalexin) synthesis is induced as a response to pathogen attack and ROS generating abiotic elicitors.[148, 149] These phytoalexins inhibit the growth of human cancer cells and thus may have a potential use as chemopreventive agents.[64] Several indole phytoalexins found in *Brassica* vegetables, brassinin, spirobrassinin, brassilexin, camalexin, 1-methoxyspirobrassinin, 1-methoxyspirobrassinol, and methoxyspirobrassinol methyl ether, have been found to possess significant antiproliferative activity against various cancer cells, while others, such as cyclobrassinin, spirobrassinin, brassinin also exhibited chemopreventive activity in models of mammary and skin carcinogenesis.[150]

Brassicaceae species contain a range of signalling and regulatory compounds known to be involved in general defence mechanisms activated by pathogen and herbivore attacks on plants.[151] These include salicylic acid, ethylene, H_2O_2, and jasmonic acid (an acid-derived oxylipin)[79] and signal peptides, such as systemin.[152-154] Some of these are bioactive compounds which exhibited anticancer activity in animals when added to experimental diets.[79] In particular, jasmonic acid and its derivatives, which represent the best characterized class of signal compounds, mediating the defence responses to wounding and herbivore attack in Brassicaceae,[155-159] have been proved to inhibit the proliferation of human prostate cancer cells, while not affecting normal human blood cells.[64, 160]

8 Phenylpropanoids, Flavonoids and Tannins

Phenylpropanoids, flavonoids and other minor compounds (**Table 3**) are considered to be among the health promoting compounds in Brassicaceae species.[161] Plant polyphenols are multifunctional, having diverse biological activities apart from acting as reducing agents.[162] Phenolics also contribute to the bitter, astringent, and unpleasant flavour of rapeseed, though the threshold of this unpleasant flavour is higher for individual phenolic compounds than for the mixture.[113] In spite of this they are considered to be beneficial and harmless components of rapeseed meal.

The contribution of *Brassica* vegetables to health improvement has generally been associated with their antioxidant capacity and, undoubtedly, phenolic compounds are the major antioxidants of *Brassica* vegetables.[89, 163, 164] Phenolics is a generic term which refers to a large number of compounds that can be classified in groups, namely, phenolic

acids, flavonoids, isoflavonoids, lignans, stilbenes, and complex phenolic polymers.[11, 165]

As mentioned above, these antioxidants have proved to be good for human health and also useful as food preservatives.[166] Mustard seeds have a chemopreventive potential and enhance the antioxidant defence system. Their inclusion in the diet may very probably contribute to reducing the risk of cancer incidence in the human population.[167] A rapeseed phenolic extract has shown a stronger antioxidant activity than many artificial antioxidants[168] and exhibited a greater efficiency on a mole-to-mole basis than natural antioxidants such as vitamin C, vitamin E, and β-carotene.[162]

Table 3 – Variation of phenolics (μg/g) among different Brassicaceae vegetables on fresh weight basis

	Quercetin	Kaempferol	Apigenin	Lutein
Brassica oleracea L var. *italica* (Broccoli)	137 [a]	46 [a]	–	6.8 [c]
Brassica oleracea L var *botrytis* L (Cauliflower)	39 [a]	12 [a]	2 [a]	1.3 [c]
Brassica campestris var *chinensis* (Chinese cabbage)	390 [a]	96 [a]	45 [a]	0.2 [c]
Brassica rapa L. *subsp. sylvestris*	102 [b]	334 [b]	–	–
Brassica oleracea var *capitata* (white cabbage)	51 [a]	–	8 [a]	1.4 [c]

a = [92]; b = [161]; c = [101].

Species of the Brassicaceae family are generally rich in polyphenols. *Brassica rapa*[113] and *B. oleracea* L. var. *botrytis* contain a high amount of phenolic compounds.[169] Phenolic contents of several species have been reported, such as Chinese cabbage (1189±125μg/g), broccoli (822±89 μg/g), cauliflower (278±15μg/g), and white cabbage (153±21μg/g) on a fresh weight basis.[92, 168] In the case of broccoli, phenylpropanoids such as ferulic, sinapic, caffeic, and protocatechuic acid were reported to be the most abundant and important bioactive compounds.[170, 171] Four phenylpropanoids (caffeic, *p*-coumaric, ferulic, and sinapic acid) were identified in the water-soluble phenolic fraction of the leaves of oilseed rape (*Brassica napus* L. var. *oleifera*)[172] and gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, salicylic, *p*-coumaric, caffeic, ferulic, and sinapic acid were identified in kale (*B. oleraceae* L. var. *acephala* DC.).[173]

The main phenolics in rapeseed meal were determined to be sinapic acid which constitutes over 73% of its free phenolic acid content,[51] while apart from sinapic acid, rapeseed oil also contains vinylsyringol.[174, 175] An efficient peroxynitrite scavenger activity has been described for sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid), which has shown to contribute to the cellular defence against this powerful cytotoxic free radical, thus avoiding peroxynitrite-mediated disorders.[176] Besides the typical seed constituent sinapic acid, large amounts of choline esters of other phenolic acids have been detected in Brassicaceae species, for example, feruloyl- and isoferuloylcholine and hydroxybenzoic acid.[177] Brassicaceae plants accumulate glucose esters (1,6-di-*O*-sinapoylglucose), gentiobiose esters (1-*O*-caffeoylgentiobiose and 1,2,6-tri-*O*-sinapoylgentiobiose) of phenolic acids and kaempferol conjugates.[178]

Flavonoids are one of the most common and widely distributed groups of plant phenolics. Over 5000 different flavonoids have been described to date and they are classified into at least 10 chemical groups. Among them, flavones, flavonols, flavanols, flavanones, anthocyanins, and isoflavones are particularly common in the human diet.[179] As these compounds have interesting biological activities, these are being used in numerous medical treatments,[180, 181] connected to cancer-prevention[182, 183] and cardiovascular system protection, including inhibition of oxidative damage.[184, 185] At higher doses, however, flavonoids may act as mutagens, pro-oxidants that generate free radicals, and as inhibitors of key enzymes involved in hormone metabolism.[186]

Flavones are involved in various interactions with other organisms, microbes as well as insects and other plants.[187] Pharmacological activities have been described for various flavonoids (for example, quercetin, apigenin, catechins) which have shown an anti-inflammatory action by inhibiting cycloxygenase-2 and inducible nitric oxide synthase.[188] The flavonols quercetin, kaempferol, and isorhamnetin are among the flavonoid derivatives present in *Brassica* species.[53, 189-191] Two main flavonol glycosides, quercetin 3-*O*-sophoroside and kaempferol 3-*O*-sophoroside, are present in broccoli florets. Three minor glucosides of quercetin and kaempferol, isoquercitrin, kaempferol 3-*O*-glucoside, and kaempferol diglucoside, have also been detected. The quercetin and kaempferol glycosides were present in florets at a level of 43 μg/g and 94 μg/g of dry weight, respectively.[192] Glycosylated

kaempferol derivatives from the external leaves of tronchuda cabbage (*B. oleracea* L. var. *costata* DC) have been reported.[193]

Total flavonoid content in Chinese cabbage, broccoli, cauliflower, and white cabbage is 944 µg/g, 316 µg/g, 172 µg/g, and 102 µg/g, on a fresh weight basis, respectively.[92] The accumulation of derivatives of flavonols such as quercetin in *Camelina sativa*; quercetin and kaempferol in *Crambe hispanica* var. *glabrata*; quercetin, kaempferol, and isorhamnetin in *Brassica napus*; kaempferol and isorhamnetin in *Sinapis alba* is reported.[190] The constitutive flavonoids of *B. napus*, isorhamnetin-3-sophoroside-7-glucoside and kaempferol-3,7-diglucoside, are effective deterrents of armyworm.[44] Analysis of *B. alba* extracts revealed the presence of 3,5,6,7,8-pentahydroxy-4-methoxy flavone in shoots, as well as 2,3,4,5,6-pentahydroxy chalcone and 3,5,6,7,8-pentahydroxy flavone in roots and root exudates. Apigenin was also found in the shoots and roots.[194]

Anthocyanins are potent antioxidants and consequently may be chemoprotective.[195] Brassicaceae plants provide a variety of anthocyanins. Cauliflower and red cabbage showed differences in their anthocyanin profiles: cyanidin-3,5-diglucoside was absent in cauliflower, while it was well represented in red cabbage, together with the characteristic anthocyanin of *Brassica* genus, cyanidin-3-sophoroside-5-glucoside. The *p*-coumaroyl and feruloyl esterified forms of cyanidin-3-sophoroside-5-glucoside were predominant in cauliflower, while the sinapoyl ester was mostly present in red cabbage.[196] Red pigmentation of red cabbage is caused by anthocyanins. Red cabbage contains more than 15 different anthocyanins which are acylglycosides of cyaniding.[197] Red radish (*Raphanus sativus* L.) contains significant amounts of anthocyanins of which 12 acylated anthocyanins were isolated and analyzed spectroscopically to determine their structure. Six of these were identified as anthocyanin glycosides with 1 or 2 phenylpropanoids.[198] Total proanthocyanidins content in brocolli was found to be 12 µg/g and 7 µg/g in cauliflower, calculated over fresh weight. [92]

Five lignans, 5 neolignans, 2 sesquilignans, and 1 dilignan were identified in a phytotoxic extract of *Brassica fruticulosa*.[199] These compounds exhibited interesting antimicrobial, antifungal, and/or herbicidal activities that are believed to participate in plant defence mechanisms.[200] These compounds also have cancer-preventive effects.[65]

Tannins have an adverse effect on the nutritive value of rapeseed meal proteins or isolated proteins.[201] These compounds suppress the availability of essential amino acids[202] and may form complexes with

essential minerals, proteins, and carbohydrates.[203] Tannins have also a profound inhibitory effect on the digestion of carbohydrates and proteins in particular.[204] In Brassicaceae vegetables different amounts of tannins have been reported.[58] Inositol hexa-phosphate (phytic acid) and condensed tannins are reported in *B. carinata*,[78] both of which play an important role in iron binding.[203] Cabbage and turnip contain various amounts of phytic acid, tannic acid, and/or oxalic acid. Tannic acid was found at 12.66 mg/g (fresh weight basis) in cabbage. Levels of both tannic acid and phytic acid can be significantly ($p < 0.05$) reduced by different blanching methods.[205] The total amount of tannins in rapeseed/canola hulls ranged from 19.13 to 62.13 mg/g of oil-free hulls. Insoluble tannins predominated in canola/rapeseed hulls and comprised from 70 to 95.8% of total tannins present. The amounts of sodium–dodecyl-sulphate-extractable tannins were comparable to those of soluble tannins but constituted only 4.7–14.1% of insoluble tannins present.[206]

9 Glucosinolates

Sulfur-containing phytochemicals of 2 different types are present in *Brassica* (Cruciferae) vegetables (cabbage, broccoli, etc.): glucosinolates and *S*-methyl cysteine sulfoxide. Glucosinolates (**Figure 3**) are thioglucosides containing a cyano group and a sulfate group.[207]

Glucosinolates are derived from amino acid biosynthesis (**Figure 1**) and are important secondary metabolites in Brassicaceae family, involved in plant defence against pests and diseases.[207] For example glucoiberin, glucoraphanin, glucoalyssin, gluconapin, glucobrassicanapin, glucobrassicin, gluconasturtiin, and neoglucobrassicin are health promoting compounds found in broccoli inflorescences (*B. oleracea* L., var. *italica*, cv. Marathon).[55]

These compounds have both positive and negative nutritional effects,[208] appearing to possess anticarcinogenic properties, but also quite different toxic effects.[10] The effects of specific glucosinolate degradation products on individual organisms vary and are not always known. If used in excessive quantity, many of these compounds can be highly toxic.[209]

Glucosinolates and their concentrations vary among the different groups of Brassicaceae (**Table 4**).[210] In Brussels sprouts, cabbage, cauliflower, and kale, the predominant glucosinolates were found to be sinigrin and glucobrassicin. Brussels sprouts also had significant amounts of gluconapin.[211] The predominant glucosinolates in broccoli are 4-methylsulfinylbutyl glucosinolate (glucoraphanin),[212] 3-butenyl

glucosinolate (gluconapin), and 3-indolylmethyl glucosinolate (glucobrassicin).[211] Cruciferous vegetables of the *Brassica* genus (for example, Brussels sprouts, cauliflower, and broccoli) contain high levels of an indolylmethyl glucosinolate commonly known as glucobrassicin.[213] A great number of glucosinolates have been identified in *B. oleracea* var. *capitata f. alba*, namely glucoiberin, progoitrin, epiprogoitrin, sinigrin, glucrafanin, gluconapoleiferin, glucoalysin, gluconapin, 4-hydroxybrassicin, glucobrassicanapin, glucobrassicin, gluconasturein, methoxyglucobrassicin, and neoglucobrassicin.[49]

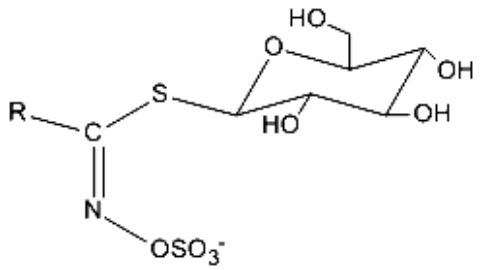

Figure 3 – Basic structure of glucosinolates

The major glucosinolates detected in different varieties of *B. oleracea* were 2-propenyl, 3-methyl-sulphinylpropyl and indol-3-yl-methyl, which accounted for an average of 35, 25, and 29%, respectively of the total glucosinolate content, while in *B. rapa*, but-3-enyl represented 86% of the total, with pent-4-enyl and 2-phenylethyl as the other major glucosinolates. The average total glucosinolate content of the flower buds was determined to be 2518 μmol 100 g^{-1} dry wt. in troncha (*B. oleracea* var. *tronchuda*) and 4979 μmol 100 g^{-1} dry wt in nabo (*B. rapa*), which is much higher than the highest amounts reported for broccoli (*B. oleracea* var. *italica*).[214] As in other Brassicaceae seeds and plants, rapeseed contains up to 5% of glucosinolates, which are partially decomposed during rapeseed processing or storage.

When plant material is crushed, as in food preparation or chewing, a thioglucosidase–mediated autolytic process is initiated, generating indole-3-carbinol, glucose, and thiocyanate.[215] These, together with other important degradation products, such as isothiocyanates, vinyl-oxazolidinethione, and nitriles, contaminate the crude rapeseed oils, impairing their hydrogenation and transesterification and ultimately may be harmful to human consumption.[216]

The main glucosinolate breakdown products of *Brassica* vegetables are the sinigrin breakdown product 1-cyano-2,3-epithiopropane, the gluconapin hydrolysis product 3-butenyl isothiocyanate, the glucobrassicin metabolite ascorbigen and low concentrations of other indole glucosinolate-derived hydrolysis products such as neoascorbigen and 3,3`-di-indolylmethane.[217] Rapeseed meal, a by-product of rapeseed oil production, also contains glucosinolates which together with phytic acid contribute to its anti-nutritional properties.[218, 219]

Table 4 – Variation of glucosinolate contents (μg/g) among different Brassicaceae vegetables on dry weight basis

	Cabbage	Broccoli	Brussels sprouts	Cauliflower	Kale
Glucoiberin	2289 ± 380 [a]	697 ± 127 [a]	42 ± 84 [c]	–	3455 ± 591 [d]
Glucoraphanin	17 [a]	3208 ± 528 [a]	3099 [c]	218 ± 131 [c]	1361 [b]
Progoitrin	452 ± 20 [a]	1017 ± 68 [a]	2922 [c]	120 ± 40 [c]	524 [b]
Gluconapin	472 ± 26 [a]	96 ± 37 [a]	4654 [c]	111 ± 74 [c]	372 ± 37 [c]
Sinigrin	3443 ± 939 [a]	35 ± 143 [c]	3261 [c]	3332 ± 36 [c]	3400 ± 322 [d]
Glucoalysin	–	90 ± 45 [c]	90 ± 45 [c]	–	–
Glucoerucin	–	–	–	–	1206 [b]
Glucobrassicin	1315 ± 13 [a]	1566 ± 130 [a]	1431 ± 89 [c]	715 ± 716 [c]	353 ± 1029 [c]
Neoglucobrass-icin	38 ± 19 [a]	458 ± 29 [a]	95 ± 48 [c]	95 ± 95 [c]	353 [d]
4-Methoxyglu-cobrassicin	214 ± 24 [a]	124 ± 5 [a]	–	–	–

a = [221]; b = [222]; c = [211]; d = [223].

* Calculation is made by conversion of μmol to μg on dry weight basis.

Goitrin, a naturally occurring compound in cruciferous vegetables, can easily be nitrosated if in contact with nitrites in gastrointestinal conditions, yielding the mutagenic compound N-nitroso-oxazolidone, with loss of sulphur.[220] Additionally, goitrin which is a decomposition product of progoitrin (**Figure 4**) is known to be strongly goitrogenic, inhibiting the synthesis of thyroid hormones, thyroxine, and tri-iodine-thyronine by a selective binding of iodine which prevents iodine intake by the thyroid gland.[63]

The other decomposition products of glucosinolates, as mentioned before, are thiocyanates, isothiocyanates and oxazolidine-2-thiones (**Figure 5**),[50, 224] and have also been shown to be goitrogenic. The benzyl-, phenethyl-, allyl-isothiocyanate, and sulforaphane are formed through the hydrolysis of their naturally occurring precursor glucosinolates, glucotropaelin, gluconasturtiin, sinigrin and glucoraphanin, respectively, by myrosinase.[225]

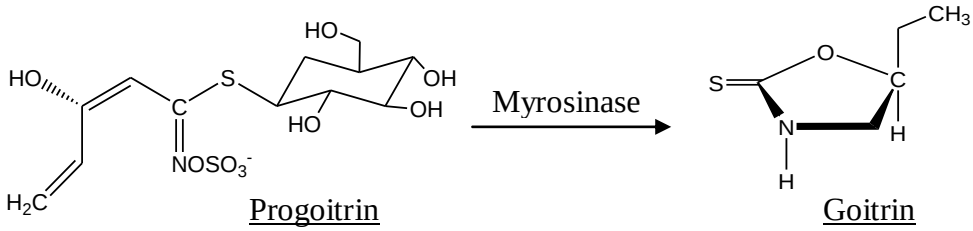

Figure 4 – Conversion of progoitrin to goitrin by myrosinase

However, under certain conditions, the glucosinolate aglycones may yield a nitrile rather than an isothiocyanate. Nitriles such as *S*-l-cyano-2-hydroxy-3-butene and 1-cyano-2-hydroxy-3,4-epithiobutane, are the most toxic of the normal glucosinolate hydrolysis products, with a human lethal dose of 170 and 178 mg/kg, respectively.[218] These negative effects of glucosinolates have led to research directed at finding methods to reduce the glucosinolate content in the seeds of some *Brassica* crops.[47] Other processes intended to avoid toxicity of the meal include heat treatment of the seeds prior to removal of the oil.

This inactivates myrosinase and subsequent breakdown of glucosinolates when the meal is consumed.[218] High or low glucosinolate contents of the seed of some varieties of *B. napus* correlate positively with glucosinolate levels in the roots, at least during the early stages of in vitro plant development.[43]

Glucosinolates are also responsible for the bitter acidic flavours of *Brassicacea* species[76] and the hydrolysis by-products of glucosinolates mentioned above, such as isothiocyanates, nitriles, and thiocyanates, are responsible for the hot and pungent taste of the mustard that is often objected to by consumers.[207] Many of these degradation products are volatile[226] and also play an important role in the characteristic aroma or off-odour of Brassicacae.[84] A great deal of research has been carried out on the volatiles of these species.

Cruciferous vegetables, for example, have been reported to contain substantial quantities of isothiocyanates.[227] Volatiles and semi-volatiles from *B. oleracea* L. var. *botrytis* seeds were identified as cyanides such as 4-(methylthio) butyl-cyanide, 3-(methylthio) propyl cyanide, and isothiocyanates such as 4-(methylthio) butyl-isothiocyanate.[228] In *B. rapa* var. *perviridis*, 6 isothiocyanates were detected in the steam volatiles and identified as sec-butylisothiocyanate, 3-butenylisothiocyanate, 4-pentenylisothiocyanate, benzyl-isothiocyanate, 2-phenylethylisothiocyanate, and 5-methylthiopentylisothiocyanate. Three nitriles were also detected and identified as 2-methyl-5-hexenenitrile, 3-phenylpropiononitrile, and 6-methylthiohexanonitrile.[84] In *Brassica oleracea* var. *Botrytis* 35 volatile and semi-volatile constituents were detected.[228]

Dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, hexanal, 3-*cis*-hexen-1-ol, nonanal, ethanol,[228, 229] and hex-3(Z)-enol were identified as major constituents representing, respectively, 30.2, 24.2, and 21.7% of the volatiles.[228] Various interesting bioactivities have also been reported for hydrolysis and breakdown products of glucosinolates,[43, 230] such as strong bactericidal, antifungal properties,[218, 231, 232] and health promoting effects for plants and humans.[47, 224] Some of these glucosinolates have a potential application in the industry, for example, an aqueous extract of *B. nigra* seeds might be included in industrial biofilms as an antimicrobial agent.[233] The breakdown products of glucosinolates assist in the activity of important naturally occurring, direct- acting antioxidants such as tocopherols and also enhance the synthesis of glutathione, one of the most abundant intracellular direct antioxidants.[234] Working on rapeseed oil cake (*Brassica campestris* L. *subsp. napus*), different antioxidant compounds (indolacetonitrile, S-1-methoxy-1-(3,5-dimethoxy-4-hydroxyphenyl) ethane, 4-hydroxy-indol-acetonitrile, and 4-hydroxy-phenyl-acetonitrile) were isolated, which showed a strong antioxidant activity as evaluated by the ferric thiocyanate method.[235]

Certain glucosinolates, particularly the isothiocyanates and nitriles, have been shown to modify both xenobiotic metabolizing enzymes and induce cell cycle arrest and apoptosis. It is likely that a combination of these responses explains the chemo-preventive characteristics of *Brassica* and that a combination of different cruciferous vegetables could provide optimal protection.[225, 236] The isothiocyanate chemopreventive activity could be due to its powerful inhibition of different enzymes such as glutathione *S*-transferases (GSTs) in

humans.[237, 238] Another potential cancer-blocking action, which was described for both intact and thioglucoside glucohydrolase-treated glucosinolates, as assessed by induction of GSTs activity, was found to be dependent on the nature of the side chain of the parent glucosinolate.[239]

Another naturally occurring isothiocyanate, sulforaphane, that is present in *Brassica* vegetables has been shown to block the formation of tumours[240] and together with 7-methylsulfinyl-heptyl isothiocyanates in broccoli (*B. oleracea* var. *italica*) extract exhibited an inhibitory effect on cancer cell invasion and matrix metallo-proteinase-9 activity in human breast cancer cells[213] and lowers the probability of acquiring colon and rectal cancers.[241] It was also proved to inhibit *Helicobacter pylori* infection, blocking gastric tumour formation. This suggests that broccoli consumption could prevent chronic atrophic gastritis induced by *H. pylori* infection and, thus, this type of stomach cancer.[242] Naturally, the wide range of glucosinolates content among different groups of *B. oleracea* would result in significant differences in their health-promoting properties.[211]

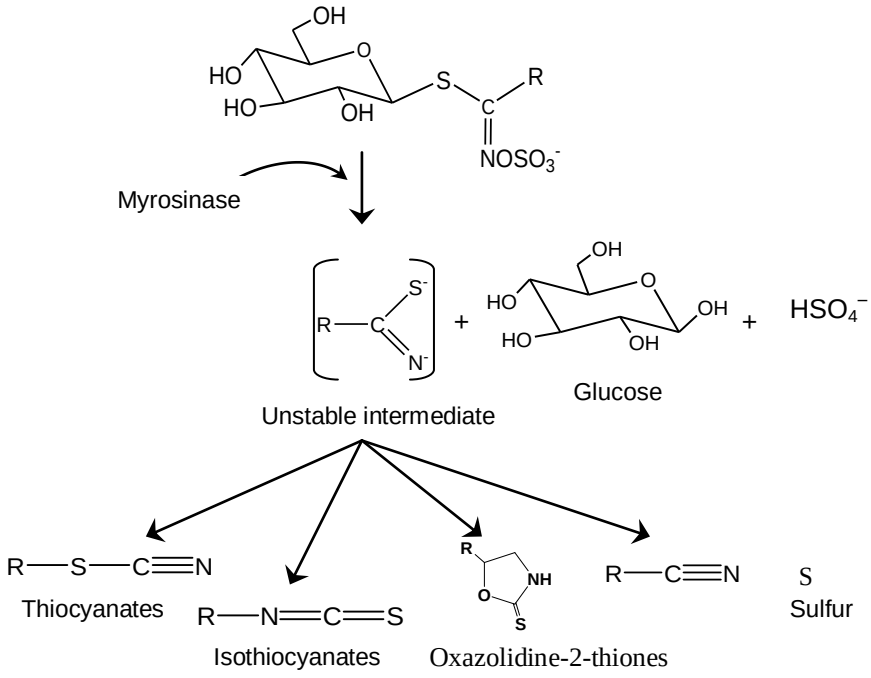

Figure 5 – Glucosinolate degradation

Indole-3-carbinol (I-3-C) is another glucosinolate breakdown product found in vegetables of the *Brassica* genus (cabbage, broccoli sprouts, Brussels sprouts, cauliflower, bok choy, and kale). Some research points to this compound as a promising anticancer agent against prostate cancer and reducing the incidence and multiplicity of mammary tumours.[243, 244] Coinciding with these studies, oral administration of I-3-C has been shown to have a possible beneficial effect on estrogen metabolism in humans and epidemiological studies support the claim that high intakes of I-3-C may have a broad chemo-preventive effect.[245] Conversely, [5,6,11,12,17,18]-hexahydrocyclonona-[1,2-b:4,5-b:7,8-b]-tri-indole (CTr), a major digestive product of indole-3-carbinol, has been proved to exhibit strong estrogenic activities increasing proliferation of estrogen-dependent breast tumour cells. Thus, the contribution of CTr to the cancer preventive or cancer-promoting effects of I-3-C remains to be established.[246] In plants, levels of secondary metabolites, such as glucosinolates are controlled by a number of factors. Although it is possible to increase levels of glucosinolates in plants by genetic manipulation, in order to enhance a particular pharmacological benefit, such a step would be premature and must await a more thorough understanding of the extremely complex interactions of these compounds and their metabolites.[50]

10 Conclusion

Brassica vegetables represent a major part of the human diet all over the world providing nutritionally significant constituents, such as phenolic compounds, vitamins, fibres, soluble sugars, minerals, fat, and carotenoids. Cruciferous vegetables are a source of some very promising chemopreventive dietary constituents, which may protect against free radical damage and LDL oxidation implicated in the pathogenesis of cardiovascular diseases, as well as DNA damage and cancer. This might be useful information from the point of view of identifying appropriate raw materials, rich in these protective components, for the development of safe food products and additives with appropriate antioxidant properties. As mentioned above, *Brassica* plants are rich in many metals including calcium and iron-containing compounds. However, there is substantial variation both within and between subspecies, which suggest, a difference in potential health benefits depending on genotype, as well as on the growth conditions and environment. This review provides a

massive body of evidence supporting the nutritional value of *Brassica* vegetables and should ultimately lead the population to better food choices.

11 Future Perspectives

Many anti-cancer agents are of plant origin, but their actual function or the mechanism behind the role they play in the plant has not yet been fully elucidated. For example, plant-derived molecules with known roles in plant cell death may be novel candidates for use in clinical oncology. But a better understanding of the molecular and cellular mechanism of action of such compounds and their structure-activity relationships is necessary for the development of new derivatives of these molecules with more favourable chemopreventive activities. Different classes of anti-cancer compounds merit continued research at a basic and pharmacological level in order to yield novel chemotherapeutic agents. However, in order to correctly evaluate the effect of such compounds in food, it is necessary to bear in mind that some constituents such as phenolic acids, tannins, and other anti-nutritional compounds may form complexes with nutritionally important compounds, reducing their bioavailability and thus lowering the nutritional value of *Brassica* products. Additional studies are needed to determine the amount of isothiocyanates or their metabolites that reach target tissues, and the concentration needed to exert biological effects.[225] Further elucidation of the protective mechanisms of food and the identification of active constituents is needed.

Enhancing the phytonutrient content of plant foods through selective breeding or genetic improvement is a powerful tool for dietary disease prevention. However, most, if not all, of these bioactive compounds confer a bitter, acid, or astringent taste to the food which is rejected by most consumers. Moreover, in the past, some of these compounds have even viewed as plant-based toxins and, as a result, the food industry routinely removes these compounds from plant foods through selective breeding and a variety of debittering processes. This poses a dilemma for the designers of functional foods because increasing the content of bitter phytonutrients for health may clash with consumer choices. Studies on phytonutrients and health, taking sensory factors and food preferences into account, constitute an important area of research.

Another aspect of these valuable *Brassica* vegetables that deserves full attention is the edaphic conditions in which they are grown.

These plants can be biofortified by growing them in a high mineral-containing medium, attaining high levels of nutritionally important minerals that can be used to produce dietary supplements. But this advantage which is due to their metal tolerance (and allows their use for phytoremediation as previously explained) can be negative as observed in crops that are irrigated with polluting metals. The excessive heavy metals (macro or micro nutrients in excess) and plant and human pathogenic microbes concentrated in the soil from this water cause stress conditions for plants. Quality parameters of *Brassicacea* vegetables are very susceptible to great changes with these stress conditions which produce different effects on the levels of *Brassica* vegetables metabolites, affecting their flavour and leading to the changes in nutritional value. Studies are needed to clarify the route of exposure, mechanisms of sensitization, and clinical importance of these phenomena.

Another question about cruciferous vegetables is their flavonoid content. Epidemiological data indicate that the present rate of consumption of these vegetables is beneficial. However, earlier studies also raised the question on the advantages of recommending an increased consumption of *Brassica* vegetables and/or phytochemical supplements. One of the reasons for this lies in the flavonoid content of these vegetables which, as explained above, is quite high in some of the species. Unfortunately, the potentially toxic effects of excessive flavonoid intake are still largely ignored. It is known that at high doses, flavonoids may act as mutagens, that is, pro-oxidants that generate free radicals, so that their adverse effects may well outweigh their beneficial ones. It is imperative that further research be conducted to learn more about the toxicological properties of flavonoids, apart from other putative health promoting compounds in *Brassica* vegetables, thus clarifying the balance of potential adverse and beneficial effects included in their mechanisms of action.

Chapter 3

Healthy and unhealthy plants: The effect of stress on the metabolism of Brassicaceae

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Abstract

Brassicaceae plants are one of the most popular vegetables consumed all over the world and considered to be a good source of bioactive phytochemicals. Additionally, *Brassica* species and varieties are increasingly becoming a research model in plant science, as a consequence of the importance of their primary and secondary metabolites. Plant interaction with environmental stress factors including animals and insects herbivory, pathogens, metal ions, light, among others, is known to lead to the activation of various defence mechanisms resulting in a qualitative and/or quantitative change in plant metabolite production. Pre-harvest and/or post-harvest conditions are also known to affect this, since plants produce signalling molecules (e.g. salicylic acid, jasmonic acid etc.), that cause a direct or indirect activation of metabolomic pathways. That ultimately affects the production of phytochemicals, such as carbohydrates (sucrose and glucose), amino acids, phenolics (phenylpropanoids and flavonoids) and glucosinolates. These phytochemicals have diverse applications due to their antimicrobial, antioxidant and anticarcinogenic properties, but on the other hand these compounds or their breakdown products can act as anti-nutritional factors in diet. In this review we report a wide range of stress induced metabolomic responses in *Brassica* plants commonly used for human consumption.

***Keywords*:** Brassicaceae, glucosinolates, metabolomics, primary and secondary metabolites, plant stress response.

1 Introduction

Brassicaceae vegetables, commonly known as Crucifers, include different genera of cabbage, broccoli, cauliflower, Brussels sprouts, kale, etc. which are consumed all over the world.[89] Genetic resemblance of *Brassica* to *Arabidopsis* has made it an alternative model system in plant science,[28] and increased the value of *Arabidopsis* plant research. Brassicaceae vegetables are a good source of antioxidants because of their high phenolics and glucosinolate content.[6, 27, 247] These compounds are generally considered to have a preventive role against cardiovascular diseases and different types of cancer[6, 211, 248, 249] but on the other hand the antinutritional effects of polyphenols, glucosinolates, *S*-methylcysteine sulfoxide, tannins and erucic acid, from Brassicaceae vegetables have also been previously reported.[250, 251]

Throughout the course of growth and development plants are ordinarily exposed to various environmental, biotic and abiotic factors,[18] to which they respond with an activation of their defence system.[252] This results in a substantial and significant variation in the plant metabolome, both within and between the subspecies.[27, 101, 253, 254] The inducing factors largely affect the primary and secondary metabolism of *Brassica* plants, resulting in the enhanced production of certain metabolites, e.g. amino acids, sugars, indoles, phenolics and glucosinolates[6, 255-257] (**Figure 1**).

In particular some biotic and abiotic elicitors can result in an enhancement of the specific secondary metabolite production.[19] Under these conditions a number of signal pathways can be pre-activated by salicylic acid (SA), jasmonic acid (JA), ethylene or abscisic acid pathways, which are generally involved in the defence responses.[18, 19, 247] As an example, due to aforementioned factors, plant cells activate the chorismate pathway (**Figure 2**) that also results in changes in the plant phenolics.[258-261]

Upon infestation by insects, plants can alter their resistance to pathogens in a complex manner (**Figure 1**).[247] For example, in a case study the presence of *Pieris brassicae* caterpillars, feeding of the lower leaves of Brussels sprouts (*Brassica oleracea* var. *gemmifera*) triggers the release of volatiles from upper leaves.[262] In *Arabidopsis*, a Brassicaceae plant, a defence reaction used by the plant against the necrotrophic fungal pathogen *Alternaria brassicicola* was ineffective but proved to avoid damage by two bacterial leaf pathogens (*Xanthomonas campestris* pv armoraciae and *Pseudomonas syringae* pv tomato). In addition the resistance was locally and systemically effective against

turnip crinkle virus (TCV) and was associated with priming for SA dependent defence responses.[247]

In general the metabolic responses of plants vary according to the type of stress. These responses can be rather specific since the metabolomic pool of plant defence is composed of a variety of constitutive and induced metabolites.[247, 263-265] The feature of the signalling is complex due to the simultaneous elicitation of several responses by the invading micro-organism.[145]

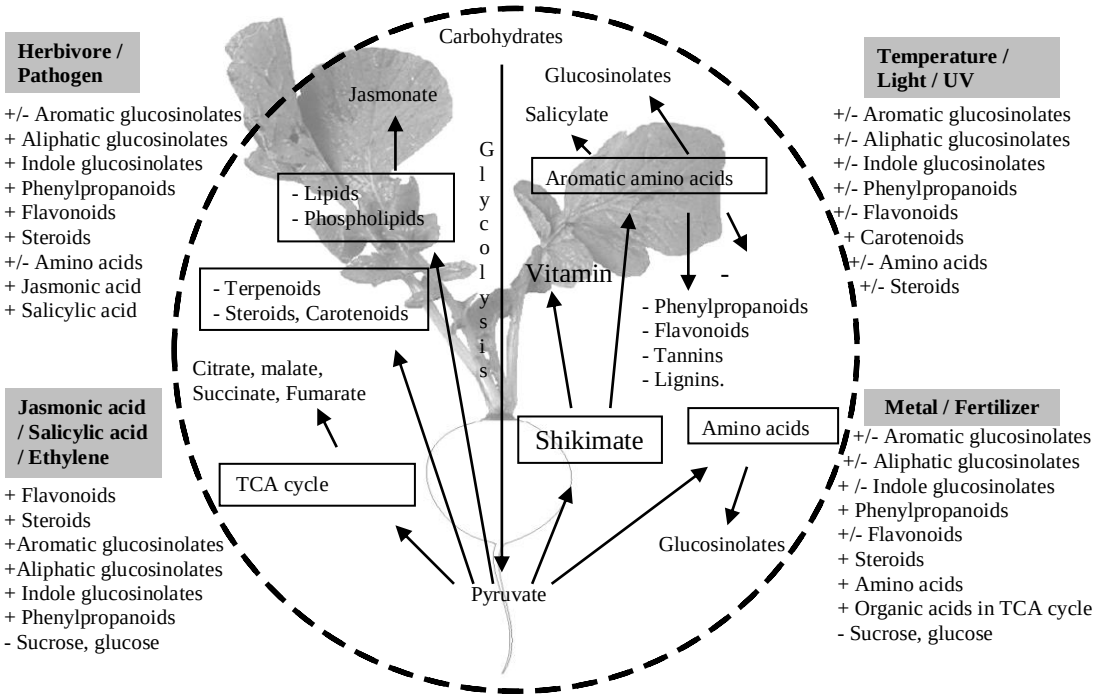

Figure 1 – Summary of the biosynthetic pathway and stress induced metabolite production. The basic metabolomic pathway is drawn in the circle and the stimuli and the compounds increased (+) or decreased (-) as a result of these are listed outside.

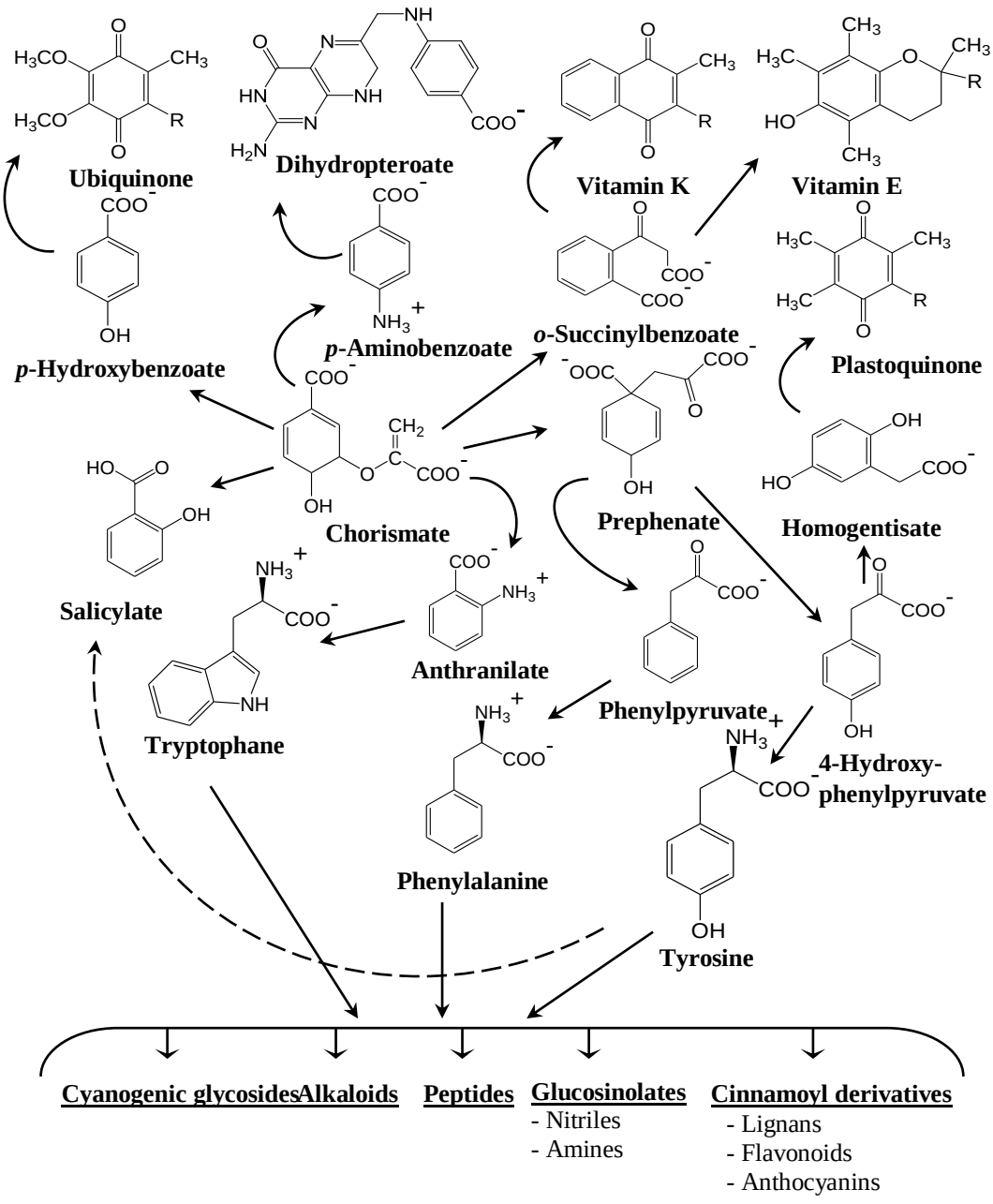

Figure 2 – Essential plant metabolites derived from chorismate.

A multitrophic interaction (**Figure 3**) could also occur, as observed in the case of a specialist parasitic wasp of *Pieris rapae* caterpillars, *Cotesia rubecula*, which is attracted to *P. rapae* caterpillar infested *Arabidopsis* plants, due to volatiles.

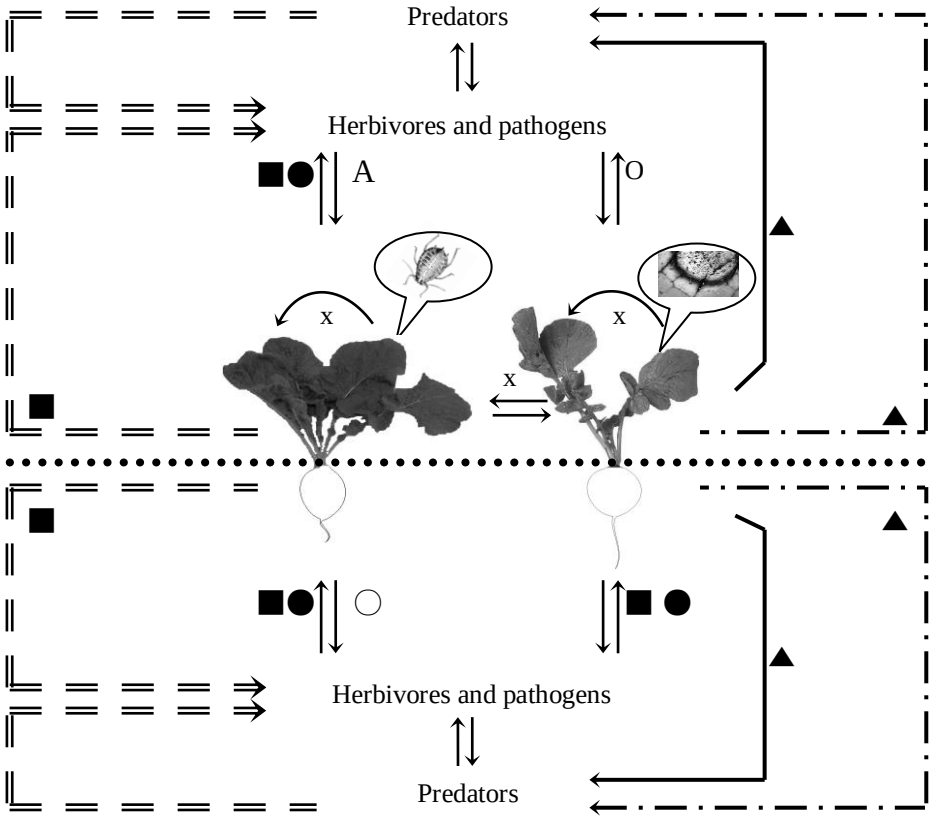

Figure 3 – Basic diagram of above and below ground stress related multitrophic interactions in *Brassica* spp. Direct interaction (▬); attraction behaviour (▬ ▪ ▬); repellence behaviour (═ ═); ground surface (• • •); (■) glucosinolates; (x) signalling molecules (jasmonic acid, salicylic acid and ethylene); (●) phenylpropanoids and flavonoids; (○) toxins and enzymes; (▲) terpenoids, nitriles and other leaf volatiles.

These volatiles are emitted as a defence against *P. rapae* attack and include metabolites from several major biosynthetic pathways, including terpenoids and green leaf volatiles.[266] Genomic information of *Arabidopsis thaliana*, and the availability of characterized mutants and transgenes, can be exploited to address functional aspects of inducible

direct and indirect responses to stress.[267] However, *Arabidopsis* is not used as a food source but have been used as a model plant. In these days, much research work is reported on the *Brassica* metabolomic alteration affected by environmental stress because *Brassica* has high genetic resemblance to *Arabidopsis* and even considered one of the most important food crops all over the world.

In this review we will focus on the interaction between the environmental factors and *Brassica* species, aiming at providing an overview of their reported metabolomic responses to pre-harvest or post-harvest stimuli.

