Metabolomic changes of Brassica rapa under biotic stress

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Chapter 3

Metabolic characterization of *Brassica rapa* cultivars leaves by NMR spectroscopy

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Abstract

The *Brassica* genus has been intensively studied due to the nutritional properties and beneficial effects on health of its members. However, a wide range of species, varieties, and cultivars included in this genus and the resulting large metabolic variation has been an obstacle for systematic research of the plants of this genus. In order to get insight in the problems posed by the biological variation, the metabolomic analysis of various cultivars of *Brassica rapa* was performed by NMR spectroscopy combined with multivariate data analysis. Discriminating metabolites in different cultivars and development stages were elucidated by diverse 2D-NMR techniques after sorting out different significant signals using ^{1}H-NMR measurements and principal component analysis. Among the elucidated metabolites, several organic and amino acids, carbohydrates, adenine, indole acetic acid (IAA), phenylpropanoids, flavonoids, and glucosinolates were found to be the metabolites contributing to the differentiation between cultivars and age of *Brassica rapa*. Based on these results the distribution of plant metabolites among different cultivars and development stages of *B. rapa* is discussed.

Keywords: Metabolomic analysis, NMR, principal component analysis, *Brassica rapa*, cultivars, biological variation

3.1 Introduction

Brassica species constitute an important source of vegetable oils (Sasaki and Takahashi, 2002; Abramson and Smith, 2003) and proteins for humans (Font *et al.,* 2005), and animal nutrition (Slominski *et al.,* 1999; Font *et al.,* 2005). Increased popularity of *Brassica* vegetables has resulted in a considerable increase in its production in Asia, Europe and the USA not only as a vegetable but also as an industrial source for seed oils and biofuel (Li, 1962; Tan, 1979; Perry and Harwood, 1993). In addition to the commercial use of *Brassica*, its high level of genetic resemblance to *Arabidopsis* has made it an alternative model system in the field of plant physiology. Because it also occurs in the wild, it is considered an important plant model to study the interaction between plant and other organisms such as insects, fungi, or bacteria (Mahuku *et al.,* 1996; Lee *et al.,* 2004; Widarto *et al.,* 2006). In terms of metabolic interaction some phenylpropanoids, flavonoids and glucosinolates have been assumed to play a role in defense of *Brassica* against other organisms (Ludwig-Müller *et al.,* 1997; Parvez *et al.,* 2004; Liang *et al.,* 2006b).

Although the *Brassica* genus has been intensively studied due to its nutritional and health benefits, the existence of numerous species, varieties, and cultivars results in a large metabolic variation which has constituted an obstacle for the systematic research of these plants. For instance, one of the major *Brassica* metabolite groups, the glucosinolates, shows a large variation among species, up to the point that there are many species-specific glucosinolates (Fahey *et al.,* 2001). In the case of flavonoids, *Brassica* leaves have been reported to accumulate flavonols (quercetin, kaempferol, and isorhamnetin) and flavones (apigenin and luteolin) (Onyilagha *et al.,* 2003). Other species, such as *B. alba,* have been found to contain three flavonoids isolated from shoots, roots and root exudates and identified as 3,5,6,7,8-pentahydroxy-4′-methoxy flavone, 2′,3′,4′,5′,6′-pentahydroxy chalcone, and 3,5,6,7,8-pentahydroxy flavone together with apigenin from the shoots and roots (Ponce *et al.,* 2004). However, isorhamnetin-3,7-*O*-di-β-D-glucopyranoside was isolated only from the corolla of *B. rapa* flowers (Sasaki and Takahashi, 2002). Recently, isorhamnetin, kaempferol, and quercetin glycosides were also identified in turnip tops of *Brassica rapa* (Romani *et al.,* 2006). Another important group of phenolic *Brassica* compounds, phenylpropanoids, shows a different metabolic profile depending on the species and tissues studied (Nilsson *et al*., 2006).

In a recent study, a new phenylpropanoid, malate conjugated 5-hydroxy ferulic acid was identified in *Brassica rapa* leaves (Liang *et al.,* 2006a). In addition to the observed variation in the level of phenylpropanoids in different species, a variation in the types of tissue in a plant was also observed, e.g. a predominance of malate derivatives in leaves but choline forms in seeds (Stout and Chapple, 2004). Furthermore changes due to the attack of insects were reported (Widarto *et al.,* 2006).