2 Primary metabolites

2.1 Effect of biotic factors

Wild *Brassica* species in general have lower amino acid content than cultivated varieties due to diverse factors in nature,[268] as the plants are exposed to different stress conditions. Response to stress is coordinated by signalling systems induced by herbivore and/or pathogen attack.[269] It is evident, for example, that a decrease of the glucose, sucrose and amino acids levels are observed after methyl jasmonate (MeJA) elicitation of *Brassica rapa* leaves,[41] but an increase in amino acid levels after their interaction with food born pathogenic bacteria.[27, 270] Similarly, aphid infestation results in the increased production of primary metabolites including amino acids as well as some secondary metabolites.[268]

2.2 Effect of abiotic factors

Abiotic factors are also known to largely affect metabolite production. This was observed in different situations such as a characteristic linear increase of amino acids produced by drought stressed *Brassica napus* leaves, followed by a reduction in concentration upon rehydration of the plants.[271] Drought stress conditions have been proved to increase sugar contents in plants as in the case of cabbages, a high level of sugars was detected in water stressed seedlings when compared with control samples.[272]

Another abiotic factor, metal exposure, was seen to transiently increase photosynthetic pigments, proteins, free amino acids and sugar contents followed by a decrease when compared with controls.[273] Wild type *Arabidopsis* plants exposed to cadmium stress were found to

generate oxidative damage, finally resulting in the significant loss of chlorophyll content.[274] An increase in total free amino acids content in the leaves of *Brassica pekinensis* exposed to copper stress indicated that free amino acids play a role in the detoxification of the copper excess.[275] The amount of low molecular weight organic acids, which are reported to be chelating agents, increased after metal stress in *Brassica*.[276] Effective accumulation of metals (Cr, Fe, Zn, and Mn) also produced an increase of oil content up to 35% in *Brassica juncea* (cv. Rohini).[273]

Temperature also affects the metabolite content of plants. Carotenoids in Brassicaceae, including β-carotene, were found to be slightly decreased after thermal treatments.[277] Fresh and processed leaves of kale, which are normally a good source of amino acids, were reported to lose 12 – 14 % of amino acid contents as compared with control, when cooked using traditional methods or when frozen.[278] Boiling of kale, Brussels sprouts, broccoli and white cauliflower was also shown to cause a large decrease in the ascorbic acid content,[277, 279] while exposure of broccoli (*Brassica oleracea* var. *italica*) to UV light or temperature of 7 – 13 ^{o}C caused an increase in its content.[253, 280]

2.3 Effect of growth and storage

It is well known that the metabolomic profile of plants varies according to its growth stage as confirmed by the increase of ascorbic acid in three *Brassica* cultivars during the development of the inflorescence.[281] Even after harvesting the metabolomic changes still continue, e.g. broccoli undergoes major losses of sugars, organic acids, and proteins within the first 6 hours after harvest. This is followed by an increase in the free amino acid pools (especially glutamine and asparagine).[128, 254] Loss of membrane fatty acids is also a feature of post-harvest broccoli senescence.[254, 282] Storage of broccoli florets for 7 days in CO_2-containing atmosphere results in an increase in free amino acids and a decrease in protein amino acids, although the total amino acid content remained unchanged.[283]

3 Secondary metabolites

3.1 Glucosinolates

Glucosinolates are one of the most important groups of Brassicaceae metabolites derived from amino acid biosynthesis (e.g. methionine, tryptophan, pheylalanine etc.).[256, 284, 285] The flavour and

odour of *Brassica* vegetables are typically related to their glucosinolate content.[31, 286, 287] These are at least partly responsible for their benefits for human health including anti-carcinogenic, cholesterol-reducing, and other pharmacological effects[6, 288-291] but on the other hand the antinutritional effects of glucosinolates are also reported.[250]

Glucosinolates are well known to be related to the plant defence response mechanisms, being induced after wounding and/or pathogen attack [268, 292], insect herbivory,[255, 293, 294] exposure to salt stress,[295] diverse environmental factors,[231, 281] or by plant signalling molecules,[296, 297] including the treatment with SA, JA and MeJA.[298, 299] Following tissue damage endogenous plant enzymes (thioglucosidases or myrosinases) hydrolyze the glucosinolates to unstable aglycones, which rearrange to yield a variety of products including isothiocyanates, thiocyanates and nitriles. The nature of the products depends on the conditions of the hydrolysis and the particular glucosinolate.[284, 299-301]

Even under drought stress the secondary metabolism continues,[295] for example the total glucosinolate content of mature rapeseed is observed to increase following water deprivation.[265]

3.1.1 Effect of herbivory and pathogens

Induced defence responses are elicited when plants are exposed to different types of biotic stress such as attack by herbivores or pathogens.[247] The activity of glucosinolates and their degraded products against various strains of microorganisms has been documented by many investigators,[224, 302] being present in the leaves of *Brassica spp*. at concentrations that can prevent the development of pathogens. A report of the effect of the inoculation of *B. rapa* seedlings with the fungal pathogen *Alternaria brassicae*, described the catabolism of glucosinolates during the infection and subsequent release of isothiocyanates together with dimethyl disulphide, dimethyl trisulphide, and 4-oxoisphorone.[303] Unusually a high concentration of 2-hydroxy-3-butenyl glucosinolate found in leaves of *B. oleracea* var *capita* cv *offenham compacta*. It may account for the greater susceptibility of this cabbage cultivar to *Brevicoryne brassicae* (L) (cabbage aphid), compared with other *B. oleracea* accessions examined. It was concluded that an increased production of 4-pentenylglucosinolate with a commensurate reduction in the 2-hydroxy-3-butenyl and 2-propenyl glucosinolates, could provide cultivated *Brassica* crops with some partial resistance to *Brevicoryne brassicae*.[268]

There is ample evidence that glucosinolate structures and levels influence host plant suitability for generalist and specialist herbivores.[304] Monitoring of the total glucosinolate content in a resistant and a susceptible variety of cabbage (*B. compestris* ssp. *pekinesis*) during the development of club root disease, caused by *Plasmodiophora brassicae* revealed that their glucosinolates content differed significantly. The susceptible varieties showed a very high content of aliphatic glucosinolates while the resistant varieties showed high contents of aromatic glucosinolates.[305]

Levels of glucosinolates increase and their composition may be altered in response to herbivory and pathogen attack as shown in studies carried out on two wild *Brassica* species, *B. nigra* and *B. oleracea*, which were infested with larvae of the cabbage root fly, *Delia radicum*.[269, 305] The systemic response in the leaves differed between plant species. While in the case of *B. nigra*, shoot glucosinolate levels steadily increased during the growth period, almost duplicating the original concentration after 14 days of infestation, *B. oleracea* plants did not show any significant changes in shoot glucosinolate levels as compared with a control group of plants.[306]

Increased glucosinolate accumulation, primarily of short-chain aliphatic methylsulfinyl glucosinolates, in response to insect feeding (generalist *Myzus persicae* and specialist *Brevicoryne brassicae*) was also observed in *A. thaliana*.[269] Increased glucosinolate levels in *B. napus* and *Sinapis alba* reduced the extent of grazing by generalist herbivores but resulted in greater damage by the glucosinolate specialist beetle *Psylliodes chrysocephala* and butterfly *P. rapae*.[307] Differences in susceptibility to herbivores among *S. alba*, *B. napus*, and *B. campestris* have been attributed to their glucosinolate content, particularly 4-hydroxybenzyl glucosinolate.[308, 309] Mechanical wounding or feeding by the flea beetle (*Phyllotreta cruciferae*) was found to produce a three-fold increase in the concentration of indole glucosinolates in the cotyledons of one-week-old seedlings of the oilseed rape *B. napus*, *B. rapa* and *B. juncea*.[310] Similarly, another study reported that damage to the *B. napus* by *Psylliodes chrysocephala* induced systemic changes to the glucosinolate profile, most noticeably an increase in the concentration of indole glucosinolates.[311]

Aliphatic glucosinolate profiles have a significant impact on the development and performance of *Brevicoryne brassicae* and *Myzus persicae* on *A. thaliania* [312], *B. napus*[313] and oilseed rape.[314] Conversely, no changes in the glucosinolate content of oilseed rape plants following

infection with Turnip Mosaic Virus were reported.[315] Decrease in glucosinolate content was observed in healthy and *Alternaria brassicae* inoculated seedlings of *B. rapa*.[303]

Indole glucosinolates degradation increased in resistant varieties after infestation with downy mildew possibly due to their conversion into other biochemical compounds involved in the resistance. These indole glucosinolates could be involved in a complex metabolomic process, in which they are not considered solely as metabolomic end-products but also as precursors of other molecules, such as phytoalexins or auxins, known for their involvement in the resistance to microorganisms.[316] The results of a study carried out on two susceptible and three resistant varieties of cauliflower plants (*Brassica oleracea* var. *botrytis*) infected with *Peronospora parasitica* to determine the correlation between glucosinolates and resistance to this microorganism showed that sinigrin content was higher in the resistant varieties than in susceptible ones and that if glucobrassicin decreased, methoxyglucobrassicin increased.[316] It is important to note that infection by a virulent bacterial pathogen was proved to induce the expression of genes responsible for indole glucosinolate biosynthesis.[284]

3.1.2 Effect of signalling molecules

It is evident that the presence of signalling molecules affects the glucosinolate profile of these species. In the case of *A. thaliana*, its responses to different stresses are coordinated by several interacting signalling systems including JA, SA and ethylene (ET) mediated pathways[269] as shown in the infection with the fungus *Alternaria brassicicola*.[317] Plant growth-promoting bacteria produce this effect through several mechanisms, including the synthesis of indoleacetic acid (IAA).[318] It was also observed that in *Arabidopsis*, the rhizobacteria-induced systemic resistance is phenotypically similar to pathogen-induced systemic acquired resistance (SAR),[26] but functions independently of SA and requires responsiveness to JA and ethylene.[319]

The concentration of gluconasturtiin was specifically increased by SA.[320] *Brassica napus* plants exposed to MeJA accumulated indole glucosinolates in their leaves, the amount of which depended on the concentration of MeJA applied.[321] Moreover, an increase in glucosinolate levels, especially indole glucosinolates, was observed in *B. rapa* leaves after MeJA elicitation.[41] The treatment of a resistant and a susceptible variety of Chinese cabbage (*B. compestris ssp. pekinesis*) with SA or JA, produced an increase in the total amount of glucosinolates, though the

response depended on the type of treatment applied. JA induced indole glucosinolates production only in the leaves, while SA induced indole glucosinolates in both leaves and roots of the cabbage.[305] However, as a result of a negative cross-talk, SA has been observed to inhibit JA induced resistance of *Arabidopsis* to *Spodoptera exigua* by inhibiting production of glucosinolates by JA.[247]

Even more conclusive results were obtained from a study carried out on the glucosinolate content of leaves and cotyledons of *B. napus*, *B. rapa* and *B. juncea*, that revealed the content of glucobrassicin and 4-hydroxyglucobrassicin increased up to 20-fold after treatment of leaves and cotyledons with JA or MeJA.[298] In contrast, treatment with abscisic acid resulted in low levels of indole glucosinolates in *B. napus*.[322]

3.1.3 Effect of fertilizers and salts

The accumulation of indole, aliphatic and aromatic glucosinolates could be enhanced by the presence of low nitrogen and high sulfur fertilizers. For example, the use of a sulfur fertilizer produced an increase in the level of glucosinolates, gluconapin, sinigrin and progoitrin.[290, 323] Similarly, the total glucosinolate level was also observed to increase in response to sulfur availability in turnip rape (*B. rapa*)[324] and kale (*B. oleracea* L. Acephala Group),[76] while three broccoli cultivars showed an increase in total glucosinolate content, only at the start of the inflorescence development followed by a rapid decrease depending on its fertilization with sulphur.[281] Another fertilizer, selenium, was observed to increase glucosinolates in general and sulforaphane in particular, when applied up to a certain doses, above which it decreased glucosinolate production.[325] Submitting broccoli to salt stress increased their glucosinolate content, indicating the involvement of these compounds in its stress response.[295] Some exceptions are also reported, as in the case of cadmium stress which produced no change in glucosinolate production in *B. rapa*.[3]

3.1.4 Effect of temperature and radiation

There is a relationship between temperature, radiation and glucosinolate content, though the synthesis of each individual glucosinolate is affected differently by each of these factors.[253, 326] Glucosinolate concentration in canola increased when submitted to a temperature stress of 40 ^{o}C for 15 days during growth.[327] A seasonal variation in aliphatic, indole and aromatic glucosinolate content was observed in three different varieties of *B. oleracea*.[223] The same effect

was observed in broccoli, in which the aliphatic glucosinolates (especially glucoraphanin) content was observed to increase when kept at daily mean temperatures between 7–13 ^{o}C combined with the daily mean radiation of 10–13 mol/m^2/day.[253] The effect of radiation can depend on the species as in the case of *N. officinale* and *S. alba*, in which different aliphatic and indole glucosinolates had been identified. The concentration of these glucosinolates was significantly more affected by UV treatment in *N. officinale* than *S. alba*.[328]

3.1.5 Effect of post-harvest storage conditions

Bioavailability of glucosinolates and related isothiocyanates of *Brassica* vegetables is influenced by storage[289] and processing, such as blanching and freezing, affecting the taste and aroma of final products.[329, 330] Postharvest physical disruption of the plants (e.g. chewing, chopping, blending, juicing, cooking, freezing / thawing and high temperature) leads to the loss of cellular compartmentalization and subsequent contact of glucosinolates with myrosinase to be degraded to isothiocyanates,[231] nitriles, thiocyanates, epithionitriles and oxazolidines[331] (**Figure 4**).

When stored at ambient temperature (12 – 22 ^{o}C), there was no significant loss in glucosinolate content. The contents of individual and total glucosinolates decreased in *Brassica* vegetables (broccoli, Brussels sprouts, cauliflower and green cabbage) when stored in a domestic refrigerator (4 – 8 ^{o}C) for 7-days, though some of them, i.e., glucoiberin, glucoraphanin and glucoalyssin, suffered higher losses than sinigrin, gluconapin and progoitrin.[289] The glucosinolates were the most affected constituents in rocket (*Eruca vesicaria* ssp. *sativa*) leaves as the content was reduced to 4 – 33%, when samples were stored in air while the decrease was 60 – 100% in low O_2 and high CO_2 conditions, after using sanitizers (chlorine, ozonated water, lactic acid, acidified sodium chlorite and peroxyacetic acid), in both cases.[287]

Significant loss of glucoerucin and glucoraphanin was observed in rocket during storage at 4 – 8 ^{o}C.[332] Storage of vegetables at –85 ^{o}C could cause significant loss of glucosinolates due to freeze–thaw fracture of plant cells, leading to enzymatic conversion of glucosinolates to isothiocyanates during thawing.[289] Also a significant decline in glucosinolates content was observed during storage of *Brassica* vegetables at 4 ^{o}C and at 20 ^{o}C.[333] On the other hand, indole and aliphatic glucosinolates in broccoli increased during storage period of 7 days at 7 – 13 ^{o}C.[253, 334]

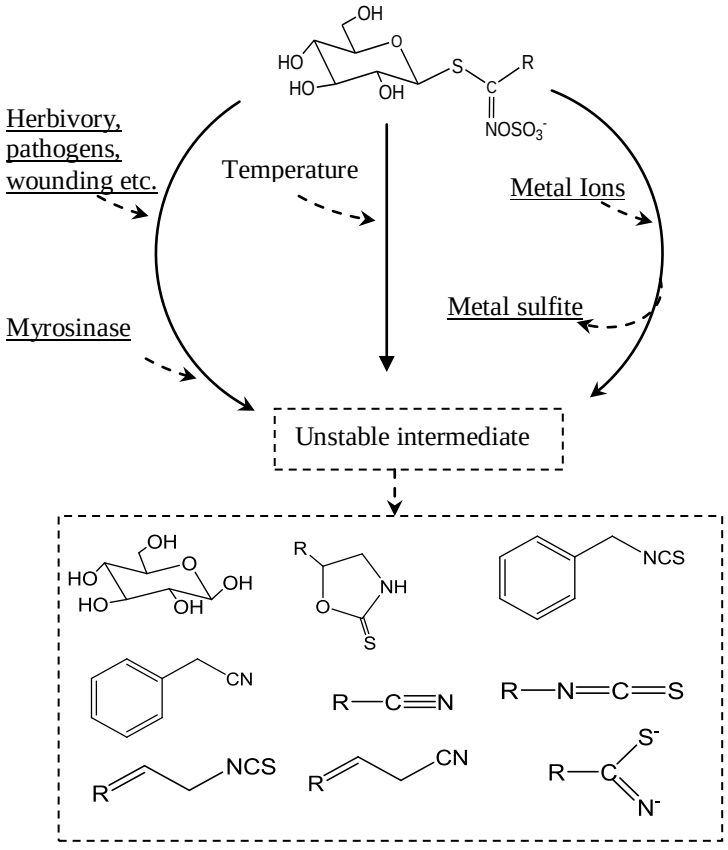

Figure 4 – Glucosinolate degradation.

3.2 Tryptophan derived phytoalexins

Phytoalexin synthesis, as a defence response of Brassicaceae plants, is induced by a number of molecular species, which can function as signal molecules including poly- and oligosaccharides, proteins, polypeptides, fatty acids,[145] and jasmonate among others.[41] Brassicaceae phytoalexins are generally biogenetically derived from tryptophan but have also different chemical structures (**Figure 5**) as well as biological activities.[335] Brassinin, a plant defence phytoalexin with antimicrobial activity, is produced by a variety of *Brassica* species in response to stress.[336, 337] Camalexin, another phytoalexin, was found in highest concentrations in or around the dead cells where bacterial growth is restricted.[338]

3.2.1 Effect of herbivory and pathogens

The differing qualitative and/or quantitative profiles in phytoalexin production are associated to the resistance of different *Brassica* species against diverse fungal attacks. *Camelina sativa* and *Capsella bursa-pastoris* exhibited strong resistance to *Alternaria brassicae* and *Brassica campestris* ssp. *rapifera* was less resistant to *A. brassicae* [339]. Another species, *Arabis lyrata* produced camalexin during its interaction with two microorganisms, *Pseudomonas syringae* pv. *Maculicola* and *Cochliobolus carbonum*[340] and the induction of two phytoalexins, wasalexin A and arvelexin (4-methoxyindolyl-3-acetonitrile) were observed after *Leptosphaeria maculans* attack on *Thlaspi arvense*[341] Further examples were observed in leaf and stem tissues of *B. napus* which accumulated two phytoalexins (methoxybrassinin and cyclobrassinin), following inoculation with *L. maculans*.[342] While *Alternaria brassicae* induced sinalexin production in *S. alba*.[343] Three phytoalexins, indole-3-acetonitrile, arvelexin, and 1-methoxyspirobrassinin, were identified in *Erucastrum gallicum* leaves after infection by *Sclerotinia sclerotiorum*[344] and *B. napus* ssp. *rapifera* produced isalexin, brassicanate A, and rutalexin, brassinin, 1-methoxybrassinin, spirobrassinin, brassicanal A and brassilexin were elicited with the phytopathogenic fungus *Rhizoctonia solani*.[345]

Canola and rapeseed (*B. rapa*) also accumulated diverse phytoalexins after inoculation with different strains of the biotroph *Albugo candida*.[263] It is important to note that most of the phytoalexins exhibit antifungal activity against the economically important pathogenic fungi *Leptosphaeria maculans*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*.[346] However, some phytopathogenic fungi may have enzymes that can detoxify the phytoanticipins or phytoalexins produced by their host.[337, 347]

3.2.2 Effect of abiotic factors

It has been proved that both biotic and abiotic elicitation can provoke a response in many *Brassica* species consisting in the production of diverse phytoalexins (**Figure 5**). This effect was observed in the leaves of the oilseed, canola and rapeseed (*B. rapa*) which accumulated the phytoalexins spirobrassinin, cyclobrassinin, rutalexin, rapalexin A and B, brassinin, brassilexin and brassianal C, apart from the phytoanticipins, indolyl-3-acetonitrile, caulilexin C, and arvelexin, after spraying with copper chloride.[263, 341]

In Brassicaceae, $CuCl_2$ has shown to be an effective inducer of phytoalexin production[343, 348] as in the case of *Thlaspi arvense* in which the production of two phytoalexins, wasalexin A and arvelexin (4-methoxyindolyl-3-acetonitrile) was detected after exposure to this abiotic elicitor.[341] Another report described the concentration of spirobrassinin to be 4 fold of that observed in compatible interactions, with the highest concentration found 2 days after spraying with copper chloride.[263]

The effect of another type of abiotic stress, UV light, was described for cauliflower (*Brassica oleracea* var. *botrytis*) florets in which the production of several phytoalexins, i.e., isalexin, (S)-spirobrassinin, 1-methoxybrassitin, brassicanal C, caulilexins A, B and C was enhanced.[346] UV light also induced isalexin, brassicanate A, and rutalexin, brassinin, 1-methoxybrassinin, spirobrassinin, brassicanal A and brassilexin synthesis in *B. napus* ssp. *Rapifera*.[345]

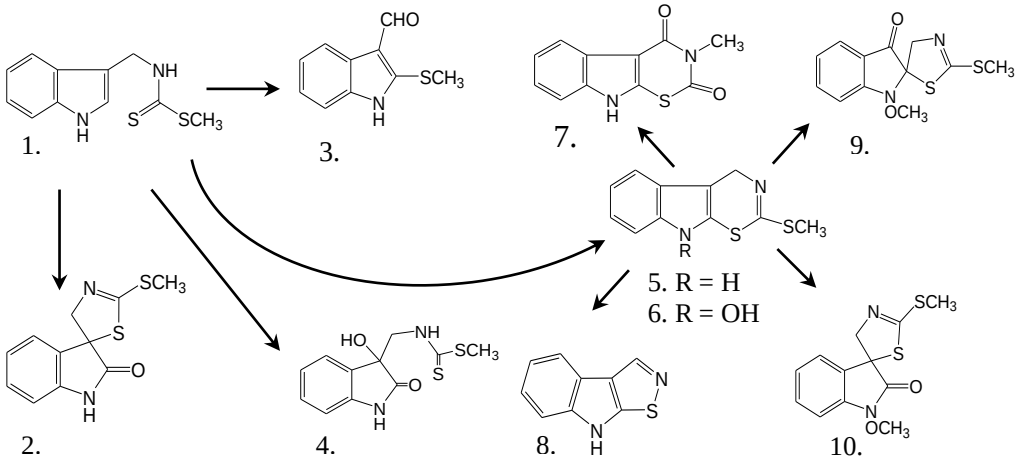

Figure 5 – Biosynthetic pathways of *Brassica* phytoalexins: Brassinin (1), spirobrassinin (2), brassicanal A (3), dioxibrassinin (4), cyclobrassinin (5), sinalbin (6), rutalexin (7), brassilexin (8), erucalexin (9), (*R*)-1-methoxyspirobrassinin (10).

3.3 Phenolics

Brassicaceae vegetables are consumed both raw and processed.[349] The content of polyphenols can be influenced by various factors such as the variety, climatic conditions, biotic and abiotic stress caused by the preharvest and postharvest conditions.[73, 89, 350, 351]

3.3.1 Effect of herbivory and pathogens

It has been proposed that phenolics play an antioxidative role in the plant defence system as a backup to the primary ascorbate-dependent detoxification system.[13] This is supported by observations such as the rapid accumulation of phenolics detected in a significant proportion of the *Arabidopsis* cells undergoing a hypersensitive response.[338] In the case of microbial infection, the set of metabolites detected differed according to the type of microorganism involved, probably reflecting the chemical environment of the invaded tissue and the mechanism of action of the attacking organism.[27, 352] For example, metabolomic changes of *Brassica*, induced by different foodborne bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium* and *Shigella flexneri*, were found to vary according to the bacterial species, though in all cases an increase in phenolic compounds including sinapoyl-malate, caffeoyl-malate was observed.[27] In the case of insect herbivory, aphid feeding involves phenolics in the formation of salivary sheaths around the penetration sites.[353]

Increases in phenolic biosynthesis gene expression or enzyme activity and accumulation of the products of these enzymes, are commonly associated with JA treatment or herbivory in many plants.[317] This was observed in MeJA treated *B. rapa* leaves, in which 5-hydroxyferuloyl malate, caffeoyl malate, coumaroyl malate, feruloyl malate, and sinapoyl malate were identified.[41, 354]

3.3.2 Effect of fertilizers

The production of phenolic metabolites responds to changes in nutrient availability in a highly complex manner.[161, 350] Nitrogen stress triggers the gene expression of flavonoid pathway enzymes and nitrate availability was shown to directly affect the enzyme activity in the phenylpropanoid pathway.[350] Sulfur fertilization increased the average phenolic contents from 96 to 111 mg/100 g of gallic acid, of fresh weight in *B. rapa*[161] and this quantitative change was confirmed by other studies. The patterns of phenolics did not change qualitatively in leaves of *B. oleracea* var. *costata* grown using different agronomical practices.[350] The most representative flavonols in *B. rapa* subsp. *Sylvestis* are kaempferol and quercetin derivates but myricetin was present only in trace amounts which was reduced rather than increased by sulfur fertilization unlike the total phenolics content. This response indicates that the total phenolics pool may be shifted towards the accumulation of different compounds based on precursor availability, presence of enzymes activators (or co-

factors) and/or other effectors, such as sulfur availability.[161] A great increase of anthocyanins in response to cadmium stress was observed in *Brassica juncea*.[355]

3.3.3 Effect of growth stage

Age also proved to affect the phenolic pattern in these plants as in the case of young *B. oleracea* var. *costata* leaves in which 15 phenolics were found. Five of aforementioned phenolics were kaempferol derivatives and 10 were cinnamic acid derivatives, while *p*-coumaroyl-*3*-*O*-quinic acid and 13 kaempferol derivatives were detected in old leaves. Only two kaempferol derivatives were found common in both types of leaves.[350] Three broccoli cultivars exhibited an increase in phenolic compounds coinciding with the inflorescence development.[281]

3.3.4 Effect of temperature and radiation

Flavonoids play an important role in plants as flower pigments and when formed as metabolites in the response to biotic or abiotic stress.[356] Temperature and radiation seem to act as a trigger for biosynthetic pathways.[253] As was also shown by the detection of higher concentrations of phenolic compounds when exposure of plants to sunlight is increased.[357] Similarly, UV-B radiation was observed to induce an enhanced production of soluble phenolics in red cabbage[358] and an increase in the flavonoid content of *S. alba* and *Nasturtium officinale*, where especially in the case of *S. alaba* the increase of quercetin was found to be 10 fold higher than in *N. officinale*.[328] Another study carried out on *B. napus* showed that irradiation with UV-B rays produced a 70–150% increase in the overall amount of flavonoids, four of which were identified as quercetin and kaempferol glucosides.[359] Another study confirmed UV-B induced accumulation of quercetin glycosides and correlated them to plant UV-B tolerance.[360] UV light (8 kJ m^{-2}) treated samples of minimally processed broccoli showed higher phenolic contents than untreated (control) plants.[280]

Post-harvest temperature conditions have also proved to influence the metabolite profile in plants. Contents of lutein in broccoli increased, when kept at daily mean temperatures between 7–13 ^{o}C.[253] Boiling of kale, Brussels sprouts, broccoli and white cauliflower was observed to cause a large decrease in its antioxidant activity, due to the loss of polyphenols.[277, 279] Freezing has been found to be one of the most effective methods of preserving the nutritive constituents of raw Brassicaceae vegetables.[277] It leads to a greater preservation of

polyphenol levels, which were even observed to increase during this process as part of the plant response to stress.[279] Also, a higher antioxidant activity was observed in broccoli plants, kept at 4 ^{o}C for 21 days as compared with control samples.[280] In the case of rocket leaves storage, the stability of quercetin derivatives differed, the glycosides showing more stability than the corresponding acylated glycosides.[287] Another study revealed a drastic reduction of the total anthocyanin content of cauliflower and the formation of isomers with all thermal pre-treatments except microwave heating.[361]

3.4 Steroids

Brassinosteroids (BRs) are a group of naturally occurring plant steroidal compounds in *Brassica* having a wide range of biological activities and the ability to confer tolerance to *Brassica* plants against a wide spectrum of biotic and abiotic stresses [362]. These stress factors include, low and high temperatures, drought, high saline concentrations, pathogen attack[362, 363] and exposure to heavy metals.[364] Sterols have been recently recognized not only as precursors of brassinosteroids (**Figure 6**) and membrane constituents, but also as modulators of plant development.[365]

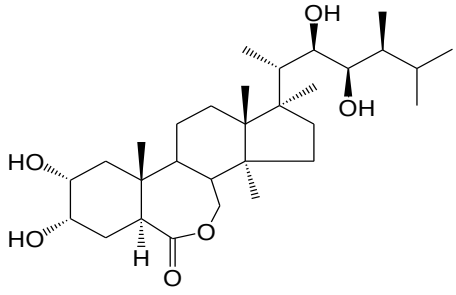

Figure 6 – Brassinosteroid.

Also, there is evidence of cross-talk between BRs and abscisic acid, JA and ethylene.[362] Treatment with 24-epibrassinolide, a brassinosteroid, increases tolerance to several environmental stresses such as basic thermo-tolerance in *B. napus*,[366] or to drought and cold stress in the case of seedlings of both *A. thaliana* and *B. napus*, aside from helping them to overcome salt-stress-induced inhibition of seed germination.[363] The expression of the *B. napus* steroid sulfotransferase genes was found to be induced by SA, suggesting that in addition to

increased accumulation of an antimicrobial protein, plants respond to pathogen infection by modulating steroid-dependent growth and developmental processes.[367]

Brassinosteroids also proved to be able to protect the membrane integrity of the radish seedling from Cd-induced oxidative stress, minimizing the impact of reactive oxygen species by increasing antioxidant enzyme activity, a possible secondary defence mechanism against oxidative stresses.[368]

4 Conclusion

Brassica species are a rich source of health affecting compounds and are widely considered as a food and model for plant science research, in diverse fields (**Figure 3**). During growth, plants are exposed to various biotic (herbivory, fungal, bacterial and/or viral infection) and abiotic (metals, UV, temperature) stresses. It leads to gene expression and biochemical changes, which finally results in an enhancement of the synthesis of primary and secondary metabolites. In this process, a number of signal pathways will be activated as *Brassica* defence responses, including SA, JA, ethylene and abscisic acid pathways[18] (**Figure 1**). On the other hand SA, JA and ethylene dependent defence pathways may also affect each other.

The systemic induced responses of different *Brassica* species led to hypothesize that plants might use a far more complex defensive strategy than a common set of biosynthetic pathways. The metabolomic changes can be quite specific, since the pool of plant defence-related compounds is composed of a variety of constitutive and induced metabolites. The set of *Brassica* metabolites observed, after infection with different microorganisms differs. It probably reflects the diverse chemical composition and mechanism of action of the invading organism, which can at the same time activate gene expression and block specific sites of a metabolomic pathway, or even metabolize the plant defence compounds.

As plants in the field are often exposed to more than one form of stress, there is selection pressure for them to evolve coordinated rather than conflicting defence mechanisms.[247] It would be interesting to understand plant-defence mechanism, when multiple stress factors are present.

5 Future Perspectives

The mechanisms by which metabolites help the plant to resist stress remain largely unclear. While there is an increasing interest in above ground interactions, there is a strong need to study underground mechanisms and interactions of plants as well, because they have not been adequately addressed yet. The complementation of the ecological approach, with the understanding of the molecular basis of plant defence strategies, employed against different attackers, should be useful to fully comprehend the extent of the integration of these mechanisms.

The study of multitrophic interactions is also an interesting area for research. This involves comparing the genomic, proteomic and metabolomic situation of the plant after being attacked by different organisms, either individually or in combination [27]. Further investigation of the systemic induced emission of volatiles, observed in different species, would also contribute to shed light on the ecological significance and regulatory mechanism, behind these defensive responses [262]. Undoubtedly, all these efforts should contribute to provide the means of controlling these different defence systems, leading to the development of more resistant plant varieties. Additionally, since *Brassica* plants are considered to be important food, it is essential to fully understand, how different environmental factors triggering mechanisms and pathways affect their metabolomic profile, since these will ultimately affect the plant's quality and its functional properties. It also attributes to taste and aroma as well as levels of health affecting compounds, which will influence consumer acceptability.

Chapter 4

Metabolomic variation in *Brassica rapa* var. *rapa* (var. raapstelen) and *Raphanus sativus* L. at different developmental stages.

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Abstract

Brassica rapa (var. raapstelen) and *Raphanus sativus* (red radish) are one of the known models in recent plant research. Metabolomic variation during the three developmental stages has been assessed in the present study. Particularly nutritional variation affected by plant growth has been evaluated with *Brassica rapa* (var. raapstelen) and red radish (*Raphanus sativus*) growth, a non-targeted and targeted metabolomic approach by NMR and HPLC, respectively, was used to identify the discrimination in nutrients for different time points of growth. Principal component analysis (PCA) has been employed to the results obtained by both techniques (^{1}H NMR and HPLC) to assess the metabolomic variation. This study shows the change in metabolites (amino acids, organic acids, chlorophyll, carotenoids, tocopherols, ascorbic acid, sucrose, phenylpropanoids and glucosinolates) during plant development. These results lead to a better understanding of the plant metabolomic changes during plant aging and show the importance of plant developmental stage with respect to the nutritional profile of vegetables.

Key words: Plant development, ^{1}H NMR and HPLC metabolomics, nutritional compounds.

1 Introduction

Among *Brassica* vegetables, *Raphanus sativus* (red radish) and *Brassica rapa* (var. raapstelen) are known for their nutritional compounds[2, 369] and are being used as model in recent plant research.[370] *Brassica* vegetables are a good source of health promoting phytochemicals, in terms of containing primary and secondary metabolites including, amino acids, glucosinolates, vitamins (ascorbic acid, carotenoids and tocopherols etc.), folate, phenolics and sugars etc.[371]

These phytochemicals play an important role in plant survival,[18] by either protecting it from cell damage by biotic and abiotic stress or playing their role in plant defence signalling pathway.[185] These are also important for human and animal nutrition.[8, 49] The health supporting role of vegetables is attributed to aforementioned compounds, including, minerals and vegetable oil content[34] as well as dietary fibres.[131]

There is a substantial and significant variation, both within and between (sub) species, for these phytochemicals.[101] A part from different infections, a range of other conditions influence the nutritional profile of Brassicaceae vegetables, including biological[49] and seasonal variation,[223] preharvest growth factors and postharvest processing conditions.[278] In the same way the harvest time also affects the phytochemicals related to quality profile of *Brassica* vegetables.[119] Plant developmental stage is considered as a crucial factor for the quantity of health promoting compounds in vegetables.[281] For example a metabolomic variation in different *Brassica* species has been observed when four week and six week developmental stages were compared. A clear change in the metabolome was observed, affecting the quality of these vegetables.[28] Similar changes were observed in broccoli with different developmental stages of the inflorescence.[281]

Metabolomics is defined as both the qualitative and quantitative analysis of the metabolites in an organism[37] so by using metabolomic approach biological systems are visualized and queried. A number of spectroscopic and chromatographic approaches are presently used for metabolomics. Most common and well known among these are, nuclear magnetic resonance spectroscopy (NMR), gas chromatography (GC) and high performance liquid chromatography (HPLC).[35]

Significant data are available on the effect of biotic and abiotic factors on the metabolome during plant developmental process. There is a need to assess the age dependent metabolomic variation in food plants,

such as *Brassica rapa* (var. raapstelen) and *Raphanus sativus* (red radish). In order to investigate the metabolomic changes during plant growth and development, metabolomic characterisation of two species of Brassicaceae (radish and *B. rapa*) leaves and roots was conducted for four, six and eight weeks old plants.

2 Materials and methods

2.1 Plant material

Seeds of both *Raphanus sativus* (red radish) and *Brassica rapa* (var. raapstelen, Groene Gewone) were sown in pots containing soil and kept in cold room (4 °C) for 2 days and then transferred to a green house in 16 : 8 hours, light : dark conditions. After 6 days of growth, the individual seedlings were transferred to separate pots and watered daily.

2.2 Sample preparation

Plants were harvested at six, eight and ten week developmental age and were washed with deionised water and dried with a tissue paper. Both the roots and leaves were separated from each other and weighed to determine fresh weight and placed immediately in separate aluminium foils to be frozen in liquid nitrogen. Three replicates were used for analysis, with one plant for each replication. All the samples were grinded to a fine powder in liquid nitrogen and freeze-dried in aluminium wrapped containers till a constant weight. After freeze-drying the samples were weighed for dry weight and then stored at –80 ^{o}C until extraction and analysis.

2.3 NMR based metabolomic assessment

NMR sample preparation, sample analysis and data processing was done as previously reported.[24] Quantitation of amino acids (alanine, valine, threonine), organic acids (fumaric acid, γ-amino butyric acid), sugars (sucrose, total glucose) was performed by calculating the relative ratio of the peak area for selected proton signals of the target compounds, to the known amount of TMSP (trimethyl silyl propionic acid sodium salt) as internal standard.

2.4 Glucosinolates assessment

Glucosinolate extraction and desulfation was carried out as previously reported.[372] For glucosinolate extraction, a 100 mg of freeze

dried sample was weighed in a 15 ml glass tube and extracted with 2 ml of boiling 70% methanol solution, desulphatased with arylsulphatase (Sigma, St Louis, MO. USA) on a DEAE-Sephadex A25 column prepared by 0.5 ml of Sephadex A25 in Pasteur pipette column, and separated on a reversed phase C-18 column (Alltima C-18, 150 × 4.6 mm, 3 μm; Alltech, Breda, The Netherlands) on HPLC with an acetonitrile–water gradient (0 – 65% acetonitrile from 0 to 30 min; flow 0.75 ml min^{-1}). Detection was performed with a photodiode array detector and peaks were integrated at 229 nm. The response factors were calculated for identification of different glucosinolates for detection at 229 nm, by using sinigrin standard curve for quantitative analysis.

2.5 Ascorbic acid (vitamin C) assessment

For vitamin C, analysis was done by HPLC-PDA as previously reported[373] by a little modification in the method by using a 10 mg of freeze dried sample, weighed in 10 ml glass tube and extracted in 2 ml of ice-cold 5 % phosphoric acid (Sigma ACS; 35 %), sonicated for 15 minutes and centrifuged at 2500 rpm for 10 min. and filtered over a 0.2 μm PTFE membrane filter into 1.8 ml HPLC vials.

2.6 Isoprenoid assessment

Freeze dried samples were weighed as 50 mg in a 15 ml glass tube. The tocopherols (α, β γ and δ) and carotenoids (lutein, β-carotene, 9-*cis*-β-carotene, violaxanthin, neoxanthin), chlorophyll (A, B) were analyzed by HPLC-photodiode array detector (PDA) as previously reported[373, 374] with some modification as we added extra 0.5 ml of deionized water during first extraction step.

2.7 Data analysis

Data processing, scaling and bucketing for ^{1}H NMR was done as reported in previous publications of our group. For the quantitative data processing, different metabolites were quantified by either NMR or HPLC. PCA and PLS-DA were performed with the SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden) based on a unit-variance scaling method as previously reported.[24]

3 Results and Discussion

Metabolomic variation has been evaluated on three different growth stages for *Brassica rapa* (var. raapstelen) and *Raphanus sativus*

(red radish). Both species differ qualitatively and quantitatively from each other. Metabolomic discrimination at different developmental stages of these two vegetables can easily be investigated by conventional metabolomic approaches, either by using an untargeted or a targeted way of analysis. At first a non-targeted NMR based metabolomic approach was followed. The ^{1}H NMR spectra have an advantage of simultaneous detection of diverse groups of compounds. As previously reported by our group,[24, 27] the amino acids (glutamate, glutamine, alanine, , serine, threonine, valine, phenylalanine and tyrosine), organic acids (acetate, γ-amino-butyric acid (GABA), malate, fumarate), carbohydrates (sucrose and glucose), phenylpropanoids (caffeoyl malate, coumaroyl malate, feruloyl malate, 5-hydroxyferuloyl malate, sinapoyl malate) were identified by ^{1}H NMR, along with 2D spectra including J-resolved, COSY and HMBC analysis. Different metabolites (alanine, valine, threonine, glucose, sucrose, fumarate, GABA) were also quantified by NMR (**Figure 6, 7**), using TMSP as internal standard. All the bucketed data of these samples, including roots and leaves were analyzed by PCA and it was found that the comparatively highest amount of sugars is present in roots, while other metabolites are higher in leaves.

Due to the relatively much higher carbohydrate content, the roots of both species are grouped together while the leaves of the both varieties are grouped together in the PCA score plot. In this case an age wise discrimination cannot be observed as the separation is based on roots and leaves only. To make it easy for the further study of variation of metabolites during developmental stage, we separated the data of roots and leaves for PCA analysis. In spite of the advantages of NMR spectroscopy, the low sensitivity of this method shows its limitation for a detailed metabolomic analysis. So a targeted approach, HPLC was used to identify and quantify glucosinolates (glucobrassicin, glucoerucin, gluconapin, gluconasturtiin, glucoraphanin, neoglucobrassicin, 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, 3-hydroxypropylglucosinolate, sinigrin), ascorbic acid (vitamin C), tocopherols (α, β, γ and δ–tocopherol), carotenoids (lutein, β-carotene, 9-*cis*-β-carotene, violaxanthin and neoxanthin) and chlorophyll (A and B). All the quantitative data either obtained by NMR or HPLC were combined and analyzed by PCA. A similar result was obtained as for NMR results only. The combined results are shown separately for leaves (**Figure 1A**) and roots (**Figure 1B**).

To confirm the results for making a conclusion of high or low contents of different metabolites, as visualized in PCA, the ^{1}H NMR spectra were superimposed and studied visually as well for some selected metabolites. The quantitative results in roots and leaves are shown in **figure 2 – 4 and 6 – 9**. Plant senescence is genetically controlled and involves the hormonally controlled reallocation of the plant's resources.[375] The photosynthetic capacity of a leaf declines with aging and so young plant leaves have more photosynthetic potential as compared with old plant leaves.[328] Plants reallocate more defence related compounds towards young leaves[376] and adjust physiologically to changes in resources availability and to extreme changes in the internal nutrient balance.[377]

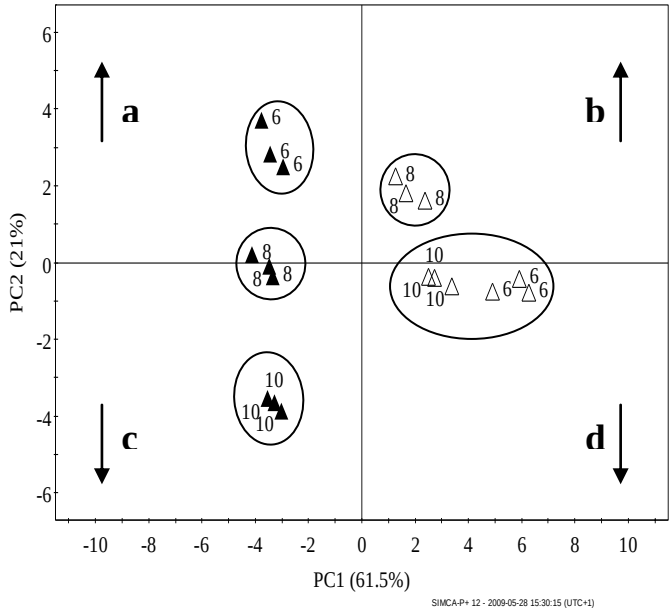


Figure 1A. Score plot of PCA for leaves of *Brassica rapa* (var. raapstelen) and *Raphanus sativus* (radish), based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0, bucketed data) and HPLC (quantitative) data. Radish, 6 weeks (6▲); 8 weeks (8▲); 10 weeks (10▲) old plants. *Brassica rapa*, 6 weeks (6Δ); 8 weeks (8 Δ); 10 weeks (10 Δ) old plants. **a** = glutamine, glutamate, alanine, threonine, valine, chlorophyll, lutein, β-carotene, 9-*cis*-β-carotene, neoxanthin, violaxanthin, 3-hydroxypropylglucosinolate. **b** = ascorbic acid, glucose, fumaric acid, 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, gluconasturtiin, gluconapin, glucoraphanin, sinigrin. **c** = phenylpropanoids, α-tocopherol, δ-tocopherol, glucobrassicin. **d** = β-tocopherol, γ-tocopherol, sucrose, dry weight, neoglucobrassicin.

Comparing the metabolome of different developmental stages of the whole plant it is observed that amino acids and organic acids (glutamine, glutamate, alanine, threonine, valine, fumaric acid, ascorbic acid), glucose, chlorophyll, carotenoids, glucosinolates (3-hydroxypropylglucosinolate, 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, gluconasturtiin, gluconapin, glucoraphanin, sinigrin), are highest in 6 weeks old total plant leaves of both species, while by further development from 6 to 10 weeks, sucrose, phenylpropanoids, tocopherol, two glucosinolates (glucobrassicin, neoglucobrassicin) are increased (**Figure 5**). Almost similar behaviour is noticed in the roots (**Figure 5**).

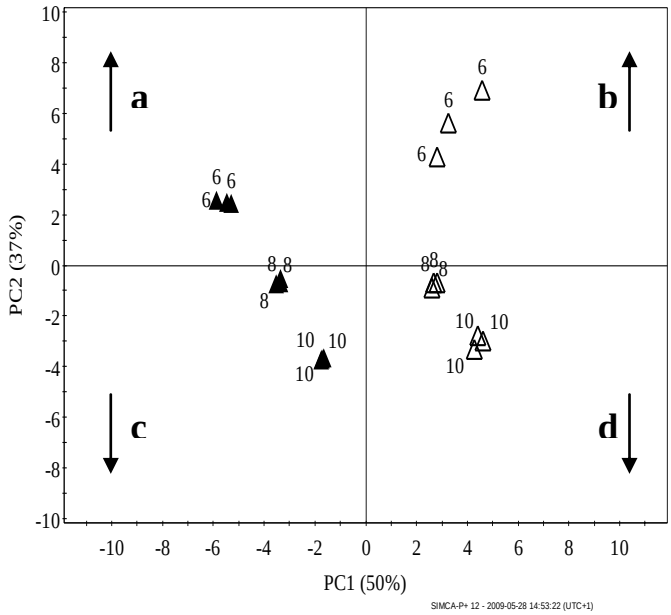
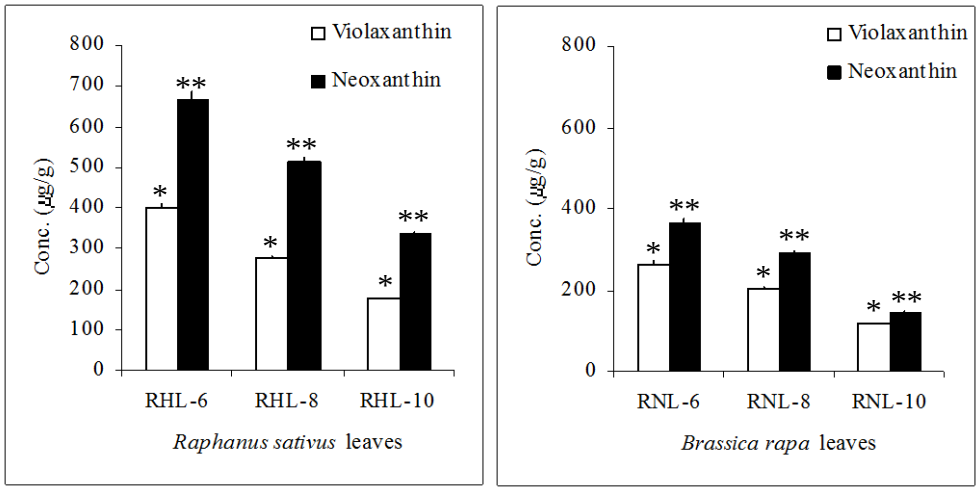
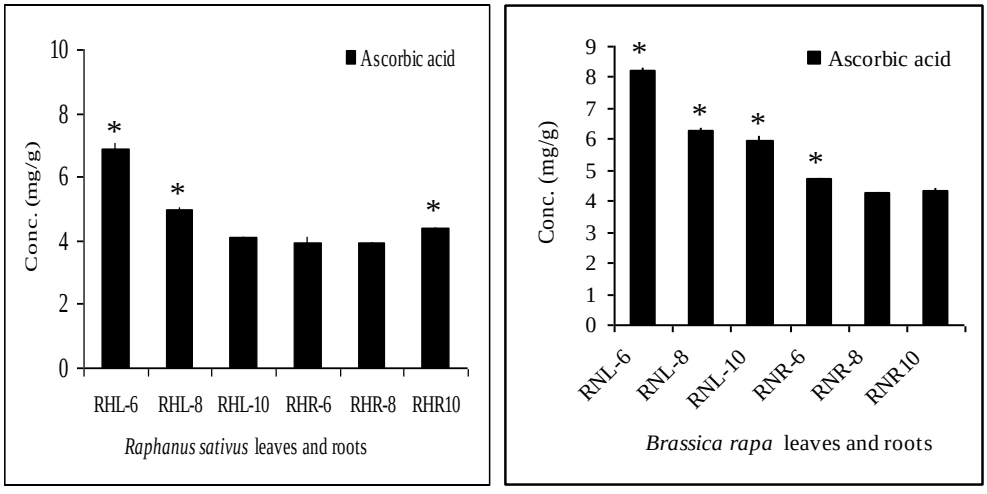


Figure 1B. Score plot of PCA for roots of *Brassica rapa* (var. raapstelen) and *Raphanus sativus* (radish), based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0, bucketed data) and HPLC (quantitative) data. Radish, 6 weeks (6▲); 8 weeks (8▲); 10 weeks (10▲) old plants. *Brassica rapa*, 6 weeks (6Δ); 8 weeks (8 Δ); 10 weeks (10 Δ) old plants. **a** = fumaric acid, β-carotene, γ-amino butyric acid. **b** = threonine, glucose. **c** = phenylpropanoids, valine, lutein, alanine, glucobrassicin. **d** = sucrose, dry weight, 3-hydroxypropylglucosinolate, glucoraphanin, sinigrin, 4-hydroxyglucobrassicin, gluconasturtiin, gluconapin, neoglucobrassicin.



A B

Figure 2 Concentration of violaxanthin and neoxanthin (µg/g of dry weight) and at different developmental stages of *Raphanus sativus* (A) and *Brassica rapa* (var. raapstelen) (B) leaves. RHL = Radish leaves; RHR = Radish roots; RNL = Raapstelen leaves; RNR = Raapstelen roots; 6 weeks old plant (6); 8 weeks old plants (8); 10 weeks old plants (10). *; ** = The value shows a significant difference (P = < 0.001).



A B

Figure 3 Concentration of ascorbic acid (mg/g) and at different developmental stages of *Raphanus sativus* (A) and *Brassica rapa* (var. raapstelen) (B) leaves. RHL = Radish leaves; RHR = Radish roots; RNL = Raapstelen leaves; RNR = Raapstelen roots; 6 weeks old plant (6); 8 weeks old plants (8); 10 weeks old plants (10). *; ** = The value shows a significant difference (P = < 0.001).

If inter-species comparison of leaves of *Brassica rapa* and *Raphanus sativus* is studied, high amounts of glutamine, glutamate, alanine, threonine, valine, chlorophyll, phenylpropanoids, carotenoids, α-tocopherol, δ-tocopherol, glucobrassicin and 3-hydroxypropyl-glucosinolate was found in *Raphanus sativus* while sucrose, glucose, ascorbic acid, fumaric acid, β-tocopherol, γ-tocopherol, 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, gluconasturtiin, gluconapin, glucoraphanin, sinigrin and neoglucobrassicin were found to be higher in *Brassica rapa* leaves (**Figure 1**). Generally a decrease in glucosinolate content is observed with increasing age of plants of both species, except for glucobrassicin and neoglucobrassicin which were found to increase during development (**Figure 4**).

In certain cases, like for sinigrin, 3-hydroxypropylglucosinolate, 4-hydroxyglucobrassicin, and glucobrassicin, a different course of the concentration is observed. These glucosinolates showed a sudden increase or decrease in concentration, in 8 week old plants, following by a decrease or increase in 10 week old plants, respectively. This irregular behaviour is observed in both leaves and roots of both species, showing that this effect occurs in whole of the plant (**Figure 4**).

The aliphatic and indole glucosinolate profiling of the leaves of both species (*B. rapa* var. raapstelen and *Raphanus sativus*) showed that 3-hydroxypropylglucosinolate, glucobrassicin are the major glucosinolates (**Figure 4A & 4C**), while *Brassica rapa* leaves were also found to contain a higher amount of neoglucobrassicin (**Figure 4A**) as comparative to other glucosinolates. Gluconasturtiin and 3-hydroxypropylglucosinolate are found as major glucosinolates in *B. rapa* roots (**Figure 4B**), while glucobrassicin is found to be the major glucosinolate in radish roots (**Figure 4D**). The violaxanthin and neoxanthin are the major carotenoids found in leaves of both species (**Figure 2**). A decrease in carotenoids is observed with increasing age of plant. The same was observed for ascorbic acid in the leaves of both species, whereas in the roots of both, the concentration of ascorbic acid is almost constant (**Figure 3**). Overall, all metabolites measured in both species showed a similar behaviour (although not so pronounced in case of *Brassica rapa* roots) in the leaves and roots.

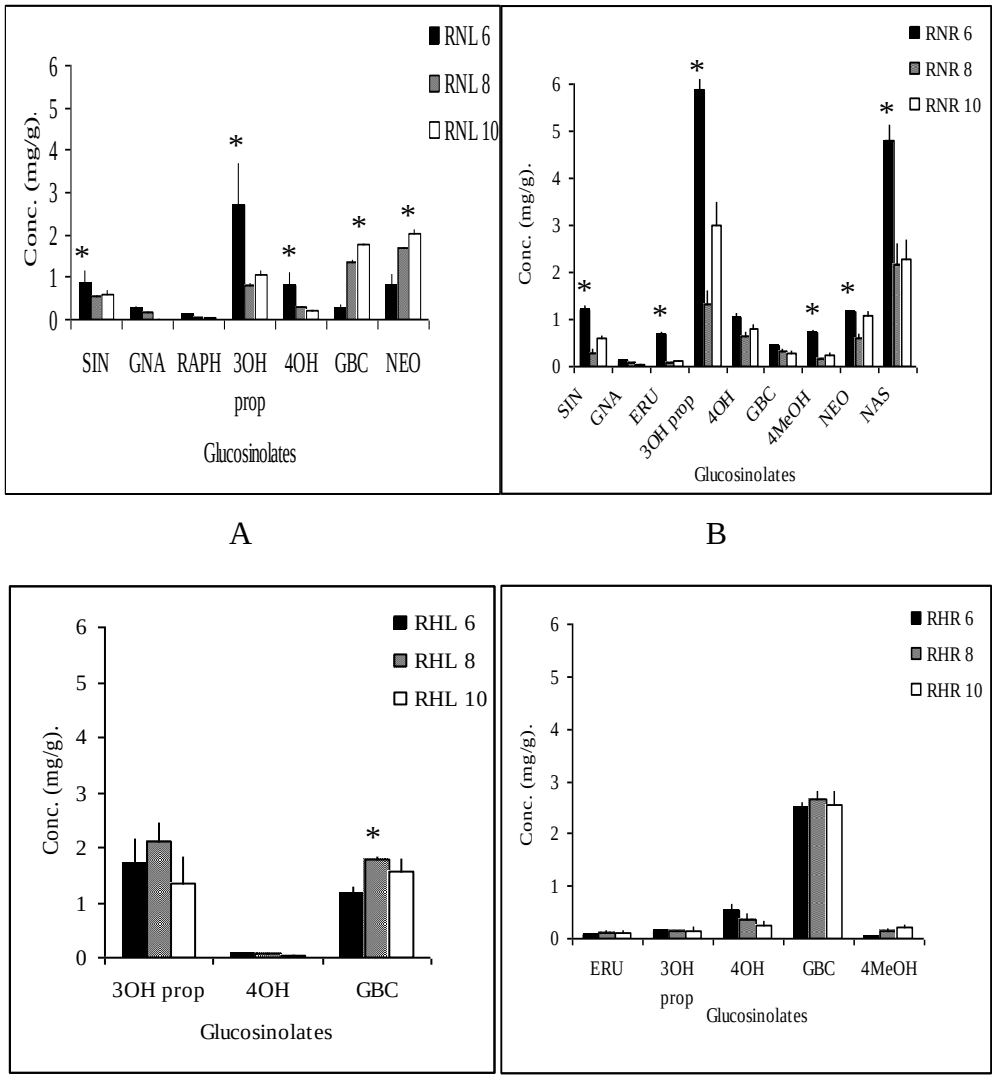


Figure 4. Concentration of glucosinolates (mg/g of dry weight) at different developmental stages of *Brassica rapa* (var. Raapstelen) and *Raphanus sativus* leaves. RNL = Raapstelen leaves (A); RNR = Raapstelen roots (B); RHL = Radish leaves (C); RHR = Radish roots (D); 6 weeks old plant (6); 8 weeks old plants (8); 10 weeks old plants (10). Glucobrassicin (GBC), Glucoerucin (EU), Gluconapin (GNA), Gluconasturtiin (NAS), Glucoraphanin (RAPH), Neoglucobrassicin (NEO), 4-hydroxyglucobrassicin (4OH), 4-methoxyglucobrassicin (4MeOH), 3-hydroxypropylglcosinolate (3OH prop), Sinigrin (SIN). * = The value shows a significant difference (P = < 0.001).

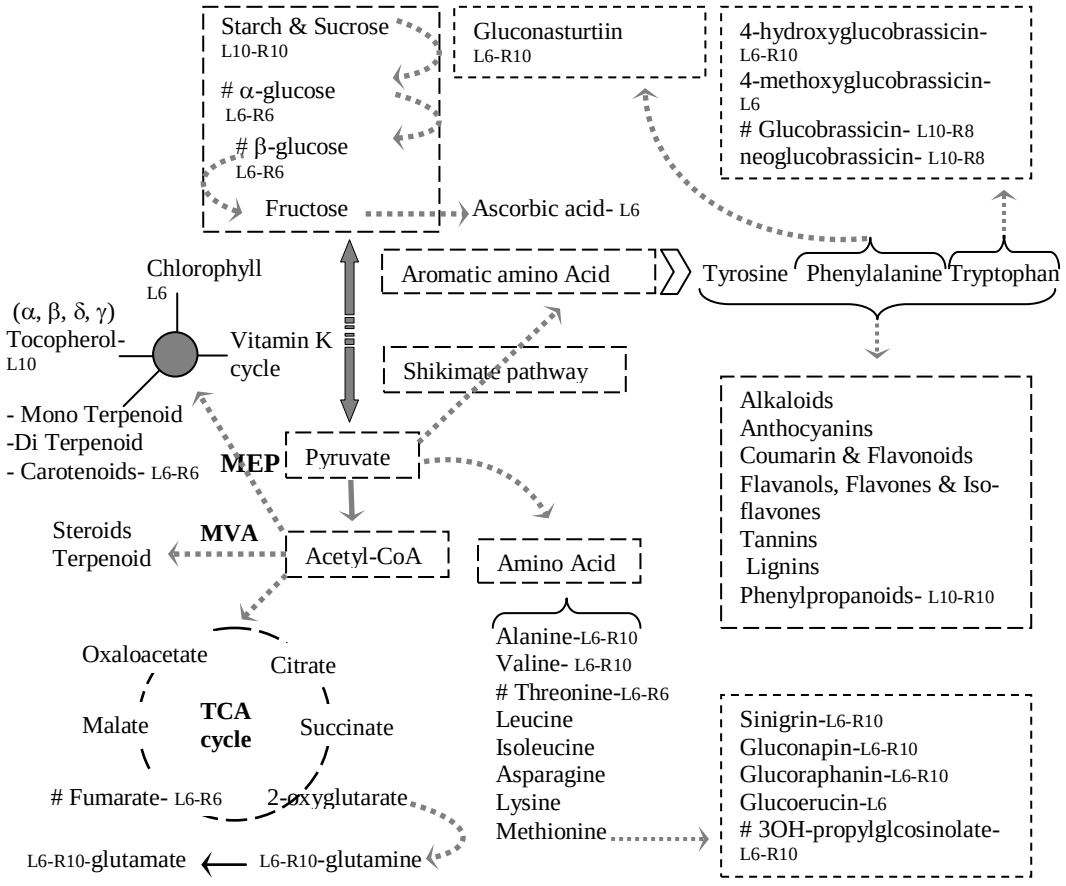


Figure 5. Change in metabolite quantities during plant growth. L6 = maximum in 6 weeks old plants leaves; L10 = maximum in 10 weeks old plant leaves; R6= maximum in 6 weeks old plant roots; R8 = maximum in 8 week old plant roots; R10 = maximum in 10 weeks old plant roots. # = The exception of the fumarate, threonine and glucose are higher in 8 weeks old roots in case of *Brassica rapa* while glucobrassicin and 3-hydroxypropylglcosinolate are higher in 8 weeks old radish leave and glucobrassicin is also higer in 8 weeks old radish roots. TCA = tricarboxylic acid cycle; MVA= mevalonate pathway; MEP = 2-C-methyl-D-erythritol 4-phosphate pathway.

4 Conclusion

Overall the young *Brassica* plants showed higher levels of amino acids, organic acids, glucose, carotenoids, glucosinolates and chlorophyll. During plant development and growth the chemical composition of the plant changes. This change in metabolite profile (**Figure 4**) during growth represents the change in metabolomic fluxes in different pathways. The present study also shows the importance of plant age as a factor for nutritional value of a plant for human consumption as the younger plants can be a better source of nutrients if compared with old plants. The results from this study provide a better understanding of plant metabolites with reference to developmental stage. In future studies plant age should be kept in mind as an important factor, especially in case of the plant interactions with its environment factors.

Acknowledgement

The help of Dr. Nicole M. van Dam, Netherlands Institute of Ecology (NIOO-KNAW), Heteren, The Netherlands, for the identification of glucosinolates is gratefully acknowledged.

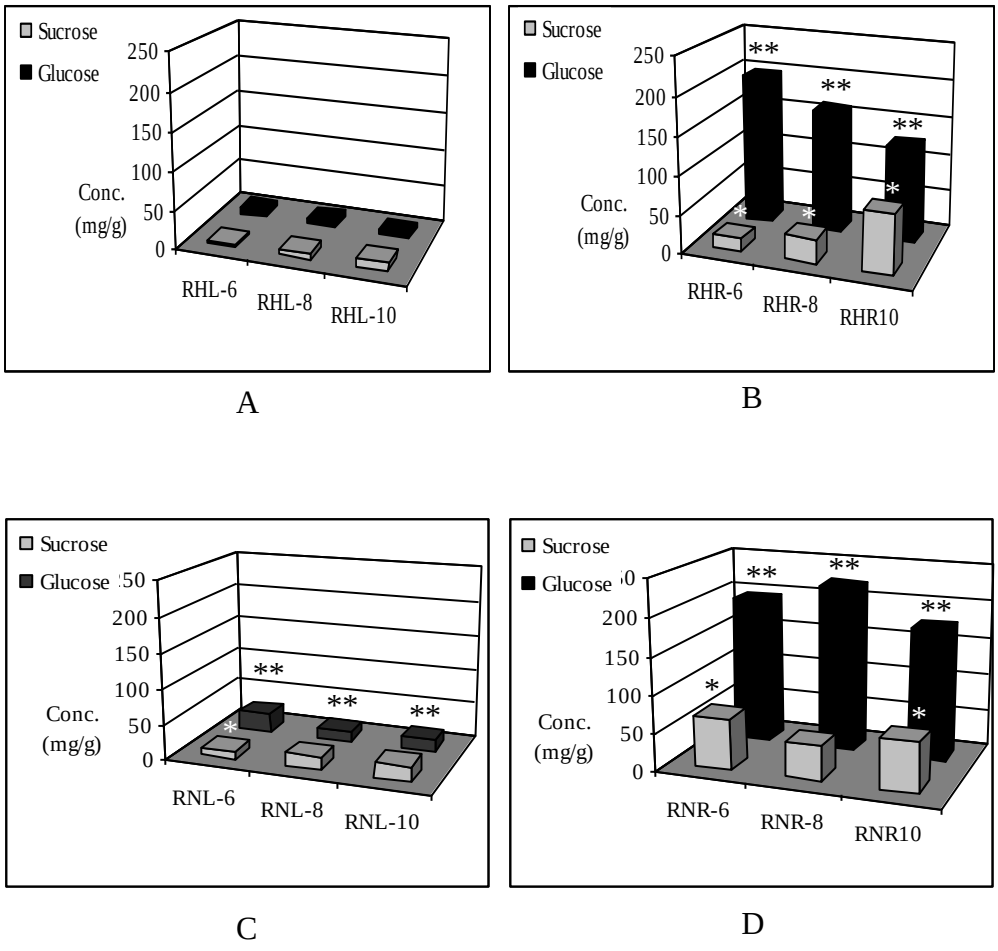


Figure 6. Concentration of sucrose and glucose (mg/g of dry weight) in radish leaves (A), radish roots (B), raapstelen leaves (C) and raapstelen roots (D) at different developmental stages. RHL = Radish leaves; RHR = Radish roots; RNL = Raapstelen leaves; RNR = Raapstelen roots; 6 weeks old plant (6); 8 weeks old plants (8); 10 weeks old plants (10). *; ** = The value shows a significant difference (P = < 0.001).

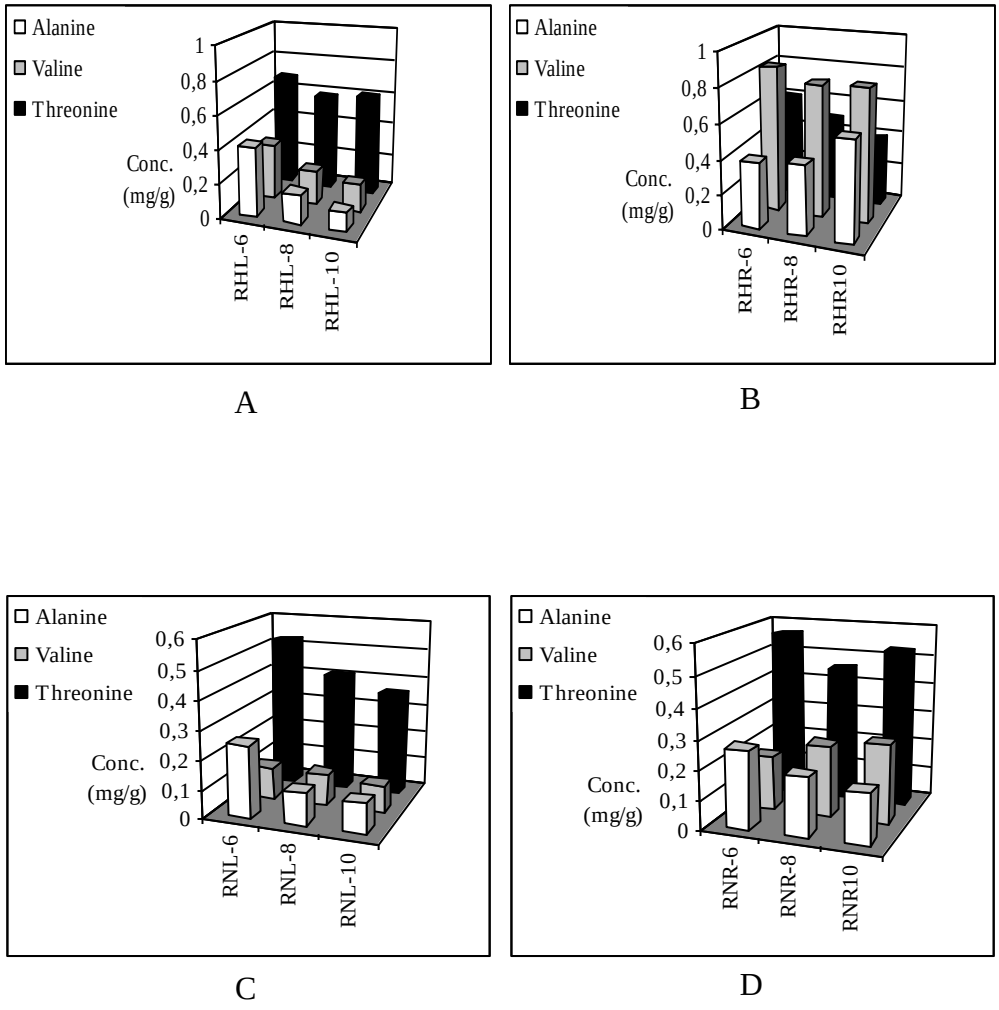


Figure 7. Concentration of alanine, valine and threonine (mg/g of dry weight) in radish leaves (A), radish roots (B), raapstelen leaves (C) and raapstelen roots (D) at different developmental stages. RHL = Radish leaves; RHR = Radish roots; RNL = Raapstelen leaves; RNR = Raapstelen roots; 6 weeks old plant (6); 8 weeks old plants (8); 10 weeks old plants (10).

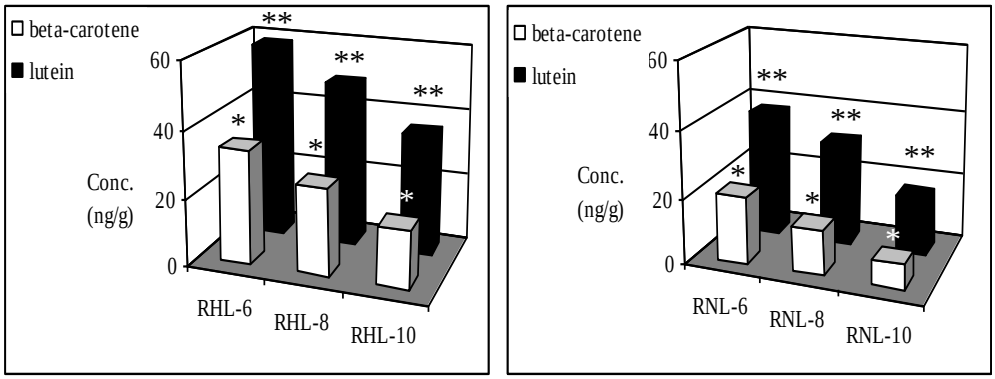


Figure 8. Concentration of β-carotene and lutein (ng/g of dry weight) in radish leaves (A) and raapstelen leaves (B). RHL = Radish leaves; RNL = Raapstelen leaves; 6 weeks old plant (6); 8 weeks old plants (8); 10 weeks old plants (10). *; ** = The value shows a significant difference (P = < 0.001).

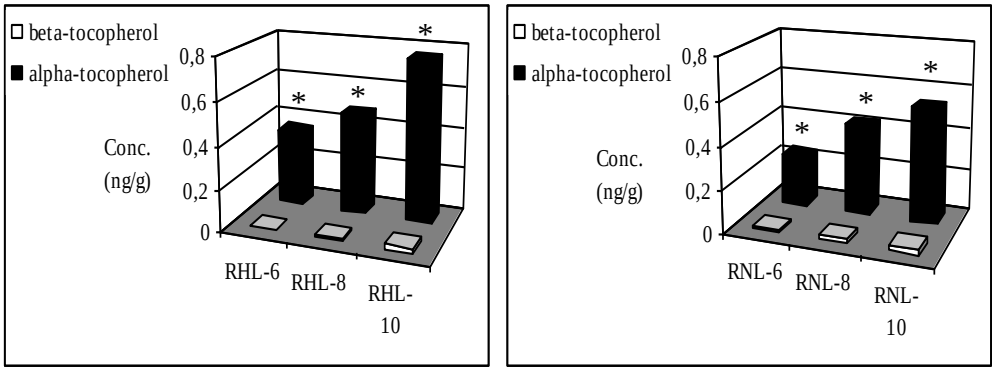


Figure 9. Concentration of β-tocopherol and α-tocopherol (ng/g of dry weight) in Radish leaves (A) and raapstelen leaves (B). RHL = Radish leaves; RNL = Raapstelen leaves; 6 weeks old plant (6); 8 weeks old plants (8); 10 weeks old plants (10). * = The value shows a significant difference (P = < 0.001).

Chapter 5

Metabolomic response of *Brassica rapa* (var. raapstelen) submitted to pre-harvest bacterial contamination

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Abstract

Plants are continuously challenged by pathogens. Among a number of environmental factors, pre-harvest contamination of plants with pathogens responsible for enteric diseases in humans is of major international concern. Despite the knowledge of how bacterial infestation can affect the biological system of plants, little is known about the effect of the interaction of these bacteria on the plant's metabolome. In order to investigate possible metabolomic changes of *Brassica rapa* induced by typically foodborne bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium* and *Shigella flexneri*, 1 dimensional (1D) and 2 dimensional (2D) NMR spectra were measured and analyzed by principal component analysis (PCA) and partial least square–discriminant analysis (PLS-DA).

The metabolomic changes were found to vary according to bacterial species; for example, Gram-positive and Gram-negative bacteria had a different effect on the *Brassica* metabolome. While threonine and GABA were found to be the discriminating metabolites in Gram-positive bacteria treated plants, those treated with Gram-negative bacteria exhibited a significant increase in sinapoyl malate, caffeoyl malate and histidine. The detailed study of the effect of type of bacteria showed that amino acids, alcohols, carbohydrates and phenols were discriminating metabolites. These results show the potential of NMR-based metabolomics as a tool to study the interaction of foodborne bacteria with vegetables.

Keywords: Metabolomic analysis, NMR, Multivariate data analysis, *Brassica rapa*, Cultivars, Biological variation, Developmental stages.

1 Introduction

Brassicaceae species have been traditionally included among the important food crops in all Asian countries.[42] Over the past decades *Brassica* production has increased, becoming an important source of oil and proteins for animal and human nutrition.[51] In addition to the nutritional benefits, they constitute a very rich source of health-promoting phytochemicals such as phenols, flavonoids, phenylpropanoids, vitamins, glucosinolates, fibres, soluble sugars, fats and carotenoids.[55] Besides this, there is growing evidence that a higher intake of *Brassica* vegetables (e.g., broccoli, cabbage, kale, mustard greens, Brussels-sprouts, cauliflower) could help to reduce the risk of cancer.[62]

In the wild, plants are constantly interacting with external environmental factors. In particular, plants are exposed to the challenge posed by pathogens. Among a number of environmental factors, pre-harvest contamination of plants with bacteria that are responsible for enteric diseases in humans is of major international concern.[378] There are many sources of pre-harvest contaminations. In recent years, biological products such as manure which might contain pathogenic microbes have been widely used as fertilizers in vegetable production.[379] Digested urban sludge and livestock waste were also found applicable as basal dressing for the growth of leafy vegetables.[380] This, added to the rapid change of environmental conditions which lead to different ecological interactions, may contribute to higher risks of contamination by food-borne pathogens.[381]

Microbial growth in food is of serious concern as it causes decay, loss of nutritional effects and organoleptic properties.[382] Bacteria are known to produce chemicals which can either promote or inhibit the growth of other organisms when interacting with plants.[25] Some of these bacteria are pathogenic for the plants, destroying or diminishing their photosynthetic output. As a result of the bacterial attack, plants generate their own defence mechanisms, triggering many complex biological processes. There are a number of reports on the changes at a genetic or protein level brought about by phytopathogenic microbes which are reflected in a profound alteration of the metabolite pool of the affected plants.[383] However, little is known about the interaction of the plants with bacteria that are pathogenic to humans, in particular, those that are responsible for enteric infections usually acquired by ingestion of fresh

fruit and vegetables, such as *Escherichia coli* and *Salmonella* species, that have the potential for reproduction prior to consumption.[384]

While there is clear evidence of an effect with plant pathogenic microorganisms infection on the plant's biosynthetic network, the interaction of plants with food-borne human pathogens is not yet clear.[385, 386] If the metabolome of host plants were quantitatively or qualitatively affected by these exogenous bacteria following a clearly distinguishable and constant pattern, this could constitute a good tool for the quality control of plants to be used as food. Thus, studies on the metabolomic interaction of plants and micro-organisms including the total heterotrophs, *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus*, *E. coli*, *Salmonella* species, and *Shigella* species are necessary.[387]

An investigation of the plant metabolome, that is, of all its metabolites, is an extremely complex task due to the large number and variety of compounds. It is unlikely that a single analytical method could provide information about all the metabolites in plants, since the diversity of their structures results in a wide range of physical properties and chemical characteristics, such as volatility, polarity, solubility, chromatographic behaviour and detectability, the use of multiple methods is required. Particularly in the case of the interaction between plants and bacteria an unbiased method that allows the simultaneous detection of as wide array of metabolites as possible, is essential. In this context the use of metabolomics, a comprehensive profiling of metabolites achieved through the analytical methods and the multivariate data analysis of results thus generated seems most suited.

Among the analytical methods appropriate for the implementation of this technique, nuclear magnetic spectroscopy (NMR) is an optimum choice for the first step, that is, the acquisition of data of general metabolite composition of the sample. It is a quick, non-destructive method which simultaneously detects all proton-bearing compounds such as carbohydrates, amino acids, fatty acids, amines, esters, lipids etc.[388] Although ^{1}H NMR has a relatively low sensitivity compared with other methods such as mass spectrometry (MS), it has the advantage of allowing the detection and easy absolute quantitation of diverse groups of plant metabolites in a single run. Therefore many researchers have chosen it as a first macroscopic approach to metabolomic studies.

The aim of the present study was to investigate the effect of certain foodborne human pathogenic bacteria on the *Brassica rapa* metabolome. To follow and detect these changes, ^{1}H NMR and two dimensional NMR spectra, coupled with principal component analysis

(PCA) and partial least square–discriminant analysis (PLS-DA) were applied.

2 Materials and Methods

2.1 Preparation of MS 0.5 media

Seeds were grown on Murashige and Skoog (MS medium – 0.5) solid medium [389] and the seedlings were transferred in conical flasks containing MS (0.5) liquid medium including vitamin B_5 and 0.3% (w/v) sucrose. Previous experiments were performed with different concentrations of auxin (2,4-dichlorophenoxyacetic acid) and cytokinin (6-benzylaminopurine) in order to select the best combination of these hormones for growth of *Brassica rapa* (var. raapstelen) seedlings in liquid media. Optimum growth was observed in control samples, so no hormones were added to the seedlings in experimental conditions.

2.2 Plant and microbial material

Brassica rapa seeds of a registered cultivar (var. raapstelen, Groene Gewone) were germinated in MS (0.5) media. After surface sterilization, the seeds were sown in MS (0.5) solid media in conical flasks under sterilized conditions and kept in cold storage (4 ^{o}C) in the dark overnight, after which they were transferred to the greenhouse and kept in 24-hour daylight conditions. Five days later, the seedlings were transferred to a MS (0.5) liquid media in sterile conditions and kept in a shaker at 75 rpm in continuous light conditions. After nine days of plant growth, the liquid media were inoculated individually with 500 µl of different bacteria cultures (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium* and *Shigella flexneri*), with a bacterial concentration of 10^8/ml. The control sample was treated with 500 µl of the sterilized liquid broth used for bacteria culture, while an untreated sample of seedlings was kept as a blank sample. After three days of the bacterial inoculation at room temperature, the plants were removed from flasks, washed thoroughly with running tap water and then with sterilized and deionized water, roots were removed and leaves were immediately frozen in liquid nitrogen. Prior to extraction all material was pulverized in liquid nitrogen using a mortar and pestle and freeze-dried. Measurement was done in three replications.

2.3 Extraction of plant material and NMR measurements

Fifty mg of freeze dried material were transferred to a microtube (2 ml) to which 1.5 ml of 50% methanol-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) containing 0.05% TMSP (trimethyl silyl propionic acid sodium salt, w/v) was added. The mixture was vortexed at room temperature for 1 min, ultrasonicated for 20 min, and centrifuged at 13,000 rpm at room temperature for 5 min. Eight hundred μl of the supernatant was transferred to a 5 mm NMR tube.

^{1}H NMR and 2D J-resolved spectra were recorded at 25 ^{o}C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. MeOH-d_4 was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 sec acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30^o (11.3 μsec), and relaxation delay (RD) = 1.5 sec. A presaturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to TMSP at 0.0 ppm, using XWIN NMR (version 3.5, Bruker). 2D J-resolved NMR spectra were acquired using 8 scans per 128 increments for F1 and 8 k for F2 using spectral widths of 5000 Hz in F2 (chemical shift axis) and 66 Hz in F1 (spin-spin coupling constant axis). A 1.5 sec relaxation delay was employed, giving a total acquisition time of 56 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. J-resolved spectra tilted by 45^o, was symmetrized about F1, and then calibrated, using XWIN NMR (version 3.5, Bruker). ^{1}H-^{1}H-correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600 MHz Bruker DMX-600 spectrometer (Bruker). The COSY spectra were acquired with 1.0 sec relaxation delay, 6361 Hz spectral width in both dimensions. Window function for COSY spectra was sine-bell (SSB = 0). The HSQC spectra were obtained with 1.0 sec relaxation delay, 6361 Hz spectral width in F2 and 27 164 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HSQC. The HMBC spectra were recorded with the same parameters as the HSQC spectrum except for 30183 Hz of spectral width in F2. The optimized coupling constants for HSQC and HMBC were 145 Hz and 8 Hz, respectively.

2.4 Data Analysis

The ^{1}H NMR spectra were automatically reduced to ASCII file. Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.3 – δ 10.0. The region of δ 4.75 – δ 4.9 and δ 3.28 – δ 3.34 was excluded from the analysis because of the residual signal of HDO and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker) with scaling on total intensity. Principal component analysis (PCA) and partial least square–discriminant analysis (PLS-DA) were performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden) with scaling based on Pareto and unit variance method, respectively.

3 Results and discussion

A number of metabolites including amino acids, organic acids, carbohydrates and phenylpropanoids were identified from bacterial infected *Brassica rapa* using ^{1}H NMR and 2D J-resolved spectra together with other 2D spectra including ^{1}H–^{1}H COSY, HSQC and HMBC spectra (**Table 1**). The amino acids and organic acids region (δ 0.8 – δ 4.00) showed ^{1}H NMR signals of alanine, threonine, valine, malate, glutamate, glutamine, acetate and GABA (γ-amino-butyric acid).

The strong signals of the amino acids and organic acids together with sugars made it easier to elucidate the signals by comparison with reference compounds. Most of the metabolites in the aromatic region (δ 6.0 – δ 8.5) were found to be secondary metabolites but low signal intensity and lack of reference compounds made it difficult to elucidate their structures. Based on previous reports, [354] diverse phenylpropanoids were identified in *Brassica* leaves. The doublets in the range of δ 6.30 - δ 6.50 (J = 16.0) having COSY correlation with doublets at δ 7.30 - δ 7.85 (J = 16.0) and coupling with carbonyl carbons at δ 171 in HMBC spectrum are typical signal of H-8 of *trans*-phenylpropanoids. Four *trans*- and three *cis*-phenylpropanoids (**Table 1**) were identified using COSY, HMQC and HMBC spectra. However, the *cis*- forms of phenylpropanoids are considered to be artifacts of their *trans*- forms possibly produced during extraction or sample storage.[354]

Clear differences were detected in the ^{1}H NMR spectra of treated plants when compared with both the blank and control samples. In the first place, fermentation products such as short chain alcohols or acids exhibited a high variability: high levels of 2,3-butanediol at δ 1.14 (d, J =

6.4) were detected in *B. subtilis* and *S. typhimurium* treated plants, while increased levels of acetate at δ 1.91 (s) were found in *B. subtilis*, *E. coli* and *S. flexneri* treated plants (**Figure 1**). The fermentation product 2,3-butanediol has been reported to cause induced systemic resistance (ISR) of plants which might play a role in the triggering of the production of plant primary and secondary metabolites.[390] These increased short chain alcohols and acids might not be products of plant biosynthesis but from the infecting bacteria and subsequently taken up by the plants. Glucosinolate levels after infection are known to be increased[306] but in present study glucosinolates could not be identified in either control or infected plants.

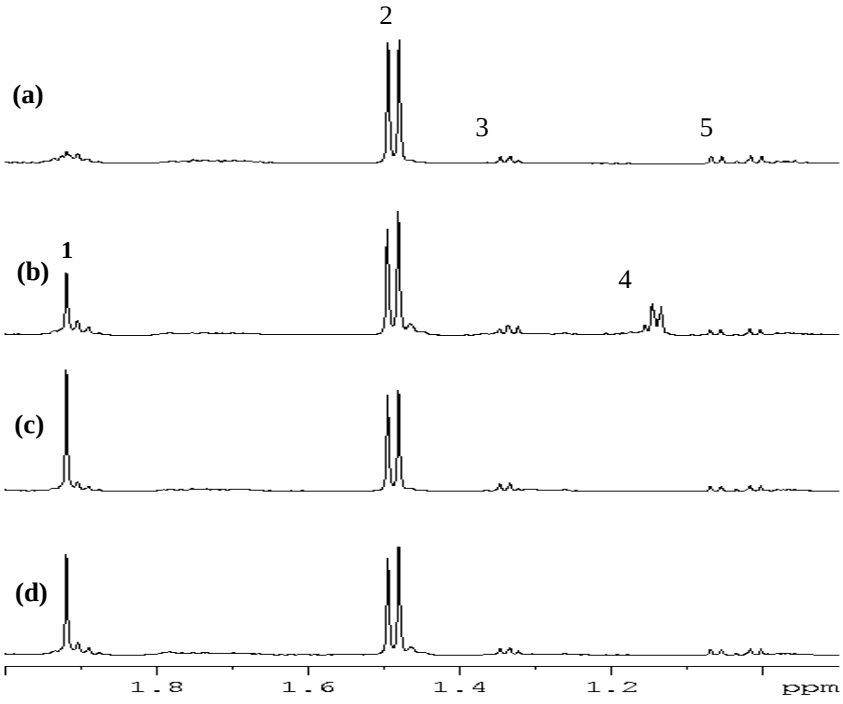

Figure 1 – ¹H NMR spectra (500 MHz,) of control *Brassica rapa* leaves (a), infected with *Bacillus subtilis* (b), *Escherichia coli* (c), *Shigella flexneri* (d) in the range of δ 0.9 – δ 2.0. 1; acetate, 2; alanine, 3; threonine, 4; 2,3-butandiol, 5; valine. The spectra were measured in the mixture of KH_2PO_4 in D_2O (pH 6.0)–methanol-d_4 (1:1).

Table 1 – Characteristic ^{1}H chemical shifts (δ) and coupling constant (Hz) of hydroponically grown *Brassica rapa* metabolites obtained from 1D and 2D NMR spectra in 50% methanol-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0).

Metabolite	Chemical (δ) shift and coupling constant (Hz)
Acetate	1.91 (s)
Alanine	1.48 (d, *J* = 7.3), 3.73 (q, *J* = 7.2)
γ-Amino-butyric acid (GABA)	1.90 (m), 2.30 (t, *J* = 7.2), 3.01 (dd, *J* = 8.4, 6.3)
trans-Caffeoyl malate	2.62 (dd, *J* = 15.3, 11.3), 5.21 (dd, *J* = 11.5, 2.7), 6.43 (d, *J* = 16.1), 6.84 (d, *J* = 8.8), 7.14 (dd, *J* = 6.9, 1.6), 7.64 (d, *J* = 16)
cis-Coumaroyl malate	2.58 (dd, *J*= 15.3, 11.3), 5.18 (dd, *J*=11.5, 2.7), 5.95 (d, *J*= 13.0), 6.80 (d, *J*= 8.8), 6.90 (d, *J*= 9.2), 6.93 (d, *J*= 13.6), 7.07 (d, *J*= 8.5), 7.60 (d, *J*= 9.2)
trans-Coumaroyl malate	2.62 (dd, *J* = 5.3, 11.3), 5.21 (dd, *J*= 11.5, 2.7), 6.49 (d, *J* = 15.9), 6.84 (d, *J* = 8.8), 7.58 (d, *J* = 9.1), 7.67 (d, *J* = 16.2)
cis-Feruloyl malate	2.58 (dd, *J* = 15.3, 11.3), 5.18 (dd, *J* = 11.5, 2.7), 5.99 (d, *J* = 13.0), 6.84 (d, *J*= 8.8), 6.94 (d, *J* = 13.6), 7.13 (dd, *J*= 9.4, 2.0), 7.83 (d, *J*= 3.0)
trans-Feruloyl malate	2.62 (dd, *J* = 15.3, 11.3), 5.21 (dd, *J* = 11.5, 2.7), 6.49 (d, *J* = 16.0), 6.89 (d, *J* = 8.2), 7.13 (dd, *J* = 8.8, 2.1), 7.27 (d, *J* = 2.3), 7.66 (d, *J* = 16.3)
Fumarate	6.53 (s)
α-Glucose	3.4 (m), 3.47 (dd, *J* = 9.8, 3.6), 5.19 (d, *J* = 3.8)
β-Glucose	4.59 (d, *J* = 8.0)
Glutamate	2.13 (m), 2.46 (m), 3.72 (t, *J* = 6.0)
Glutamine	2.14 (m), 2.47 (m)
Histidine	3.16 (dd, *J* = 6.5, 1.5), 3.25 (d, *J* = 8.2, 1.5), 7.12 (d, *J* = 1.6), 7.93 (d, *J* = 1.6)
Malate	2.68 (dd, *J* = 15.3, 3.2), 2.38 (dd, *J* = 16.3, 6.8), 4.28 (dd, *J* = 9.8, 3.3)
cis-Sinapoyl malate	2.58 (dd, *J* = 15.3, 11.3), 5.18 (dd, *J* = 11.5, 2.7), 5.93 (d, *J* = 13.0), 7.15 (s), 6.93 (d, *J* = 13.6)
trans-Sinapoyl malate	2.62 (dd, *J* = 15.3, 11.3), 5.21 (dd, *J* = 11.5, 2.7), 6.93 (s), 6.50 (d, *J* = 16.0), 7.67 (d, *J* = 16.3)
Sucrose	3.44 (dd, *J* = 9.9, 8.5), 3.51 (m), 3.73 (d, *J* = 6.8), 4.05 (d, *J* = 6.7), 4.17 (d, *J* = 8.5), 5.4 (d, *J* = 4.0)
Threonine	1.33 (d, *J* = 7.0)
Valine	1.00 (d, *J* = 7.1), 1.05 (d, *J* = 7.1), 2.29 (m)

The constitutive plant metabolite content showed a very significant change. In particular, the level of GABA was clearly increased in *B. subtilis*, *S. flexneri*, and *S. aureus* infected plants as compared with other treatments and control/blank. An increase in sucrose, α-glucose, β-glucose, alanine, threonine (**Figure 2**), and some phenylpropanoids was also observed in bacteria treated samples. Grouping the observed metabolomic alterations, it was found that there was a difference in the plants infected by Gram (+) and Gram (–) bacteria. While phenolic metabolites increased in all treated samples, high levels of histidine, feruloyl-malate and caffeoyl-malate were detected in Gram (–) infected plants only (*E.coli*, *S. flexneri, and S. typhimurium* in this study) whereas Gram (+) bacteria (*B. subtilis and S. aureus*) infected plants displayed increased levels of coumaroyl-malate and fumarate. The increase of phenolic compounds might be explained as a generic response of all the plants to the infection by microorganisms.[41, 354]

First principal component analysis (PCA) was used to identify metabolomic changes, after bacterial treatment of *Brassica* plants in an unbiased manner. In the PCA score plot, control and blank samples were grouped together, showing that the broth by itself had no effect on the metabolome, while different bacterial treated plants were clearly discriminated from the control and blank samples. Additionally, each treated plant displayed metabolomic changes which differed for each type of infecting bacteria as can be observed in **Figure 2**.

In the score plot there were four well defined groups corresponding to blank and control; *B. subtilis* and *E. coli*; *S. flexneri* and *S. aureus*; and *S. typhimurium* treated plants, all separated by PC1 and PC2 (**Figure 2**). For the investigation of differentiating metabolites, a loading plot was used in which the correlation between grouping and correlated metabolites was shown. The primary metabolites contributing to the discrimination were found to be glutamic acid, glutamine, glucose, sucrose, alanine, threonine, GABA and acetate (**Figure 2**). The highest glutamine and glutamate content was observed in control and blank plants but the treatment with *S. flexneri* and *S. aureus* was observed to increase the level of sucrose in *Brassica* leaves. In the case of *S. typhimurium*, glucose proved to be differentiating metabolite from control and other treated plants. Acetate, threonine, and GABA were responsible for the discrimination of *B. subtilis* and *E. coli* treated plants from other treatments both in PC1 and PC2 (**Figure 2**).

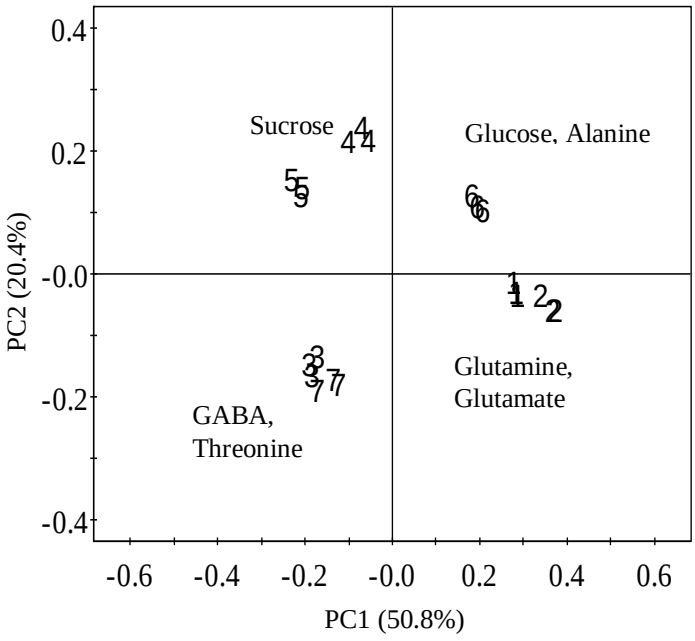

Figure 2 – Score plot of PCA based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0). 1; Control (with 500 µl of sterilized broth), 2; blank (without bacteria and broth), and plants treated by 3; *Bacillus subtilis*, 4; *Staphylococcus aureus*, 5; *Shigella flexneri*, 6; *Salmonella typhimurium*, and 7; *Escherichia coli*.