In recent years, metabolomics has become prominent as a part of systems biology with the expectation that functional genomics will uncover unknown gene functions. Moreover, metabolomics is of interest in chemical classification of plants for chemotaxonomy, and for following perturbations of living organisms by external factors. The term metabolome has been used to describe the observable chemical profile or fingerprint of the metabolites present in whole cells, tissues or whole organism (Ott *et al.,* 2003; Verpoorte *et al*., 2008). Any significant change brought about by conditions that affect living organisms are expected to show up in the profile of its metabolome. However, measuring the metabolome as an indicator for the biological interactions poses some problems, particularly the large natural biological variation in a species, inability to comprehensively profile all the metabolome due to their chemical complexity, also metabolomic profiling in crude extracts is not an easy task to perform since natural products display a wide range of structural diversity, and plant contain a large number of compounds with very different times of synthesis and quantities (Sumner *et al*., 2003; Liang *et al*., 2006b). A metabolomics study can provide significant results if the metabolic changes in the target group exceed the biological variation of the control group. The metabolome is dynamic. Some metabolites have both spatial and temporal variation in plant species and even under a given experimental condition, i.e., in a one or two week period, the metabolome of a plant can completely change. For example, it has been reported that the glucosinolate content in *Brassica* is dependent on several factors such as cultivar, age, seasonal and environmental factors (temperature, soil type, and fertilizer application). Glucosinolates may even differ in quantity within the same organ during the day (Booth *et al.,* 1991; Rosa *et al.,* 1996; Rosa, 1997b; Kushad *et al.,* 1999; Brown *et al.,* 2003; Nilsson *et al.,* 2006; Zhang *et al*., 2008; Blaževic and Mastelić, 2009).

Indeed there are so many factors that affect the metabolome that, particularly in the case of *Brassica* metabolomics, there is an urgent need to investigate the influence of the most important factors such as species, varieties, cultivars, growing conditions, and age on the metabolic differentiation.

In this study, NMR spectroscopy coupled with multivariate data analysis was applied to *Brassica* metabolomics in order to investigate the metabolic differentiation of various cultivars and ages of *Brassica rapa* leaves. Four and six week-old plants of four cultivars of *B. rapa* (Raapstelen, Witte Mei, Herfstraap and Oleifera) were examined for their metabolomic discrimination.

3.2 Materials and methods

3.2.1 Plant materials

Different *Brassica rapa* cultivars were submitted to uniform culture conditions. Seeds from registered cultivars including Raapstelen (Groene Gewone), Witte Mei (Witte Mei), Herfstraap (Goldana) and Oleifera were germinated in soil in the cold room (4 ^{o}C) for two days. The pots were placed in a box, provided with sufficient water and covered. These were then transferred to the greenhouse and kept at 25 ºC, 50-60% relative humidity with 16 h of daylight and 8 h of darkness per day. Seven-day old seedlings were transferred to 10 cm-diameter pots with substrate and placed in the same greenhouse and watered daily. Three individual plants were used as replicates from each cultivar. Four upper leaves from each plant were harvested four and six weeks after germination at around 13.00 h and plunged directly into liquid nitrogen before freeze drying to avoid possible enzyme degradation. The samples were then ground to a fine powder with a pestle and mortar and stored in the cold room until analyzed.

3.2.2 Methods

3.2.2.1 Extraction

Freeze dried plant material (50 mg) was transferred to a 2-ml microtube. A volume of 1.5 ml of a mixture of KH_2PO_4 buffer (pH 6.0) in D_2O containing 0.05% trimethyl silyl propionic acid sodium salt, w/w) (TMSP) and methanol-d_4 (1:1) was added to the plant samples. The mixture was vortexed at room temperature for 1 min, ultrasonicated for 20 min and centrifuged at 13,000 rpm for 10 min. An aliquot of 0.8 ml was used for NMR analysis.

3.2.2.2 NMR measurements

^{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). 2-D-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}, were 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 30 183 Hz of spectral width in F2. The optimized coupling constants for HSQC and HMBC were 145 Hz and 8 Hz, respectively.

3.2.2.3 Data analysis

^{1}H-NMR projection spectra were automatically reduced to ASCII (v. 3.7, Bruker Biospin). Intensities were scaled to total intensities and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.4-δ 10.0. The region

of 4.8-4.9 was excluded from the analysis because of the residual signal of water as well as δ 3.28 - δ 3.34 for residual methanol. Principal component analysis (PCA) was performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden). ANOVA for ^{1}H-NMR signals were performed using Minitab version 12.21.

3.3 Results and discussion

Four cultivars of *Brassica rapa* (Raapstelen, Herfstraap, Witte Mei, and Oleifera) of two different ages, four and six weeks old in this case, were analyzed by NMR to evaluate the effect of cultivar type and age on metabolic variation. Of the possible analytical techniques generally used in metabolomics, MS and NMR-based methods are usually acknowledged to be the optimum choice for a metabolic study. NMR, particularly ^{1}H-NMR, has advantages unsurpassed by other methods such as the ease of simultaneous detection of diverse groups of metabolites in a relatively short measuring time. Here, the study was performed on the basis of known compounds recognized in NMR spectra. Therefore, influence of cultivar and age can easily be examined with respect to these compounds. In spite of the relative ease of NMR method implementation, signal overlapping does constitute an obstacle to identify each metabolite in the extract. As shown in **Figures 3.1** and **3.2** this problem was partly solved by acquiring 2D-^{1}H-^{1}H-*J*-resolved NMR spectra, which provided additional information and the splitting pattern of each signal with the exact coupling constant (Ward *et al*., 2007). In fact, the *J*-resolved spectra together with correlation 2D-NMR spectra including COSY, HMQC, and HMBC considerably increased the number of identified metabolites beyond that from a 1D-^{1}H-NMR spectra (Choi *et al*., 2006).

In the amino acid region (δ 0.8 – δ 4.0), alanine, glutamic acid, glutamine, threonine, γ-amino butyric acid (GABA) and valine were identified (**Table 3.1**). Organic acids such as acetic, formic, fumaric, lactic, malic, succinic, as well as choline and signals of the terminal CH_3 of fatty acids or lipids were identified (**Table 3.1**). For sugars, the anomeric proton of β-glucose at δ 4.58 (d, J = 7.8 Hz), α-glucose at δ 5.18 (d, J = 3.7 Hz), sucrose at δ 5.4 (d, J = 3.6 Hz) and fructose moiety with sucrose at δ 4.16 (d, J = 7.8 Hz) were assigned. In this region, the anomeric signals at δ 4.7, δ 4.97, δ 5.03 and δ 5.05 with the characteristic large coupling constants (J = 9.8–10.0 Hz) different from those of common anomeric sugar protons (1.0-8.0 Hz),

indicated the presence of the glucose moiety of aliphatic or aromatic glucosinolates (Liang *et al.,* 2006b). Of these glucosinolates, progoitrin was identified by 2D-NMR (**Figure 3.1**). The anomeric proton of progoitrin was detected at δ 5.03 and correlated in HMBC with the carbon at δ 163.3. The signal of H-2 of progoitrin was detected at δ 2.87 (dd, *J* = 16.0, 10.0 Hz), H-3 at δ 4.63 (m), H-4 at δ 5.96 (m), H-5a at δ 5.34 (dt, *J* = 16.0, 2.0 Hz), and H-5b at δ 5.21 (dt, *J* = 11.0, 2.0 Hz). Correlation between H-3 and the carbon at δ 118.0 in HMBC confirmed the position of OH group. In the COSY spectrum H-2, H-3, H-4 and H-5 were correlated continuously with each other. These signals correlated with carbons in HSQC at δ 68.2, δ 94.0, δ 115.0, and δ 114.0, respectively. Neoglucobrassicin, an indolic glucosinate was identified by using *J*-resolved and COSY spectra. Additionally, indole acetic acid (IAA) was detected as a minor compound in the plant mixture (**Table 3.1**). Both neoglucobrassicin and indole acetic acid were found to increase in *B. rapa* after treatment with methyl jasmonate (Liang *et al.,* 2006b).

In the aromatic region, the presence of five major doublets with the same coupling constants (d, *J* = 16.0 Hz) in the range of δ 6.39 – δ 6.49 indicated the presence of the *trans* olefinic protons of the phenylpropanoids (**Figure 3.2**). This was confirmed by the fact that H-8′ of the phenylpropanoids correlated with the H-7′ protons at δ 7.59 - δ 7.66 in the COSY spectrum and with the carboxyl group at δ 171.3 in the HMBC spectrum. Five *trans*-phenylpropanoids and two *cis* forms were elucidated by two dimensional NMR. However, the c*is*-phenylpropanoids are believed to be artifacts of *trans*-phenylpropanoids produced during the sample extraction (Pauli *et al*., 1998; Liang *et al*., 2006b). These compounds were already detected in methyl jasmonate treated plants, together with another three *cis*-phenylpropanoids (Liang *et al.,* 2006a).