A clear separation in PCA was observed between Gram (+) (*S. aureus* & *B. subtilis*) bacteria treated plants. The separation of *S. aureus* was due to coumaroyl malate, sucrose, α-glucose, β-glucose and glutamic acid. Separation of *B. subtilis* in PCA was determined by the presence of feruloyl-malate, sinapoyl-malate, threonine, alanine, GABA and histidine (**Figure 3**). Similarly, a clear discrimination of Gram (–) (*E.coli, S. flexneri* and *S. typhimurium*) bacteria-treated plants was observed. Separation of *S. typhimurium* was due to sinapoyl malate and *S. flexneri* was separated due to signals of caffeoyl-malate, coumaroyl-malate and histidine, while *E. coli* was separated due to feruloyl-malate and fumarate signals in PCA (**Figure 4**).

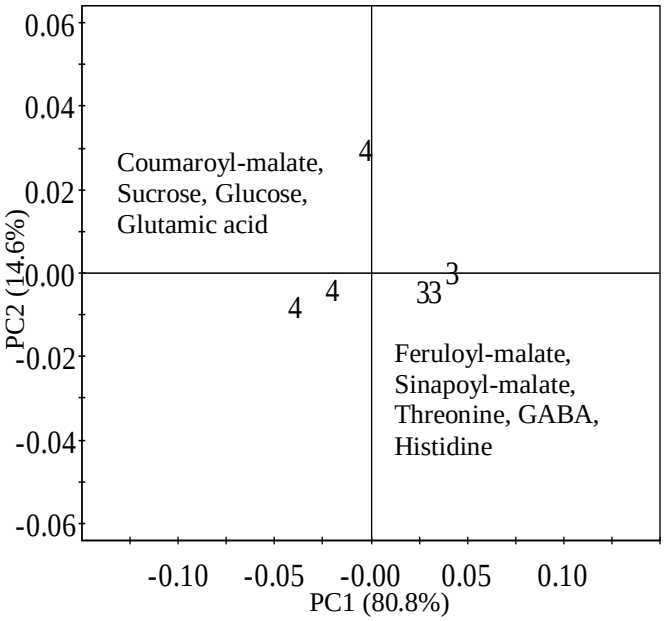

Figure 3 – Score plot of PCA based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0). Plants treated by Gram positive bacteria, 3; *Bacillus subtilis* and 4; *Staphylococcus aureus.*

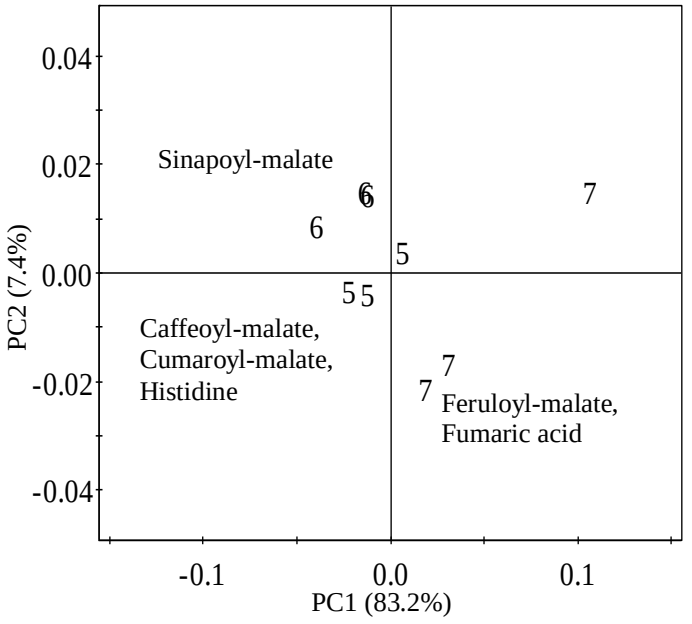

Figure 4 – Score plot of PCA based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0). Plants treated by Gram negative bacteria 5; *Shigella flexneri*, 6; *Salmonella typhimurium*, and 7; *Escherichia coli*.

As next step for metabolomic analysis, the partial least square–discriminant analysis (PLS-DA). PLS-DA is performed based on pre-input information, i.e. unlike the unbiased system used for PCA, information is classified prior to its input. The most important information obtained from PLS-DA is the correlation between two data sets, in this case, the investigation of ^{1}H NMR signals and their classification such as control, blank, Gram(+) and Gram (–) treated plants. For the classification required by PLS-DA three groups were applied. Group 1 was for control and blank samples, group 2 for Gram (+) bacteria treated *Brassica*, and group 3 for Gram (–) treated ones. In the PLS-DA score plot those three groups were well separated (**Figure 5a**). The identification of characteristic metabolites in each group loading plot of PLS-DA is displayed in **Figure 5b**. Threonine and GABA were found to be the discriminating metabolites in Gram (+) bacterial treated plants. However, in the case of Gram (–) bacterial treated ones, sinapoyl-malate, caffeoyl-malate and histidine were clearly increased, while sugar, glucose, glutamine and glutamate levels were decreased.

Plant disease resistance to pathogens such as fungi, bacteria, and viruses often depends on whether the plant is able to recognize the pathogen. Recognition of pathogens triggers a large range of inducible defence mechanisms that are believed to contribute to overall resistance in the plant.[391] Plants, for example, can synthesize secondary metabolites as a defensive response. It has been observed that the level of production of phenolic compounds is particularly sensitive to the type of attacking bacteria.[392] This is of course, a relevant factor for a plant that will be included in human diet, since these compounds are particularly bioactive and have pronounced effects.[393] By comparing different spectra the increase in the production of GABA, phenylpropanoids, glutamine, glutamate, sugars and amino acids was confirmed. A clear separation in PCA of plants submitted to the different treatments shows that bacterial strains differ in their ability to induce resistance in *Brassica rapa* leaves.

These pathogens have developed sophisticated mechanisms to interact with their hosts through a specialized protein secretion system, which has been identified in several Gram (–) pathogenic bacteria including the plant pathogens *Pseudomonas* spp., *Erwinia* spp. and *Xanthomonas* spp. and the animal pathogens *Salmonella* spp., *Pseudomonas aeruginosa*, *Shigella* spp., *Yersinia* spp., and *E. coli* spp.[394]

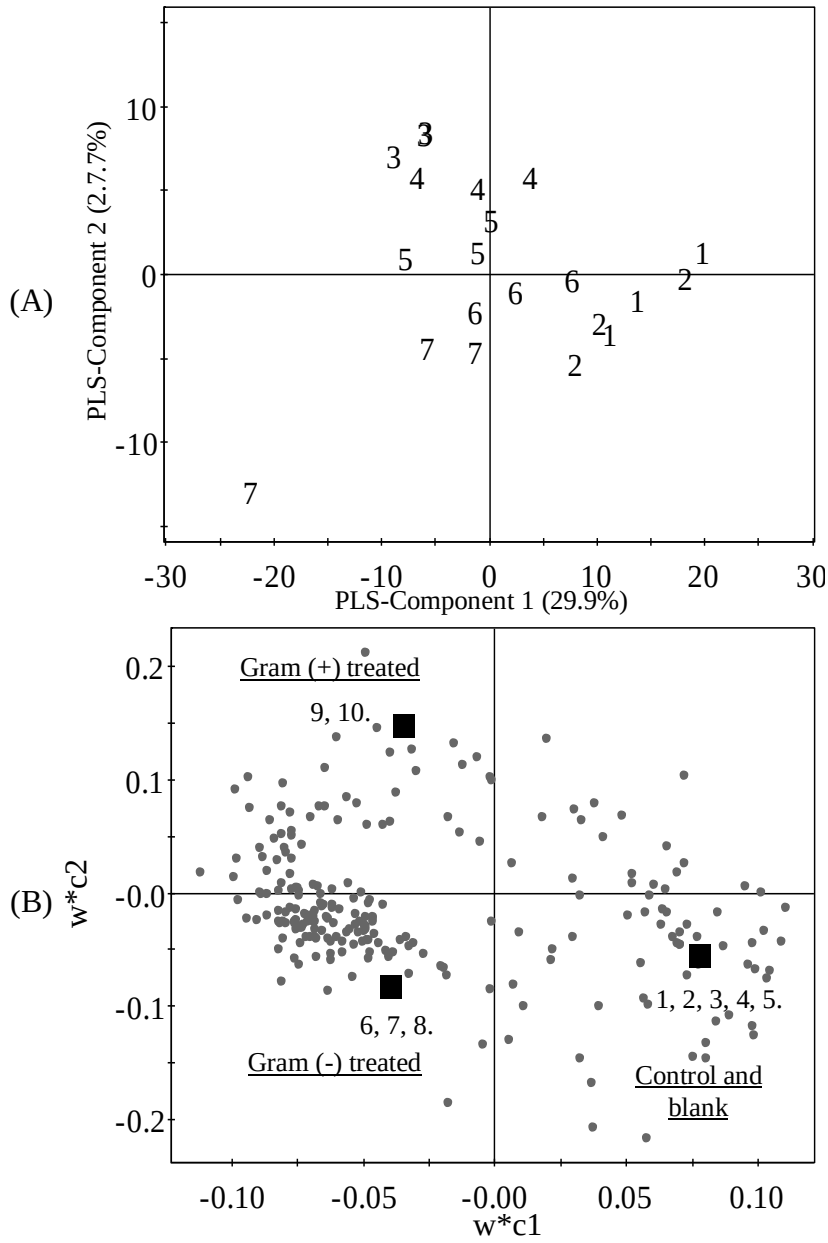

Figure 5 – Score (A) and loading plot (B) of PLS-DA based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0). 1; Control, 2; blank, and plants treated by 3; *Bacillus subtilis*, 4; *Staphylococcus aureus*, 5; *Shigella flexneri*, 6; *Salmonella typhimurium*, and 7; *Escherichia coli*. Control and blank region in loading plot contains glucose (1), sucrose (2), valine (3), glutamine (4), glutamate (5). Region of gram negative bacterial treatment in PLS-DA loading plot contains sinapoyl malate (6), caffeoyl malate (7) and histidine (8). Region of gram positive bacterial treatment in PLS-DA loading contains threonine (9) and γ-Amino-butyric acid (GABA) (10).

The decrease in the quantity of sugars produced in all bacteria-treated plants can be attributed to the relocation of plant resources and utilization of sugars for primary and secondary metabolism,[376, 395] especially in the case of *B. subtilis* and *E. coli* treated plants. Production of GABA, a non protein producing amino acid derived from glutamate catabolism after abiotic and biotic stresses was also observed in all treatments. It is thought that it acts as a signal molecule.[396]

Increase of primary and secondary metabolites in infected plants suggested that biotic stress by the tested human pathogenic bacteria could cause induced systemic resistance (ISR) in *Brassica* leaves. In general, these compounds are either absent or present in very low concentrations in healthy plants. However, upon infection their concentration increases considerably to prevent infection by the invading bacteria.

4 Conclusion

Plant response to bacterial stress depends on the type of invading bacteria. The set of metabolites affected by different microorganisms differed, probably reflecting the chemical environment of the invaded tissue and the mechanism of action of the infecting bacteria.[352] The present results show the potential of NMR to study the interaction of foodborne bacteria and vegetables. NMR- based metabolomics seems a promising tool for studying preharvest conditions on the quality of vegetables.

More insight in the plant response to microorganisms may lead to the identification of specific biomarkers for plants infected with certain types of microorganisms as this study indicate that there might be a difference between plant infected with Gram+ and Gram– bacteria. The biomarkers found need to be confirmed by validation with a larger set of bacteria.

Acknowledgement

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Chapter 6

Metal ions-inducing metabolites accumulation in *Brassica rapa* (var. raapstelen)

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Abstract

Plants always face biotic and abiotic environmental stress factors during growth. Among the abiotic factors, particularly, great attention has been paid to metals not only because of their increasing amounts in the environment due to rapid industrial development but also because of the variation of metal composition in soil. Cultivation of crops close to industrial areas or irrigation with contaminated water may result in both growth inhibition and tissue accumulation of metals. *Brassica* species are well known as metal accumulators and are being used for phytoremediation of contaminated soils. However, the metal tolerance mechanism in the plant still remains unclear. In order to investigate the metabolomic changes induced by metal ions in *Brassica*, plants were subjected to concentrations 50, 100, 250 and 500 mmol of copper (Cu), iron (Fe) and manganese (Mn) in separate treatments. ^{1}H NMR and two-dimensional NMR spectra coupled with principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were applied to investigate the metabolomic change of *Brassica rapa* (var. raapstelen). Based on the ^{1}H NMR analysis followed by the application of chemometric methods, a manifold of metabolomic consequences was observed. For the treatments phenylpropanoids, glucosinolates as well as the primary metabolites like carbohydrates and amino acids were found to be the discriminating metabolites. This study shows that the effect of Cu and Fe on the plant metabolome was larger than that of Mn and that the metabolomic changes varied not only according to the type of metal but also to its concentration.

Keywords: *Brassica rapa*, Metabolomic analysis, Metal ions, NMR spectroscopy, Partial least square-discriminant analysis.

1 Introduction

The high level of genetic resemblance between *Brassica* and *Arabidopsis* has allowed it to be considered as an alternative model system in the field of plant physiology. Additionally, its wide distribution in nature has led to the existence of very different ecotypes, due to which, *Brassica* species are considered one of the most important plant models to study the interaction between the plant and diverse environmental factors including metals in soil, UV and drought, as well as living organisms such as insects, fungi, or bacteria.[397-399] Economically, *Brassica* species are becoming important food crops and are considered to be an invaluable source of vegetable oil and proteins for human nutrition.[51] Moreover, *Brassica* vegetables are well known for their varied nutrients such as vitamins, glucosinolates, soluble sugars, fats, and carotenoids as well as fibres.[400] In terms of other secondary metabolites, the plants are good sources of health-promoting phytochemicals including phenolics, flavonoids and phenylpropanoids.[55] *Brassica* plants have been mainly used for their nutritional qualities but recently, phytoextraction combined with biofuel production is becoming a profitable enterprise.[401] Consequently, further improving the efficient production of *Brassica* crops is of great interest due to their current large demand in the market.

Throughout their growth, plants have frequently to cope with unfavourable environmental conditions, such as pesticides, drought, UV, light deficit, salt, metals as well as herbivores and infection with bacteria, fungi and viruses. Among the exogenous factors, metals have particularly attracted the attention of researchers, not only because they are part of the soil components but also because the rapid increase in industrial activities might bring about a larger exposure and consequently uptake of metals.

Application of sewage sludge to the soil provides a significant amount of the plant nutrients including copper (Cu), manganese (Mn), iron (Fe), zinc (Zn), phosphorus (P) and potassium (K).[23] If applied in excessive amounts however, Cu, Fe and Mn may become major contaminants as is the case with drainage and agricultural soils treated with sludge.[23, 402] In this study, the metabolomic response of *Brassica* to the presence of some of these metals was evaluated, subjecting its leaves and roots to NMR spectroscopy and analysing the data with multivariate data analysis.

Cultivation of crops close to contaminated sites may lead to both growth inhibition and tissue accumulation of metals, resulting in possible risks to humans or livestock if these tissues are ingested.[403] As an example, the growth of *Brassica* species has been reported to be greatly retarded by the accumulation of cadmium (Cd), copper (Cu), lead (Pb), zinc (Zn).[403] *Brassica juncea* has been reported to accumulate high levels chromium (Cr) and nickel (Ni). Such metal accumulating plants show a remarkable degree of selectivity in their uptake and protection mechanisms exhibiting a high specialization according to the species and the metal. [404] Soluble metals may enter the root by either active or passive transport and likely to move from the roots to the shoots during transpiration. Transport increases with the solubility of the metal complex.[404]

On the other hand *Brassica* crops can be enriched by growing on different metal concentrations and administrated as specific mineral supplements for human consumption.[107] The presence of redox active metals like Fe and Cu results in H_2O_2 and hydroxide ion production via Fenton type reactions, causing cellular injury in plants.[21] Even micronutrients such as Mn may cause oxidative stress when available in excess.[20] In this situation plant survival depends on its capacity to increase specific pathways for reactive oxygen species (ROS) removal.[405]

Response to metal toxicity is expressed in a variety of different ways. These include immobilization, exclusion, chelation and compartmentalization of the metal ions aside from the expression of more general stress response mechanisms through organic acids, such as citrate, malate, and some amino acids. Histidine is particularly important in the chelation of metal ions.[406] Increased production of reactive oxygen species followed by primary defence reactions also result in the increased production of secondary metabolites[407] such as phenylpropanoids, terpenoids and alkaloids.[408] However, despite these efforts, more research is needed to completely understand the metal tolerance mechanism in the plant system. So far, metal and plant interaction in terms of metabolomic response, has not been thoroughly studied.[409]

Metabolomic studies are always considered to be complex due to the large number of metabolites involved. Though it is almost impossible for one single analytical method to provide information about all the metabolites in plants, nuclear magnetic spectroscopy (NMR) constitutes an optimum choice for the first step of a metabolomic study from a macroscopic viewpoint. It is a non-destructive method and can simultaneously detect and quantify all proton-bearing compounds such as

phenolics, carbohydrates, amino acids, fatty acids, amines, esters, lipids etc., in a short time.[36, 37] Although ^{1}H NMR is rather insensitive compared with other methods such as mass spectrometry (MS), it has the advantage of allowing the detection of diverse groups of plant metabolites in a single run, thus motivating researchers to use it as a macroscopic approach for metabolomics.

The aim of the present research was to examine the metabolomic changes in *Brassica rapa* leaves and roots subjected to Cu, Fe and Mn stress. For this purpose ^{1}H NMR and two dimensional NMR spectra, with unsupervised and supervised multivariate data analysis including principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were applied.

2 Materials and Methods

2.1 Solvents and chemicals

CH_3OH-d_4 (99.96%) and D_2O (99.00%) were purchased from Cambridge Isotope Laboratories Inc (Miami, FL, USA) and NaOD was purchased from Cortec (Paris, France).

2.2 Plant material

Brassica rapa seeds of a registered cultivar (var. raapstelen, Groene Gewone) were sown in pots containing soil and kept in cold room (4 ^{o}C) for two days and then transferred to a green house in 24-hour light conditions. After six days of growth the seedlings were transferred to small pots and watered daily, until four weeks.

2.3 Metal application

Stock solutions of 50 mM for each of $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$ was prepared. For each metal treatment increasing volumes (1, 2, 5 and 10 ml) of each solution was applied to the soil near the plant root to achieve amounts of 50, 100, 250 and 500 mmol of metals respectively. One week after this, the plants were harvested and washed thoroughly with deionized water. Leaves and roots were separated and immediately frozen in liquid nitrogen. Prior to extraction all material was pulverized under liquid nitrogen using a mortar and pestle and freeze dried.

2.4 Extraction of plant material

Three replicates were used for analysis, with one plant for each replication. Fifty milligrams of freeze dried material was transferred to a microtube (2 ml) to which 1.5 ml of 50% CH_3OH-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) containing 0.05% TMSP (trimethylsilylpropionic acid sodium salt, w/v) was added. The mixture was vortexed at room temperature for 1 min, sonicated for 20 min, and centrifuged at 13,000 rpm at room temperature for 5 min. Eight hundred microliters of the supernatant was transferred to a 5 mm-NMR tube.

2.5 NMR measurement

^{1}H NMR, 2D-J-resolved spectra were recorded at 25 ^{o}C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany). ^{1}H-^{1}H-correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600 MHz Bruker DMX-600 spectrometer (Bruker). All the NMR parameters were the same to those of our previous reports. [27, 28]

2.6 Data analysis

Spectral intensities of ^{1}H NMR spectra were scaled to total intensity and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.4- δ 10.0. The regions of δ 4.8 – δ 4.9 and δ 3.28 – δ 3.40 were excluded from the analysis because of the residual signal of the deuterated solvents. Principal component analysis (PCA) and partial least square-discrimination analysis (PLS-DA) were performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden) based on unit-variance scaling method. ANOVA test for ^{1}H NMR signals were performed by MultiExperiment Viewer (v. 4.0) [410].

3 Results and discussion

Each metabolomic study starts with establishing a database with the metabolome of the plants under well defined condition in order to learn more about the natural biological variability. In this part of the study also the identification of metabolites by means of various spectrometric methods is included.

Thus, in the previous papers of our group we reported the identification of a number of metabolites from *Brassica rapa*. This included amino acids, organic acids, carbohydrates, glucosinolates, and

phenylpropanoids. Identification was made by ^{1}H NMR together with diverse 2D spectra including J-resolved, COSY, HSQC, and HMBC spectra.[27, 28, 41, 354] The amino and organic acids region (δ 0.80 – δ 4.00) in the spectra showed the signals of acetate, alanine, GABA (γ-amino-butyric acid), glutamate, glutamine, malate, serine, threonine and valine, while fumarate, phenylalanine and tyrosine were identified in the aromatic region. Besides the primary metabolites, diverse glucosinolates and phenylproponoids were detected in the ^{1}H NMR spectra. In particular, more than five phenylpropanoids were clearly detected in J-resolved spectra using the chemical shift of H-8´ [28, 354] (**Figure 1**).

In the present study, we further identified signals due to glucosinolates. The typical signal of the anomeric protons of glucosinolates is a doublet at δ 4.70 - δ 5.00 with a coupling constant *J* = 10 Hz (Abdel-Farid et al., 2007). Three glucosinolates, progoitrin at δ 2.87 (dd, *J* = 16.0, 10.0 Hz), δ 4.63 (m), δ 5.21 (dt, *J* = 11.0, 2.0 Hz), δ 5.34 (dt, *J* = 16.0, 2.0 Hz), and δ 5.96 (m), gluconapoleiferin at δ 2.79 (dd, *J* = 15.6, 8.5 Hz), δ 4.20 (dd, *J* = 10.0, 7.8 Hz), δ 2.34 (m), δ 5.89 (m), δ 5.14 (dd, *J* = 10.0, 2.0 Hz), δ 5.04 (dd, *J* = 10.0, 2.0 Hz), and glucobrassicanapin δ 2.7 (m), δ 2.56 (m), δ 2.14 (m), δ 5.88 (m), δ 5.18 (dd, *J* = 17.2, 2.5), δ 5.10 (dd, *J* = 17.2, 2.5) were assigned with the help of ^{1}H NMR spectra (**Figure 1**), COSY, HSQC and HMBC spectra.

Using our extensive database of the *B. rapa* metabolome we studied the effect of different metal ions on the metabolome. A large difference was observed between the metabolomic profiles obtained by ^{1}H NMR analysis of roots and leaves (**Figure 1**). Sugars, glucosinolates and some free amino acids were found to be in higher amounts in roots while phenylpropanoids were clearly more concentrated in leaves. In order to assess the effect of metal ions on the *Brassica* metabolome of different organs, principal component analysis (PCA) was performed separately on leaves and roots. In both cases clear changes in the level of amino acids, organic acids, sugars, glucosinolates, and phenylpropanoids were observed in all the metal treated *Brassica* plants when compared with control plants (**Figure 2**).

The metals evaluated in this study affected the *Brassica rapa* metabolome differently, Cu and Fe having a higher effect than Mn. As shown in **Figure 2** the PC scores of the plants treated with Cu and Fe are more separated than those of manganese from the control. However, in all cases, the sugar level evidently decreased in metal treated plants. Apparently the plant reallocates its resources in defence metabolism.[377]

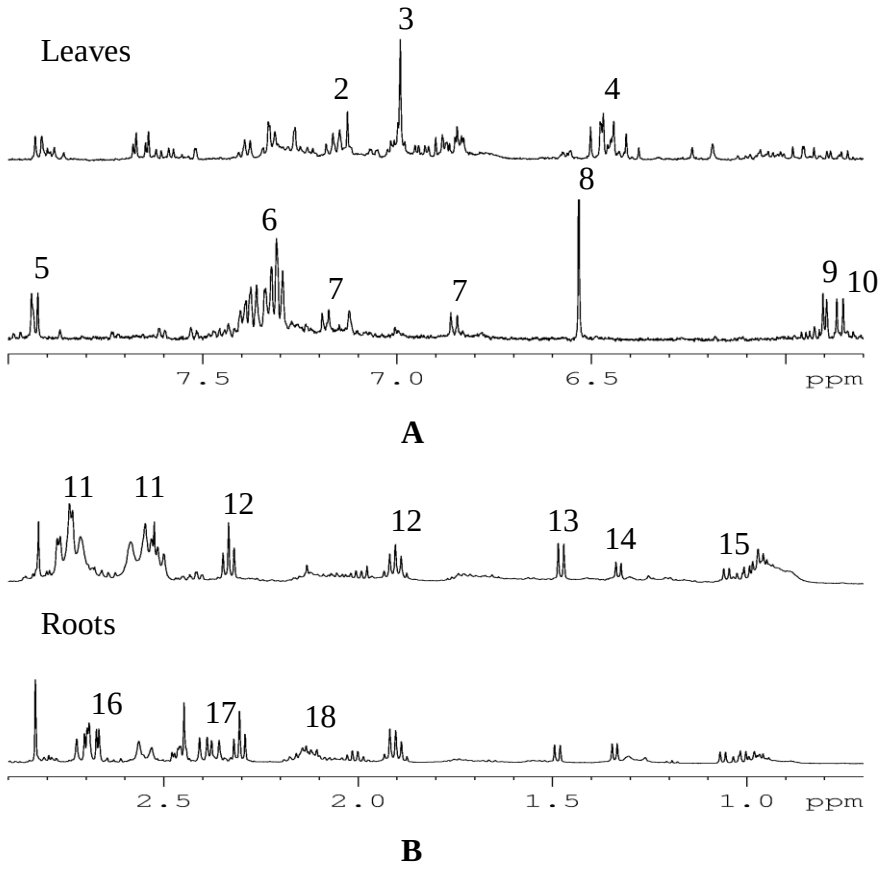


Figure 1 – ^{1}H NMR spectra of *Brassica rapa* leaves and roots in aromatic (A) and amino acid region (B). 1; H-7' of phenylpropanoids, 2; phenolic signal of caffeoyl malate, 3; phenolic signal of sinapoyl malate, 4; H-8' of phenylpropanoids, 5; a cytosine analogue, 6; phenylalanine, 7; tyrosine, 8; fumarate, 9; progoitrin, 10; a cytosine analogue, 11; malate, 12; γ-amino butyric acid, 13; alanine, 14; threonine, 15; valine, 16; glucobrassicanapin, 17; gluconapoleiferin, 18; glutamate and glutamine.

The comparison of the results obtained with treated and untreated (control) plants, showed that not only the type of metal but also their concentration largely affects the level of metabolites. Depending on the concentration and type of metal applied, expression of primary and secondary metabolites is clearly different (**Figure 2**). Cu (100 mmol) and Fe (100 mmol) treated leaves were grouped together and are well separated from control and other treatments by PC1 and PC2 scores, associated to a high production of the primary metabolites, alanine,

threonine, valine, glutamic acid, glutamine, the malate conjugates of ferulic, sinapic, 5-hydroxyferulic and caffeic acid, and of the glucosinolates, progoitrin, gluconapoleiferin and glucobrassicanapin.

Similarly, in roots, Cu (100 mmol) and Fe (100 mmol) treatments produced the largest separation of the metabolome of treated and control samples. Discrimination in PC1 and PC2 of the results of the 100 mmol Cu treatment seems to be due to the level of progoitrin, gluconapoleiferin, glucobrassicanapin, fumarate and serine while in Fe (100 mmol) treatment the separation was related to amino acids such as alanine, threonine, valine, glutamic acid, glutamine and the malate conjugates of ferulic, sinapic, 5-hydroxyferulic, and caffeic acid.

Although a separation among different metal treatments is observed in the PCA score plot there is an overlap of different treatments which makes it difficult to clearly visualize and understand the effect of metal concentration. Therefore, the effect of different concentrations of each metal was separately analyzed by PCA. The results of these experiments show that increasing the concentration of each metal greatly affected the accumulation of sugars, amino acids, phenolic compounds and glucosinolates.

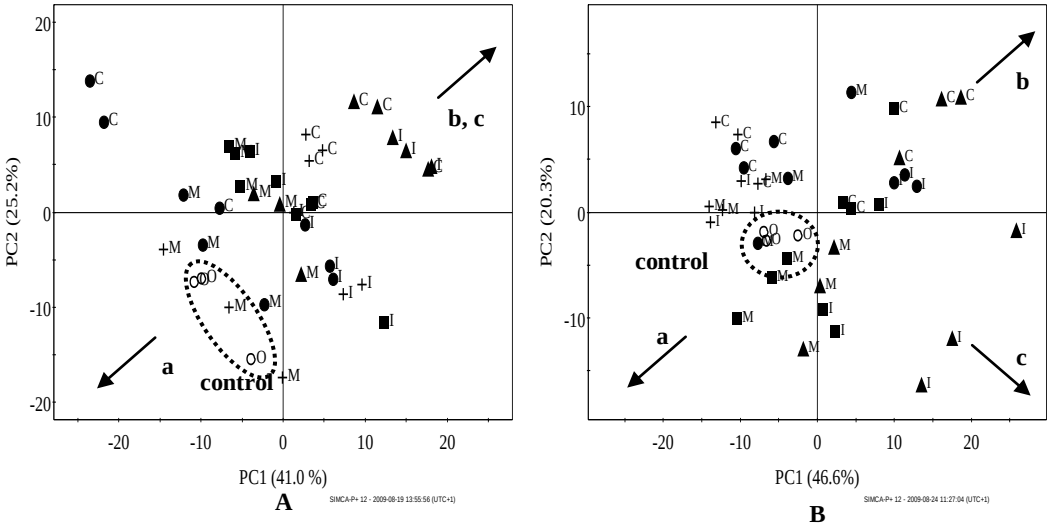


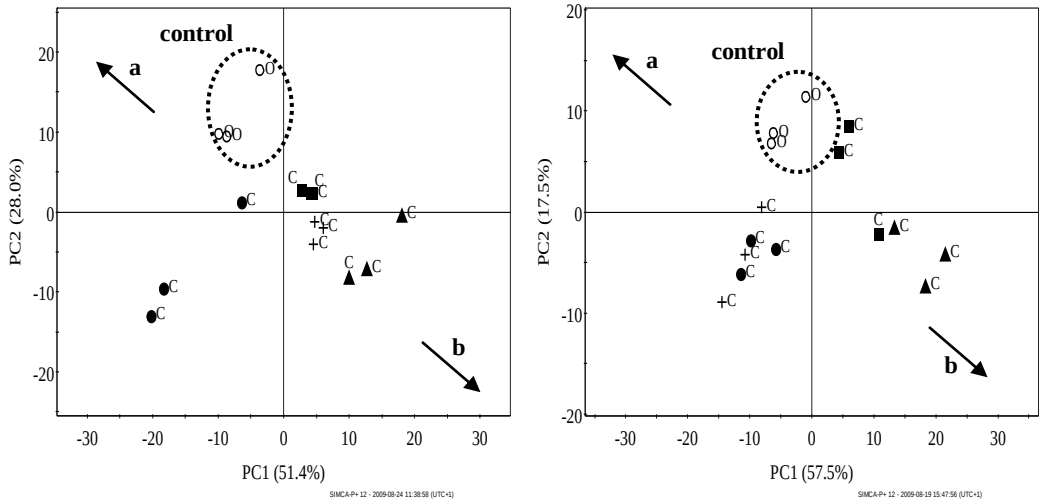
Figure 2 – Score plot (PC1 vs PC2) of PCA for *Brassica rapa* leaves (A) and roots (B), based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0). Control (O), Cu (C), Fe (I), Mn (M); Control (black-open circle); 50 mmol (●); 100 mmol (▲); 250mmol (+); 500 mmol (■). **a** = glucose, sucrose; **b** = progoitrin, gluconapoleiferin, glucobrassicanapin, fumarate, serine; **c** = alanine, threonine, valine, glutamate, glutamine, feruloyl malate, sinapoyl malate, 5-hydroxyferuloyl malate, caffeoyl malate.

A high amount of amino acids, phenolics, and glucosinolates were observed in *Brassica* leaves and roots treated with Cu (100 mmol) as compared with the control samples (**Figure 3**). The same behaviour was detected in the case of Fe (100 mmol) treated leaves and roots (**Figure 4**). Conversely, the sucrose and glucose level decreased in the treated plants. In the case of Mn, treatments with different concentrations were not well separated.

In order to confirm the results obtained by PCA, another multivariate data analysis method, PLS-DA was employed. Although PCA is a typical unsupervised method in which all tested samples can be grouped on the basis of maximum variation within evaluated samples, a minor change might be ignored, particularly when each group exhibits a large biological variation compared with that between groups. In this context, a type of supervised multivariate data analysis targeting on covariance between two datasets is required to investigate minor changes responsible for separation between interesting groups.

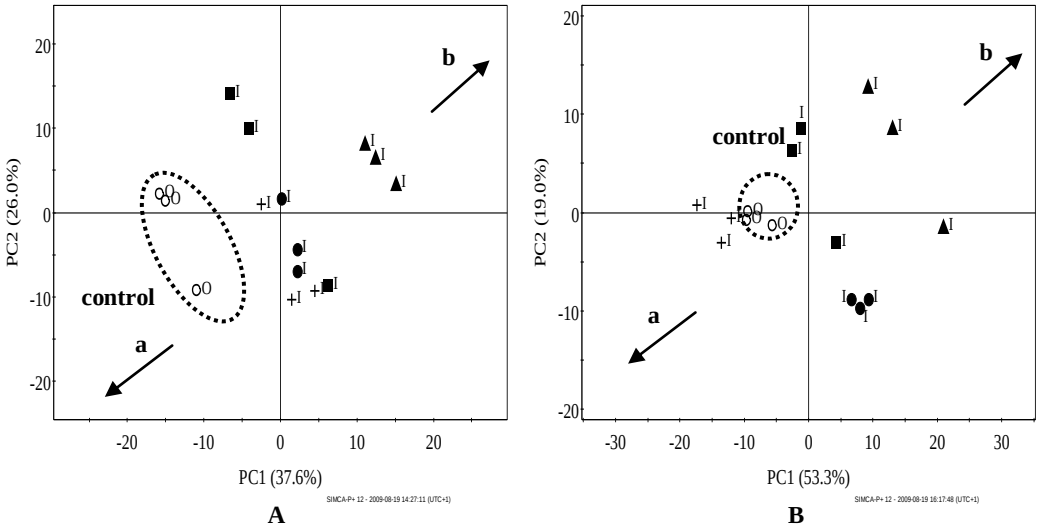
Thus in order to review the effect of the type of metal ion, the common supervised multivariate data analysis, PLS-DA was applied. The samples were grouped into four classes, Cu, Fe, and Mn treated *Brassica* and control plants. As a result of this, a better separation in the metabolome of different metal treated plants is observed in PLS-DA as compared with PCA.

For example, in PCA Cu (100 mmol) and Fe (100 mmol) treated *Brassica* were grouped together but in PLS-DA a manifest separation was observed even between Cu and Fe. Additionally signals of discriminating metabolites were much more clearly visualized. Different concentrations of the same metal were grouped together to study the principle discriminating metabolites among different treatments and control. In PLS score plot (**Figure 5 and 6**) a clear discrimination was observed between iron, copper, control and manganese treated plants. The discrimination in leaves (**Figure 5**) of Cu treated plants was observed to be due to alanine, threonine, valine, glutamate, glutamine and glucobrassicanapin, while the discrimination in the leaves of Fe treated plants was due to feruloyl malate, sinapoyl malate, 5-hydroxyferuloyl malate, and caffeoyl malate. Progoitrin and gluconapoleiferin were also found to be responsible for the separation of both the Cu and Fe groups from control and Mn treatments.



A **B**

Figure 3 – Score plot (PC1 vs PC2) of PCA for *Brassica rapa* leaves (A) and roots (B), based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0). Control (O), Cu (C); Control (black-open circle); 50 mmol (●); 100 mmol (▲); 250mmol (+); 500 mmol (■). **a** = glucose, sucrose; **b** = progoitrin, gluconapoleiferin, glucobrassicanapin, fumarate, serine; **c** = alanine, threonine, valine, glutamate, glutamine, feruloyl malate, sinapoyl malate, 5-hydroxyferuloyl malate, caffeoyl malate.

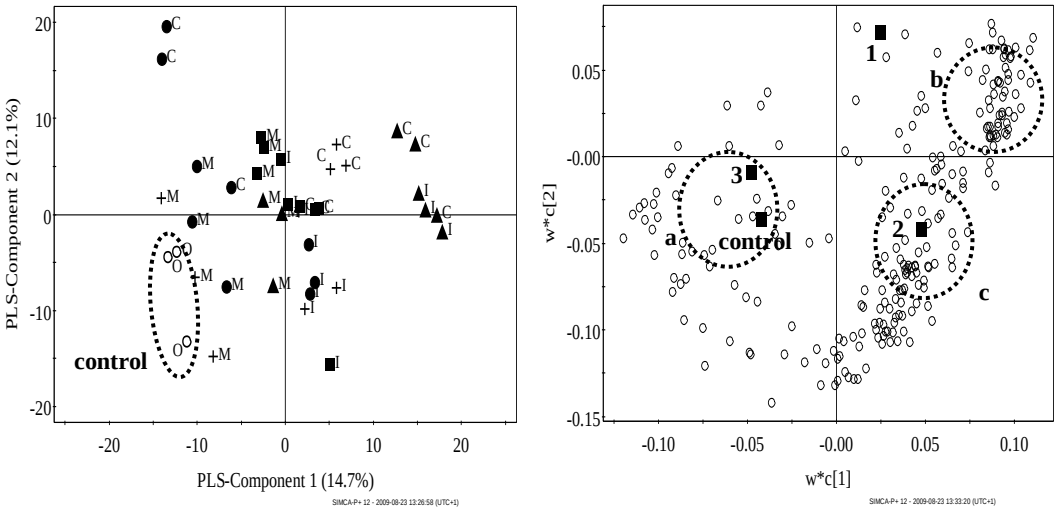


A **B**

Figure 4 – Score plot (PC1 vs PC2) of PCA for *Brassica rapa* leaves (A) and roots (B), based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0). Control (O), Fe (I); Control (black-open circle); 50 mmol (●); 100 mmol (▲); 250mmol (+); 500 mmol (■). **a** = glucose, sucrose; **b** = alanine, threonine, valine, glutamate, glutamine, feruloyl malate, sinapoyl malate, 5-hydroxyferuloyl malate, caffeoyl malate.

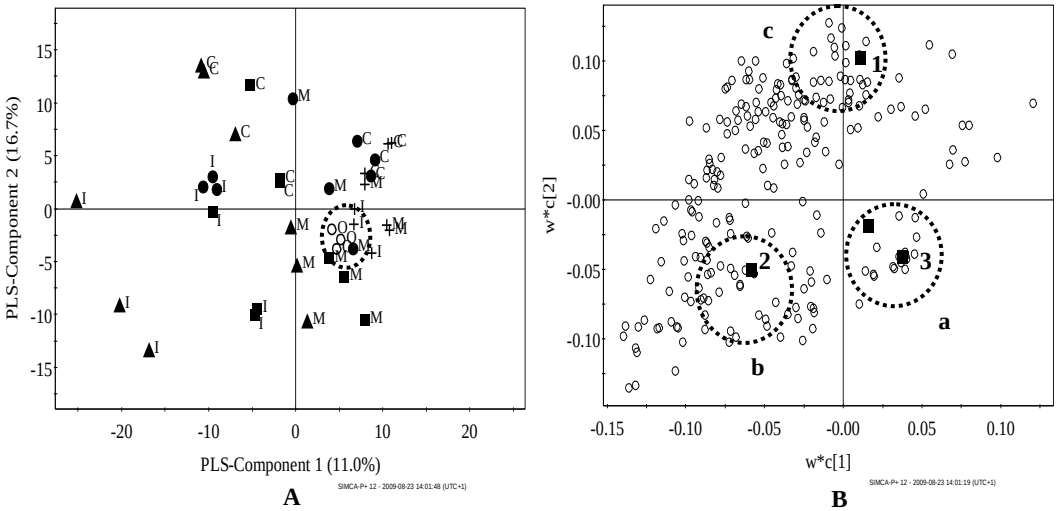
Meanwhile in the PLS-DA loading scatter plot of root samples (**Figure 6**) a clear separation was observed in Cu treatments due to feruloyl malate, alanine, threonine, glutamate, glutamine, glucose, progoitrin, gluconapoleiferin and glucobrassicanapin. In Fe treatments the separation was found to be due to sinapoyl malate, 5-hydroxyferuloyl malate, caffeoyl malate, valine, and γ-amino-butyric acid.

Our results indicate that upon metal exposure, especially in case of Cu and Fe treatments, plant produces more amino acids, phenolics and glucosinolates. Amino acids and phenolics are reported to have a metal chelating effect[275] indicating that the observed increase in amino acids and phenylpropanoids might be a detoxification response of the plant. However, high metal concentrations (500 mmol) produced a decrease in primary and secondary metabolites as compared with moderate concentrations (50 mmol & 100 mmol) which might point to an adverse effect of metal ions on primary and secondary metabolism proving the toxic effect of metals at high concentrations.



A **B**

Figure 5 – Score plot (PLS-Component 1 vs PLS-Component 2) (A) (Control – **O**; Cu – **C**; Fe – **I**; Mn – **M**); Control (black-open circle); 50 mmol (●); 100 mmol (▲); 250mmol (+); 500 mmol (■); and loading plot (B) (Control, Cu – **1**; Fe – **2**; Mn – **3**) of PLS-DA for *Brassica rapa* leaves, based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0). **a** = glucose, sucrose; **b** = progoitrin, gluconapoleiferin, feruloyl malate, sinapoyl malate, 5-hydroxyferuloyl malate, caffeoyl malate; **c** = alanine, threonine, valine, glutamate, glutamine, glucobrassicanapin.



A **B**

Figure 6 – Score plot PLS-Component 1 vs PLS-Component 2) (A) (Control – **O**; Cu – **C**; Fe – **I**; Mn – **M**); Control (black-open circle); 50 mmol (●); 100 mmol (▲); 250mmol (+); 500 mmol (■); and loading plot (B) (Control, Cu – **1**; Fe – **2**; Mn – **3**) of PLS-DA for *Brassica rapa* roots, based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0). Control (black), Cu (blue), Fe (red), Mn (purple). **a** = sucrose, malic acid; **b** = valine, γ-amino-butyric acid, sinapoyl malate, 5-hydroxyferuloyl malate, caffeoyl malate; **c** = alanine, threonine, glutamate, glutamine, glucose, feruloyl malate, progoitrin, gluconapoleiferin, glucobrassicanapin.

Plants are known to tolerate metals to some extent but high concentrations of available metals affect and induce disorders in the plant metabolism.[411] Disturbance of the metabolism by excessive metal appears to happen in multiple ways, causing a reduction of chlorophyll content, inhibiting plant growth and respiration, altering the activity and quantity of the key enzymes of various metabolomic pathways.[411] Under metal stress, plants produce primary and secondary metabolites which increase with increasing metal concentrations up to a certain point, beyond which a decrease in primary and secondary metabolite concentration was observed.[409] In general, the primary and the secondary metabolites have three major functions, i.e., metal binding, antioxidant defence, and signalling.[33] The increase of phenolic compounds is dependent on both the type of metal and its concentration, which correlates with their chelating activity, hydroxyl (OH^-) scavenging capacity, reduction potential and cytoprotectivity.[409]

4 Conclusion

Plants can absorb and distribute metals internally in many different ways and may localize selected metals mostly in leaves and roots.[412] As a mechanism of metal tolerance or accumulation in plants, apparently the response to metal stress is observed in both leaves and roots. This response and accumulation of metals is more dependent on type of metal rather than metal concentration.[412] Primary and secondary metabolites play a crucial role in stress induced responses of *Brassica* plants, in case of metal ion stresses. These results lead to the better understanding of the role of plant metabolites in stress conditions, ultimately describing the role of health affecting compounds in plant, under stressed conditions.

Acknowledgments

The Higher Education Commission (HEC) of Pakistan is gratefully acknowledged for the support of M. Jahangir. We also thank Ms. E. G. Wilson for reviewing the manuscript and providing helpful comments and suggestions.

Chapter 7

Postharvest storage stability of red radish (*Raphanus sativus* L.) at different temperatures

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Abstract

Radish (*Raphanus sativus* L.) is a well known and commonly consumed vegetable all over the world. Its bioactive or nutritional constituents consist of a wide range of metabolites including, glucosinolates, phenolics, amino acids, organic acids, and sugars. However, many of these metabolites are not stable and easily degraded or modified during storage. In order to investigate the metabolomic changes occurring during post harvest storage, radish samples were subjected to four different storage temperatures (20 ^{o}C, 4 ^{o}C, –20 ^{o}C, and –80 ^{o}C) in 28 days storage time course. ^{1}H nuclear magnetic resonance (NMR) and two-dimensional NMR spectra data were subjected to principal component analysis (PCA) followed by partial least square-discriminant analysis (PLS-DA) to investigate the metabolomic changes.

A profound chemical alteration, both in primary and secondary metabolites was observed. Glucosinolates, phenylpropanoids, amino acids, organic acids, and sugars were found to be the discriminating metabolites for storage effects. At the start of the storage an increase in secondary metabolites (phenylpropanoids, flavonoids and glucosinolates) is observed followed by a decrease in these compounds in later stages. This loss is high at room temperature while lower at cold and freezing temperatures. During the later storage stages, still a high amount of primary metabolites is observed.

Keywords: radish (*Raphanus sativus* L.), storage effect, postharvest storage, ^{1}H NMR, multivariate data analysis.