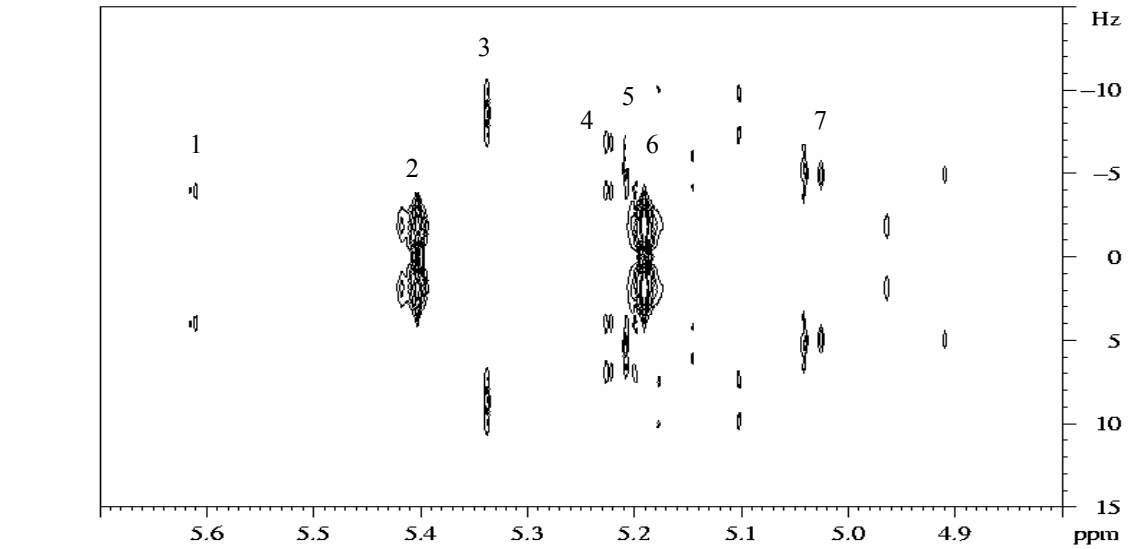

Figure 3.1. Two dimensional ^{1}H-^{1}H *J*-resolved spectrum of *Brassica rapa* Raapstelen upper leaves in the range of δ 4.8 – δ 5.7. 1: anomeric protons of flavonoid glycosides, 2: sucrose, 3: H-5a of progoitrin, 4: H-2 of malic acid conjugated to *trans*-phenylpropanoids, 5: H-5b of progoitrin, 6: α-glucose, 7: anomeric proton of progoitrin.

The 2D-*J*-resolved spectrum of the plant extract showed characteristic double doublet signals at δ 5.22 (dd, J = 11.0, 3.0 Hz) and at δ 5.19 (dd, J = 11.0, 3.0 Hz). These signals were assigned as the H-2 of malic acid conjugated with phenylpropanoids, based on the correlation in the COSY spectrum with the signals of H-3 of malic acid at δ 2.70 and δ 2.85 (dd, J = 16.0, 12.0 and 16.0, 3.0 Hz conjugation with *trans* phenylpropanoids) and at δ 2.65 and δ 2.83 (dd, J = 16.0, 11.0 Hz and 16.0, 3.0 Hz conjugation with *cis* forms).

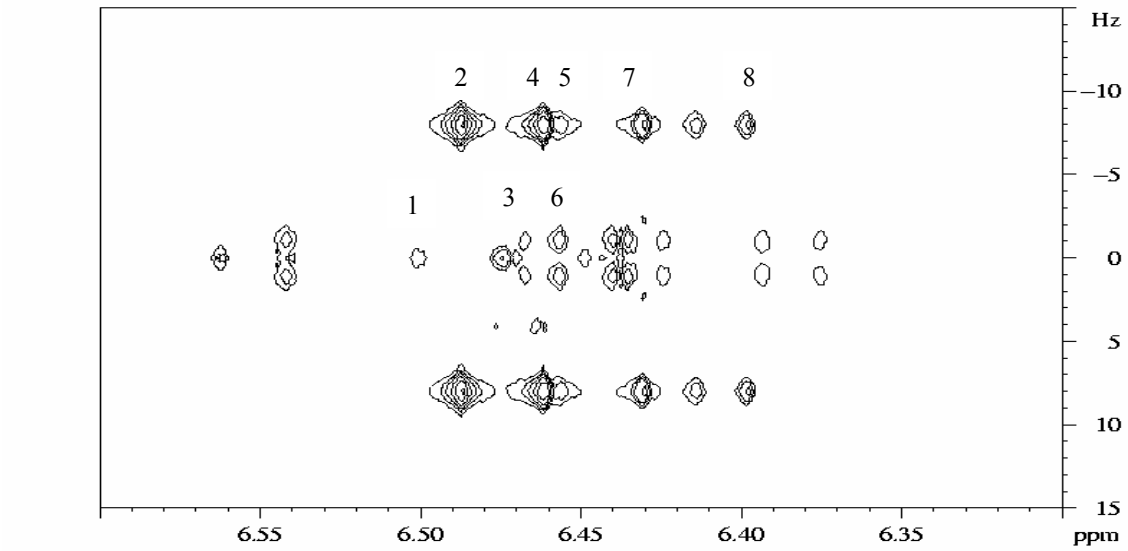

Figure 3.2. Two dimensional ^{1}H-^{1}H *J*-resolved spectrum of *Brassica rapa* Raapstelen upper leaves in the range of δ 6.3 – δ 6.6. 1: fumaric acid, 2: H-8 of *trans*-sinapoyl malate, 3: H-6 of quercetin glycoside, 4: H-8 of *trans*-feruloyl malate, 5: H-8 of *trans*-coumaroyl malate, 6: H-6 of kaempferol glycoside, 7: H-8 of *trans*-5-hydroxyferuloyl malate, 8: H-8 of *trans*-caffeoyl malate.