1 Introduction

Different post-harvest processing and storage methods are reported to retard the degradation of bioactive compounds and to retain quality attributes in different vegetables.[89, 413-415] Post-harvest storage (e.g. low temperature storage, freezing, as well as fresh vegetable storage at room temperature), industrial processing (drying, blanching, canning etc.), and different cooking methods play an important role in degradation of *Brassica* bioactive components.[89] Effect of post harvest storage conditions especially cold storage on plant metabolome has always been a controversial issue. Changes may occur due to pure chemical conversion (fastest at higher temperature) or to residual metabolism of the plants and plant cells after harvesting. The later includes the plant response to wounding, infection, drought and temperature, which is quite complex and unpredictable as one or more responses may be active depending on the conditions. In this study radish presents a special case as roots and green parts remain intact as a whole plant.

It is also possible to prevent loss of nutritional value by using a lower storage temperature,[35, 416] as it results in decreased rate of metabolism, thus preserving radish quality.[417] But storage at chilling temperature can have both positive and negative effects on vegetables, depending on the commodity and the storage temperature.[11] Altered gene expression, e.g. in the response of plants to environmental stimuli, results in qualitative and quantitative changes in the metabolome.[35] The constitutive activation of several stress-inducible pathways and different kinetics in the accumulation of several metabolites may represent an advantage to prepare plants to face low temperature stress conditions.[418]

The evidence for the importance of health-promoting bioactive compounds present in *Brassica* vegetables has increased in the last few years.[419] Because of the nutritional importance of *Brassica* metabolites, there has been an increasing interest in the evaluation of these compounds in postharvest treatments.[420] Radish is one of the most important and worldwide well-known food crop belonging to the Brassicacae.[369, 421] Radish is considered to have health benefits due to the presence of sugars, amino acids, organic acids, phenolics, and glucosinolates.[101, 125, 400, 422] Also it is a well established model system for plant research.[370, 423, 424] Like many other vegetables, radish can be preserved by storage, pickling, canning or drying[2] but the chemical composition of its nutritional constituents, especially the phenolics is

easily affected by such pre-harvest agronomic and post-harvest processing and the storage conditions.[11, 416]

Phenolics, such as phenylpropanoids play an important role in vegetable food quality, such as in appearance, flavour, and antioxidant properties.[11] The key enzyme (phenylalanine ammonia lyase, PAL) in the biosynthesis of phenylpropanoids activity is regulated by a diverse array of pre-harvest and post-harvest factors.[11] These phenolic compounds can be degraded into other simple phenolics such as vanillic and protocatechuic acid[425] along with other degradation products including oxalic, glyoxylic, oxaloacetic, mesoxalic and formic acid.[426] In addition to aforementioned degradation products, *Brassica* vegetables include ascorbigens, which are formed as the result of the reaction between ascorbic acid and degradation products of indol-3-yl-methyl-glucosinolates produced in the myrosinase-catalysed degradation.[427, 428]

The comprehensive quantitative and qualitative analysis of all metabolites within a cell, tissue or organism is a very ambitious goal.[35] The components of the metabolome can be viewed as the end products of gene expression and define the biochemical environment of a cell or tissue. Metabolomics provides a broad view of the biochemical status of an organism[429] and is largely used to study the phytochemical changes in plants.[35] At present no single analytical method can provide information about all the metabolites in plants, since the diversity in plant metabolites is too large; volatility, polarity, solubility, and chromatographic behaviour and detectability differ largely.[27] So the selection of the most suitable analytical method is generally a compromise between speed, selectivity and sensitivity.[35] Nuclear magnetic spectroscopy (NMR) based metabolomics is becoming increasingly recognized in research and development as a highly reproducible and quantitative method. Although NMR is not as sensitive as some other analytical methods, like HPLC, MS, etc. But it is a non destructive method and can detect a large number and diverse groups of compounds in a single-run.[35, 37]

A lot of work has been done on the storage stability of vegetables after pre-treatment (e.g. blanching, packing, UV etc.) but there is still need to study the effect of different temperatures with respect to storage time, and without pre-treatment. NMR seems an optimum choice to study the overall major changes in the metabolome during cold storage.

The objective of the present study was to investigate the phytochemical changes in radish metabolome at different time points and temperatures, with particular focus on compounds related to quality of the food plant. To analyze these changes, ^{1}H NMR and two-dimensional

NMR spectra were used in combination with principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA).

2 Materials and methods

2.1 Preparation of plant material

Red radish (*Raphanus sativus* L.) plants were purchased from local market. Fresh and healthy plants were selected and washed thoroughly with de-ionized water and kept in open air at room temperature for half an hour to remove surface water from plant. The aerial parts (leaves and petioles) and roots were kept intect during storage.

2.2 Storage and sample collection

Plants were stored at four different temperatures [20 ^{o}C (room temperature), 4 ^{o}C, –20 ^{o}C, and –80 ^{o}C] in open plastic bags and kept in dark. Samples from each treatment were collected after each 2 h, 4 h, 6 h, 12 h, 24 h, 2 d, 3 d, 7 d, 14 d, 21 d and 28 d. After 3 days plants stored at room temperature started decaying so further sampling was stopped from this condition. Three replicates were used for analysis, with one plant for each replication. The aerial parts (mentioned as Leaves) were separated from roots and both roots and leaves were immediately frozen in liquid nitrogen. Leaves and roots were ground separately in liquid nitrogen and freeze-dried, to obtain a fine powder.

2.3 Extraction of plant material and NMR measurements

Fifty mg of freeze dried material were transferred to a microtube (2 ml) to which 1.5 ml of 50% methanol-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) containing 0.05% TMSP (trimethyl silyl propionic acid sodium salt, w/v) was added. The mixture was vortexed at room temperature for 1 min, ultrasonicated for 20 min, and centrifuged at 13,000 rpm at room temperature for 5 min. Eight hundred µl of the supernatant was transferred to a 5 mm NMR tube. NMR measurements were done as mentioned in our previous studies.[27, 28]

2.4 Data analysis

The ^{1}HNMR spectra were automatically reduced to ASCII (v. 3.7, Bruker Biospin). Spectral intensities were scaled to trimethylsilyl propionid acid sodium salt (TMSP) and reduced to integrated regions of

equal width (0.04) corresponding to the region of δ 0.4 – δ 10.0. The data were normalised to total intensity. The region of δ 4.7 – δ 4.9 was excluded from the analysis because of the possible residual signal of water. Principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden) by using unit variance (UV) scaling method.

3 Results and Discussion

The ^{1}H NMR spectra of methanol water extracts for the healthy aerial parts (leaves and petioles) and roots of red radish (*Raphanus sativus*) were studied. Based on NMR spectroscopy of roots and leaves, a number of metabolites were identified including amino acids, organic acids, carbohydrates, flavanoids, phenylpropanoids and glucosinolates. The signals of alanine, threonine, valine, fumaric acid, adenine, gallic acid, malic acid, glutamate, glutamine, acetate and GABA (γ-amino-butyric acid), were identified in the spectral region of organic and amino acids. Five phenylpropanoids (feruloyl malate, caffeoyl malate, sinapoyl malate, 5-hydroxyferuloyl malate, coumaroyl malate) and two glucosinolates (glucobrassicin and neoglucobrassicin) were identified in aromatic region as previously reported by our group.[24, 28, 354] In addition, flavonoids, quercetin-7-glucoside (quercimeritrin) at δ 6.47 (d, *J* = 2.2), δ 6.75 (d, *J* = 2.2), δ 7.07 (dd, *J* = 8.0, 2.1), δ 6.81 (d, *J* = 8.3), δ 7.21 (d, *J* = 2.2), and two kaempferol analogues at δ 8.05 (d, *J* = 9.2), δ 6.99 (d, *J* = 9.2), δ 6.80 (d, *J* = 2.1), δ 6.58 (d, *J* = 2.2) and δ 8.06 (d, *J* = 9.2), δ 7.03 (d, *J* = 9.2), δ 7.26 (d, *J* = 2.2), δ 7.15 (d, *J* = 2.2) were identified. These assignments were confirmed by diverse 2D NMR spectra including J-resolved, COSY, HSQC, HMBC, and by the comparison with reference compounds.

Principal component analysis (PCA) enables straightforward visualization of data similarities or differences in the data set.[35] Our study results were assessed by PCA and also by direct visual comparison of ^{1}H NMR spectra of different treatments. Using the data collected during this study, we examined the effect of storage time and temperature on the radish metabolome. Amino acids, organic acids, sugars, phenylpropanoids, flavonoids and glucosinolates were found to be the major discriminating metabolites (**Table 1**).

The leaves and roots spectra are compared in PCA. From these PCA results it is also concluded that the change in metabolites of leaves

is much higher as compared with the change in metabolites of roots, due to the diversity of compounds in leaves. This shows a clear separation of leaves and roots in two different groups. Carbohydrates are present in high amounts in roots as compared with leaves; on the other hand leaves show a high concentration of other primary and secondary metabolites.[27] So for the comparison of metabolite profiles, ^{1}H NMR spectra of radish roots and leaves were analyzed separately by PCA. A clear difference in both cases, among different treatments was observed, but still due to a large number of samples, there was a massive overlapping of different treatments in PCA score plot. So to extract the common factors, the samples stored at room temperature and at 4 ^{o}C along with control, were separately investigated (**Figure 1 A, B**). While the samples stored at –20 ^{o}C and –80 ^{o}C were assessed together, along with control in PCA (**Figure 2 A, B**).

After three days of storage at room temperature, yellowing of radish leaves was observed, showing the loss of pigments, similar results were reported for rocket salad (*Eruca sativa* Mill.).[128] At 4 ^{o}C yellowing of leaves was less discernible until 28 days of storage, while at chilling temperatures (–20 ^{o}C and –80 ^{o}C) it was negligible as compared with samples stored at room temperature. Plants integrate roots in resistance and tolerance mechanisms of leaf defence. Roots are not only a storage site for plant metabolites but also provide a backup supply of primary metabolites to the plant for secondary metabolite production [430]. Overall the roots of radish plants stored at different temperatures showed similar changes during storage as that to plant leaves, except those stored at 4 ^{o}C. At 4 ^{o}C an increase in phenylpropanoids, and a decrease in fumaric acid and adenine is observed with increasing storage time (**Figure 1 A, B**).

The metabolomic changes still continue even after harvesting the plants, e.g. broccoli undergoes losses of sugars, organic acids, and proteins within the first 6 hours of harvest, which is followed by increase in the free sugars, amino acids, and organic acids.[41, 254] A continuous change in radish metabolites is observed during storage at 4 ^{o}C and room temperature storage, as the result of postharvest physiological stress due to non-availability of nutrients. Leaves show high amount of phenylpropanoids, gallic acid and malic acid at early storage, while amino acids, organic acids and glucosinolates are found to be discriminating metabolites for late storage at room temperature and 4 ^{o}C. In roots, phenolic compounds are found in high amounts at late storage, but interestingly in both types of samples glucosinolates are found to increase in late storage (**Figure 1**).

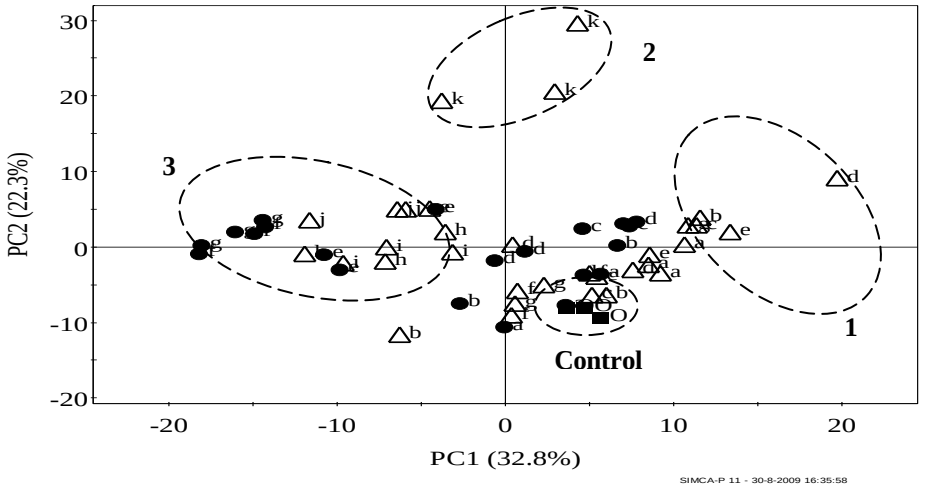

Figure 1 (A). Score plot (PC1 vs PC2) of PCA for radish leaves, based on whole range of ^{1}H NMR signals (δ 0.0 – δ10.0); control (■), samples stored at 4 ^{o}C (Δ) and room temperature (●);control (O); 2 h (a); 4 h (b); 6 h (c); 12 h (d); 24 h (e); 2 d (f); 3 d (g); 7 d (h); 14 d (i); 21 d (j); 28 d (k); **1** = feruloyl malate, 5-hydroxyferuloyl malate, sinapoyl malate, coumaroyl malate, and caffeoyl malate, kaempferol, quercetin, gallic and malic acid; **2** = Fumaric acid, glucose, sucrose, adenine; **3** = threonine, alanine, valine, glutamine, glutamate, glucobrassicin, and neoglucobrassicin.

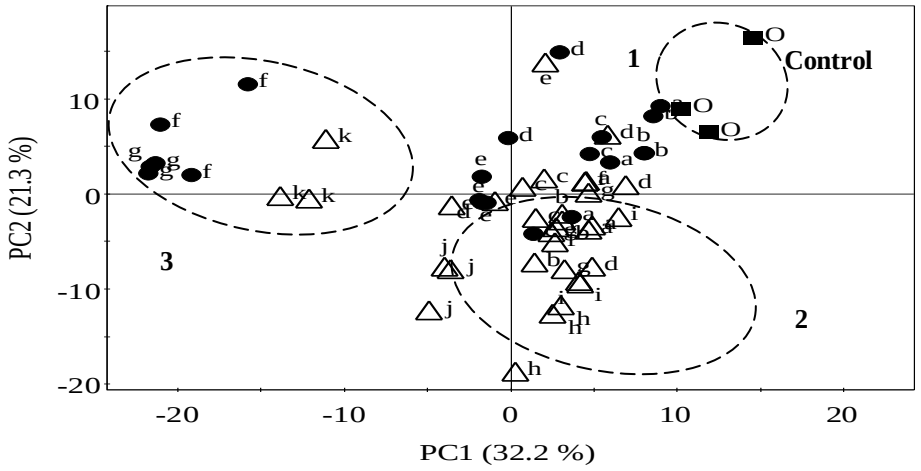

Figure 1 (B). Score plot (PC1 vs PC2) of PCA for radish roots, based on whole range of ^{1}H NMR signals (δ 0.0 – δ10.0); control (■), samples stored at 4 ^{o}C (Δ) and room temperature (●);control (O); 2 h (a); 4 h (b); 6 h (c); 12 h (d); 24 h (e); 2 d (f); 3 d (g); 7 d (h); 14 d (i); 21 d (j); 28 d (k); **1** = Fumaric acid, adenine; **2** = glucose, malic acid, sinapoyl malate; **3** = feruloyl malate, 5-hydroxyferuloyl malate, coumaroyl malate, and caffeoyl malate, kaempferol, quercetin, gallic acid, sucrose, threonine, alanine, valine, glutamine, glutamate, glucobrassicin, and neoglucobrassicin.

In case of cold stored samples (–20 ^{o}C and –80 ^{o}C) discrimination in metabolites is observed between early storage and final day storage due to the presence of high amounts of sucrose, phenolics and glucosinolates in early storage samples, and high amounts of amino acids, organic acids at late storage. An increase in sucrose and amino acids is known as response to cold stress.[418] A decrease of glucose is also observed during storage.

From the PCA of the results of this study, it can be concluded that the decrease in phenolic content differs for different storage temperatures. In the PCA score plot, all the samples of the first 24 hours were found near to control in PC1 and PC2.If focusing only at the phenolic region the PCA shows that a high amount of phenylpropanoids and flavonoids is characteristic for the control and early storage (e.g. **Figure 2**).

When only the initial storage hours are analysed, it is clear that by increasing time an increase in phenolics and glucosinolates occurs during the early storage. The increase of phenolics and glucosinolates in cold stored samples may be correlated to the physiological stress due to the chilling injury and non-availability of the nutrients in the start of cold storage.[11, 128] The phenylpropanoids, especially ferulic acid accumulation are thought to result in cell wall rigidity to protect it from chilling injury.[431] After some time production of phenolics and glucosinolates stalls,[128] though still an increase of amino acids, and glucose is observed (**Figure 3**).

A similar behaviour was observed for leaves and roots. Cold storage may improve phenolics related quality[11] as in some cases low temperature increases anthocyanins, and hydroxycinnamic acid derivatives.[11] Accumulation of phenylpropanoids in *Arabidopsis* at low temperature is also reported.[418] An increase in phenylpropanoids during early chilling (–20 ^{o}C and –80 ^{o}C) storage may protect cells against frost-induced oxidative stress, scavenging hydrogen peroxide diffusing across membranes.[431]

At later storage the plant tissue gets frozen and biological activity may stop but chemical activity continues and leads to further metabolomic changes. Although a separation for different treatments is observed in PCA, still some clear markers for the different treatments and for the time course would be important. Therefore a supervised multivariate analysis method PLS-DA was applied to the data. The grouping was made on the basis of different time periods (twelve groups) (**Figure 4, 5**) and temperatures (five groups) (**Figure 6, 7**).

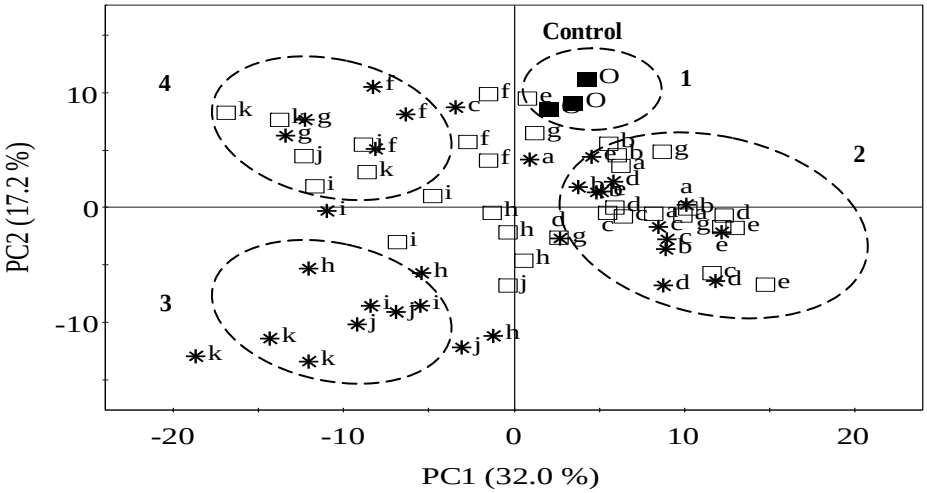

Figure 2 (A). Score plot (PC1 vs PC2) of PCA for radish leaves, based on whole range of ^{1}H NMR signals (δ 0.0 – δ10.0); control (■), samples stored at –20 ^{o}C (*) and –80 ^{o}C (□); control (O); 2 h (a); 4 h (b); 6 h (c); 12 h (d); 24 h (e); 2 d (f); 3 d (g); 7 d (h); 14 d (i); 21 d (j); 28 d (k); **1** = glucose; **2** = feruloyl malate, 5-hydroxyferuloyl malate, sinapoyl malate, coumaroyl malate, and caffeoyl malate, kaempferol, quercetin, sucrose, adenine, glucobrassicin and neoglucobrassicin; **3** = alanine and threonine, **4** = valine, glutamine, glutamate, and malic acid.

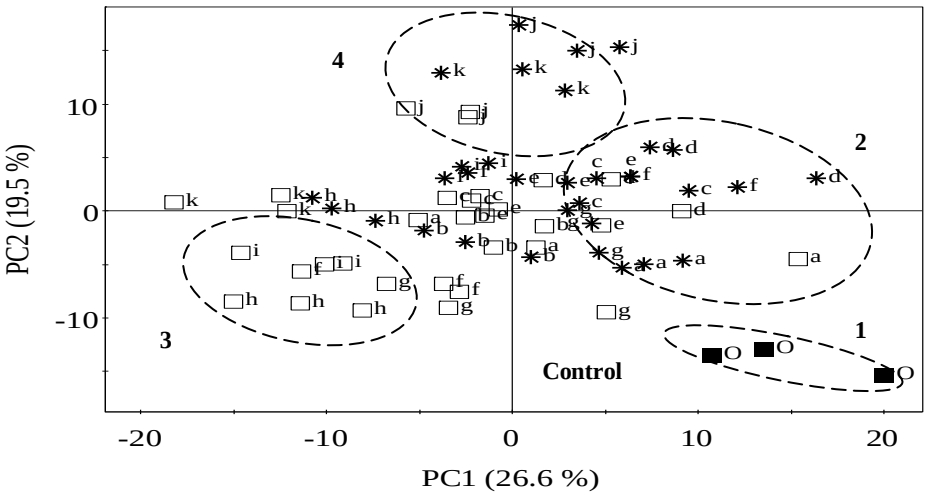

Figure 2 (B). Score plot (PC1 vs PC2) of PCA for radish roots, based on whole range of ^{1}H NMR signals (δ 0.0 – δ10.0); control (■), samples stored at –20 ^{o}C (*) and –80 ^{o}C (□); control (O); 2 h (a); 4 h (b); 6 h (c); 12 h (d); 24 h (e); 2 d (f); 3 d (g); 7 d (h); 14 d (i); 21 d (j); 28 d (k); **1** = glucose; **2** = feruloyl malate, 5-hydroxyferuloyl malate, sinapoyl malate, coumaroyl malate, and caffeoyl malate, adenine, kaempferol, quercetin, glucobrassicin and neoglucobrassicin; **3** = sucrose; **4** = alanine, threonine, valine, glutamine, glutamate, and malic acid.

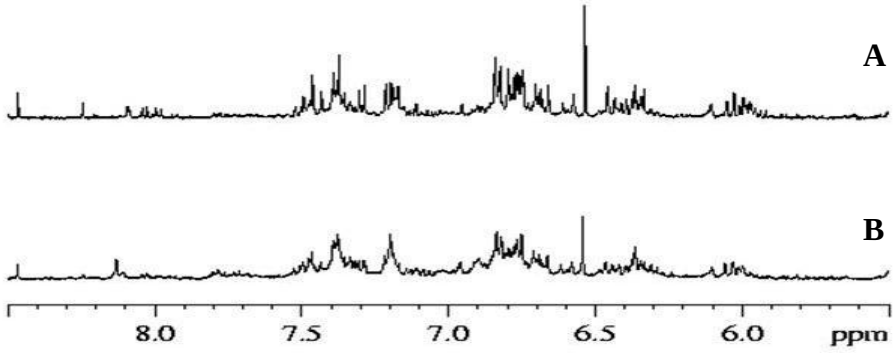

Figure 2. ^{1}H NMR spectra of aerial parts of red radish (petioles and leaves) stored at 4 ^{o}C for 12 hours (A) and 28 days (B).

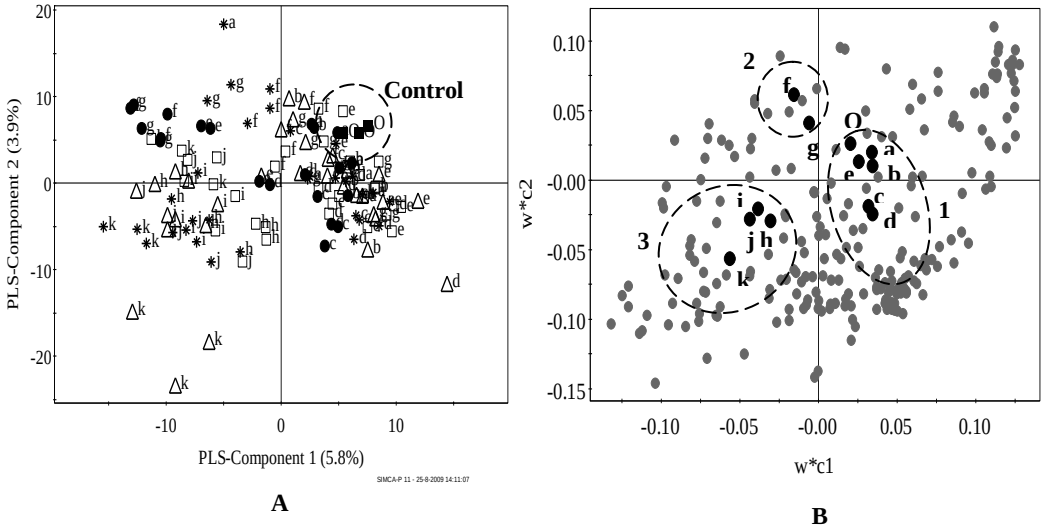

Figure 4. Score plot (PLS-Component 1 vs PLS-Component 2) (A) and loading (B) of PLS-DA, for effect of storage time on radish leaves, based on whole range of ^{1}H NMR signals (δ 0.0 – δ10.0); control (■), samples stored at room temperature (●); at 4 ^{o}C (Δ);at –20 ^{o}C (*) and –80 ^{o}C (□); control (O); 2 h (a); 4 h (b); 6 h (c); 12 h (d); 24 h (e); 2 d (f); 3 d (g); 7 d (h); 14 d (i); 21 d (j); 28 d (k); **1** = valine; feruloyl malate, 5-hydroxyferuloyl malate, sinapoyl malate, coumaroyl malate, and caffeoyl malate, kaempferol, quercetin, and neoglucobrassicin, adenine, glucose, glucobrassicin; **2** = alanine, glutamine, glutamate; **3** = threonine, sucrose, and malic acid.

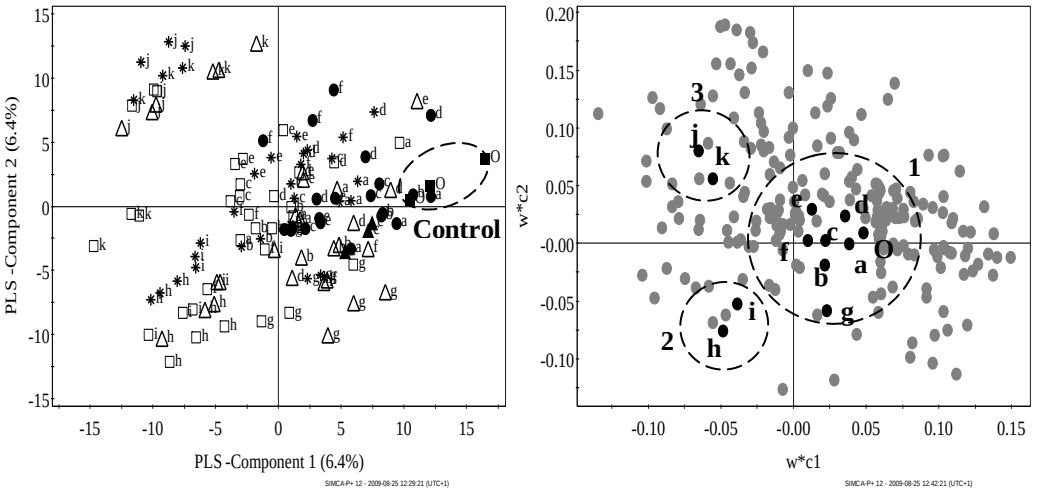

Figure 5. Score plot (PLS-Component 1 vs PLS-Component 2) (A) and loading (B) of PLS-DA, for effect of storage time on radish roots, based on whole range of ^{1}H NMR signals (δ 0.0 – δ10.0); control (■), samples stored at room temperature (●); at 4 °C (Δ); at –20 °C (*) and –80 °C (□); control (O); 2 h (a); 4 h (b); 6 h (c); 12 h (d); 24 h (e); 2 d (f); 3 d (g); 7 d (h); 14 d (i); 21 d (j); 28 d (k); **1** = adenine, glucose, fumaric acid, feruloyl malate, 5-hydroxyferuloyl malate, sinapoyl malate, and caffeoyl malate, kaempferol, quercetin, glucobrassicin and neoglucobrassicin; **2** = alanine, glutamine, glutamate, valine, and sucrose; **3** = threonine, malic

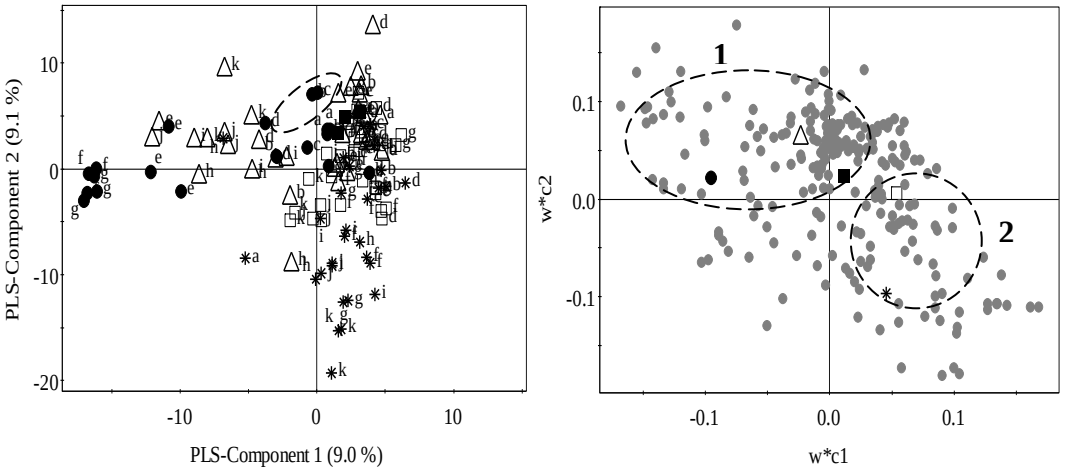

Figure 6. Score plot (PLS-Component 1 vs PLS-Component 2) (A) and loading (B) of PLS-DA, for effect of storage temperature on radish leaves, based on whole range of ^{1}H NMR signals (δ 0.0 – δ10.0); control (■),samples stored at room temperature (●); at 4 °C (Δ); at –20 °C (*) and –80 °C (□); control (O); 2 h (a); 4 h (b); 6 h (c); 12 h (d); 24 h (e); 2 d (f); 3 d (g); 7 d (h); 14 d (i); 21 d (j); 28 d (k); **1** = alanine, valine, threonine, glutamine, glutamate, glucobrassicin, sucrose, α-glucose, feruloyl malate, 5-hydroxyferuloyl malate, sinapoyl malate, coumaroyl malate, and caffeoyl malate, kaempferol, quercetin, and neoglucobrassicin; **2** = adenine, β-glucose, and malic acid.

The results of PLS-DA for leaves (**Figure 4 A, B**) and roots (**Figure 5 A, B**) supports the conclusions of the PCA and further explain that with increasing time first an increase in secondary metabolites is observed, followed by a decrease in these compounds. This decrease may be correlated with the release of breakdown products of plant metabolites.[128] High amounts of primary metabolites including sugars, amino acids and organic acids are observed in late storage samples (**Figure 4, 5**).

The samples stored at 4 ^{o}C show less separation in PLS-DA from control as compared with that stored at room temperature, while samples stored at –80 ^{o}C shows less separation in PLS-DA from control as compared with that at –20 ^{o}C. Comparison of leaves stored at different temperatures (**Figure 6**) shows discrimination for samples stored at room temperature, due to glutamine, glutamate, threonine, alanine and glucobrassicin, while –20 ^{o}C and –80 ^{o}C samples were separated by the samples at room and 4 ^{o}C by adenine, malic acid, and β-glucose signals. A high amount of phenylpropanoids, flavonoids, neoglucobrassicin, malic acid, and sucrose is characteristic for the leaf samples stored at 4 ^{o}C (**Figure 6**).

Low temperature acclimation induces sucrose and organic acids in plant. This increases the plant cell tolerance against dehydration and freezing injury.[432, 433] The increase of organic acids and amino acids in later storage shows the selectivity and shift of plant metabolomic pathways, most suited for its survival. Loss of glucose and secondary metabolites in the late storage may also results in the increase in amino acids under storage.[417]

Storage at –80 ^{o}C exhibits less metabolomic changes (**Figure 3 A, B**) by decreasing tissue respiration at cold temperature.[96] The root samples stored at room temperature, 4 ^{o}C and control show separation in PLS-DA (**Figure 7**) due to glucosinolates, phenylpropanoids, sucrose, valine, glutamine, glutamate, and fumaric acid, while the samples stored at –20 ^{o}C and –80 ^{o}C show discrimination due to alanine, threonine and adenine. Different preharvest and postharvest factors, including storage temperature, significantly affect phenolic degradation or stability.[11] A decrease in anthocyanins in red radish was observed during storage, both in light and dark conditions,[434] while decreasing the storage temperatures the loss of phenolic is reduced.[11] With the results of NMR based metabolomic characterization and visual appearance of radish leaves and roots, the samples stored at low temperature were found closest to

control. By visual assessment the quality of radish stored at 4 °C were found of acceptable quality, even after 28 days of storage, but samples stored at room temperature were not acceptable any more after three days of storage. Samples stored at –20 °C and –80 °C looked fresh but after defrosting due to thawing effect, radish roots and leaves were not acceptable for consumption.

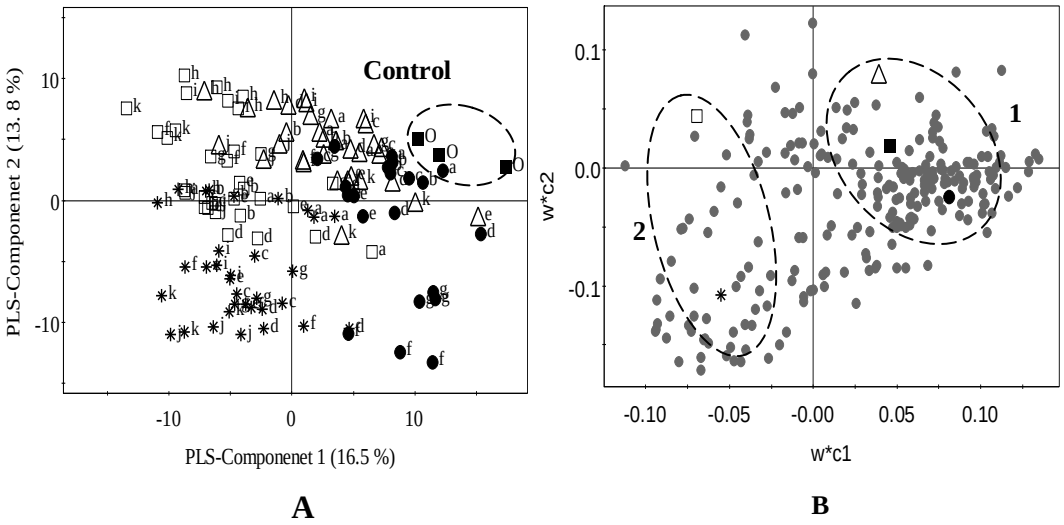

Figure 7. Score plot (PLS-Component 1 vs PLS-Component 2) (A) and loading (B) of PLS-DA, for effect of storage temperature on radish roots, based on whole range of ^{1}H NMR signals (δ 0.0 – δ10.0); control (■); room temperature (●); 4 °C (Δ); –20 °C (*); –80 °C (□); control (O); 2 h (a); 4 h (b); 6 h (c); 12 h (d); 24 h (e); 2 d (f); 3 d (g); 7 d (h); 14 d (i); 21 d (j); 28 d (k); **1** = glucose, and malic acid, sucrose, valine, glutamine, glutamate, fumaric acid, feruloyl malate, 5-hydroxyferuloyl malate, sinapoyl malate, coumaroyl malate, and caffeoyl malate, kaempferol, quercetin, glucobrassicin, neoglucobrassicin; **2** = alanine, threonine, adenine.

4 Conclusion

Multivariate data analysis method is a promising method to find biomarker compounds relative to storage at different time and storage. Radish roots show least metabolomic changes as compared with leaves, when compared in PCA. While focusing on effect of temperature, least metabolomic changes were observed in the samples stored at –20 ^{o}C and –80 ^{o}C, these temperature conditions are found comparatively best for experimental purpose and it is advantageous to keep the vegetable samples at –80 ^{o}C, for research sampling. But in this case the post-storage physical quality of radish is not acceptable as freezing destroys the structure and after thawing massive changes in the metabolome occur. Adenine, malic acid in leaves while adenine, alanine and threonine in roots, are found as discriminating metabolites for the storage temperature of –20 ^{o}C and –80 ^{o}C. A rapid increase of glucosinolates until the later storage time is studied at room temperature and 4 ^{o}C. While at –20 ^{o}C and –80 ^{o}C an increase in these compounds is observed at early storage only. *Brassica* vegetables offer a good basis for the development of healthy food, however vegetable storage has a critical role in the preservation of these compounds so further research is needed to study these compounds in relation to their breakdown products.

Table 1 – A: Effect of different temperatures on the metabolites of red radish aerial parts (petioles and leaves) at 20 °C, 4 °C, –20 °C and –80 °C. Increase until late storage (+); decrease until late storage (–); increase in initial storage and decrease in late storage (+–).

	20 °C	4 °C	–20 °C	–80 °C
Adenine	+	+	+	+ –
Fumaric acid	+	+	+ –	+ –
Galic acid	–	+ –	–	–
Glutamine and glutamate	+	+	+ –	+ –
Malic acid	–	–	+ –	+ –
Alanine and threonine	+	+	+	+ –
Valine	+	+	+ –	+ –
Glucose	+	+	–	–
Sucrose	+	+	+ –	+ –
Glucobrassicin and neoglucobrassicin	+	+	+ –	+ –
5-hydroxyferuloyl -, caffeoyl -, coumaroyl -, feruloyl -, and sinapoyl malate	+ –	+ –	+ –	+ –
Flavonoids	+ –	+ –	+ –	+ –

Table 1 – B: Effect of different temperatures on the metabolites radish roots at 20 °C, 4 °C, –20 °C and –80 °C. Increase until late storage (+); decrease until late storage (–); increase in initial storage and decrease in late storage (+ –).

	20 °C	4 °C	–20 °C	–80 °C
Adenine	–	–	–	–
Fumaric acid	–	–	–	–
Galic acid	+	+	+	–
Glutamine and glutamate	+	+	+	+ –
Malic acid	+	+	+	+ –
Alanine and threonine	+	+	+	+ –
Valine	+	+	+	+ –
Glucose	+ –	+ –	–	–
Sucrose	+	+	+ –	+
Glucobrassicin and neoglucobrassicin	+	+	+ –	+ –
Sinapoyl malate	+ –	+ –	+ –	+ –
5-Hydroxyferuloyl -, caffeoyl -, coumaroyl -, and feruloyl malate	+	+	+ –	+ –
Flavonoids	+	+	+ –	+ –

Chapter 8

Study of different biological activities of *Raphanus sativus* L. (red radish)

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Abstract

Brassicaceae vegetables are a good source of food around the world. Previously a diverse range of metabolites have been reported from this genus with regard to human nutrition. Extensive data are available on the biological activities of primary and secondary metabolites of *Brassica* plants, such as antioxidant, anticancer, and antimicrobial activity. For the active compounds include among others are phenolics, glucosinolates, and sterols. Among other vegetables *Raphanus sativus* L. (red radish) is an important member of Brassicaceae, well known as food and as a model system for plant research. Red radish extracts were evaluated for antibacterial, acetylcholine esterase inhibition, CB1 and Adenosine A1 receptor binding activity. Although showing to be little active, it is concluded that radish extract did not show significant activity for any of aforementioned assays. We can conclude that radish can be used as a food, rather than considering as a medicinal plant with higher pharmacological value.

1 Introduction

Several species belonging to the *Brassica* family have been used as food since time immemorial. In recent years it has been shown that these plants have a high content of diverse health-promoting antioxidant metabolites such as polyphenols [58], cinnamic acid derivatives,[354] flavonoids, vitamins etc.[55, 56, 89, 190] A part from nutritional value to human and animal populations, these phytochemicals have a role in the plant response to different stress factors.[19] Throughout the course of growth and development, plants are exposed to various environmental factors, both of biotic and abiotic character,[18, 27] which ultimately results in the quantitative and/or qualitative change in various primary and secondary metabolites.[19]

There is currently much interest in identifying phytochemicals with health promoting biological activity in food.[60] Glucosinolates and their hydrolysis products, common in cruciferous plants (Brassicaceae family) including *Brassica* species, were found to possess antitumor activities.[167, 223] Also phenolics as antioxidants and antimicrobial compounds are now well-known,[29, 173, 392] while antifungal activity of *Brassica* phytoalexins was also reported.[341, 346]

Radish (*Raphanus sativus* L.) contains such health promoting (poly)-phenolics, flavonoids and glucosinolates. In the present study radish leaves and roots extracts were tested for some biological activities with general interest, acetylcholine esterase inhibition in connection with treating the symptoms of alzheimer disease, CB1 and adenosine A1 receptor binding in connection with possible affects on appetite, obesity, anorexia, bulimia and also antimicrobial activity which has several potential uses. The aim of present work is to investigate the potential of bioactive compounds present in radish, for different biological activities.

2 Materials and methods

2.1 Preparation of plant material

Red radish (*Raphanus sativus* L.) plants were purchased from local market for acetylcholine esterase assay, while for the other bioactivities, the plant material was grown in green house conditions, until six week plant age. Fresh and healthy plants were selected and washed thoroughly with de-ionized water and kept in open air at room temperature for half an hour to remove water from plant surface. Roots as commonly consumed food were used for further fractionation.

2.2 Extraction of plant material

The dried plant material was extracted 3 times with 100% MeOH by ultrasonication at room temperature for 30 min. Extract was dried by rotary evaporator, resulting in 148 g of dry methanol extract. It was suspended in deionized water. This was partitioned successively with *n*-hexane and chloroform. The resulting fractions were taken to dryness. For different bioactivity assays, fresh plant material was produced every time. In case of acetycholinesterase inhibitors isolation a large scale extraction was done, with plant material purchased from the market. Leave and root extracts (chloroform and *n*-hexane) were analyzed for adenosine A1 and CB1 receptor binding activities.

2.3 Acetylcholine esterase inhibitory activity

Acetylcholine esterase inhibitory activity was evaluated by using the Ellman's reagent in combination with thin layer chromatography (TLC) as previously reported.[435] To determine the false positive activity the same method with a modification is used as previously reported.[436] Microplate reader (HTS 7000 Bio Assay Reader, Perkin Elmer, USA) was used to measure the absorbance at 405 nm for the enzyme reaction in the microplate assay as previously reported.[437] Fluorimetric method is used for the confirmation of the activity.[438]

2.3.1 Bioassay guided fractionation for acetylcholine esterase inhibitory activity.

The chloroform fraction was sub-fractioned on a silica gel column by a stepwise gradient of chloroform – ethyl acetate (1: 0 → 0:1), and final elution with methanol in the end for chloroform fraction. For the *n*-hexane fraction the eluent was a gradient of *n*-hexane – ethyl acetate (1:0 → 0:1), and final elution with methanol. Chloroform extract resulted in 142 sub-fractions, *n*-hexane extract provided 45 sub-fractions. These fractions were pooled according to evaluation with analytical TLC. The fractions showing AChE inhibition in the TLC method were further processed by preparative TLC. After each step an analytical TLC analysis was performed to check the activity including a test for false positives.

2.4 Adenosine A1 and cannabinoid CB1 receptors binding activities of radish extract.

Radish leaves and roots were evaluated for adenosine A1 (Table-1) and cannabinoid CB1 (Table-2) receptor binding activities. Extraction of plant material was done similar as for acetylcholine esterase inhibitory activity. Adenosine A1 receptor binding activity was done by the method as previously reported by our group,[439] while CB1 activity was done as reported by Horswill and others.[440]

2.5 Antibacterial activity

Bacterial growth inhibition by the extract (methanol-water: 1-1) of radish leaves and roots was evaluated by spectrophotometer (HTS 7000 Bio Assay Reader, Perkin Elmer, USA) at 590 nm by using 96 well plates, as reported in literature.[441] Methanol-water extract was evaporated and 80 mg/ml stock solution was prepared in milli-pore water. Series of dilutions (40, 20, 10, 5 and 2.5 mg/ml) were prepared to test the antibacterial activity. Different bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium* and *Bacillus subtilis*) were used.

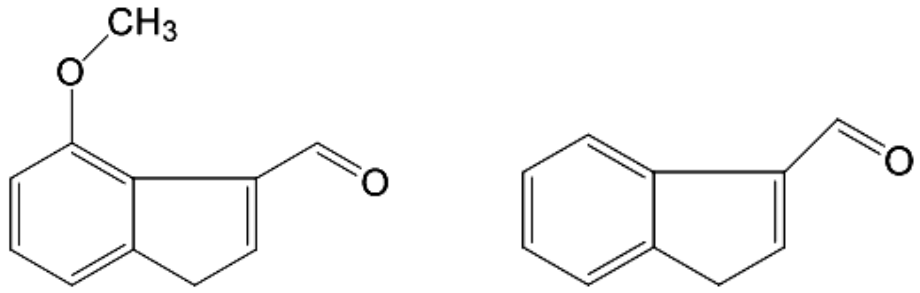
3 Results and Discussion

Brassica vegetables are considered to be a good source of phytochemicals for different bioactivities,[49] including antioxidant,[279] anti-inflammatory and antibacterial properties.[173] In our previous studies we found that particularly radish was rich in plant metabolites (see **chapter 4** and **7**, and **Table 1** in **chapter 8**) comparing to some other *Brassica* species. Therefore this species was chosen for further studies on biological activities.

Acetylcholine esterase inhibitory activity was done on radish roots and leaves. Chloroform and *n*-hexane fractions were found active after fractionation of the methanol (100%) extract of red radish roots. The TLC false positive test was done throughout the fractionation and isolation work. Two spots from chloroform fraction were identified as real positive, while all the spots from *n*-hexane fraction were found to be false positive for acetylcholine esterase inhibitory activity. These two compounds in chloroform fraction were identified as 4-methoxy-1*H*-indole-3-carboxaldehyde (4methoxy-I-3-C) (**Figure 1 A**) and 1*H*-indole-3-carboxaldehyde (I-3-C) (**Figure 1 B**), by using ^{1}H NMR, J-resolved, COSY and HMBC spectra. To validate the method galanthamine was

used as reference compound, showing IC50 of 4.55 μM concentration. At the same conditions the IC50 of 4-methoxy-I-3-C was 6.604 mM and of I-3-C was 3.07 μM.

Acetylcholine esterase inhibitory activity is well reported for alkaloids,[442] but the presence of an aldehyde group in these compounds make the results suspicious, so these compounds were further assayed for the confirmation of the acetylcholine esterase activity by using the fluorimetric method as previously reported.[443] The identified compounds didn't show any activity by this method. So it is concluded that the activity assayed by Ellman's method was false positive, which was not detected by the false positive activity test, showing the limitation of Ellman's reagent. So at the end no active compound for acetylcholine esterase inhibitory activity was found in red radish.



A **B**

Figure 1: 4-methoxy-1*H*-indole-3-carboxaldehyde (A) and 1*H*-indole-3-carboxaldehyde (B)

Table 1 – Dry weight of plant material, methanol as crude extract, and extracts of radish leaves, radish roots, Brussels sprout and broccoli, after fractioned with *n*-hexane, chloroform and water.

	Radish leaves	Radish roots	Brussels sprout	Broccoli
Dry Weight (g)	50	50	50	50
Methanol (crude extract) (g)	12.675	25.1	18.046	14.941
n-Hexane (g)	2.531	0.410	0.536	01.007
Chloroform (g)	0.046	0.271	0.359	0.040
Water (g)	8.364	23.796	15.859	11.228

For the antimicrobial analysis, different concentrations of the radish root and the leave extract (Methanol: water – 1:1) have been evaluated. Positive (ampicillin) and negative (without any extract and ampicillin) controls were used to compare the results. Only a slight

decrease in bacterial growth was observed in case of radish root extract, for *Staphylococcus aureus* (**Figure 2**).

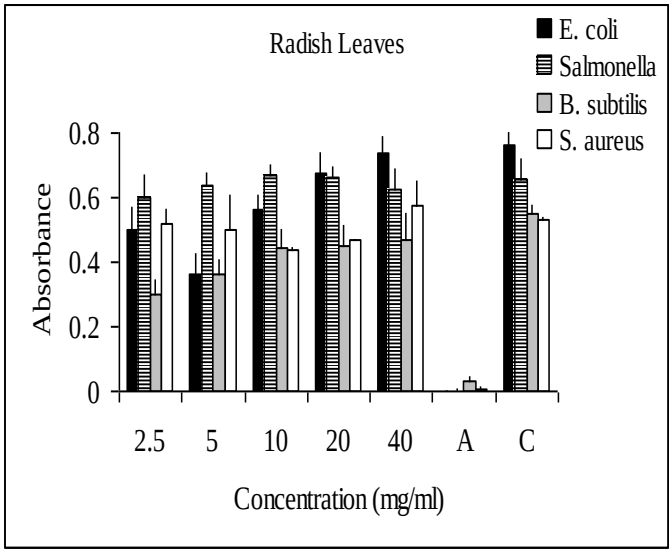


Figure 2 A: Antibacterial activity of *Raphanus sativus* (red radish) leaves, for four different strains of bacteria; *Escherichia coli*; *Salmonella typhimurium*; *Bacillus subtilis*; *Staphylococcus aureus*.

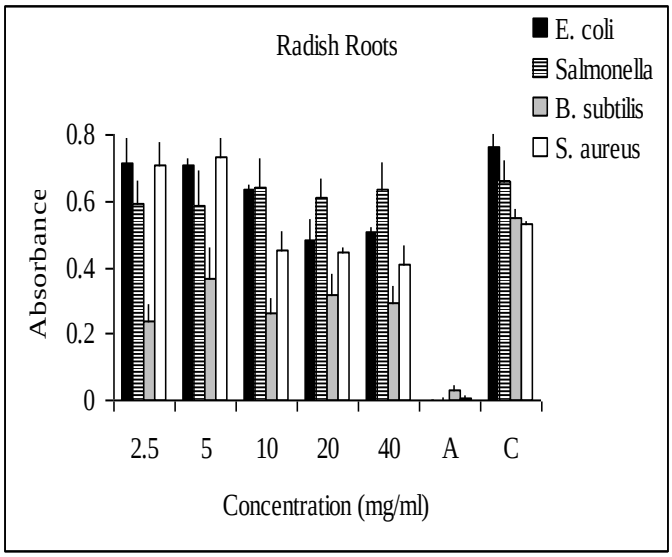


Figure 2 B: Antibacterial activity of *Raphanus sativus* (red radish) roots, for four different strains of bacteria; *Escherichia coli*; *Salmonella typhimurium*; *Bacillus subtilis*; *Staphylococcus aureus*.

The reduced growth as compared with negative control is not considered as significant as still at 40 mg/ml concentration bacterial growth was observed. Even in some cases, an increase in bacterial growth is observed for different extracts. May be a pure isolated compound or a group of certain compounds may show an antibacterial

activity, but from these results it can be concluded that red radish is not a good source of antibacterial compounds.

Possible CB1 and adenosine receptor binding activities were evaluated as these activities are related with regulation of food intake and thus with obesity, and other food related diseases. Although some activity was observed in methanol crude extracts. The factions obtained by liquid-liquid partitioning showed relatively higher activities, with a relatively high activity in the *n*-hexane fraction. But from the ^{1}H NMR spectra of the *n*-hexane fraction it is concluded that it contains a high amount of unsaturated fatty acids, that may be responsible for the high activity of this fraction, as unsaturated fatty acids can bind to membranes, showing false positive results in receptor binding assays (**Table 2**).[444]

Table 2 – Adenosine (A1) and CB1 receptor inhibition activity for different fractions of radish leaves and roots.

	Adenosine A1 inhibition (%)		CB1 inhibition (%)	
	Radish leaves	Radish roots	Radish leaves	Radish roots
Methanol (crude extract)	8.5	9.3	3.1	13.1
n-Hexane	76.7	73.4	74.6	42.2
Chloroform	25.8	7.7	34	35
Water	28.2	36.1	48	10.6

4 Conclusion

It is concluded that although radish may have some bioactive compounds, quantitatively these compounds are not present in a major concentration. Either these compounds may not be present in sufficient amounts to ascribe radish for any medicinal use. But radish may affect human health by providing a good source of vitamins, sugars, amino acids and glucosinolates (**chapter 4**).

Acknowledgement

The help of Ms. Nancy Dewi Yuliana and Ms. Andrea Lubbe, Division of Pharmacognosy, Institute of Biology, Leiden University, The Netherlands, for the evaluation of biological activities of different samples is gratefully acknowledged.

Chapter 9

General discussion, conclusion and future perspectives

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General Discussion and Conclusion of the Thesis

Brassica vegetables are a rich source of health affecting compounds and are widely used as food, moreover they are a model for plant science research. These vegetables represent a major part of the human diet all over the world providing nutritionally significant constituents, such as phenolic compounds, vitamins, fibres, soluble sugars, minerals, fats, and carotenoids.

The metabolome changes during plant growth, which represents the changes in metabolomic fluxes through different pathways. The present study shows the importance of plant age as a factor for nutritional value of vegetables for human consumption and suggests that young plants are a better source of nutrients as compared with old plants.

During growth, plants are exposed to various biotic (e.g. herbivory, fungal, bacterial and/or viral infection) and abiotic (e.g. metals, UV, temperature, drought) stresses. The metabolomic changes can be quite specific, since the plant defence-related compounds are composed of a variety of constitutive and induced metabolites. The set of *Brassica* metabolites differs after infection with different microorganisms. Plant response to bacterial stress depends on the type of invading bacteria. It probably reflects the diverse chemical composition and mechanism of action of the invading organism, which can at the same time may activate gene expression and block specific steps of a metabolomic pathway in the plant, or even metabolize the plant defence compounds.

Under metal stress, plants produce primary and secondary metabolites which increase with increasing metal concentrations up to a certain point, beyond which primary and secondary metabolite concentration decreases. The increase of phenolic compounds is dependent on both the type of metal and its concentration. Apparently the response to metal stress is observed in the both leaves and roots, which could be due to metal tolerance mechanisms or metal accumulation in the whole plant. This response and accumulation of metals is more dependent on type of metal rather than metal concentration.

Postharvest storage temperature is also crucial for the metabolomic variation in vegetables. By decreasing the temperature the rate of metabolomic variation decreases. As compared with room temperature, lower metabolomic variation happens in the vegetables stored at 4 ^{o}C, but least metabolomic changes are observed in the samples stored at –20 ^{o}C and –80 ^{o}C, although due to freezing injury higher

amount of phenolics are observed, but with further increasing the time this stabilizes. The storage temperature of 4 ^{o}C is comparatively better for consumption purpose, but for experimental purpose it is advantageous to keep the vegetable samples at –80 ^{o}C.

As aforementioned, in nature plants have multitrophic interactions during growth and developmental processes.[255] The power of metabolomics analytical methods is the analysis of wide spectra of compounds resulting in a huge data set in an unbiased and comprehensive manner.[445] These enormous metabolomic data sets can be assessed by multivariate analysis, usually stating with an unsupervised method such as principal component analysis (PCA). To understand the specificity of the interactions of the plant and its environment a large amount of data on to *Brassica* was obtained concerning the effects of the defence signal compounds, such as jasmonic acid, salicylic acid, and furthermore of infection with pathogenic and non pathogenic fungi, as well as human pathogenic bacteria and metals. Effects were measured at different developmental stages of the plant. Finally also the effect of storage for different periods and temperatures was evaluated. An overlap of different treatments was observed that needs to be studied in more detail.

To try to make a clear overall picture, as a next step data from all experiments described in this thesis for leaves (**Figure 1**) and roots (**Figure 2**), covering a period of 4 years were subjected to PCA analysis. A clear discrimination in the PCA score plot is observed for the plant species. But also the metabolomic changes observed for the different treatments of the same species are visible in these PCA score plots. For the loading plot it was concluded that serine, glucose and sucrose are higher in the plants grown in hydroponic conditions and infected by food born bacteria (in accordance with the analysis made in **chapter 5**), while glucosinolates and phenylpropanoids were found in higher amounts in radish stored at different storage temperatures for different time period (in accordance with the analysis made in **chapter 7**). When focusing on stored radish, the initial storage samples were found to be higher in glucosinolate and phenylpropanoids. GABA and alanine are found to be as discriminating metabolites for *B. rapa* samples treated with metals (conform analysis in **chapter 6**).

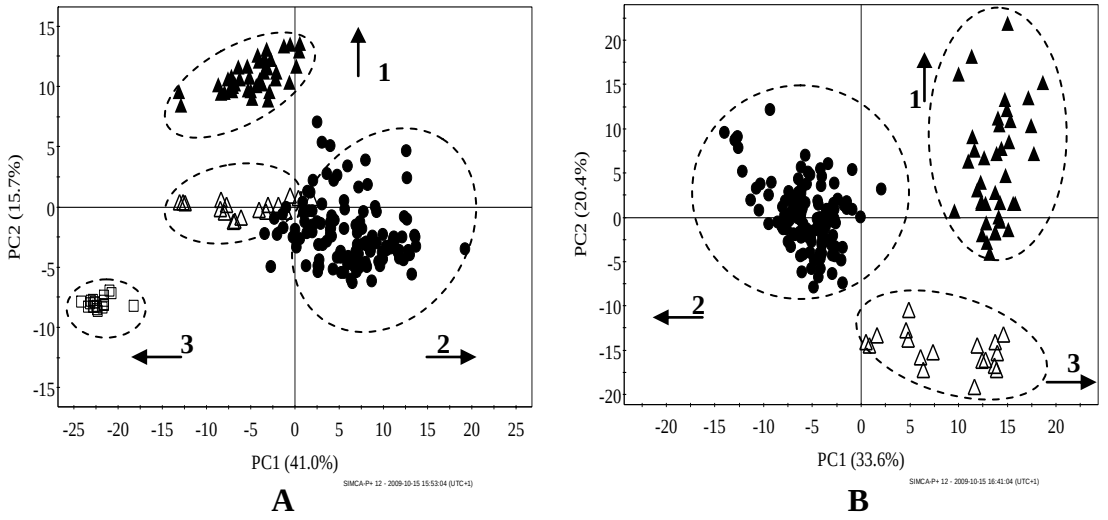

Figure 1: Score plot (PC1 vs PC2) of PCA for leaves (A) and roots (B). *Brassica rapa* stressed with metals (as in **chapter 6**) (▲), Effect of post harvest storage time and temperature on *Raphanus sativus* (as in **chapter 7**) (●), Change in *Brassica rapa* and *Raphanus sativus* metabolome at different developmental stages (as in **chapter 4**) (Δ), Effect of food born bacteria on *Brassica rapa* in hydroponic conditions (as in **chapter 5**) (□). **1** = GABA, alanine, acetate, threonie ; **2** = feruloyl malate, 5-hydroxyferuloyl malate, sinapoyl malate, coumaroyl malate, and caffeoyl malate, kaempferol, quercetin, gallic and malic acid, fumaric acid, valine, glutamine, glutamate, glucobrassicin, gluconapolefrin, progoitrin and neoglucobrassicin; **3** = Serine, glucose, sucrose.

The samples for different developmental stage (conform **chapter 4**) were grouped together near post harvest stored radish. The discrimination of these samples from post harvest stored radish and metal affected plants is due to the higher amount of sucrose, serine and glucose. A similar result is obtained for root samples of all experiments, except that hydroponically grown plants are not included, as in that case we could not generate root samples. Partial least square-discriminant analysis (PLSDA) as a supervised method of analysis was used to analyse the same data. The grouping was made on the classes based on experimental conditions. A similar result was obtained as with the PCA analysis (**Figure 1**). From the results it is obvious that the different treatments applied did result in different responses of the plant, though in part they do overlap. Plants apparently have specific responses to different forms of stress. Signal compounds like methyljasmonate and salicylate, also have overlap with these responses. The good news of this final overall analysis is that a metabolomics approach does allow datamining in results obtained in different experiments done over the

years and results in the conclusion that plants differ in specific responses to different forms of stress. The "bad" news of this discovery is that to learn for understanding the regulation of plant defence is even more complex than originally anticipated.

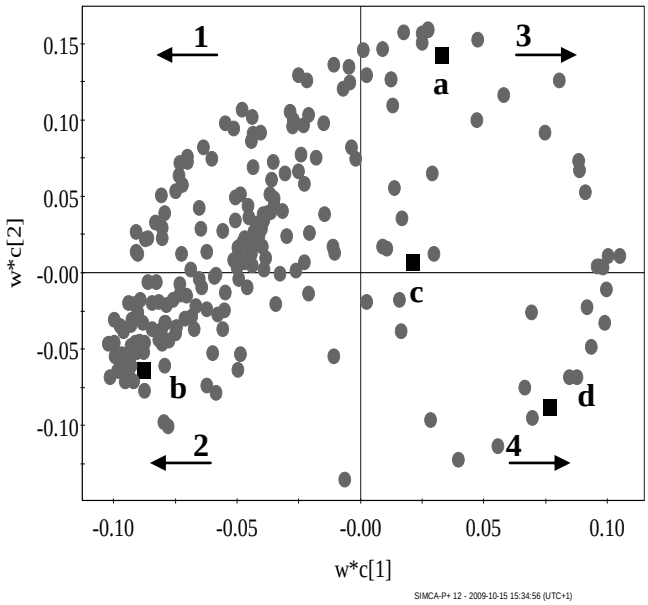

Figure 1-A: Loading plot (PC1 vs PC2) of PLSDA for leaves (A) and roots (B). *Brassica rapa* stressed with metals (as in **chapter 6**) (a), Effect of post harvest storage time and temperature on *Raphanus sativus* (as in **chapter 7**) (b), Change in *Brassica rapa* and *Raphanus sativus* metabolome at different developmental stages (as in **chapter 4**) (c), Effect of food born bacteria on *Brassica rapa* in hydroponic conditions (as in **chapter 5**) (d). **1** = GABA, alanine, acetate ; **2** = feruloyl malate, 5-hydroxyferuloyl malate, sinapoyl malate, coumaroyl malate, and caffeoyl malate, kaempferol, quercetin, gallic and malic acid, fumaric acid, valine, threonie, glutamine, glutamate, glucobrassicin, gluconapolefrin, progoitrin and neoglucobrassicin; **3** = α-glucose; **4** = β-glucose, sucrose, serine.

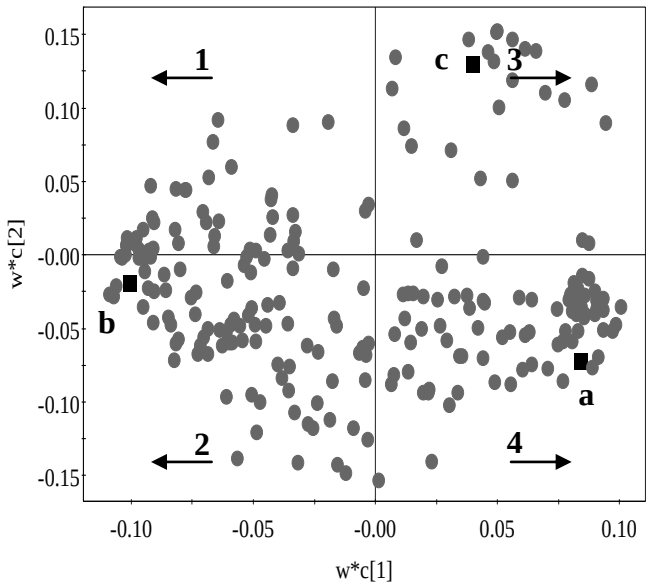

Figure 1-B: Loading plot (PC1 vs PC2) of PLSDA for leaves (A) and roots (B). *Brassica rapa* stressed with metals (as in **chapter 6**) (a), Effect of post harvest storage time and temperature on *Raphanus sativus* (as in **chapter 7**) (b), Change in *Brassica rapa* and *Raphanus sativus* metabolome at different developmental stages (as in **chapter 4**) (c), Effect of food born bacteria on *Brassica rapa* in hydroponic conditions (as in **chapter 5**) (d). **1** = GABA, alanine, acetate ; **2** = feruloyl malate, 5-hydroxyferuloyl malate, sinapoyl malate, coumaroyl malate, and caffeoyl malate, kaempferol, quercetin, gallic and malic acid, fumaric acid, valine, threonie, glutamine, glutamate, glucobrassicin, and neoglucobrassicin, glucose; **3** = Serine; **4** = sucrose, gluconapolefrin, progoitrin.

The presence of health promoting constituents, such as phenolics, glucosinolates, amino acids, vitamins, sugars, fibre etc. makes Brassicaceae plants healthy food. The combined presence of antioxidant, anticancer and antimicrobial compounds seems to distinguish *Brassica* vegetables from many other vegetables, however, the active compounds are present only at low levels and certainly not at a level that could make these vegetables a medicinal plant. Rather one should see the presence of the various biologically active compounds as proof for the health sustaining, or supporting character of *Brassica* vegetables.

Metabolomics aims at both the qualitative and quantitative analysis of all metabolites, and but no single method can analyse all the metabolites in a single run. In this scenario we have to select a good method by keeping resolution, reproducibility, sensitivity, sample preparation and handling into account.[37]

Although HPLC and MS are highly sensitive analytical techniques and used for plant metabolomic studies but in my view NMR is an excellent tool to have a macroscopic view on the plant metabolome due to its high resolution, reproducibility, ease of sample preparation and sample handling. Thus the use of NMR is of great interest in functional genomics and systems biology studies of biological processes. For the identification of metabolites the use of various 2D NMR methods can overcome the problem of signals overlapping in ^{1}H NMR.[37, 39]

In order to be able to draw conclusions from a systems biology study using metabolomics, it is in particular important to use both the analytical method and the data analysis in an unbiased holistic manner.[445] NMR analysis coupled with multivariate data analysis techniques are well known recently for metabolomic studies in plants.[388] As NMR is used to study a wide range of diverse group of metabolites to analyse in a single run, it is advantageous to use multivariate data analysis tools for NMR data processing and analysis.

Future Perspectives

Clear evidence for health promotion by phytochemicals and/or their breakdown products is still missing, so further research work is needed by using in vivo experiment. A major constraint is the question of the required dose to have optimal effect. This is a matter of content of the active compounds in the vegetables, the amount eaten, and the actual amount absorbed in the body. All are quite variable and consequently food will never become a medicine, at best health supporting items in our diet. *Brassica* plants can be biofortified and further research work can be carried out in this aspect, but metabolic variation during biofortification should monitored constantly during this process.

Studies on phytonutrients and health, taking sensory factors and food preferences into account, constitute an important area of research. Through selective breeding or genetic improvement these compounds may be enhanced in plants.

However environmental effects will cause changes in the metabolome, particularly multitrophic interactions of *Brassica*, under stress conditions are still unclear. Further research work is needed to study the above ground and below ground multitrophic interactions of plants in connection with phytonutrients.

An increased insight into plant-microbe interaction may allow the detection of contaminants, in this case, pathogenic bacteria in vegetables and fruits, thus helping to ensure safety for human consumption. In plant–microbe interactions, it is very important to analyze the microbial volatiles that play a role for immediate activation of the defence mechanisms in plant tissues.

After post-harvest storage at freezing temperatures, the physical quality of radish was not acceptable anymore and thawing caused vast metabolomic changes. Proper drying method should be used to deal with such expected metabolomic changes. Further research is needed to study the production and degradation of primary and secondary metabolites during storage, with relation to their breakdown products.

As an important group of health affecting compounds in Brassicaceae, the glucosinolate content as response of plant to external stimuli should be evaluated in more detail, in relation to the response of plant to external stimuli.

Brassica phytochemicals, especially glucosinolates are well known for biological activities, including anticancer and antioxidant activities. But their actual function or the mechanism has not yet been

fully elucidated. Glucosinolates are known for their anti-nutritional affects, and also certain compounds like phenolic acids, tannins etc. may so form anti-nutritional complexes. A better understanding of molecular and cellular mechanism for the phytochemicals and their breakdown products will be helpful for considering these compounds as health affecting compounds, in positive or negative manner, particularly in connection with obesities.

Undoubtedly, all these efforts should contribute to provide the means of controlling these different defence systems, leading to the development of more resistant plant varieties ultimately providing higher yields along with higher nutritional value.

Summary of the Thesis

Vegetables have always been considered as healthy food. *Brassica* vegetables are well known all over the world as a common food containing health affecting compounds (**Chapter 2**). A vast amount of data is available for health promoting compounds in Brassicaceae vegetables. These health promoting affects are due to a range of phytochemicals including primary (carbohydrates, amino acids and organic acid) and secondary metabolites (phenolics and glucosinolates), along with vitamins and minerals. These metabolites are interconnected through different biosynthetic pathways and are affected by different external stimuli. Plant metabolic responses are specific for different kinds of stress, but use in part similar metabolic pathways (**Chapter 3**). Certain internal or external factors play an important role for the metabolite profile of vegetables, thus changing the nutritional value for human (**Chapter 4**). These factors are related to the plant response to external stress factors (**Chapter 5**, **6**, **7**), helping the plant to survive. The aim of this thesis was to study the *Brassica* phytochemicals and their response to stress factors by using a holistic analytical approach.

General overview of stress induced metabolomic response of Brassicaceae

A significant quantitative metabolomic variation among different species of *Brassica* is reported, showing a nutritional distinction of these species. During growth and development, the plant has to cope with a number of stress factors. These include attack of animals, insects, pathogens, and/or metal, UV, temperature, and drought stress. These external stimuli or the internal physiological growth regulation is known to lead to the inactivation or enhancement of various metabolic pathways resulting in qualitative and/or quantitative changes in plant metabolite production. In nature plants are exposed to multiple stresses at the same time, so there is natural selection pressure for them to evolve coordinated rather than conflicting defence mechanisms [247] (**Chapter 3**).

Although an enhancement of secondary metabolite production is observed when plants are under stress, this change seems specific in terms of the type of interaction. It might depend on the nature of stimuli and/or invading microorganism, that could specifically activate or supress gene expression and induce or block specific sites of a metabolomic pathway, or even result in catabolism of the plant defence compounds (**Chapter 5**, **6**).

Metabolomic assessment of *Brassica rapa* (va. raapstelen) and *Raphanus sativus* L.

Brassica rapa (var. raapstelen) and *Raphanus sativus* (red radish) are well known model systems in recent plant research. Metabolomic data can be misleading if not all the factors affecting the plant growth are taken into consideration. The concentration of primary and secondary metabolites is also dependent on the plant age.[28] Metabolomic variation during the three developmental stages has been assessed (**Chapter 4**). Metabolomic characterisation was done by following a two way analytical approach, i.e. a non targeted metabolomic study by NMR, and a targeted study of certain metabolites by HPLC. All the results either by NMR or HPLC were statistically analyzed by PCA, showing metabolomic changes for both species at different growth stages. Amino acids, organic acids, chlorophyll, carotenoids, tocopherols, ascorbic acid, sucrose, phenylpropanoids and glucosinolates are evidently the discriminating metabolites in this study. In plant species, the metabolomic variation is both spatial and temporal, and different trends under similar conditions may be observed for the species (**Chapter 4**). These results confirm the change in plant metabolomic pool with respect to increase in plant age previously reported by our group.[28]

Metabolomic response of *Brassica* to preharvest bacterial contamination

An inducible cell defence response activates different metabolomic pathways leading to altered production of certain primary and/or secondary metabolites in *Brassica*. These plant self-protective responses,[392] are activated upon recognition of signal molecules from pathogens[391] or the stimuli from the chemical environment.[352]

Although the interaction of plants to plant pathogens is extensively studied, this is not the case for interaction with human pathogens, which may spread in the environment, especially in land irrigated directly or indirectly by drainage / sewage water. In this scenario we cannot exclude the possibility of the interaction of these microbes with vegetables, even if visual symptoms for this interaction cannot be observed. In order to investigate *Brassica* interaction with typically foodborne bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium* and *Shigella flexneri*, ^{1}H NMR in combination with PCA and PLS-DA treatment of the data

was applied to *B. rapa* which had been subjected to these pathogens (**Chapter 5**).

A microbial class (gram-positive and gram-negative) dependent response of *B. rapa* was easily seen by PCA analysis and even by a visual comparison in case of differentially treated plant samples. A higher production of secondary metabolites was observed in the plants under stress by bacteria. Specifically notable is the higher production of GABA in *Bacillus* infested plants, while generally higher amount of phenolics were observed in bacterial infected plants as compared with control and blank. A systemic induced resistance thus to be induced by the bacteria. Generally threonine and γ-amino butyric acid (GABA) were found to be the discriminating metabolites in gram-positive bacteria treated plants, as compared with those treated with gram-negative bacteria that on the other hand exhibited a significant increase in sinapoyl malate, caffeoyl malate and histidine (**Chapter 5**). In case of microorganisms certain volatiles are produced that play a pivotal role in SAR, especially in case of *Bacillus spp.* it is quite clear from the literature that 2,3-butandiol, produced by these bacteria plays an important role in systemic induced resistance of plants. Such microbial volatiles may trigger certain biochemical changes in primary and secondary metabolism of plants, causing elicitation of plant defence.[390]

Metabolomic response to metal stress

Rapid industrial development along with extreme changes in atmospheric conditions around the globe made the environment threatening for plant development because of contaminated air and soil. In these circumstances, plants may have to deal with high concentration of metal ions, resulting in changes in plant metabolism.[411] This metabolomic alteration results in the activation of multiple metabolomic ways, by varying the activity and quantity of key enzymes of different metabolomic pathways.[411] Apparently this response of plant to stress is observed in the both leaves and roots.[412]

Results of the present study indicate that plants grown in higher levels of metal ionic conditions increase the production of amino acids, phenolics and glucosinolates. Among these amino acids and phenolics are reported to have a metal chelating effect[275] indicating that the observed increase in amino acids and phenylpropanoids might be a detoxification response of the plant. However, high metal concentrations (500 mmol) produced a decrease in primary and secondary metabolites as

compared with moderate concentrations (50 mmol & 100 mmol) which might point to an adverse effect of metal ions on primary and secondary metabolism due to the toxic effect of metals at high concentrations (**Chapter 6**).

Metabolomic response to post harvest storage time and temperature

Vegetables travel long ways from field to cooking pan. The preharvest growth conditions are known to severely affect the plant metabolome, but postharvest storage conditions also play a crucial role for the nutritional character of vegetables. During the storage period radish plants show an increase in glucosinolates and phenolics with a subsequent decrease in these compounds during long term storage (**Chapter 7**). The decline of secondary metabolites might be due to the degradation of these products. This phenomenon depends on the storage time and temperature.[11, 434] In postharvest storage of *Brassica* vegetables, either with the purpose of consumption or for experimental purposes, low temperature is known as an important factor. In our study, least metabolomic changes were observed in –80 ^{o}C and –20 ^{o}C, followed by 4 ^{o}C and room temperature storage conditions. The samples stored at room temperature started to get spoiled after three days of storage. Samples stored at 4 ^{o}C were still looking fresh after 28 days storage time, although yellowing of leaves was visible. Samples stored at –20 ^{o}C and –80 ^{o}C remained unchanged.

Brassicaceae vegetables are consumed all over the world. The presence of a range of phytochemicals (amino acids, glucosinolates, flavonoids, vitamins and mineral nutrients), made these vegetables as an important source of food for humans and animals. The response of *Brassicaceae* metabolites to preharvest and postharvest factors was studied by using a systems biology approach. It is observed that different environmental, storage and/or growth factors induce metabolic responses in terms of changes of primary and/or secondary plant metabolites profiles It is concluded that the aforementioned factors cause changes in plant metabolites finally leading to change in quality.

Samenvatting van de Thesis

Groenten zijn altijd beschouwd als gezond voedsel. Groenten van het genus *Brassica* worden over de hele wereld als voedsel gebruikt (**Hoofdstuk 2**). Veel kennis is inmiddels beschikbaar over de gezondheidsbevorderende stoffen in Brassicaceae groenten. Het gezondheidsbevorderende effect hangt samen met een reeks van primaire (koolhydraten, aminozuren en organische zuren) en secundaire metabolieten (fenolen en glucosinolaten), samen met vitaminen en mineralen. De productie van deze metabolieten is met elkaar verbonden in biosynthetische netwerken en wordt beïnvloed door verschillende externe stimuli. Metabole reacties van de plant hierop zijn specifiek voor verschillende soorten van stress, maar gebruiken voor een deel vergelijkbare metabole routes (**Hoofdstuk 3**). Zowel interne als externe factoren spelen een belangrijke rol voor de metabolietenprofielen van groenten, en veranderen daarmee de voedingswaarde voor de mens (**Hoofdstuk 4**). Met name externe stressfactoren beinvloeden het metabolisme van de plant (**Hoofdstuk 5, 6, 7**). Dit hangt samen met de overleving van de plant in zijn omgeving. Het doel van dit proefschrift was om de fytochemicaliën in *Brassica* te onderzoeken, met name in relatie tot de reactie van de plant op stressfactoren, gebruikmakend van een holistische analytisch-chemische benadering.

Algemeen overzicht van stress-geïnduceerd metabolisme in Brassicaceae

Er zijn duidelijk kwantitatieve en kwalitatieve metabole verschillen tussen verschillende *Brassica*-soorten en varieteiten, die daarmee ook een verschil in voedingswaarde hebben. Tijdens de groei en ontwikkeling heeft de plant te kampen met een aantal stressfactoren. Deze omvatten herbivorie door dieren en insecten, ziekteverwekkers, omgevingsfactoren zoals metalen uit de bodem, UV-straling, temperatuur en droogtestress. Van het effect van deze externe prikkels op de interne fysiologische groeiregulatie is bekend dat ze leiden tot het verhogen of verlagen van de flow door verschillende metabole routes, resulterend in kwalitatieve en/of kwantitatieve veranderingen in de metabolietproductie. In de natuur zijn planten tegelijkertijd blootgesteld aan meervoudige stressfactoren, hetgeen resulteert in een continue natuurlijke selectiedruk die leidt tot de evolutie van gecoördineerde afweermechanismen (**Hoofdstuk 3**).

In het algemeen wordt een verhoging van de productie van secundaire metabolieten waargenomen bij planten onder stress, en deze verandering blijkt specifiek te zijn voor de aard van de interactie. De verschillen hangen af van de aard van de stimuli en/of type van micro-organismen of herbivoren die specifiek genexpressie induceren of onderdrukken en daarmee de bijbehorende metabole routes. Katabolisme van de plantenafweerstoffen bij infectie door microorganismen kan ook een rol spelen (**Hoofdstuk 5, 6**).

Metabole veranderingen in *Brassica rapa* (raapstelen Va) en *Raphanus sativus* L

Brassica rapa (var. raapstelen) en *Raphanus sativus* (rode radijs) zijn bekende modelsystemen in plantenonderzoek. Voordat onderzoek gedaan kan worden naar veranderingen in het metaboloom (het metabolietenprofiel) onder verschillende experimentele condities moet men de veranderingen in het metaboloom kennen tijdens de ontwikkeling van de plant. De verschillen in het metaboloom in drie ontwikkelingsstadia van de plant zijn daarom eerst bepaald voordat verschillende experimenten uitgevoerd werden (**Hoofdstuk 4**).

Het metaboloom werd gemeten met twee verschillende analytisch-chemische benaderingen, een niet-gerichte analyse met behulp van NMR, en een op bepaalde metabolieten gerichte analyse met HPLC. Alle resultaten, van zowel NMR als HPLC zijn met behulp van multivariaatanalyse bewerkt, waarmee metabole veranderingen voor beide soorten in verschillende groeifasen in kaart zijn gebracht. Aminozuren, organische zuren, chlorofyl, carotenoïden, tocoferolen, ascorbinezuur, sucrose, fenylpropanoïden en glucosinolaten zijn de discriminerende metabolieten. Metabole verschillen zijn er zowel in ruimte (deel van de plant) als tijd, en er kunnen onder vergelijkbare condities verschillende trends worden waargenomen voor soorten (**Hoofdstuk 4**). De gevonden resultaten bevestigen eerdere resultaten van ons onderzoek naar verandering van het plantenmetaboloom tijdens de groei.

Metabole reactie van *Brassica* op "pre-harvest" bacteriële besmetting

Wanneer de afweer van een cel wordt geinduceerd dan resulteert dat in het activeren van verschillende biosynthesewegen van primaire en/of secundaire metabolieten in *Brassica*. De afweerreactie wordt geinduceerd door signaalmoleculen uit de plant zelf, uit pathogenen of

herbivoren, maar het is ook mogelijk dat chemische signalen uit de omgeving deze reactie kunnen opwekken.

De interactie van planten met plantpathogene microorganismen is in het verleden uitgebreid bestudeerd, maar dit is niet het geval voor interactie met menselijke pathogenen Deze kunnen zich verspreiden in het milieu, met name in een veld dat wordt geïrrigeerd met water waar riool op geloosd is. In dat geval kan niet uitgesloten worden dat deze microben groenten besmetten. Mogelijkerwijs kan een dergelijke besmetting leiden tot een afweerreactie van de plant. Om dit te onderzoeken werd *Brassica* behandeld met bacteriën zoals *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium* en *Shigella flexneri*, die via voedsel overgedragen worden. Het effect van een dergelijke behandeling op *Brassica rapa* werd met ^{1}H-NMR in combinatie met multivariate data-analyse van de gegevens onderzocht (**Hoofdstuk 5**).

Een van de microbiële klasse (Gram-positieve/Gram-negatieve) afhankelijke reactie van *B. rapa* was duidelijk te zien in de PCA-analyse en zelfs door een visuele vergelijking van verschillend behandelde planten. Een hogere productie van secundaire metabolieten werd waargenomen in de planten onder stress door de bacteriën. Met name opvallend is de hogere productie van GABA in door *Bacillus* aangetaste planten, terwijl in het algemeen hogere concentraties van fenolen werden waargenomen bij de geïnfecteerde planten in vergelijking met de controle en blanco. De humane pathogene bacteriën induceren dus een systemische afweerreactie in de plant.

Threonine en γ-aminoboterzuur (GABA) zijn de discriminerende metabolieten die verhoogd zijn in met Gram-positieve bacteriën behandelde planten, in het geval van Gram-negatieve bacteriën zijn het sinapoylmalaat, caffeoylmalaat en histidine (**Hoofdstuk 5**). Micro-organismen kunnen bepaalde vluchtige stoffen produceren die een cruciale rol spelen in de systemische afweer, met name in het geval van *Bacillus* spp. is het bekend dat 2,3-butaandiol wordt geproduceerd dat een belangrijke rol speelt in geïnduceerde systemische resistentie van planten. Dergelijke microbiële vluchtige stoffen kunnen leiden tot biochemische veranderingen in het primair en secundair metabolisme van planten, als onderdeel van de afweerreactie van de plant.

Metabole reactie op stress met metaalionen

Snelle industriële ontwikkelingen, samen met veranderingen in atmosferische omstandigheden over de hele wereld zijn een bedreiging voor de groei van planten, onder andere als gevolg van de verontreinigde lucht en bodem. Planten moeten zich dan ook vaak aanpassen aan hoge concentraties van metaalionen, wat resulteert in veranderingen in het plantenmetabolisme. De metabolome verandering wordt veroorzaakt door veranderingen in genexpressie die leiden tot veranderingen in de activiteit van enzymen in biosynthesewegen. Deze verandering onder stress met metaal-ionen wordt waargenomen in zowel de bladeren als de wortels.

Uit de resultaten van deze studie blijkt dat in planten bij hogere metaalionconcentraties de productie van aminozuren, fenolen en glucosinolaten verhoogd is. Deze aminozuren en fenolen hebben mogelijk een metaalion-chelaatvormend effect en kunnen dus een rol spelen in de ontgiftingsreactie van de plant. Een hoge concentratie van metalen (500 mmol) resulteerde in een daling van de primaire en secundaire metabolieten in vergelijking met lagere concentraties (50 mmol & 100 mmol). Dit zou kunnen wijzen op een negatief (toxisch) effect van metaalionen op het primaire en secundaire metabolisme (**Hoofdstuk 6**).

Metaboloomveranderingen tijdens opslag na oogsten

Groenten leggen een lange weg af van het veld naar de pan. Van de groeicondities is het bekend dat zij het plantenmetaboloom bepalen, maar ook de bewaarcondities na het oogsten spelen een cruciale rol voor het metaboloom en dus de voedingswaarde van groenten. Tijdens de opslag van radijsplanten blijkt er eerst een toename van de glucosinolaten en fenolen te zijn, maar bij langere opslag dalen de gehaltes weer (**Hoofdstuk 7**). De daling van secundaire metabolieten zou te wijten kunnen zijn aan de afbraak van deze producten. Dit fenomeen hangt af van de opslagtijd en temperatuur. Voor het bewaren van *Brassica*-groenten, voor consumptie of voor experimentele doeleinden, zijn lage temperaturen bekend als een belangrijke factor. In onze studie werden de minste metabolome veranderingen waargenomen bij –80^{o}C en –20^{o}C, gevolgd door 4^{o}C en kamertemperatuur. De bij kamertemperatuur bewaarde monsters beginnen te bederven na drie dagen van opslag. Monsters bewaard bij 4^{o}C leken nog steeds vers na 28 dagen opslag, hoewel geelverkleuring van de bladeren zichtbaar was. Opslag bij -20 en –80‘C geeft geen visuele veranderingen, alleen na het ontdooien is het

materiaal niet meer geschikt als groente. Voor analyse is deze bewaarwijze echter de meest geschikte.

Brassicaceae groenten worden gebruikt over de hele wereld. Het spectrum aan fytochemicaliën (aminozuren, glucosinolaten, flavonoïden, vitamines en minerale voedingsstoffen) maken van deze groenten een belangrijke bron als menselijke en dierlijke voeding. Daarom is de reactie van het metabolisme in Brassicaceae planten op verschillende condites voor en na oogsten onderzocht met behulp van een systeembiologische benadering.

Verschillende milieu, opslag-en groeifactoren induceren verschillende veranderingen in zo wel primaire en secundaire stofwisseling van de planten. De bovengenoemde factoren kunnen daarmee ook veranderingen veroorzaken in de gehaltes van de belangrijke nutrienten, en daarmee dus de kwaliteit van de groente beinvloeden.

References

1 Grusak MA, DellaPenna D. Improving the nutrient composition of plants to enhance human nutrition and health. *Annu Rev Plant Physiol Plant Mol Biol*. 1999; 50: 133-61.

2 Curtis IS. The noble radish: past, present and future. *Trends Plant Sci*. 2003; 8: 305-07.

3 Siemens DH, Garner SH, Mitchell-Olds T, Callaway RM. Cost of defense in the context of plant competition: *Brassica rapa* may grow and defend. *Ecology*. 2002; 83: 505-17.

4 Verkerk R, vanderGaag MS, Dekker M, Jongen WMF. Effects of processing conditions on glucosinolates in cruciferous vegetables. *Cancer Lett*. 1997; 114: 193-94.

5 Gomes MH, Rosa E. Free amino acid composition in primary and secondary inflorescences of 11 broccoli (*Brassica oleracea* var *italica*) cultivars and its variation between growing seasons. *J Sci Food Agric*. 2001; 81: 295-99.

6 Moreno DA, Carvajal M, Lopez-Berenguer C, Garcia-Viguera C. Chemical and biological characterisation of nutraceutical compounds of broccoli. *J Pharm Biomed Anal*. 2006; 41: 1508-22.

7 Rickman JC, Barrett DM, Bruhn CM. Nutritional comparison of fresh, frozen and canned fruits and vegetables. Part 1. Vitamins C and B and phenolic compounds. *J Sci Food Agric*. 2007; 87: 930-44.

8 Barnes S. Nutritional genomics, polyphenols, diets, and their impact on dietetics. *J Am Diet Assoc*. 2008; 108: 1888-95.

9 Halkier BA, Gershenzon J. Biology and biochemistry of glucosinolates. *Annu Rev Plant Biol*. 2006; 57: 303.

10 Stoewsand GS. Bioactive organosulfur phytochemicals in *Brassica oleracea* vegetables - a review. *Food Chem Toxicol*. 1995; 33: 537-43.

11 Tomas-Barberan F, Espin JC. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *J Sci Food Agric*. 2001; 81: 853-76.

12 Tung YT, Wu JH, Huang CY, Kuo YH, Chang ST. Antioxidant activities and phytochemical characteristics of extracts from *Acacia confusa* bark. *Bioresour Technol*. 2009; 100: 509-14.

13 Sakihama Y, Cohen MF, Grace SC, Yamasaki H. Plant phenolic antioxidant and prooxidant activities: Phenolics-induced oxidative damage mediated by metals in plants. *Toxicology*. 2002; 177: 67-80.

14 Sun J, Liu RH. Apple phytochemical extracts inhibit proliferation of estrogen-dependent and estrogen-independent human breast cancer cells through cell cycle modulation. *J Agric Food Chem*. 2008; 56: 11661-67.

15 Habib SHM, Makpol S, Hamid NAA, Das S, Ngah WZW, Yusof YAM. Ginger extract (*Zingiber officinale*) has anti-cancer and anti-inflammatory effects on ethionine-induced hepatoma rats. *Clinics*. 2008; 63: 807-13.

16 Girish C, Pradhan SC. Drug development for liver diseases: Focus on picroliv, ellagic acid and curcumin. *Fundam Clin Pharmacol*. 2008; 22: 623-32.

17 Mattson MP, Cheng A. Neurohormetic phytochemicals: Low-dose toxins that induce adaptive neuronal stress responses. *Trends Neurosci*. 2006; 29: 632-39.

18 Zhao TJ, Liu Y, Yan YB, Feng F, Liu WQ, Zhou HM. Identification of the amino acids crucial for the activities of drought responsive element binding factors (DREBs) of *Brassica napus*. *FEBS Lett*. 2007; 581: 3044-50.

19 Sudha G, Ravishankar GA. Involvement and interaction of various signaling compounds on the plant metabolic events during defense response, resistance to stress factors, formation of secondary metabolites and their molecular aspects. *Plant Cell Tissue Organ Cult*. 2002; 71: 181-212.

20 Gratao PL, Polle A, Lea PJ, Azevedo RA. Making the life of heavy metal-stressed plants a little easier. *Funct Plant Biol*. 2005; 32: 481-94.

21 Schutzendubel A, Polle A. Plant responses to abiotic stresses: Heavy metal-induced oxidative stress and protection by mycorrhization. *J Exp Bot*. 2002; 53: 1351-65.

22 Salt DE, Blaylock M, Kumar NPBA, Dushenkov V, Ensley BD, Chet I, *et al.* Phytoremediation: A novel strategy for the removal of toxic metals from the environment using plants. *Nat Biotech*. 1995; 13: 468-74.

23 Parkpain P, Sreesai S, Delaune RD. Bioavailability of heavy metals in sewage sludge-amended Thai soils. *Water Air Soil Pollut*. 2000; 122: 163-82.

24 Jahangir M, Abdel-Farid IB, Choi YH, Verpoorte R. Metal ion-inducing metabolite accumulation in *Brassica rapa*. *J Plant Physiol*. 2008; 165: 1429-37.

25 Kennedy AC. Bacterial diversity in agroecosystems. *Agriculture, Ecosystems & Environment*. 1999; 74: 65-76.

26 van Loon LC, Bakker P, Pieterse CMJ. Systemic resistance induced by rhizosphere bacteria. *Annu Rev Phytopathol*. 1998; 36: 453-83.

27 Jahangir M, Kim HK, Choi YH, Verpoorte R. Metabolomic response of *Brassica rapa* submitted to pre-harvest bacterial contamination. *Food Chem*. 2008; 107: 362-68.

28 Abdel-Farid BB, Kim HK, Choi YH, Verpoorte R. Metabolic characterization of *Brassica rapa* leaves by NMR spectroscopy. *J Agric Food Chem*. 2007; 55: 7936-43.

29 Roy MK, Juneja LR, Isobe S, Tsushida T. Steam processed broccoli (*Brassica oleracea*) has higher antioxidant activity in chemical and cellular assay systems. *Food Chem*. 2009; 114: 263-69.

30 Trischuk RG, Schilling BS, Wisniewski M, Gusta LV. Freezing stress: system biology to study of cold tolerance. In: Gusta LV (ed.). *Physiol Mol Biol Plants Stress Tol Plants* 2006; 131-55.

31 Jones RB, Faragher JD, Winkler S. A review of the influence of postharvest treatments on quality and glucosinolate content in broccoli (*Brassica oleracea* var. *italica*) heads. *Postharvest Biol Technol*. 2006; 41: 1-8.