A flavonoid was detected and identified as kaempferol glycoside. The signals at δ 6.46 and δ 6.77 correlated with each other in the COSY spectrum with a *meta* coupling constant (J = 2.0 Hz) and were assigned to be H-6 and H-8 of kaempferol glycoside. The presence of the flavonoid was confirmed by HSQC spectrum in which the carbons of H-6 and H-8 showed upfield shifts (97.9 and 93.2 ppm), respectively. The correlation between the signals at δ 7.00 (H-2′ H-6′, d, J = 8.8 Hz) and δ 8.07 (H-3′ H-5′, d, J = 8.8 Hz) led to the elucidation of the B-ring protons of kaempferol. The downfield shift of H-8 showed the attachment of glucose at the C-7 position. Another flavonoid, quercetin glycoside, was also detected. A signal at δ 6.47 of H-6 (d, J = 2.0 Hz) was correlated in the COSY spectrum with the signal at δ 6.77 of H-8 (d, J = 2.0 Hz) and a signal at δ 6.90 of H-5′ (d, J = 8.8 Hz) with one at δ 7.54 of H-6′ (dd, J = 6.5, 3.0 Hz). The signal of H-2′ was detected at δ 7.82 (d, J = 2.0 Hz). An anomeric proton of β-glucose in flavonoid glycosides was detected in the *Brassica* extracts at δ 5.61 (d, J = 7.4). This signal correlated with the carbons at δ 166.0 and 179.6.