32 Jia C-G, Xu C-J, Wei J, Yuan J, Yuan G-F, Wang B-L, *et al.* Effect of modified atmosphere packaging on visual quality and glucosinolates of broccoli florets. *Food Chem*. 2009; 114: 28-37.

33 Sharma SS, Dietz K-J. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J Exp Bot*. 2006; 57: 711-26.

34 Huber LS, Hoffmann-Ribani R, Rodriguez-Amaya DB. Quantitative variation in Brazilian vegetable sources of flavonols and flavones. *Food Chem*. 2009; 113: 1278-82.

35 Sumner LW, Mendes P, Dixon RA. Plant metabolomics: Large-scale phytochemistry in the functional genomics era. *Phytochemistry*. 2003; 62: 817-36.

36 Choi YH, Kim HK, Linthorst HJM, Hollander JG, Lefeber AWM, Erkelens C, *et al.* NMR metabolomics to revisit the Tobacco Mosaic Virus infection in *Nicotiana tabacum* leaves. *J Nat Prod*. 2006; 69: 742-48.

37 Verpoorte R, Choi YH, Kim HK. NMR-based metabolomics at work in phytochemistry. *Phytochem Rev*. 2007; 6: 3-14.

38 Ratcliffe RG, Shachar-Hill Y. Revealing metabolic phenotypes in plants: Inputs from NMR analysis. *Biol Rev*. 2005; 80: 27-43.

39 Dowlatabadi R, Weljie AM, Thorpe TA, Yeung EC, Vogel HJ. Metabolic footprinting study of white spruce somatic embryogenesis using NMR spectroscopy. *Plant Physiol Biochem*. 47: 343-50.

40 Ahrens CH, Wagner U, Rehrauer HK, Turker C, Schlapbach R. Current challenges and approaches for the synergistic use of systems biology data in the scientific community. *Plant syst Biol*. 2007: 277-307.

41 Liang YS, Choi YH, Kim HK, Linthorst HJM, Verpoorte R. Metabolomic analysis of methyl jasmonate treated *Brassica rapa* leaves by 2-dimensional NMR spectroscopy. *Phytochemistry*. 2006; 67: 2503-11.

42 Sasaki K, Takahashi T. A flavonoid from *Brassica rapa* flower as the UV-absorbing nectar guide. *Phytochemistry*. 2002; 61: 339-43.

43 O'Callaghan KJ, Stone PJ, Hu XJ, Griffiths DW, Davey MR, Cocking EC. Effects of glucosinolates and flavonoids on colonization of the roots of *Brassica napus* by *Azorhizobium caulinodans* ORS571. *Appl Environ Microbiol*. 2000; 66: 2185-91.

44 Onyilagha JC, Lazorko J, Gruber MY, Soroka JJ, Erlandson MA. Effect of flavonoids on feeding preference and development of the crucifer pest *Mamestra configurata* Walker. *J Chem Ecol*. 2004; 30: 109-24.

45 Kaushik N, Agnihotri A. GLC analysis of Indian rapeseed-mustard to study the variability of fatty acid composition. *Biochem Soc T*. 2000: 581-83.

46 Ferreres F, Sousa C, Valentao P, Seabra RM, Pereira JA, Andrade PB. Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) seeds: Phytochemical characterization and antioxidant potential. *Food Chem*. 2007; 101: 549-58.

47 Font R, del Rio-Celestino M, Cartea E, de Haro-Bailon A. Quantification of glucosinolates in leaves of leaf rape (*Brassica napus* ssp *pabularia*) by near-infrared spectroscopy. *Phytochemistry*. 2005; 66: 175-85.

48 Sardi E, Tordai E. Determination of fully N-methylated compound in different cabbage and beetroot varieties. *Acta Biol Szeg*. 2005; 49: 43-45.

49 Kusznierewicz B, Bartoszek A, Wolska L, Drzewiecki J, Gorinstein S, Namiesnik J. Partial characterization of white cabbages (*Brassica oleracea* var *capitata f. alba*) from different regions by glucosinolates, bioactive compounds, total antioxidant activities and proteins. *LWT-Food Sci Technol*. 2008; 41: 1-9.

50 Heaney RK, Fenwick GR. Natural toxins and protective factors in *Brassica* species, including rapeseed. *Nat Toxins*. 1995; 3: 233-37.

51 Thiyam U, Kuhlmann A, Stockmann H, Schwarz K. Prospects of rapeseed oil by-products with respect to antioxidative potential. *Les Comptes Rendus Chimie*. 2004: 611-16.

52 Dekker M, Verkerk R, Jongen WMF. Predictive modelling of health aspects in the food production chain: a case study on glucosinolates in cabbage. *Trends Food Sci Technol*. 2000; 11: 174-81.

53 Vallejo F, Tomas-Barberan FA, García-Viguera C. Potential bioactive compounds in health promotion from broccoli cultivars grown in Spain. *J Sci Food Agric*. 2002; 82: 1293-97.

54 Vallejo F, Tomas-Barberan F, Garcia-Viguera C. Health-promoting compounds in broccoli as influenced by refrigerated transport and retail sale period. *J Agric Food Chem*. 2003; 51: 3029-34.

55 Vallejo F, Gil-Izquierdo A, Perez-Vicente A, Garcia-Viguera C. In vitro gastrointestinal digestion study of broccoli inflorescence phenolic compounds, glucosinolates, and vitamin C. *J Agric Food Chem*. 2004; 52: 135-38.

56 Pedroche J, Yust MM, Lqari H, Giron-Calle J, Alaiz M, Vioque J, *et al.* *Brassica carinata* protein isolates: chemical composition, protein characterization and improvement of functional properties by protein hydrolysis. *Food Chem*. 2004; 88: 337-46.

57 Fowke JH, Chung FL, Jin F, Qi D, Cai QY, Conaway C, *et al.* Urinary isothiocyanate levels, *Brassica*, and human breast cancer. *Cancer Res*. 2003; 63: 3980-86.

58 Heimler D, Vignolini P, Dini MG, Vincieri FF, Romani A. Antiradical activity and polyphenol composition of local *Brassicaceae* edible varieties. *Food Chem*. 2006; 99: 464-69.

59 Zakaria-Rungkat F, Djaelani M, Setiana, Rumondang E, Nurrochmah. Carotenoid bioavailability of vegetables and carbohydrate-containing foods measured by retinol accumulation in rat livers. *J Food Compos Anal*. 2000; 13: 297-310.

60 Rice-Evans C, Miller N, Paganga G. Antioxidant properties of phenolic compounds. *Trends Plant Sci*. 1997; 2: 152-59.

61 Kristal AR, Lampe JW. *Brassica* vegetables and prostate cancer risk: A review of the epidemiological evidence. *Nutr Cancer*. 2002; 42: 1 - 9.

62 Wang LI, Giovannucci EL, Hunter D, Neuberg D, Su L, Christiani DC. Dietary intake of cruciferous vegetables, glutathione S-transftrase (GST) polymorphisms and lung cancer risk in a Caucasian population. *Cancer Causes Control*. 2004; 15: 977-85.

63 Zukalova H, Vasak J. The role and effects of glucosinolates of *Brassica* species - a review. *Rostl Vyroba*. 2002; 48: 175-80.

64 Samaila D, Ezekwudo DE, Yimam KK, Elegbede JA. Bioactive plant compounds inhibited the proliferation and induced apoptosis in human cancer cell lines, *in vitro*. *Trans Int Biomed: Inform Enabl Tech Symp J*. 2004; 1: 34-42.

65 Hanf V, Gonder U. Nutrition and primary prevention of breast cancer: Foods, nutrients and breast cancer risk. *Eur J Obstet Gynecol Reprod Biol*. 2005; 123: 139-49.

66 Staub RE, Feng C, Onisko B, Bailey GS, Firestone GL, Bjeldanes LF. Fate of indole-3-carbinol in cultured human breast tumor cells. *Chem Res Toxicol*. 2002; 15: 101-09.

67 Cover CM, Hsieh SJ, Tran SH, Hallden G, Kim GS, Bjeldanes LF, *et al.* Indole-3-carbinol Inhibits the expression of cyclin-dependent kinase-6 and

Induces a G1 cell cycle arrest of human breast cancer cells independent of estrogen receptor signaling. *J Biol Chem.* 1998; 273: 3838-47.

68 Steinkellner H, Rabot S, Freywald C, Nobis E, Chabicovskyk M, Knasmuller S, *et al.* Effects of cruciferous vegetables and their constituents on drug metabolizing enzymes involved in the bioactivation of DNA-reactive dietary carcinogens. *Mutat Res-Fund Mol M*. 2001; 480: 285-97.

69 O'Connor SE, Maresh JJ. Chemistry and biology of monoterpene indole alkaloid biosynthesis. *ChemInform.* 2006; 37.

70 Dixon RA. Natural products and plant disease resistance. *Nature*. 2001; 411: 843-47.

71 Winkel-Shirley B. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol*. 2001; 126: 485-93.

72 Graser G, Schneider B, Oldham NJ, Gershenzon J. The methionine chain elongation pathway in the biosynthesis of glucosinolates in *Eruca sativa* (*Brassicaceae*). *Arch Biochem Biophys*. 2000; 378: 411-19.

73 Dixon RA, Paiva NL. Stress-induced phenylpropanoid metabolism. *Plant Cell.* 1995; 7: 1085-97.

74 Azuma K, Ippoushi K, Ito H, Higashio H, Terao J. Evaluation of antioxidative activity of vegetable extracts in linoleic acid emulsion and phospholipid bilayers. *J Sci Food Agric*. 1999; 79: 2010-16.

75 Ferguson LR. Prospects for cancer prevention. *Mutat Res-Fund Mol M.* 1999; 428: 329-38.

76 Kopsell DE, Kopsell DA, Randle WA, Coolong TW, Sams CE, Curran-Celentano J. Kale carotenoids remain stable while flavor compounds respond to changes in sulfur fertility. *J Agric Food Chem.* 2003; 51: 5319-25.

77 Mithen RF, Dekker M, Verkerk R, Rabot S, Johnson IT. The nutritional significance, biosynthesis and bioavailability of glucosinolates in human foods. *J Sci Food Agric*. 2000; 80: 967-84.

78 Matthaus B, Angelini LG. Anti-nutritive constituents in oilseed crops from Italy. *Indus. Crops Prod*. 2005; 21: 89-99.

79 Kurilich AC, Tsau GJ, Brown A, Howard L, Klein BP, Jeffery EH, *et al.* Carotene, tocopherol, and ascorbate contents in subspecies of *Brassica oleracea*. *J Agric Food Chem.* 1999; 47: 1576-81.

80 Verhoeven DTH, Goldbohm RA, vanPoppel G, Verhagen H, vandenBrandt PA. Epidemiological studies on *Brassica* vegetables and cancer risk. *Cancer Epidemiol Biomark Prev*. 1996; 5: 733-48.

81 Kurilich AC, Jeffery EH, Juvik JA, Wallig MA, Klein BP. Antioxidant capacity of different broccoli (*Brassica oleracea*) genotypes using the oxygen radical absorbance capacity (ORAC) assay. *J Agric Food Chem.* 2002; 50: 5053-57.

82 Eberhardt MV, Kobira K, Keck AS, Juvik JA, Jeffery EH. Correlation analyses of phytochemical composition, chemical, and cellular measures of antioxidant activity of broccoli (*Brassica oleracea* L. var. *italica*). *J Agric Food Chem.* 2005; 53: 7421-31.

83 Granado F, Olmedilla B, Herrero C, Perez-Sacristan B, Blanco I, Blazquez S. Bioavailability of carotenoids and tocopherols from broccoli: In vivo and in vitro assessment. *Exp Biol Med*. 2006; 231: 1733-38.

84 Miyazawa M, Nishiguchi T, Yamafuji C. Volatile components of the leaves of *Brassica rapa* L. var. *perviridis* Bailey. *Flavour Fragr J*. 2005; 20: 158-60.

85 Wills RBH, Rangga A. Determination of carotenoids in Chinese vegetables. *Food Chem*. 1996; 56: 451-55.

86 Riso P, Brusamolino A, Ciappellano S, Porrini M. Comparison of lutein bioavailability from vegetables and supplement. *Int J Vitam Nutr Res*. 2003; 73: 201-05.

87 Khachik F, Steck A, Pfander H. Isolation and structural elucidation of(13Z,13 ' Z,3R,3 ' R,6 ' R)-lutein from marigold flowers, kale, and human plasma. *J Agric Food Chem*. 1999; 47: 455-61.

88 Lefsrud MG, Kopsell DA. Kale carotenoids are unaffected by, whereas biomass production, elemental concentrations, and selenium accumulation respond to, changes in selenium fertility. *J Agric Food Chem*. 2006; 54: 1764-71.

89 Podsedek A. Natural antioxidants and antioxidant capacity of *Brassica* vegetables: A review. *LWT-Food Sci Technol*. 2007; 40: 1-11.

90 Piironen V, Syvaoja EL, Varo P, Salminen K, Koivistoinen P. Tocopherols and tocotrienols in Finnish foods: Vegetables, fruits, and berries. *J Agric Food Chem*. 1986; 34: 742-46.

91 Goffman FD, Mollers C. Changes in tocopherol and plastochromanol-8 contents in seeds and oil of oilseed rape (*Brassica napus* L.) during storage as influenced by temperature and air oxygen. *J Agric Food Chem.* 2000; 48: 1605-09.

92 Bahorun T, Luximon-Ramma A, Crozier A, Aruoma OI. Total phenol, flavonoid, proanthocyanidin and vitamin C levels and antioxidant activities of Mauritian vegetables. *J Sci Food Agric*. 2004; 84: 1553-61.

93 Goldoni JS, Bonassi IA, Conceicao FA. Comparative study of vitamin C of cabbage cultivars (*Brassica oleraceae* L., var. *capitata* L.), before and after their processing in sauerkraut. *Arch Latinoam Nutr*. 1983; 33: 45-56.

94 McKillop DJ, Pentieva K, Daly D, McPartlin JM, Hughes J, Strain JJ, *et al.* The effect of different cooking methods on folate retention in various foods that are amongst the major contributors to folate intake in the UK diet. *Br J Nutr*. 2002; 88: 681-88.

95 Melse-Boonstra A, Verhoef P, Konings EJM, van Dusseldorp M, Matser A, Hollman PCH, *et al.* Influence of processing on total, monoglutamate and polyglutamate folate contents of leeks, cauliflower, and green beans. *J Agric Food Chem*. 2002; 50: 3473-78.

96 Puupponen-Pimia R, Hakkinen ST, Aarni M, Suortti T, Lampi AM, Eurola M, *et al.* Blanching and long-term freezing affect various bioactive compounds of vegetables in different ways. *J Sci Food Agric*. 2003; 83: 1389-402.

97 Devi R, Arcot J, Sotheeswaran S, Ali S. Folate contents of some selected Fijian foods using tri-enzyme extraction method. *Food Chem.* 2008; 106: 1100-04.

98 Bailey LB, Rampersaud GC, Kauwell GPA. Folic acid supplements and fortification affect the risk for neural tube defects, vascular disease and cancer: Evolving science. *J Nutr*. 2003; 133: 1961S-68S.

99 Cornel MC, Smit DJd, de Jong-van den Berg LTW. Folic acid - The scientific debate as a base for public health policy. *Reprod Toxicol*. 2005; 20: 411-15.

100 Bollheimer LC, Buettner R, Kullmann A, Kullmann F. Folate and its preventive potential in colorectal carcinogenesis: How strong is the biological and epidemiological evidence? *Crit Rev Oncol Hematol*. 2005; 55: 13-36.

101 Singh J, Upadhyay AK, Prasad K, Bahadur A, Rai M. Variability of carotenes, vitamin C, E and phenolics in *Brassica* vegetables. *J Food Compos Anal*. 2007; 20: 106-12.

102 Singh G, Kawatra A, Sehgal S. Nutritional composition of selected green leafy vegetables, herbs and carrots. *Plant Foods Hum Nutr*. 2001; 56: 359-64.

103 Tirasoglu E, Cevik U, Ertugral B, Apaydin G, Baltas H, Ertugrul M. Determination of trace elements in cole (*Brassica oleraceae* var. *acephale*) at Trabzon region in Turkey. *J Quant Spectrosc Ra*. 2005; 94: 181-87.

104 Finley JW. Reduction of cancer risk by consumption of selenium-enriched plants: Enrichment of broccoli with selenium increases the anticarcinogenic properties of broccoli. *J Med Food*. 2003; 6: 19-26.

105 Heaney RK, Weaver CM, Hinders SM, Martin B, Packard PT. Absorbability of calcium from *Brassica* vegetables: Broccoli, bok choy, and kale. *J Food Sci*. 1993; 58: 1378-80.

106 Glew RS, VanderJagt DJ, Bosse R, Huang YS, Chuang LT, Glew RH. The nutrient content of three edible plants of the Republic of Niger. *J Food Compos Anal*. 2005; 18: 15-27.

107 Elless MP, Blaylock MJ, Huang JW, Gussman CD. Plants as a natural source of concentrated mineral nutritional supplements. *Food Chem*. 2000; 71: 181-88.

108 Kmiecik W, Lisiewska Z, Korus A. Retention of mineral constituents in frozen brassicas depending on the method of preliminary processing of the raw material and preparation of frozen products for consumption. *Eur Food Res Technol*. 2007; 224: 573-79.

109 Kawashima LM, Valente Soares LM. Mineral profile of raw and cooked leafy vegetables consumed in southern Brazil. *J Food Compos Anal*. 2003; 16: 605-11.

110 He ZL, Yang XE, Stoffella PJ. Trace elements in agroecosystems and impacts on the environment. *J Trace Elem Med Biol*. 2005; 19: 125-40.

111 Banuelos GS. Phyto-products may be essential for sustainability and implementation of phytoremediation. *Environ Pollut*. 2006; 144: 19-23.

112 Dudka S, Miller WP. Permissible concentrations of arsenic and lead in soils based on risk assessment. *Water Air Soil Pollut*. 1999; 113: 127-32.

113 Naczk M, Amarowicz R, Sullivan A, Shahidi F. Current research developments on polyphenolics of rapeseed/canola: A review. *Food Chem*. 1998; 62: 489-502.

114 Choudhury AR, Das T, Sharma A. Mustard oil and garlic extract as inhibitors of sodium arsenite-induced chromosomal breaks in vivo. *Cancer Lett*. 1997; 121: 45-52.

115 Dwivedi C, Muller LA, Goetz-Parten DE, Kasperson K, Mistry VV. Chemopreventive effects of dietary mustard oil on colon tumor development. *Cancer Lett*. 2003; 196: 29-34.

116 Ahuja KL, Batta SK, Raheja RK, Labana KS, Gupta ML. Oil content and fatty acid composition of promising Indian *Brassica campestris* L. (Toria) genotypes. *Plant Foods Hum Nutr*. 1989; 39: 155-60.

117 Vermunt SHF, Beaufrere B, Riemersma RA, Sebedio J-L, Chardigny J-M, Mensink RP. Dietary trans-α-linolenic acid from deodorised rapeseed oil and plasma lipids and lipoproteins in healthy men: the *Trans*LinE Study. *Br J Nutr*. 2001; 85: 387-92.

118 Adamska E, Cegielska-Taras T, Kackzmarek Z, Szala L. Multivariate approach to evaluating the fatty acid composition of seed oil in a doubled haploid population of winter oilseed rape (*Brassica napus* L.). *J Appl Genetics* 2004; 45: 419-25.

119 Scalzo RL, Bianchi G, Genna A, Summa C. Antioxidant properties and lipidic profile as quality indexes of cauliflower (*Brassica oleracea* L. var. *botrytis*) in relation to harvest time. *Food Chem*. 2007; 100: 1019-25.

120 Mcewan M, Smith WHM. Identification of volatile organic compounds emitted in the field by oilseed rape (*Brassica napus* ssp. *oleifera*) over the growing season. *Clin Exp Allergy*. 1998; 28: 332-38.

121 Vuorinen T, Reddy GVP, Nerg A-M, Holopainen JK. Monoterpene and herbivore-induced emissions from cabbage plants grown at elevated atmospheric CO_2 concentration. *Atmos Environ*. 2004; 38: 675-82.

122 Lechner M, Reiter B, Lorbeer E. Determination of tocopherols and sterols in vegetable oils by solid-phase extraction and subsequent capillary gas chromatographic analysis. *J Chromatogr A*. 1999; 857: 231-38.

123 Appelqvist L-AD, Kornfeld AK, Wennerholm JE. Sterols and steryl esters in some *Brassica* and sinapis seeds. *Phytochemistry*. 1981; 20: 207-10.

124 Hobbs DH, Hume JH, Rolph CE, Cooke DT. Changes in lipid composition during floral development of *Brassica campestris*. *Phytochemistry*. 1996; 42: 335-39.

125 Ayaz FA, Glew RH, Millson M, Huang HS, Chuang LT, Sanz C, *et al.* Nutrient contents of kale (*Brassica oleraceae* L. var. *acephala* DC.). *Food Chem*. 2006; 96: 572-79.

126 Rule DC, Busboom JR, Kercher CJ. Effect of dietary canola on fatty acid composition of bovine adipose tissue, muscle, kidney, and liver. *J Anim Sci*. 1994; 72: 2735-44.

127 Rosa E, David M, Gomes MH. Glucose, fructose and sucrose content in broccoli, white cabbage and Portuguese cabbage grown in early and late seasons. *J Sci Food Agric*. 2001; 81: 1145-49.

128 King GA, Morris SC. Early compositional changes during postharvest senescence of broccoli. *J Am Soc Hortic Sci*. 1994; 119: 1000-05.

129 Simbaya J, Slominski BA, Rakow G, Campbell LD, Downey RK, Bell JM. Quality characteristics of yellow-seeded *Brassica* seed meals: Protein, carbohydrate, and dietary fiber components. *J Agric Food Chem*. 1995; 43: 2062-66.

130 Knudsen KEB. The nutritional significance of "dietary fibre" analysis. *Anim Feed Sci Technol*. 2001; 90: 3-20.

131 Rodriguez R, Jimenez A, Fernandez-Bolanos J, Guillen R, Heredia A. Dietary fibre from vegetable products as source of functional ingredients. *Trends Food Sci Technol*. 2006; 17: 3-15.

132 Wennberg M, Engqvist G, Nyman M. Effects of harvest time and storage on dietary fibre components in various cultivars of white cabbage (*Brassica oleracea* var *capitata*). *J Sci Food Agric*. 2002; 82: 1405-11.

133 Wennberg M, Ekvall J, Olsson K, Nyman M. Changes in carbohydrate and glucosinolate composition in white cabbage (*Brassica oleracea* var. *capitata*) during blanching and treatment with acetic acid. *Food Chem*. 2006; 95: 226-36.

134 Slominski BA, Simbaya J, Campbell LD, Rakow G, Guenter W. Nutritive value for broilers of meals derived from newly developed varieties of yellow-seeded canola. *Anim Feed Sci Technol*. 1999; 78: 249-62.

135 Jensen CR, Mogensen VO, Mortensen G, Fieldsend JK, Milford GFJ, Andersen MN, *et al.* Seed glucosinolate, oil and protein contents of field-grown rape (*Brassica napus* L.) affected by soil drying and evaporative demand. *Field Crops Res*. 1996; 47: 93-105.

136 Mahajan A, Dua S. Nonchemical approach for reducing antinutritional factors in rapeseed (*Brassica campestris* Var. *Toria*) and characterization of enzyme phytase. *J Agric Food Chem*. 1997; 45: 2504-08.

137 Rozan P, Lamghari R, Linder M, Villaume C, Fanni J, Parmentier M, *et al.* In Vivo and in vitro digestibility of soybean, lupine, and rapeseed meal proteins after various technological processes. *J Agric Food Chem*. 1997; 45: 1762-69.

138 Berot S, Compoint JP, Larre C, Malabat C, Gueguen J. Large scale purification of rapeseed proteins (*Brassica napus* L.). *J Chromatogr B*. 2005; 818: 35-42.

139 Aluko RE, McIntosh T. Polypeptide profile and functional properties of defatted meals and protein isolates of canola seeds. *J Sci Food Agric*. 2001; 81: 391-96.

140 Ghodsvali A, Khodaparast MHH, Vosoughi M, Diosady LL. Preparation of canola protein materials using membrane technology and evaluation of meals functional properties. *Food Res Int*. 2005; 38: 223-31.

141 Puumalainen TJ, Poikonen S, Kotovuori A, Vaali K, Kalkkinen N, Reunala T, *et al*. Napins, 2S albumins, are major allergens in oilseed rape and turnip rape. *J Allergy Clin Immunol*. 2006; 117: 426-32.

142 Suido H, Tanaka T, Tabei T, Takeuchi A, Okita M, Kishimoto T, *et al.* A mixed green vegetable and fruit beverage decreased the serum level of low-density lipoprotein cholesterol in hypercholesterolemic patients. *J Agric Food Chem*. 2002; 50: 3346-50.

143 Morrissey JP, Osbourn AE. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiol Mol Biol Rev*. 1999; 63: 708-24.

144 Grayer RJ, Harborne JB. A survey of antifungal compounds from higher plants, 1982-1993. *Phytochemistry*. 1994; 37: 19-42.

145 Smith CJ. Accumulation of phytoalexins: Defence mechanism and stimulus response system. *New Phytol*. 1996; 132: 1-45.

146 Rogers EE, Glazebrook J, Ausubel FM. Mode of action of *Arabidopsis thaliana* phytoalexin camalexin and its role in *Arabidopsis* - pathogen interactions. *Mol Plant Microbe Interact*. 1996; 9: 748-57.

147 Pedras MSC, Okanga FI, Zaharia IL, Khan AQ. Phytoalexins from crucifers: Synthesis, biosynthesis, and biotransformation. *Phytochemistry*. 2000; 53: 161-76.

148 Reuber TL, Plotnikova JM, Dewdney J, Rogers EE, Wood W, Ausubel FM. Correlation of defense gene induction defects with powdery mildew susceptibility in *Arabidopsis* enhanced disease susceptibility mutants. *Plant J*. 1998; 16: 473-85.

149 Roetschi A, Si-Ammour A, Belbahri L, Mauch F, Mauch-Mani B. Characterization of an *Arabidopsis-Phytophthora* pathosystem: resistance requires a functional PAD2 gene and is independent of salicylic acid, ethylene and jasmonic acid signalling. *Plant J*. 2001; 28: 293-305.

150 Mezencey R, Mojzis J, Pilatova M, Neoplasma KP. Antiproliferative and cancer chemopreventive activity of phytoalexins: focus on indole phytoalexins from crucifers. *Neoplasma*. 2003; 50: 239-45.

151 Kaplan F, Kopka J, Haskell DW, Zhao W, Schiller KC, Gatzke N, *et al.* Exploring the temperature-stress metabolome of *Arabidopsis*. *Plant Physiol*. 2004; 136: 4159-68.

152 Ryan C, Pearce G, Scheer J, Moura DS. Polypeptide hormones. *Plant Cell*. 2002; 14: 251-64.

153 Ryan CA, Pearce G. Systemins: A functionally defined family of peptide signals that regulate defensive genes in *Solanaceae* species. *Proc Natl Acad Sci* USA 2003; 14577-80.

154 Halitschke R, Baldwin IT. Jasmonates and related compounds in plant-insect interactions. *J Plant Growth Regul*. 2004; 23: 238-45.

155 Creelman RA, Mullet JE. Biosynthesis and action of jasmonates in plants. *Annu Rev Plant Physiol Plant Mol Biol*. 1997; 48: 355-81.

156 Beale MH, Ward JL. Jasmonates: Key players in the plant defense. *Nat Prod Rep*. 1998; 15: 533-48.

157 Blee E. Phytooxylipins and plant defense reactions. *Prog Lipid Res*. 1998; 37: 33-72.

158 Devoto A, Turner JG. Regulation of jasmonate-mediated plant responses in *Arabidopsis*. *Ann Bot*. 2003; 92: 329-37.

159 Farmer EE, Almeras E, Krishnamurthy V. Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. *Curr Opin Plant Biol*. 2003; 6: 372-78.

160 Flescher E. Jasmonates-a new family of anti-cancer agents. *Anticancer Drugs*. 2005; 16: 911-16.

161 Pascale SD, Maggio A, Pernice R, Fogliano V, Barbieri G. Sulphur fertilization may improve the nutritional value of *Brassica rapa* L. subsp. sylvestris. *Eur J Agron*. 2007; 26: 418-24.

162 Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med*. 1996; 20: 933-56.

163 Ninfali P, Bacchiocca M. Polyphenols and antioxidant capacity of vegetables under fresh and frozen conditions. *J Agric Food Chem*. 2003; 51: 2222-26.

164 Singh J, Upadhyay AK, Bahadur A, Singh B, Singh KP, Rai M. Antioxidant phytochemicals in cabbage (*Brassica oleracea* L. var. *capitata*). *Sci Hortic*. 2006; 108: 233-37.

165 Clifford MN. Chlorogenic acids and other cinnamates - nature, occurrence, dietary burden, absorption and metabolism. *J Sci Food Agric*. 2000; 80: 1033-43.

166 Kroon PA, Williamson G. Hydroxycinnamates in plants and food: Current and future perspectives. *J Sci Food Agric*. 1999; 79: 355-61.

167 Gagandeep, Dhiman M, Mendiz E, Rao AR, Kale RK. Chemopreventive effects of mustard (*Brassica compestris*) on chemically induced tumorigenesis in murine forestomach and uterine cervix. *Hum Exp Toxicol*. 2005; 24: 303-12.

168 Wanasundara U, Shahidi F. Canola extract as an alternative natural antioxidant for canola oil. *J Am Oil Chem Soc*. 1994; 71: 817-22.

169 Llorach R, Espin JC, Tomas-Barberan FA, Ferreres F. Valorization of cauliflower (*Brassica oleracea* L. var. *botrytis*) by-products as a source of antioxidant phenolics. *J Agric Food Chem*. 2003; 51: 2181-87.

170 Robbers JE, Speedie MK, Tyler VE (eds). Pharmacognosy and pharmacobiotechnology. Williams and Wilkins: Baltimore 1996.

171 Robbins RJ, Keck A-S, Banuelos G, Finley JW. Cultivation conditions and selenium fertilization alter the phenolic profile, glucosinolate, and sulforaphane content of broccoli. *J Med Food*. 2005; 8: 204-14.

172 Solecka D, Boudet A-M, Kacperska A. Phenylpropanoid and anthocyanin changes in low-temperature treated winter oilseed rape leaves. *Plant Physiol Biochem*. 1999; 37: 491-96.

173 Ayaz FA, HayIrlIoglu-Ayaz S, Alpay-Karaoglu S, Gruz J, Valentova K, Ulrichova J, *et al*. Phenolic acid contents of kale (*Brassica oleraceae* L. var. *acephala* DC.) extracts and their antioxidant and antibacterial activities. *Food Chem*. 2008; 107: 19-25.

174 Vuorela S, Meyer A, Heinonen M. Quantitative analysis of the main phenolics in rapeseed meal and oils processed differently using enzymatic hydrolysis and HPLC. *Eur Food Res Technol*. 2003; 217: 517-23.

175 Vuorela S, Kreander K, Karonen M, Nieminen R, Hamalainen M, Galkin A, *et al*. Preclinical evaluation of rapeseed, raspberry, and pine bark phenolics for health related effects. *J Agric Food Chem*. 2005; 53: 5922-31.

176 Zou Y, Kim AR, Kim JE, Choi JS, Chung HY. Peroxynitrite scavenging activity of sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) isolated from *Brassica juncea*. *J Agric Food Chem*. 2002; 50: 5884-90.

177 Regenbrecht J, Strack D. Distribution of 1-sinapoylglucose: Choline sinapoyltransferase activity in the *Brassicaceae*. *Phytochemistry*. 1985; 24: 407-10.

178 Baumert A, Milkowski C, Schmidt J, Nimtz M, Wray V, Strack D. Formation of a complex pattern of sinapate esters in *Brassica napus* seeds, catalyzed by enzymes of a serine carboxypeptidase-like acyltransferase family? *Phytochemistry*. 2005; 66: 1334-45.

179 Berlin J (ed). Secondary products from plant cell cultures. 2nd edn. VCH: Federal Republic of Germany 1997.

180 Caporale LH. Chemical ecology: A view from the pharmaceutical industry. *Proc Natl Acad Sci U S A*. 1995; 92: 75-82.

181 Morton LW, Caccetta RA-A, Puddey IB, Croft KD. Chemistry and biological effects of dietary phenolic compounds: Relevance to cardiovascular disease. *Clin Exp Pharmacol Physiol*. 2000; 27: 152-59.

182 Chu Y-H, Chang C-L, Hsu H-F. Flavonoid content of several vegetables and their antioxidant activity. *J Sci Food Agric*. 2000; 80: 561-66.

183 Birt DF, Hendrich S, Wang W. Dietary agents in cancer prevention: Flavonoids and isoflavonoids. *Pharmacol Ther*. 2001; 90: 157-77.

184 Omenn GS. What accounts for the association of vegetables and fruits with lower incidence of cancers and coronary heart disease? *Ann Epidemiol*. 1995; 5: 333-35.
185 Williams RJ, Spencer JPE, Rice-Evans C. Flavonoids: Antioxidants or signalling molecules? *Free Radic Biol Med*. 2004; 36: 838-49.
186 Skibola CF, Smith MT. Potential health impacts of excessive flavonoid intake. *Free Radic Biol Med*. 2000; 29: 375-83.
187 Siqueira JO, Safir GR, Nair MG. Stimulation of vesicular-arbuscular mycorrhiza formation and growth of white clover by flavonoid compounds. *New Phytol*. 1991; 118: 87-93.
188 Le Marchand L. Cancer preventive effects of flavonoids - a review. *Biomed Pharmacother*. 2002; 56: 296-301.
189 Nielsen JK, Nørbæk R, Olsen CE. Kaempferol tetraglucosides from cabbage leaves. *Phytochemistry*. 1998; 49: 2171-76.
190 Onyilagha J, Bala A, Hallett R, Gruber M, Soroka J, Westcott N. Leaf flavonoids of the cruciferous species, *Camelina sativa*, *Crambe spp.*, *Thlaspi arvense* and several other genera of the family *Brassicaceae*. *Biochem Syst Ecol*. 2003; 31: 1309-22.
191 Chun OK, Smith N, Sakagawa A, Lee CY. Antioxidant properties of raw and processed cabbages. *Int J Food Sci Nutr*. 2004; 55: 191 - 99.
192 Price KR, Casuscelli F, Colquhoun IJ, Rhodes MJC. Composition and content of flavonol glycosides in broccoli florets (*Brassica olearacea*) and their fate during cooking. *J Sci Food Agric*. 1998; 77: 468-72.
193 Ferreres F, Valentao P, Llorach R, Pinheiro C, Cardoso L, Pereira JA, *et al.* Phenolic compounds in external leaves of Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC). *J Agric Food Chem*. 2005; 53: 2901-07.
194 Ponce MA, Scervino JM, Erra-Balsells R, Ocampo JA, Godeas AM. Flavonoids from shoots, roots and roots exudates of *Brassica alba*. *Phytochemistry*. 2004; 65: 3131-34.
195 Giusti MM, Wrolstad RE. Acylated anthocyanins from edible sources and their applications in food systems. *Biochem Eng J*. 2003; 14: 217-25.
196 Scalzo RL, Genna A, Branca F, Chedin M, Chassaigne H. Anthocyanin composition of cauliflower (*Brassica oleracea* L. var. *botrytis*) and cabbage (*B. oleracea* L. var. *capitata*) and its stability in relation to thermal treatments. *Food Chem*. 2008; 107: 136-44.
197 Dyrby M, Westergaard N, Stapelfeldt H. Light and heat sensitivity of red cabbage extract in soft drink model systems. *Food Chem*. 2001; 72: 431-37.
198 Otsuki T, Matsufuji H, Takeda M, Toyoda M, Goda Y. Acylated anthocyanins from red radish (*Raphanus sativus* L.). *Phytochemistry*. 2002; 60: 79-87.
199 Cutillo F, D'Abrosca B, DellaGreca M, Fiorentino A, Zarrelli A. Lignans and neolignans from *Brassica fruticulosa*: Effects on seed germination and plant growth. *J Agric Food Chem*. 2003; 51: 6165-72.
200 Erdemoglu N, Sener B, Choudhary MI. Bioactivity of lignans from *Taxus baccata*. *Z Naturforsch C*. 2004; 59: 494-98.
201 Durkee AB. The nature of tannin in rapeseed (*Brassica campestris*). *Phytochemistry*. 1971; 10: 1583-85.

202 Sadeghi MA, Rao AGA, Bhagya S. Evaluation of mustard (*Brassica juncea*) protein isolate prepared by steam injection heating for reduction of antinutritional factors. *Food Sci Technol*. 2006; 39: 911-17.

203 Shahidi F (ed). Antinutrients and phytochemical in food. American Chemical Society: Washington, DC 1995.

204 McSweeney CS, Gough J, Conlan LL, Hegarty MP, Palmer B, Krause DO. Nutritive value assessment of the tropical shrub legume *Acacia angustissima*: Anti-nutritional compounds and in vitro digestibility. *Anim Feed Sci Technol*. 2005; 121: 175-90.

205 Mosha TC, Gaga HE, Pace RD, Laswai HS, Mtebe K. Effect of blanching on the content of antinutritional factors in selected vegetables. *Plant Foods Hum Nutr*. 1995; 47: 361-67.

206 Naczk M, Amarowicz R, Pink D, Shahidi F. Insoluble condensed tannins of canola/rapeseed. *J Agric Food Chem*. 2000; 48: 1758-62.

207 Zrybko CL, Fukuda EK, Rosen RT. Determination of glucosinolates in domestic and wild mustard by high-performance liquid chromatography with confirmation by electrospray mass spectrometry and photodiode-array detection. *J Chromatogr A*. 1997; 767: 43-52.

208 Mithen RF. Glucosinolates and their degradation products. *Adv Bot Res*. 2001; 35: 213-32.

209 Brown J, Morra MJ. Glucosinolate-containing seed meal as a soil amendment to control plant pests. 2000-2002. University of Idaho: Idaho, Moscow 2005; 1-99.

210 Windsor AJ, Reichelt M, Figuth A, Svatos A, Kroymann J, Kliebenstein DJ, *et al*. Geographic and evolutionary diversification of glucosinolates among near relatives of *Arabidopsis thaliana* (*Brassicaceae*). *Phytochemistry*. 2005; 66: 1321-33.

211 Kushad MM, Brown AF, Kurilich AC, Juvik JA, Klein BP, Wallig MA, *et al*. Variation of glucosinolates in vegetable crops of *Brassica oleracea*. *J Agric Food Chem*. 1999; 47: 1541-48.

212 Iori R, Barillari J, Rollin P. Comment on In vitro gastrointestinal digestion study of broccoli inflorescence phenolic compounds, glucosinolates, and vitamin C. *J Agric Food Chem*. 2004; 52: 7432-33.

213 Rose P, Huang Q, Ong CN, Whiteman M. Broccoli and watercress suppress matrix metalloproteinase-9 activity and invasiveness of human MDA-MB-231 breast cancer cells. *Toxicol Appl Pharmacol*. 2005; 209: 105-13.

214 Rosa EAS. Glucosinolates from flower buds of Portuguese *Brassica* crops. *Phytochemistry*. 1997; 44: 1415-19.

215 Bradfield CA, Bjeldanes LF. Modification of carcinogen metabolism by indolylic autolysis products of *Brassica oleraceae*. *Adv Exp Med Biol*. 1991; 289: 153-63.

216 Velisek J, Davidek J, Michova J, Pokorny J. Rapid gas chromatographic determination of volatile degradation products of glucosinolates in rapeseed oil. *J Chromatogr A*. 1990; 502: 167-70.

217 Smith TK, Lund EK, Clarke RG, Bennett RN, Johnson IT. Effects of Brussels sprout juice on the cell cycle and adhesion of human colorectal carcinoma cells (HT29) in vitro. *J Agric Food Chem*. 2005; 53: 3895-901.

218 Fenwick GR, Heaney RK. Glucosinolates and their breakdown products in cruciferous crops, foods and feedingstuffs. *Food Chem*. 1983; 11: 249-71.

219 Tripathi MK, Mishra AS. Glucosinolates in animal nutrition: A review. *Anim Feed Sci Technol*. 2007; 132: 1-27.

220 Luthy J, Carden B, Friederich U, Bachmann M. Goitrin - a nitrosatable constituent of plant foodstuffs. *Experientia*. 1984; 40: 452-53.

221 Verkerk R, Dekker M, Jongen WMF. Post-harvest increase of indolyl glucosinolates in response to chopping and storage of *Brassica* vegetables. *J Sci Food Agric*. 2001; 81: 953-58.

222 Kushad MM, Cloyd R, Babadoost M. Distribution of glucosinolates in ornamental cabbage and kale cultivars. *Sci Hortic*. 2004; 101: 215-21.

223 Cartea ME, Velasco P, Obregon S, Padilla G, de Haro A. Seasonal variation in glucosinolate content in *Brassica oleracea* crops grown in northwestern Spain. *Phytochemistry*. 2008; 69: 403-10.

224 Wittstock U, Halkier BA. Glucosinolate research in the *Arabidopsis* era. *Trends Plant Sci*. 2002; 7: 263-70.

225 Smith TJ, Yang CS. Effect of organosulfur compounds from garlic and cruciferous vegetables on drug metabolism enzymes. *Drug Metabol Drug Interact*. 2000; 17: 23-49.

226 Valette L, Fernandez X, Poulain S, Lizzani-Cuvelier L, Loiseau A-M. Chemical composition of the volatile extracts from *Brassica oleracea* L. var. *botrytis* 'Romanesco' cauliflower seeds. *Flavour Fragr J*. 2006; 21: 107-10.

227 Kawanishi S, Oikawa S, Murata M. Evaluation for safety of antioxidant chemopreventive agents. *Antioxid Redox Signal*. 2005; 7: 1728-39.

228 Valette L, Fernandez X, Poulain S, Loiseau AM, Lizzani-Cuvelier L, Levieil R, *et al.* Volatile constituents from Romanesco cauliflower. *Food Chem.* 2003; 80: 353-58.

229 Jacobsson A, Nielsen T, Sjoholm I. Influence of temperature, modified atmosphere packaging, and heat treatment on aroma compounds in broccoli. *J Agric Food Chem*. 2004; 52: 1607-14.

230 Griffiths DW, Deighton N, Birch ANE, Patrian B, Baur R, Städler E. Identification of glucosinolates on the leaf surface of plants from the Cruciferae and other closely related species. *Phytochemistry*. 2001; 57: 693-700.

231 Rosa EAS, Heaney RK, Fenwick GR, Portas CAM. Glucosinolates in crop plants. *Hortic Rev*. 1997; 19: 99–215.

232 Tierens KFMJ, Thomma BPHJ, Brouwer M, Schmidt J, Kistner K, Porzel A, *et al.* Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of *Arabidopsis* to microbial pathogens. *Plant Physiol.* 2001; 125: 1688-99.

233 de Saravia SGG, Gaylarde CC. The antimicrobial activity of an aqueous extract of *Brassica negra*. *Int Biodeterior Biodegrad*. 1998; 41: 145-48.

234 Fahey JW, Talalay P. Antioxidant functions of sulforaphane: a potent inducer of phase II detoxication enzymes. *Food Chem Toxicol*. 1999; 37: 973-79.

235 Nagatsu A, Sugitani T, Mori Y, Okuyama H, Sakakibara J, Mizukami H. Antioxidants from rape (*Brassica campestris* var. *Japonica Hara*) oil cake. *Nat Prod Res.* 2004; 18: 231-9.

236 Lund E. Non-nutritive bioactive constituents of plants: Dietary sources and health benefits of glucosinolates. *Int J Vitam Nutr Res*. 2003; 73.

237 Seow A, Yuan J-M, Sun C-L, Van Den Berg D, Lee H-P, Yu MC. Dietary isothiocyanates, glutathione S-transferase polymorphisms and colorectal cancer risk in the Singapore Chinese health study. *Carcinogenesis*. 2002; 23: 2055-61.

238 Ambrosone CB, McCann SE, Freudenheim JL, Marshall JR, Zhang Y, Shields PG. Breast cancer risk in premenopausal women is inversely associated with consumption of broccoli, a source of isothiocyanates, but is not modified by GST genotype. *J Nutr*. 2004; 134: 1134-38.

239 Tawfiq N, Heaney RK, Plumb JA, Fenwick GR, Musk SRR, Williamson G. Dietary glucosinolates as blocking agents against carcinogenesis: Glucosinolate breakdown products assessed by induction of quinone reductase activity in murine hepa1c1c7 cells. *Carcinogenesis*. 1995; 16: 1191-94.

240 Liang H, Yuan Q, Xiao Q. Purification of sulforaphane from *Brassica oleracea* seed meal using low-pressure column chromatography. *J Chromatogr B*. 2005; 828: 91-96.

241 Branca F, Li G, Goyal S, Quiros CF. Survey of aliphatic glucosinolates in Sicilian wild and cultivated Brassicaceae. *Phytochemistry*. 2002; 59: 717-24.

242 Sato K, Kawakami N, Ohtsu T, Tsutsumi A, Miyazaki S, Masumoto T, *et al.* Broccoli consumption and chronic atrophic gastritis among Japanese males: an epidemiological investigation. *Acta Med Okayama*. 2004; 58: 127-33.

243 Hsu JC, Zhang J, Dev A, Wing A, Bjeldanes LF, Firestone GL. Indole-3-carbinol inhibition of androgen receptor expression and downregulation of androgen responsiveness in human prostate cancer cells. *Carcinogenesis*. 2005; 26: 1896-904.

244 Rahman KW, Sarkar FH. Inhibition of nuclear translocation of nuclear factor-kB contributes to 3,3'-diindolylmethane-induced apoptosis in breast cancer cells. *Cancer Res*. 2005; 65: 364-71.

245 Brignall MS. Prevention and treatment of cancer with indole-3-carbinol. *Altern Med Rev*. 2001; 6: 580-89.

246 Xue L, Schaldach CM, Janosik T, Bergman J, Bjeldanes LF. Effects of analogs of indole-3-carbinol cyclic trimerization product in human breast cancer cells. *Chem Biol Interact*. 2005; 152: 119-29.