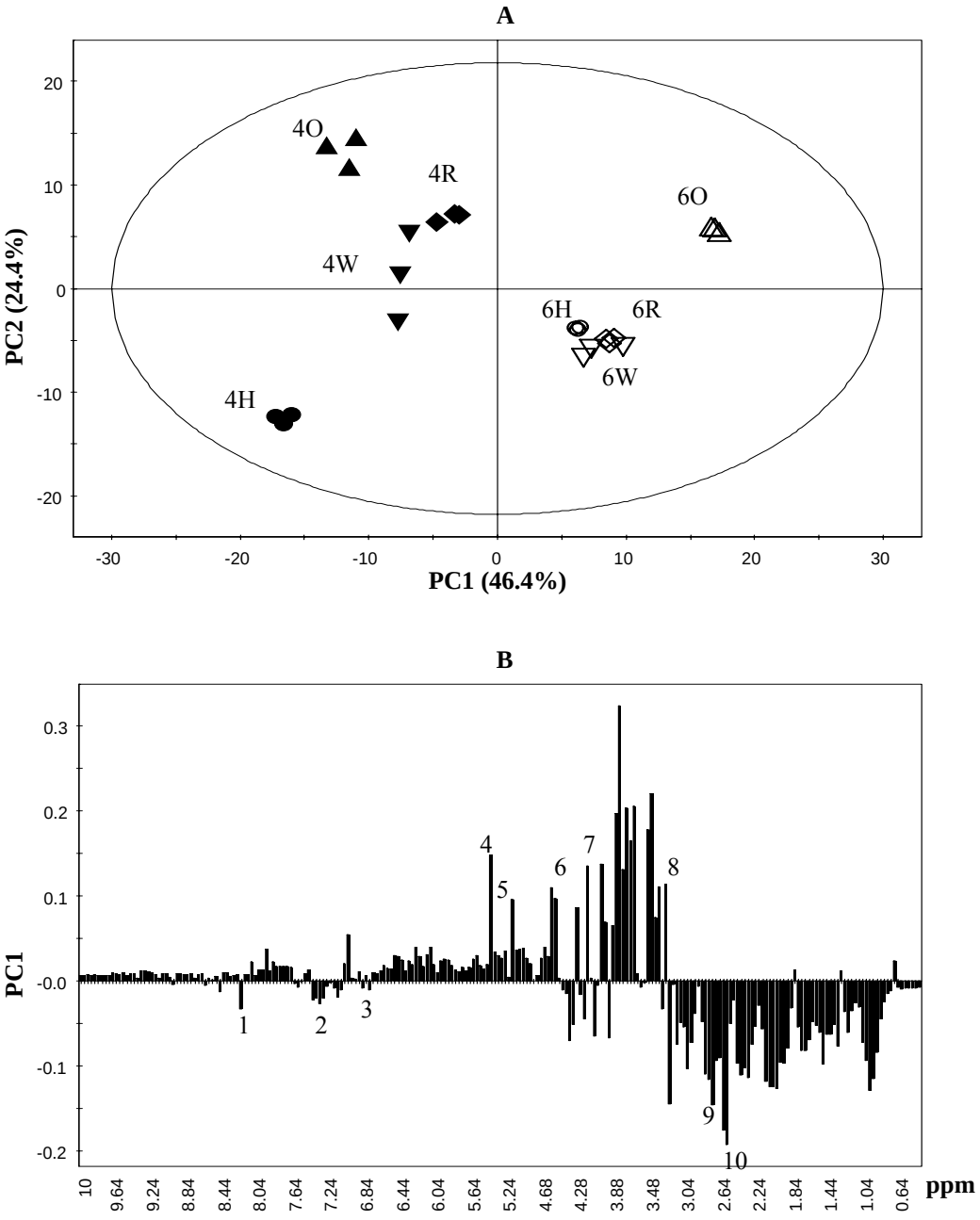

Figure 3.3. Score (PC1 vs. PC2, **A**) and loading (PC1, **B**) plot of PCA results obtained from ^{1}H-NMR spectra of four cultivars of *Brassica rapa* upper leaves of four and six weeks old. 4H: 4 weeks old Herfstraap (●), 4O: 4 weeks old Oleifera (▲), 4R: 4 weeks old Raapstelen (♦), 4W: 4 weeks old Witte Mei (▼), 6H: 6 weeks old Herfstraap (○), 6O: 6 weeks old Oleifera (Δ), 6R: 6 weeks old Raapstelen (◊), 6W: 6 weeks old Witte Mei (∇). 1: adenine, 2: indole acetic acid (IAA), 3: flavonoid glycoside, 4: sucrose, 5: α- glucose, 6: β-glucose, 7: fructose moiety of sucrose, 8: choline, 9: malic acid, 10: succinic acid.

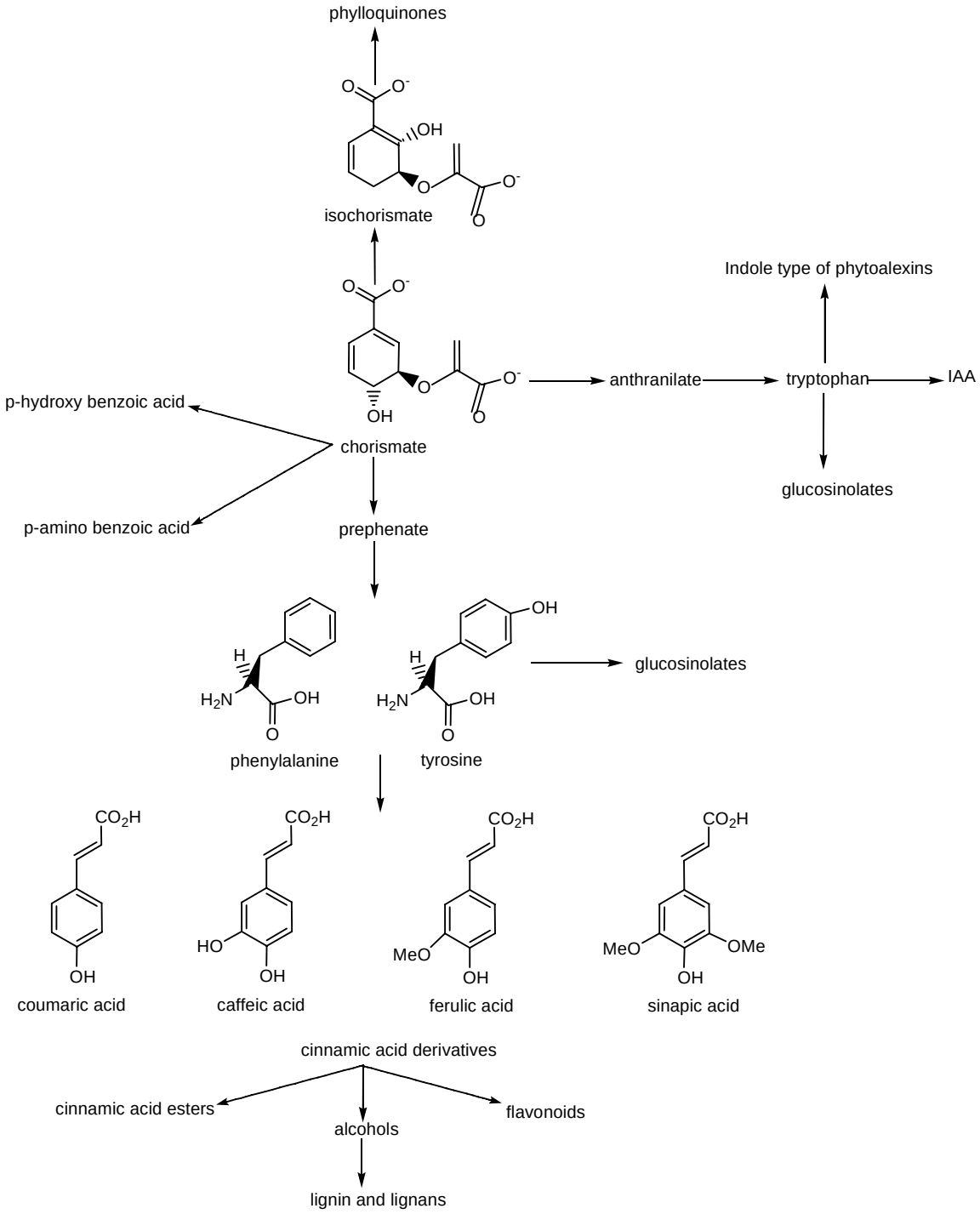

Figure 3.4. General pathways for formation of secondary metabolites in plants.