247 Bruce TJ, Pickett JA. Plant defence signalling induced by biotic attacks. *Curr Opin Plant Biol*. 2007; 10: 387-92.

248 Verhoeven DTH, Verhagen H, Goldbohm RA, vandenBrandt PA, vanPoppel G. A review of mechanisms underlying anticarcinogenicity by *Brassica* vegetables. *Chem Biol Interact*. 1997; 103: 79-129.

249 Byers T, Perry G. Dietary carotenes, vitamin C, and vitamin E as protective antioxidants in human cancers. *Annu Rev Nutr*. 1992; 12: 139-59.

250 Griffiths DW, Birch ANE, Hillman JR. Antinutritional compounds in the *Brassicaceae*: Analysis, biosynthesis, chemistry and dietary effects. *J Hortic Sci Biotechnol*. 1998; 73: 1-18.

251 Lotito SB, Frei B. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: Cause, consequence, or epiphenomenon? *Free Radic Biol Med*. 2006; 41: 1727-46.

252 Hayat S, Ali B, Hasan SA, Ahmad A. Brassinosteroid enhanced the level of antioxidants under cadmium stress in *Brassica juncea*. *Environ Exp Bot*. 2007; 60: 33-41.

253 Schonhof I, Klaring HP, Krumbein A, Claussen W, Schreiner M. Effect of temperature increase under low radiation conditions on phytochemicals and ascorbic acid in greenhouse grown broccoli. *Agric Ecosyst Environ*. 2007; 119: 103-11.

254 Eason JR, Ryan D, Page B, Watson L, Coupe SA. Harvested broccoli (*Brassica oleracea*) responds to high carbon dioxide and low oxygen atmosphere by inducing stress-response genes. *Postharvest Biol Technol*. 2007; 43: 358-65.

255 Gols R, Raaijmakers CE, van Dam NM, Dicke M, Bukovinszky T, Harvey JA. Temporal changes affect plant chemistry and tritrophic interactions. *Basic Appl Ecol*. 2007; 8: 421-33.

256 Bellostas N, Sorensen AD, Sorensen JC, Sorensen H. Genetic variation and metabolism of glucosinolates. *Adv Bot Res*. 2007; 45: 369 - 415.

257 Petersen IL, Hansen HCB, Ravn HW, Sorensen JC, Sorensen H. Metabolic effects in rapeseed (*Brassica napus* L.) seedlings after root exposure to glyphosate. *Pestic Biochem Physiol*. 2007; 89: 220-29.

258 Shomerilan A, Jones GP, Paleg LG. Invitro thermal and salt stability of pyruvate-kinase are increased by proline analogs and trigonelline. *Aust J Plant Physiol*. 1991; 18: 279-86.

259 Rathinasabapathi B. Metabolic engineering for stress tolerance: Installing osmoprotectant synthesis pathways. *Ann Bot*. 2000; 86: 709-16.

260 Qasim M, Ashraf M, Ashraf MY, Rehman SU, Rha ES. Salt-induced changes in two canola cultivars differing in salt tolerance. *Biol Plant*. 2003; 46: 629-32.

261 Martinez-Ballesta MC, Martinez V, Carvajal M. Osmotic adjustment, water relations and gas exchange in pepper plants grown under NaCl or KCl. *Environ Exp Bot*. 2004; 52: 161-74.

262 Mattiacci L, Rocca BA, Scascighini N, D'Alessandro M, Hern A, Dorn S. Systemically induced plant volatiles emitted at the time of "danger". *J Chem Ecol*. 2001; 27: 2233-52.

263 Pedras MSC, Zheng QA, Gadagi RS, Rimmer SR. Phytoalexins and polar metabolites from the oilseeds canola and rapeseed: Differential metabolic responses to the biotroph *Albugo candida* and to abiotic stress. *Phytochemistry*. 2008; 69: 894-910.

264 Chinnusamy V, Schumaker K, Zhu JK. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *J Exp Bot*. 2004; 55: 225-36.

265 Bouchereau A, ClossaisBesnard N, Bensaoud A, Leport L, Renard M. Water stress effects on rapeseed quality. *Eur J Agron*. 1996; 5: 19-30.

266 Van Poecke RMP, Posthumus MA, Dicke M. Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: Chemical, behavioral, and gene-expression analysis. *J Chem Ecol*. 2001; 27: 1911-28.

267 Dicke M, van Poecke RMP, de Boer JG. Inducible indirect defence of plants: from mechanisms to ecological functions. *Basic Appl Ecol*. 2003; 4: 27-42.

268 Cole RA. The relative importance of glucosinolates and amino acids to the development of two aphid pests *Brevicoryne brassicae* and *Myzus persicae* on wild and cultivated *Brassica species*. *Entomol Exp Appl*. 1997; 85: 121-33.

269 Mewis I, Appel HM, Hom A, Raina R, Schultz JC. Major signaling pathways modulate *Arabidopsis* glucosinolate accumulation and response to both phloem-feeding and chewing insects. *Plant Physiol*. 2005; 138: 1149-62.

270 Brandl MT. Fitness of human enteric pathogens on plants and implications for food safety. *Annu Rev Phytopathol*. 2006; 44: 367-92.

271 Good AG, Zaplachinski ST. The effects of drought stress on free amino-acid accumulation and protein-synthesis in *Brassica napus*. *Physiol Plant*. 1994; 90: 9-14.

272 Sasaki H, Ichimura K, Okada K, Oda M. Freezing tolerance and soluble sugar contents affected by water stress during cold-acclimation and de-acclimation in cabbage seedlings. *Sci Hortic*. 1998; 76: 161-69.

273 Singh S, Sinha S. Accumulation of metals and its effects in *Brassica juncea* (L.) Czern. (cv. Rohini) grown on various amendments of tannery waste. *Ecotoxicol Environ Saf*. 2005; 62: 118-27.

274 Zawoznik MS, Groppa MD, Tomaro ML, Benavides MP. Endogenous salicylic acid potentiates cadmium-induced oxidative stress in *Arabidopsis thaliana*. *Plant Sci*. 2007; 173: 190-97.

275 Xiong ZT, Liu C, Geng B. Phytotoxic effects of copper on nitrogen metabolism and plant growth in *Brassica pekinensis* Rupr. *Ecotoxicol Environ Saf*. 2006; 64: 273-80.

276 Seth CS, Chaturvedi PK, Misra V. The role of phytochelatins and antioxidants in tolerance to Cd accumulation in *Brassica juncea* L. *Ecotoxicol Environ Saf*. 2008; 71: 76-85.

277 Gebczynski P, Lisiewska Z. Comparison of the level of selected antioxidative compounds in frozen broccoli produced using traditional and modified methods. *Inn Food Sci Emerg Technol*. 2006; 7: 239-45.

278 Lisiewska Z, Kmiecik W, Korus A. The amino acid composition of kale (*Brassica oleracea* L. var. *acephala*), fresh and after culinary and technological processing. *Food Chem*. 2008; 108: 642-48.

279 Sikora E, Cieslik E, Leszczynska T, Filipiak-Florkiewicz A, Pisulewski PM. The antioxidant activity of selected cruciferous vegetables subjected to aquathermal processing. *Food Chem.* 2008; 107: 55-59.

280 Lemoine ML, Civello PM, Martinez GA, Chaves AR. Influence of postharvest UV-C treatment on refrigerated storage of minimally processed broccoli (*Brassica oleracea* var. *Italica*). *J Sci Food Agric*. 2007; 87: 1132-39.

281 Vallejo F, Garcia-Viguera C, Tomas-Barberan FA. Changes in broccoli (*Brassica oleracea* L. var. *italica*) health-promoting compounds with inflorescence development. *J Agric Food Chem.* 2003; 51: 3776-82.

282 Page T, Griffiths G, Buchanan-Wollaston V. Molecular and biochemical characterization of postharvest senescence in broccoli. *Plant Physiol*. 2001; 125: 718-27.

283 Hansen ME, Sorensen H, Cantwell M. Changes in acetaldehyde, ethanol and amino acid concentrations in broccoli florets during air and controlled atmosphere storage. *Postharvest Biol Technol*. 2001; 22: 227-37.

284 Chen S, Andreasson E. Update on glucosinolate metabolism and transport. *Plant Physiol Biochem*. 2001; 39: 743-58.

285 Halkier BA, Du LC. The biosynthesis of glucosinolates. *Trends Plant Sci*. 1997; 2: 425-31.

286 Padilla G, Cartea ME, Velasco P, de Haro A, Ordas A. Variation of glucosinolates in vegetable crops of *Brassica rapa*. *Phytochemistry*. 2007; 68: 536-45.

287 Martinez-Sanchez A, Allende A, Bennett RN, Ferreres F, Maria IG. Microbial, nutritional and sensory quality of rocket leaves as affected by different sanitizers. *Postharvest Biol Technol*. 2006; 42: 86–97.

288 Cieslik E, Leszczynska T, Filipiak-Florkiewicz A, Sikora E, Pisulewski PM. Effects of some technological processes on glucosinolate contents in cruciferous vegetables. *Food Chem*. 2007; 105: 976-81.

289 Song LJ, Thornalley PJ. Effect of storage, processing and cooking on glucosinolate content of *Brassica* vegetables. *Food Chem Toxicol*. 2007; 45: 216-24.

290 Xin-juan C, Zhu-jun Z, Xiao-lei N, Qong-qiu Q. Effect of nitrogen and sulfur supply on, glucosinolates in *Brassica campestris* ssp. *chinensis*. *Agr Sci China*. 2006; 8: 603–08.

291 van Poppel G, Verhoeven DTH, Verhagen H, Goldbohm RA. *Brassica* vegetables and cancer prevention - Epidemiology and mechanisms. In: Zappia VDFBARGLDR (ed.). Kluwer Academic / Plenum Publ: 1999; 159-68.

292 Doughty KJ, Porter AJR, Morton AM, Kiddle G, Bock CH, Wallsgrove R. Variation in the glucosinolate content of oilseed rape (*Brassica napus* L.) leaves II. Response to infection by *Alternaria brassicae* (berk) Sacc. *Ann Appl Biol*. 1991; 118: 469-77.

293 Burow M, Zhang ZY, Ober JA, Lambrix VM, Wittstock U, Gershenzon J, *et al*. ESP and ESM1 mediate indol-3-acetonitrile production from indol-3-ylmethyl glucosinolate in *Arabidopsis*. *Phytochemistry*. 2008; 69: 663-71.

294 Martin N, Muller C. Induction of plant responses by a sequestering insect: Relationship of glucosinolate concentration and myrosinase activity. *Basic Appl Ecol*. 2007; 8: 13-25.

295 Lopez-Berenguer C, Martinez-Ballesta MC, Garcia-Viguera C, Carvajal M. Leaf water balance mediated by aquaporins under salt stress and associated glucosinolate synthesis in broccoli. *Plant Sci*. 2008; 174: 321-28.

296 Kliebenstein DJ, Figuth A, Mitchell-Olds T. Genetic architecture of plastic methyl jasmonate responses in *Arabidopsis thaliana*. *Genetics*. 2002; 161: 1685-96.

297 Mikkelsen MD, Petersen BL, Glawischnig E, Jensen AB, Andreasson E, Halkier BA. Modulation of CYP79 genes and glucosinolate profiles in *Arabidopsis* by defense signaling pathways. *Plant Physiol*. 2003; 131: 298-308.

298 Bodnaryk RP. Potent effect of jasmonates on indole glucosinolates in oilseed rape and mustard. *Phytochemistry*. 1994; 35: 301-05.

299 Mithen R. Glucosinolates - biochemistry, genetics and biological activity. *Plant Growth Regul*. 2001; 34: 91-103.

300 Fenwick GR, Heaney RK, Mullin WJ. Glucosinolates and their breakdown products in food and food plants. *CRC Crit Rev Food Sci Nutr*. 1983; 18: 123-201.

301 Manici LM, Lazzeri L, Baruzzi G, Leoni O, Galletti S, Palmieri S. Suppressive activity of some glucosinolate enzyme degradation products on *Pythium irregulare* and *Rhizoctonia solani* in sterile soil. *Pest Manag Sci*. 2000; 56: 921-26.

302 Sisti M, Amagliani G, Brandi G. Antifungal activity of *Brassica oleracea* var. botrytis fresh aqueous juice. *Fitoterapia*. 2003; 74: 453-58.

303 Doughty KJ, Blight MM, Bock CH, Fieldsend JK, Pickett JA. Release of alkenyl isothiocyanates and other volatiles from *Brassica rapa* seedlings during infection by *Alternaria brassicae*. *Phytochemistry*. 1996; 43: 371-74.

304 Agrawal AA, Kurashige NS. A role for isothiocyanates in plant resistance against the specialist herbivore *Pieris rapae*. *J Chem Ecol*. 2003; 29: 1403-15.

305 Ludwig-Muller J, Schubert B, Pieper K, Ihmig S, Hilgenberg W. Glucosinolate content in susceptible and resistant Chinese cabbage varieties during development of clubroot disease. *Phytochemistry*. 1997; 44: 407-14.

306 van Dam NM, Raaijmakers CE. Local and systemic induced responses to cabbage root fly larvae (*Delia radicum*) in *Brassica nigra* and *B. oleracea*. *Chemoecology*. 2006; 16: 17-24.

307 Giamoustaris A, Mithen R. The effect of modifying the glucosinolate content of leaves of oilseed rape (*Brassica napus* Ssp. *Oleifera*) on its interaction with specialist and generalist pests. *Ann Appl Biol*. 1995; 126: 347-63.

308 Bodnaryk RP. Developmental profile of sinalbin (p-hydroxybenzyl glucosinolate) in mustard seedlings, *Sinapis alba* L, and its relationship to insect resistance. *J Chem Ecol*. 1991; 17: 1543-56.

309 Hopkins RJ, Ekbom B, Henkow L. Glucosinolate content and susceptibility for insect attack of three populations of *Sinapis alba*. *J Chem Ecol*. 1998; 24: 1203-16.

310 Bodnaryk RP. Effects of wounding on glucosinolates in the cotyledons of oilseed rape and mustard. *Phytochemistry*. 1992; 31: 2671-77.

311 Bartlet E, Kiddle G, Williams I, Wallsgrove R. Wound-induced increases in the glucosinolate content of oilseed rape and their effect on subsequent herbivory by a crucifer specialist. *Entomol Exp Appl*. 1999; 91: 163-67.

312 Mithen R, Clarke J, Lister C, Dean C. Genetics of aliphatic glucosinolates .III. side chain structure of aliphatic glucosinolates in *Arabidopsis thaliana*. *Heredity*. 1995; 74: 210-15.

313 Magrath R, Herron C, Giamoustaris A, Mithen R. The inheritance of aliphatic glucosinolates in *Brassica napus*. *Plant Breed*. 1993; 111: 55-72.

314 Giamoustaris A, Magrath R, Mithen R. Modifying the aliphatic glucosinolate content of oilseed rape and its effect upon pest and pathogen interactions. *Aspects Appl Biol*. 1994; 39: 125-32.

315 Spak J, Lewis J, Fenwick GR. Changes in the glucosinolate content of oilseed rape plants following infection with Turnip Mosaic-Virus. *Physiol Mol Plant Pathol*. 1993; 43: 437-42.

316 Menard R, Larue JP, Silue D, Thouvenot D. Glucosinolates in cauliflower as biochemical markers for resistance against downy mildew. *Phytochemistry*. 1999; 52: 29-35.

317 Moran PJ, Thompson GA. Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. *Plant Physiol*. 2001; 125: 1074-85.

318 Farwell AJ, Vesely S, Nero V, Rodriguez H, McCormack K, Shah S, *et al.* Tolerance of transgenic canola plants (*Brassica napus*) amended with plant growth-promoting bacteria to flooding stress at a metal-contaminated field site. *Environ Pollut*. 2007; 147: 540-45.

319 Leon-Kloosterziel KM, Verhagen BWM, Keurentjes JJB, VanPelt JA, Rep M, VanLoon LC, *et al.* Colonization of the *Arabidopsis rhizosphere* by fluorescent *Pseudomonas* spp. activates a root-specific, ethylene-responsive PR-5 gene in the vascular bundle. *Plant Mol Biol*. 2005; 57: 731-48.

320 Kiddle GA, Doughty KJ, Wallsgrove RM. Salicylic acid-Induced accumulation of glucosinolates in oilseed rape (*Brassica napus* L.) leaves. *J Exp Bot*. 1994; 45: 1343-46.

321 Doughty KJ, Kiddle GA, Pye BJ, Wallsgrove RM, Pickett JA. Selective induction of glucosinolates in oilseed rape leaves by methyl jasmonate. *Phytochemistry*. 1995; 38: 347-50.

322 Mollers C, Nehlin L, Glimelius K, Iqbal MCM. Influence of in vitro culture conditions on glucosinolate composition of microspore-derived embryos of *Brassica napus*. *Physiol Plant*. 1999; 107: 441-46.

323 Kaur S, Gupta SK, Sukhija PS, Munshi SK. Accumulation of glucosinolates in developing mustard (*Brassica juncea* L.) seeds in response to sulfur application. *Plant Sci*. 1990; 66: 181-84.

324 Kim SJ, Matsuo T, Watanabe M, Watanabe Y. Effect of nitrogen and sulphur application on the glucosinolate content in vegetable turnip rape (*Brassica rapa* L.). *Soil Sci Plant Nutr*. 2002; 48: 43-49.

325 Robbins RJ, Keck AS, Banuelos G, Finley JW. Cultivation conditions and selenium fertilization alter the phenolic profile, glucosinolate, and sulforaphane content of broccoli. *J Med Food*. 2005; 8: 204-14.

326 Volden J, Borge GIA, Bengtsson GB, Hansen M, Thygesen IE, Wicklund T. Effect of thermal treatment on glucosinolates and antioxidant-related parameters in red cabbage (*Brassica oleracea* L. ssp. *capitata* f. *rubra*). *Food Chem*. 2008; 109: 595-605.

327 Aksouh NM, Jacobs BC, Stoddard FL, Mailer RJ. Response of canola to different heat stresses. *Aust J Agric Res*. 2001; 52: 817-24.

328 Reifenrath K, Muller C. Species-specific and leaf-age dependent effects of ultraviolet radiation on two *Brassicaceae*. *Phytochemistry*. 2007; 68: 875-85.

329 McNaughton SA, Marks GC. Development of a food composition database for the estimation of dietary intakes of glucosinolates, the biologically active constituents of cruciferous vegetables. *Br J Nutr*. 2003; 90: 687-97.

330 Schone F, Kirchheim U, Schumann W. Glucosinolate degradation by rapeseed myrosinase and effect on rapeseed acceptability by growing pigs. *Anim Feed Sci Technol*. 1994; 48: 229-35.

331 Bones AM, Rossiter JT. The enzymic and chemically induced decomposition of glucosinolates. *Phytochemistry*. 2006; 67: 1053-67.

332 Force LE, O'Hare TJ, Wong LS, Irving DE. Impact of cold storage on glucosinolate levels in seed-sprouts of broccoli, rocket, white radish and kohl-rabi. *Postharvest Biol Technol*. 2007; 44: 175-78.

333 Winkler S, Faragher J, Franz P, Imsic M, Jones R. Glucoraphanin and flavonoid levels remain stable during simulated transport and marketing of broccoli (*Brassica oleracea* var. *italica*) heads. *Postharvest Biol Technol*. 2007; 43: 89-94.

334 Hansen M, Moller P, Sorensen H, de Trejo MC. Glucosinolates in broccoli stored under controlled-atmosphere. *J Am Soc Hortic Sci*. 1995; 120: 1069-74.

335 Pedras MSC, Jha M, Ahiahonu PWK. The synthesis and biosynthesis of phytoalexins produced by cruciferous plants. *Curr Org Chem*. 2003; 7: 1635-47.

336 Pedras ASC, Jha M, Minic Z, Okeola OG. Isosteric probes provide structural requirements essential for detoxification of the phytoalexin brassinin by the fungal pathogen *Leptosphaeria maculans*. *Bioorg Med Chem*. 2007; 15: 6054-61.

337 Pedras MSC, Ahiahonu PWK. Metabolism and detoxification of phytoalexins and analogs by phytopathogenic fungi. *Phytochemistry*. 2005; 66: 391-411.

338 Soylu S. Accumulation of cell-wall bound phenolic compounds and phytoalexin in *Arabidopsis thaliana* leaves following inoculation with pathovars of *Pseudomonas syringae*. *Plant Sci*. 2006; 170: 942-52.

339 Conn KL, Tewari JP, Dahiya JS. Resistance to *Alternaria brassicae* and phytoalexin elicitation in rapeseed and other crucifers. *Plant Sci*. 1988; 56: 21-25.

340 Zook M, Leege L, Jacobson D, Hammerschmidt R. Camalexin accumulation in *Arabis lyrata*. *Phytochemistry*. 1998; 49: 2287-89.

341 Pedras MSC, Chumala PB, Suchy M. Phytoalexins from *Thlaspi arvense*, a wild crucifer resistant to virulent *Leptosphaeria maculans*: structures, syntheses and antifungal activity. *Phytochemistry*. 2003; 64: 949-56.

342 Dahiya JS, Rimmer SR. Phytoalexin accumulation in tissues of *Brassica napus* inoculated with *Leptosphaeria maculans*. *Phytochemistry*. 1988; 27: 3105-07.

343 Pedras MSC, Smith KC. Sinalexin, a phytoalexin from white mustard elicited by destruxin B and *Alternaria brassicae*. *Phytochemistry*. 1997; 46: 833-37.

344 Pedras MSC, Ahiahonu PWK. Phytotoxin production and phytoalexin elicitation by the phytopathogenic fungus *Sclerotinia sclerotiorum*. *J Chem Ecol*. 2004; 30: 2163-79.

345 Pedras MSC, Montaut S, Suchy M. Phytoalexins from the crucifer rutabaga: Structures, syntheses, biosyntheses, and antifungal activity. *J Org Chem*. 2004; 69: 4471-76.

346 Pedras MSC, Sarwar MG, Suchy M, Adio AM. The phytoalexins from cauliflower, caulilexins A, B and C: Isolation, structure determination, syntheses and antifungal activity. *Phytochemistry*. 2006; 67: 1503-09.

347 Van-Etten HD, Sandrock RW, Wasmann CC, Soby SD, McCluskey K, Wang P. Detoxification of phytoanticipins and phytoalexins by phytopathogenic fungi. *Nat Res Council Ca*: 1995; S518-S25.

348 Rouxel T, Kollmann A, Boulidard L, Mithen R. Abiotic elicitation of indole phytoalexins and resistance to *Leptosphaeria maculans* within *Brassicae*. *Planta*. 1991; 184: 271-78.

349 Kusznierewicz B, Smiechowska A, Bartoszek A, Namiesnik J. The effect of heating and fermenting on antioxidant properties of white cabbage. *Food Chem*. 2008; 108: 853-61.

350 Sousa C, Pereira DM, Pereira JA, Bento A, Rodrigues MA, Dopico-Garcia S, *et al.* Multivariate analysis of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) phenolics: Influence of fertilizers. *J Agric Food Chem*. 2008; 56: 2231-39.

351 Grace SC, Logan BA. Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. *Phil Trans Royal Soc Lon Ser B-Biol Sci*. 2000; 355: 1499-510.

352 Vereecke D, Messens E, Klarskov K, DeBruyn A, VanMontagu M, Goethals K. Patterns of phenolic compounds in leafy galls of tobacco. *Planta*. 1997; 201: 342-48.

353 Miles PW. Aphid saliva. *Biol Rev*. 1999; 74: 41-85.

354 Liang YS, Kim HK, Lefeber AWM, Erkelens C, Choi YH, Verpoorte R. Identification of phenylpropanoids in methyl jasmonate treated *Brassica rapa* leaves using two-dimensional nuclear magnetic resonance spectroscopy. *J Chromatogr A*. 2006; 1112: 148-55.

355 Mobin M, Khan NA. Photosynthetic activity, pigment composition and antioxidative response of two mustard (*Brassica juncea*) cultivars differing in photosynthetic capacity subjected to cadmium stress. *J Plant Physiol*. 2007; 164: 601-10.

356 Gidda SK, Varin L. Biochemical and molecular characterization of flavonoid 7-sulfotransferase from *Arabidopsis thaliana*. *Plant Physiol Biochem*. 2006; 44: 628-36.

357 Vallejo F, Tomas-Barberan FA, Garcia-Viguera C. Effect of climatic and sulphur fertilisation conditions, on phenolic compounds and vitamin C, in the inflorescences of eight broccoli cultivars. *Eur Food Res Technol*. 2003; 216: 395-401.

358 Gitz DC, Liu L, McClure JW. Phenolic metabolism, growth, and UV-B tolerance in phenylalanine ammonia-lyase-inhibited red cabbage seedlings. *Phytochemistry*. 1998; 49: 377-86.

359 Olsson LC, Veit M, Weissenbock G, Bornman JF. Differential flavonoid response to enhanced UV-B radiation in *Brassica napus*. *Phytochemistry*. 1998; 49: 1021-28.

360 Wilson KE, Thompson JE, Huner NPA, Greenberg BM. Effects of ultraviolet-A exposure on ultraviolet-B-induced accumulation of specific flavonoids in *Brassica napus*. *Photochem Photobiol*. 2001; 73: 678-84.

361 Lo Scalzo R, Genna A, Branca F, Chedin M, Chassaigne H. Anthocyanin composition of cauliflower (*Brassica oleracea* L. var. botrytis) and cabbage (*B. oleracea* L. var. capitata) and its stability in relation to thermal treatments. *Food Chemistry*. 2008; 107: 136-44.

362 Krishna P. Brassinosteroid-mediated stress responses. *J Plant Growth Regul*. 2003; 22: 289-97.

363 Kagale S, Divi UK, Krochko JE, Keller WA, Krishna P. Brassinosteroid confers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. *Planta*. 2007; 225: 353-64.

364 Janeczko A, Koscielniak J, Pilipowicz M, Szarek-Lukaszewska G, Skoczowski A. Protection of winter rape photosystem 2 by 24-epibrassinolide under cadmium stress. *Photosynthetica*. 2005; 43: 293-98.

365 Fujioka S, Yokota T. Biosynthesis and metabolism of brassinosteroids. *Annu Rev Plant Biol*. 2003; 54: 137-64.

366 Dhaubhadel S, Chaudhary S, Dobinson KF, Krishna P. Treatment with 24-epibrassinolide, a brassinosteroid, increases the basic thermotolerance of *Brassica napus* and tomato seedlings. *Plant Mol Biol*. 1999; 40: 333-42.

367 Rouleau M, Marsolais F, Richard M, Nicolle L, Voigt B, Adam G, *et al.* Inactivation of brassinosteroid biological activity by a salicylate-inducible steroid sulfotransferase from *Brassica napus*. *J Biol Chem*. 1999; 274: 20925-30.

368 Anuradha S, Rao SSR. The effect of brassinosteroids on radish (*Raphanus sativus* L.) seedlings growing under cadmium stress. *Plant Soil Environ*. 2007; 53: 465-72.

369 Zhao-liang L, Li-wang L, Xiao-yan L, Yi-qin G, Xi-lin H, Xian-wen Z, *et al.* Analysis and evaluation of nutritional quality in chinese radish (*Raphanus sativus* L.). *Agri Sci China*. 2008; 7: 823-30.

370 Klinger T, Elam DR, Ellstrand NC. Radish as a model system for the study of engineered gene escape rates via crop-weed mating. *Conserv Biol*. 1991; 5: 531-35.

371 Jahangir M, Kim HK, Choi YH, Verpoorte R. Health-affecting compounds in *Brassicaceae*. *Comp Rev Food Sci Food Saf*. 2009; 8: 31-43.

372 van Dam NM, Raaijmakers CE, van der Putten WH. Root herbivory reduces growth and survival of the shoot feeding specialist *Pieris rapae* on *Brassica nigra*. *Entomol Exp Appl*. 2005; 115: 161-70.

373 Helsper JPFG, de Vos CHR, Maas FM, Jonker HH, van den Broeck HC, Jordi W, *et al.* Response of selected antioxidants and pigments in tissues of *Rosa hybrida* and *Fuchsia hybrida* to supplemental UV-A exposure. *Physiol Plant*. 2003; 117: 171-78.

374 Bino RJ, de Vos CHR, Lieberman M, Hall RD, Bovy A, Jonker HH, *et al.* The light-hyperresponsive high pigment-2^{dg} mutation of tomato: alterations in the fruit metabolome. *New Phytol*. 2005; 166: 427-38.

375 Thomas H, Stoddart JL. Leaf senescence. *Annu Rev Plant Phys*. 1980; 31: 83-111.

376 Iwasa Y, Kubo T, van Dam N, de Jong TJ. Optimal level of chemical defense decreasing with leaf age. *Theor Popul Biol*. 1996; 50: 124-48.

377 Bloom AJ, Chapin FS, Mooney HA. Resource limitation in plants - An economic analogy. *Annu Rev Ecol Syst*. 1985; 16: 363-92.

378 Beuchat LR. Vectors and conditions for preharvest contamination of fruits and vegetables with pathogens capable of causing enteric diseases. *Br Food J*. 2006; 108: 38-53.

379 Ingham SC, Losinski JA, Andrews MP, Breuer JE, Breuer JR, Wood TM, *et al.* *Escherichia coli* contamination of vegetables grown in soils fertilized with noncomposted bovine manure: Garden-scale studies. *Appl Environ Microbiol*. 2004; 70: 6420-27.

380 Chui VWD, Lam-Leung SY, Cheung M, Wu VKC. The use of sewage sludge as basal dressing for vegetable cultivation. *Environ Int*. 1992; 18: 201-09.

381 Anderson GL, Kenney SJ, Millner PD, Beuchat LR, Williams PL. Shedding of foodborne pathogens by *Caenorhabditis elegans* in compost-amended and unamended soil. *Food Microbiol*. 2006; 23: 146-53.

382 Duarte IF, Delgadillo I, Gil AM. Study of natural mango juice spoilage and microbial contamination with *Penicillium expansum* by high resolution ^{1}H NMR spectroscopy. *Food Chem*. 2006; 96: 313-24.

383 Dangle JL, Jones JDG. Plant pathogens and integrated defense responses to infection. *Nature*. 2001; 411: 826-33.

384 Buck JW, Walcott RR, Beuchat LR. Recent trends in microbiological safety of fruits and vegetables. *Online: Plant Health Progress doi: 101094/PHP-2003-0121-01-RV*. 2003.

385 Hirano SS, Upper CD. Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* - a pathogen, ice nucleus, and epiphyte. *Microbiol Mol Biol Rev*. 2000; 64: 624-53.

386 Wachtel MR, Whitehand LC, Mandrell RE. Prevalence of *Escherichia coli* associated with a cabbage crop inadvertently irrigated with partially treated sewage wastewater. *J Food Prot*. 2002; 65: 471-75.

387 Banerjee M, Sarkar PK. Microbiological quality of some retail spices in India. *Food Res Int*. 2003; 36: 469-74.

388 Choi YH, Kim HK, Hazekamp A, Erkelens C, Lefeber AWM, Verpoorte R. Metabolomic differentiation of *Cannabis sativa* cultivars using ^{1}H NMR spectroscopy and principal component analysis. *J Nat Prod*. 2004; 67: 953-57.

389 Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*. 1962; 15: 473-97.

390 Ryu C-M, Farag MA, Hu C-H, Reddy MS, Kloepper JW, Pare PW. Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiol*. 2004; 134: 1017-26.

391 Mehdy MC. Active oxygen species in plant defense against pathogens. *Plant Physiol*. 1994; 105: 467-72.

392 Vaquero MJR, Alberto MR, de Nadra MCM. Antibacterial effect of phenolic compounds from different wines. *Food Control*. 2007; 18: 93-101.

393 Williamson G, Day AJ, Plumb GW, Couteau D. Human metabolic pathways of dietary flavonoids and cinnamates. *Biochem Soc Trans*. 2000; 28: 16-22.

394 Boquet P, Lemichez E. Bacterial virulence factors targeting Rho GTPases: Parasitism or symbiosis? *Trends Cell Biol*. 2003; 13: 238-46.

395 Hodges CF, Robinson PW. Sugar and amino acid content of *Poa pratensis* infected with *Ustilago striiformis* and *Urocystis agropyri*. *Physiol Plant*. 1977; 41: 25-28.

396 Beuve N, Rispail N, Laine P, Cliquet J-B, Ourry A, Le Deunff E. Putative role of γ-aminobutyric acid (GABA) as a long-distance signal in up-regulation of nitrate uptake in *Brassica napus* L. . *Plant Cell Environ*. 2004; 27: 1035-46.

397 Mahuku GS, Hall R, Goodwin PH. Co-infection and induction of systemic acquired resistance by weakly and highly virulent isolates of *Leptosphaeria maculans* in oilseed rape. *Physiol Mol Plant Pathol*. 1996; 49: 61-72.

398 Lee M-K, Kim H-S, Kim J-S, Kim S-H, Park Y-D. Agrobacterium-mediated transformation system for large-scale producion of transgenic chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) plants for insertional mutagenesis. *J Plant Biol*. 2004; 47: 300-06.

399 Widarto HT, Van Der Meijden E, Lefeber AWM, Erkelens C, Kim HK, Choi YH, *et al.* Metabolomic differentiation of *Brassica rapa* following herbivory

by different insect instars using two-dimensional nuclear magnetic resonance spectroscopy. *J Chem Ecol*. 2006; 32: 2417-28.

400 Bellostas N, Kachlicki P, Sorensen JC, Sorensen H. Glucosinolate profiling of seeds and sprouts of *B. oleracea* varieties used for food. *Sci Hortic*. 2007; 114: 234-42.

401 Grispen VMJ, Nelissen HJM, Verkleij JAC. Phytoextraction with *Brassica napus* L.: A tool for sustainable management of heavy metal contaminated soils. *Environ Pollut*. 2006; 144: 77-83.

402 Galan E, Gomez-Ariza JL, Gonzalez I, Fernandez-Caliani JC, Morales E, Giraldez I. Heavy metal partitioning in river sediments severely polluted by acid mine drainage in the Iberian Pyrite Belt. *Appl Geochem*. 2003; 18: 409-21.

403 Gisbert C, Clemente R, Navarro-Avino J, Baixauli C, Giner A, Serrano R, *et al*. Tolerance and accumulation of heavy metals by *Brassicaceae* species grown in contaminated soils from Mediterranean regions of Spain. *Environ Exp Bot*. 2006; 56: 19-27.

404 Van Engelen DL, Sharpe-Pedler RC, Moorhead KK. Effect of chelating agents and solubility of cadmium complexes on uptake from soil by *Brassica juncea*. *Chemosphere*. 2007; 68: 401-08.

405 DeGara L, dePinto M, Locato V. Changes in the ascorbate metabolism during programmed cell death and moderate oxidative stress in TBY-2 cells. *Comp Biochem Physiol Part A Mol Integr Physiol*. 2007; 146: S261-S61.

406 Cobbett CS. Phytochelatins and their roles in heavy metal detoxification. *Plant Physiol*. 2000; 123: 825-32.

407 Narula A, Kumar S, Srivastava PS. Abiotic metal stress enhances diosgenin yield in *Dioscorea bulbifera* L. *cultures*. *Plant Cell Rep*. 2005; 24: 250-54.

408 Mithofer A, Schulze B, Boland W. Biotic and heavy metal stress response in plants: Evidence for common signals. *FEBS Lett*. 2004; 566: 1-5.

409 Psotova J, Lasovsky J, Vicar J. Metal chelating properties, electrochemical behaviour, scavenging and cytoprotective activities of six natural phenolics. *Biomedical Paper*. 2003; 147: 147-53.

410 Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, *et al.* TM4: A free, open-source system for microarray data management and analysis. *Biotechniques*. 2003; 34: 374-78.

411 Guo TR, Zhang GP, Zhang YH. Physiological changes in barley plants under combined toxicity of aluminum, copper and cadmium. *Colloid Surface B*. 2007; 57: 182-88.

412 Memon AR, Aktoprakligul D, Zdemur A, Vertii A. Heavy metal accumulation and detoxification mechanisms in plants. *Turk J Bot*. 2001; 25: 111-21.

413 Sablani SS. Drying of fruits and vegetables: Retention of nutritional/functional quality. *Drying Technol*. 2006; 24: 123-35.

414 Bahceci KS, Serpen A, Gokmen V, Acar J. Study of lipoxygenase and peroxidase as indicator enzymes in green beans: change of enzyme activity, ascorbic acid and chlorophylls during frozen storage. *J Food Eng*. 2005; 66: 187-92.

415 Toor RK, Savage GP. Changes in major antioxidant components of tomatoes during post-harvest storage. *Food Chem*. 2006; 99: 724-27.

416 Asami DK, Hong YJ, Barrett DM, Mitchell AE. Processing-induced changes in total phenolics and procyanidins in clingstone peaches. *J Sci Food Agric*. 2003; 83: 56-63.

417 del Aguila JS, Sasaki FF, Heiffig LS, Ortega EMM, Jacomino AP, Kluge RA. Fresh-cut radish using different cut types and storage temperatures. *Postharvest Biol Technol*. 2006; 40: 149-54.

418 Mattana M, Biazzi E, Consonni R, Locatelli F, Vannini C, Provera S, *et al.* Overexpression of Osmyb4 enhances compatible solute accumulation and increases stress tolerance of *Arabidopsis thaliana*. *Physiol Plant*. 2005; 125: 212-23.

419 Ciska E, Honke J, Kozlowska H. Effect of light conditions on the contents of glucosinolates in germinating seeds of white mustard, red radish, white radish, and rapeseed. *J Agric Food Chem*. 2008; 56: 9087-93.

420 Kujala TS, Loponen JM, Klika KD, Pihlaja K. Phenolics and betacyanins in red beetroot (*Beta vulgaris*) root: Distribution and effect of cold storage on the content of total phenolics and three individual compounds. *J Agric Food Chem*. 2000; 48: 5338-42.

421 Chattopadhyay SB, Sahu PK, Thapa U, Niranjan KM. Effect of microbial amendments on root and seed yield of radish. *J Vegetable Sci* 2007; 12: 89-104

422 Martínez-Villaluenga C, Frias J, Gulewicz P, Gulewicz K, Vidal-Valverde C. Food safety evaluation of broccoli and radish sprouts. *Food Chem Toxicol*. 2008; 46: 1635-44.

423 Kostkarick R, Manning WJ. Radish (*Rapahnus sativus* L) - A model for studying plant responses to air pollutants and other environmental stresses. *Environ Pollut*. 1993; 82: 107-38.

424 Hegde SG, Nason JD, Clegg JM, Ellstrand NC. The evolution of California's wild radish has resulted in the extinction of its progenitors. *Evolution*. 2006; 60: 1187-97.

425 Keppler K, Humpf HU. Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora. *Bioorg Med Chem*. 2005; 13: 5195-205.

426 Vassilakis C, Pantidou A, Psillakis E, Kalogerakis N, Mantzavinos D. Sonolysis of natural phenolic compounds in aqueous solutions: Degradation pathways and biodegradability. *Water Res*. 2004; 38: 3110-18.

427 Buskov S, Hansen LB, Olsen CE, Sorensen JC, Sorensen H, Sorensen S. Determination of ascorbigens in autolysates of various *Brassica* species using supercritical fluid chromatography. *J Agric Food Chem*. 2000; 48: 2693-701.

428 Hrncirik K, Valusek J, Velisek J. Investigation of ascorbigen as a breakdown product of glucobrassicin autolysis in *Brassica* vegetables. *Eur Food Res Technol*. 2001; 212: 576-81.

429 Fiehn O, Kopka J, Dormann P, Altmann T, Trethewey RN, Willmitzer aL. Metabolite profiling for plant funtional genomics. *Nature Biotech*. 2000; 18: 1157-61.

430 Ian Kaplan RH, Andre Kessler, Brian J. Rehill, Sandra Sardanelli, Robert F. Denno,. Physiological integration of roots and shoots in plant defense strategies links above- and belowground herbivory. *Ecology Letters*. 2008; 11: 841-51.

431 Kim SJ, Ishii G. Effect of storage temperature and duration on glucosinolate, total vitamin C and nitrate contents in rocket salad (*Eruca sativa* Mill.). *J Sci Food Agric*. 2007; 87: 966-73.

432 Galindo FG, Herppich W, Gekas V, Sjoholm I. Factors affecting quality and postharvest properties of vegetables: Integration of water relations and metabolism. *Crit Rev Food Sci Nutr*. 2004; 44: 139 - 54.

433 Danyluk J, Perron A, Houde M, Limin A, Fowler B, Benhamou N, *et al.* Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. *Plant Cell*. 1998; 10: 623-38.

434 Bednarek P, Schneider B, Svatos A, Oldham NJ, Hahlbrock K. Structural complexity, differential response to infection, and tissue specificity of indolic and phenylpropanoid secondary metabolism in *Arabidopsis* roots. *Plant Physiol*. 2005; 138: 1058-70.

435 Rhee IK, van de Meent M, Ingkaninan K, Verpoorte R. Screening for acetylcholinesterase inhibitors from *Amaryllidaceae* using silica gel thin-layer chromatography in combination with bioactivity staining. *J Chromatogr A*. 2001; 915: 217-23.

436 Rhee IK, van Rijn RM, Verpoorte R. Qualitative determination of false-positive effects in the acetylcholinesterase assay using thin layer chromatography. *Phytochem Anal*. 2003; 14: 127-31.

437 Ingkaninan K, de Best CM, van der Heijden R, Hofte AJP, Karabatak B, Irth H, *et al.* High-performance liquid chromatography with on-line coupled UV, mass spectrometric and biochemical detection for identification of acetylcholinesterase inhibitors from natural products. *J Chromatogr A*. 2000; 872: 61-73.

438 In Kyung Rhee NA, Teus Luijendijk, Hubertus Irth, Robert Verpoorte,. Determining acetylcholinesterase inhibitory activity in plant extracts using a fluorimetric flow assay. *Phytochem. Anal.* 2003; 14: 145-49.

439 Yuliana ND, Khatib A, Link-Struensee AMR, IJzerman AP, Choi YH, Verpoorte R. Adenosine A1 receptor binding activity of methoxy flavonoids from *Orthosiphon stamineus*. *Planta Med*. 2009; 75: 132-6.

440 Horswill JG, Bali U, Shaaban S, Keily JF, Jeevaratnam P, Babbs AJ, *et al.* PSNCBAM-1, a novel allosteric antagonist at cannabinoid CB_1 receptors with hypophagic effects in rats. *Br J Pharmacol*. 2007; 152: 805-14.

441 Devienne KF, Raddi MSG. Screening for antimicrobial activity of natural products using a microplate photometer. *Braz J Microbiol*. 2002; 33: 166-68.

442 Mukherjee PK, Kumar V, Mal M, Houghton PJ. Acetylcholinesterase inhibitors from plants. *Phytomedicine*. 2007; 14: 289-300.

443 Rhee IK, Appels N, Luijendijk T, Irth H, Verpoorte R. Determining acetylcholinesterase inhibitory activity in plant extracts using a fluorimetric flow assay. *Phytochem Anal*. 2003; 14: 145-49.

444 Ingkaninan K, von Frijtag Drabbe Kunzel JK, Ijzerman AP, Verpoorte R. Interference of linoleic acid fraction in some receptor binding assays. *J Nat Prod*. 1999; 62: 912-14.

445 Tikunov Y, Lommen A, de Vos CHR, Verhoeven HA, Bino RJ, Hall RD, *et al.* A novel approach for nontargeted data analysis for metabolomics. Large-scale profiling of tomato fruit volatiles. *Plant Physiol*. 2005; 139: 1125-37.

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Curriculum Vitae

Muhammad Jahangir was born in 1979 in Haripur (Hazara), Pakistan. He obtained his high school education from Jinnah Jam-e High School in 1995, and completed higher secondary education from Telecom Boy's Public School and College, Haripur, in 1997. For further studies he got a merit scholarship from the provincial government of N.W.F.P. for a BSc Agriculture study at the University of Arid Agriculture, Rawalpindi. For his BSc research project, he studied the "Effect of stabilizers on tomato concentrate". After graduation in 2001, he got admission in the N.W.F.P. Agricultural University, Peshawar, in the MSc Food Science and Technology program, which he finished in 2003. His MSc project concerned "The development and storage study of low caloric mango squash".

He started his career with a job as executive trainee in Qarshi Industries, one of the leading industries in Pakistan for herbal medicines and food products. After six months working with Qarshi, he moved to a food processing industry, Salman Corporation, Islamabad and worked there as "Food Technologist Product Development" for almost a year. He decided to continue his academic career for PhD study in the department of Pharmacognosy and Metabolomics, Institute of Biology, Leiden University, for which he received a scholarship from the Higher Education Commission (HEC) of Pakistan in 2007. He joined the *Brassica* project and worked on the "Stress response and health affecting compounds in Brassicaceae".

Apart from attending international, national and internal trainings, conferences, and seminars, until now he has three poster publications, and eight journal publications (articles and reviews).

List of publications

1. Jahangir M., Abdel-Farid I. B., Jonker H. H., Vos C. H. de., Choi Y. H., Verpoorte R. Metabolomic profiling of *Brassica rapa* and *Raphanus sativus* on different growth stages. ***In process***.

2. Jahangir M., Abdel-Farid I. B., Choi Y. H., Verpoorte R. Effect of postharvest storage time and temperature on red radish (*Raphanus sativus*). ***In process***.

3. Jahangir M., Abdel-Farid I. B., Kim H. K., Choi Y. H., Verpoorte R. 2009. Healthy and unhealthy plants: The effect of stress on the metabolism of Brassicaceae. Environ. Exp. Bot. 67 (1): 23 – 33.
4. Jahangir M., Kim H. K., Choi Y. H., Verpoorte R. 2009. Health-affecting compounds in *Brassicaceae*. Comp. Rev. Food Sci. Food Saf. 8: 31–43.

5. Jahangir M., Abdel-Farid I. B., Choi Y. H., Verpoorte R. 2008. Metal ion-inducing metabolite accumulation in *Brassica rapa*. J Plant Physiol. 165 (14): 1429–1437.

6. Jahangir M., Kim H. K., Choi Y. H., Verpoorte R. 2008. Metabolomic response of *Brassica rapa* submitted to pre-harvest bacterial contamination. Food Chem. 107 (1): 362–368.

7. Abdel-Farid I. B., Jahangir M., van den Hondel C. A. M. J. J., Kim H.K., Choi Y.H., Verpoorte R. 2009. Fungal infection-induced metabolites in *Brassica rapa*. Plant Sci. 176 (5): 608 – 615.