PCA was done on the basis of small integrated regions on the processed raw data. The separation seen in the PCA is explained in terms of the identified compounds. Six week-old *Brassica* plant leaves showed a higher level of carbohydrates but less organic and amino acids compared to 4 week-old samples. In the aromatic region, indole acetic acid (IAA) and flavonoid signals at δ 7.1 to δ 7.6 and δ 6.76 were found to be higher in 4 week-old samples (**Figure 3.3A and 3.3B**). The two increased singlets at δ 8.2 in the 4 week old samples were assigned to adenine by comparison with the spectra of a reference compound. Other aromatic signals including phenylpropanoids and glucosinolates were higher in the older plants. The higher level of carbohydrates observed in the upper leaves of six week-old plants may be attributed to the increase in all leaves total surface area with age, which in turn increases the rate of photosynthesis, thus producing a greater accumulation of sugars. Leaves contribute actively to plant fitness so they are expected to contain a higher concentration of compounds such as phenylpropanoids or glucosinolates associated to defense mechanisms. The high concentration of these compounds in the top leaves is thought to reflect the need of these tissues to increase their defensive potential (Brown *et al.,* 2003). The build-up of these secondary metabolites which are derived from the organic and amino acids may explain the decrease of the latter observed in six week-old plants. The differences in flavonoid and phenylpropanoids levels at the different developmental stages might be due to a different channeling of the phenylpropanoid precursors (cinnamic acid derivatives) (**Figure 3.4**). In *Arabidopsis*, for example, the accumulation of one or more flavonoids correlates with the decrease in level of cinnamic acid derivatives (Li *et al.,* 1993). The higher concentrations of indole acetic acid (IAA), an important plant hormone contributing to a variety of developmental processes (Weiler *et al*., 1993; Liang *et al*., 2006b) and of adenine in four week-old plants might be due to a higher rate of metabolic activity connected with growth in younger plants. The glucosinolate concentration was reported to increase rapidly during the first 40 days after planting, reaching a maximum at 50 days (Porter *et al.,* 1991). Thus, six week-old leaves showed a higher concentration of glucosinolates than four-week-old leaves.

The PCA of four week-old plants alone showed a better separation of Herfstraap and Witte Mei leaves from those of Oleifera and Raapstelen than that obtained when both age groups were combined (**Figures 3.3A and 3.5A**).

The loading plot of PC1 revealed that Herfstraap and Witte Mei were well separated by the higher concentration of all detected amino acids, organic acids, glucosinolates, phenylpropanoids, flavonoids and adenine, from Oleifera and Raapstelen which were separated in PC1 by free malic acid, glucose and choline (**Figure 3.5B**). Raapstelen and Witte Mei were separated quite well in PC2 by alanine, succinic acid and glucose. PCA of six week-old plants separated all cultivars evaluated (**Figure 3.6A**). Herfstraap was separated by a higher content of amino and organic acids, glucosinolates, phenylpropanoids, and flavonoids, while Oleifera was found to have a higher concentration of carbohydrates (glucose, sucrose) and choline (**Figure 3.6B**). Witte Mei and Raapstelen were separated in PC2 from Oleifera and Herfstraap by a higher concentration of sucrose, threonine, neoglucobrassicin and the *cis*-form of phenylpropanoids (**Figure 3.6C**).

Both four and six week-old Herfstraap and Witte Mei *Brassica* leaves showed a higher concentration of glucosinolates, while Oleifera showed the lowest concentration among the studied cultivars. These results are in agreement with the HPLC quantitation of glucosinolates (**Figure 3.7**). The characteristic low level of glucosinolates of the Oleifera cultivar might be the reason for the ease of transformation of this *Brassica* with *Agrobacterium* (Simoh, 2008).

Table 3.1. ^{1}H chemical shifts (δ) and coupling constants (Hz) of *Brassica rapa* metabolites identified by references and using 1D and 2D NMR spectra (CD_3OD-KH_2PO_4 in D_2O (pH 6.0)

Compound	Chemical shifts and coupling constants
Fatty acids	0.91 (t, *J* = 7.5 Hz), 0.94 (t, *J* = 7.5 Hz)
Valine	1.00 (d, *J* = 7.0 Hz), 1.05 (d, *J* = 7.0 Hz), 2.3 (m)
Threonine	1.32 (d, *J* = 7.0 Hz), 3.52 (d, *J* = 12.0 Hz), 4.23 (m)
Lactic acid	1.36 (d, *J* = 7.0 Hz), 4.14 (d, *J* = 7.0 Hz)
Alanine	1.48 (d, *J* = 7.4 Hz), 3.73 (q)
GABA	1.91 (m), 2.31 (t, *J* = 7.2 Hz)
Acetic acid	1.94 (s)
Glutamic acid	2.13 (m), 2.42 (m), 3.71 (t, *J* = 6.8 Hz)
Glutamine	2.14 (m), 2.43 (td, *J* = 16.5, 7.4 Hz)
Succinic acid	2.53 (s)
Malic acid (free)	2.58 (dd, *J* = 16.0, 7.4 Hz), 2.76 (dd, *J* = 16.0, 4.6 Hz), 4.32 (dd, *J* = 7.2, 4.0 Hz)
Malic acid conjugated with *cis*-phenylprop.	2.65 (dd, *J* = 16.0, 11.0 Hz), 2.83 (dd, *J* = 16.0, 3.0 Hz), 5.19 (dd, *J* = 11.0, 3.0 Hz)
Malic acid conjugated with *trans*-phenylprop.	2.7 (dd, *J* = 16.0, 12.0 Hz), 2.85 (dd, *J* = 16.0, 3.0 Hz), 5.22 (dd, *J* = 11.0, 3.0 Hz)
Choline	3.24 (s)
Fructose or sucrose	4.16 (d, *J* = 7.8 Hz)
Sucrose	5.4 (d, *J* = 3.6 Hz)
β-glucose	4.58 (d, *J* = 7.8 Hz)
α-glucose	5.18 (d, *J* = 3.7 Hz)
Progoitrin	2.87 (dd, *J* = 16.0, 10.0 Hz), 4.63 (m), 5.21 (dt, *J* = 11.0, 2.0 Hz), 5.34 (dt, *J* = 16.0, 2.0 Hz), 5.96 (m)
Neoglucobrassicin	4.09 (s, MeO), 7.17 (t, *J* = 7.8 Hz), 7.30 (t, *J* = 7.8 Hz), 7.49 (d, *J* = 7.8 Hz), 7.48 (s), 7.73 (d, *J* = 7.8)
Indole acetic acid	3.25 (d, *J* = 16.0 Hz), 3.39 (d, *J* = 16.0 Hz), 7.12 (s), 7.13 (t, *J* = 7.8 Hz), 7.21 (t, *J* = 7.8 Hz), 7.47 (d, *J* = 7.8 Hz), 7.72 (d, *J* = 7.8 Hz)
Adenine	8.2 (s), 8.21 (s)
Fumaric acid	6.51 (s)
Kaempferol analogues	6.46 (d, *J* = 2.1 Hz), 6.77 (d, *J* = 2.0 Hz), 7.00 (d, *J* = 8.8 Hz), 8.07 (d, *J* = 8.8 Hz)
Quercetin analogues	6.47 (d, *J* = 2.1 Hz), 6.77 (d, *J* = 2.0 Hz), 6.9 (d, *J* = 8.8 Hz), 7.54 (dd, *J* = 6.5, 3.0 Hz), 7.82 (d, *J* = 2.0 Hz)
cis-sinapoyl malate	5.94 (d, *J* = 13.0 Hz), 6.95 (d, *J* = 13.0 Hz), 7.12 (s)
trans-sinapoyl malate	6.49 (d, *J* = 16.0 Hz), 6.97 (s), 7.65 (d, *J* = 16.0 Hz)
cis-caffeoyl malate	5.93 (d, *J* = 13.0 Hz), 6.84 (d, *J* = 8.8 Hz), 6.94 (d, *J* = 13.0 Hz), 7.06 (dd, *J* = 8.4, 2.0 Hz), 7.84 (d, *J* = 2.0 Hz)
trans-caffeoyl malate	6.4 (d, *J* = 16.0 Hz), 6.83 (d, *J* = 8.8 Hz), 7.13 (dd, *J* = 8.4, 2.0 Hz), 7.15 (d, *J* = 2.0 Hz), 7.61 (d, *J* = 16.0 Hz)
cis-feruloyl malate	5.97 (d, *J* = 13.0 Hz), 6.84 (d, *J* = 8.8 Hz), 6.94 (d, *J* = 13.0 Hz), 7.13 (dd, *J* = 8.4, 2.0 Hz), 7.83 (d, *J* = 2.0 Hz)
trans- feruloyl malate	6.47 (d, *J* = 16.0 Hz), 6.87 (d, *J* = 8.4 Hz), 7.06 (dd, *J* = 8.4, 2.3 Hz), 7.26 (d, *J* = 2.0 Hz), 7.65 (d, *J* = 16.0)
cis-coumaroyl malate	5.97 (d, *J* = 13.0 Hz), 6.95 (d, *J* = 13.0 Hz), 7.00 (bd, *J* = 9.2 Hz), 7.89 (bd, *J* = 9.2 Hz)
trans-coumaroyl malate	6.46 (d, *J* = 16.0 Hz), 6.84 (bd, *J* = 8.8 Hz), 7.57 (bd, *J* = 9.2 Hz), 7.66 (d, *J* = 16.0 Hz)
cis-5-hydroxyferuloyl malate	5.94 (d, *J* = 13.0 Hz), , 6.95 (d, *J* = 13.0 Hz), 7.13 (d, *J* = 2.0 Hz), 7.51 (d, *J* = 2.0 Hz)
trans-5-hydroxyferuloyl malate	6.43 (d, *J* = 16.0 Hz), 6.84 (d, *J* = 2.0 Hz), 6.87 (d, *J* = 2.0 Hz), 7.59 (d, *J* = 16.0 Hz)

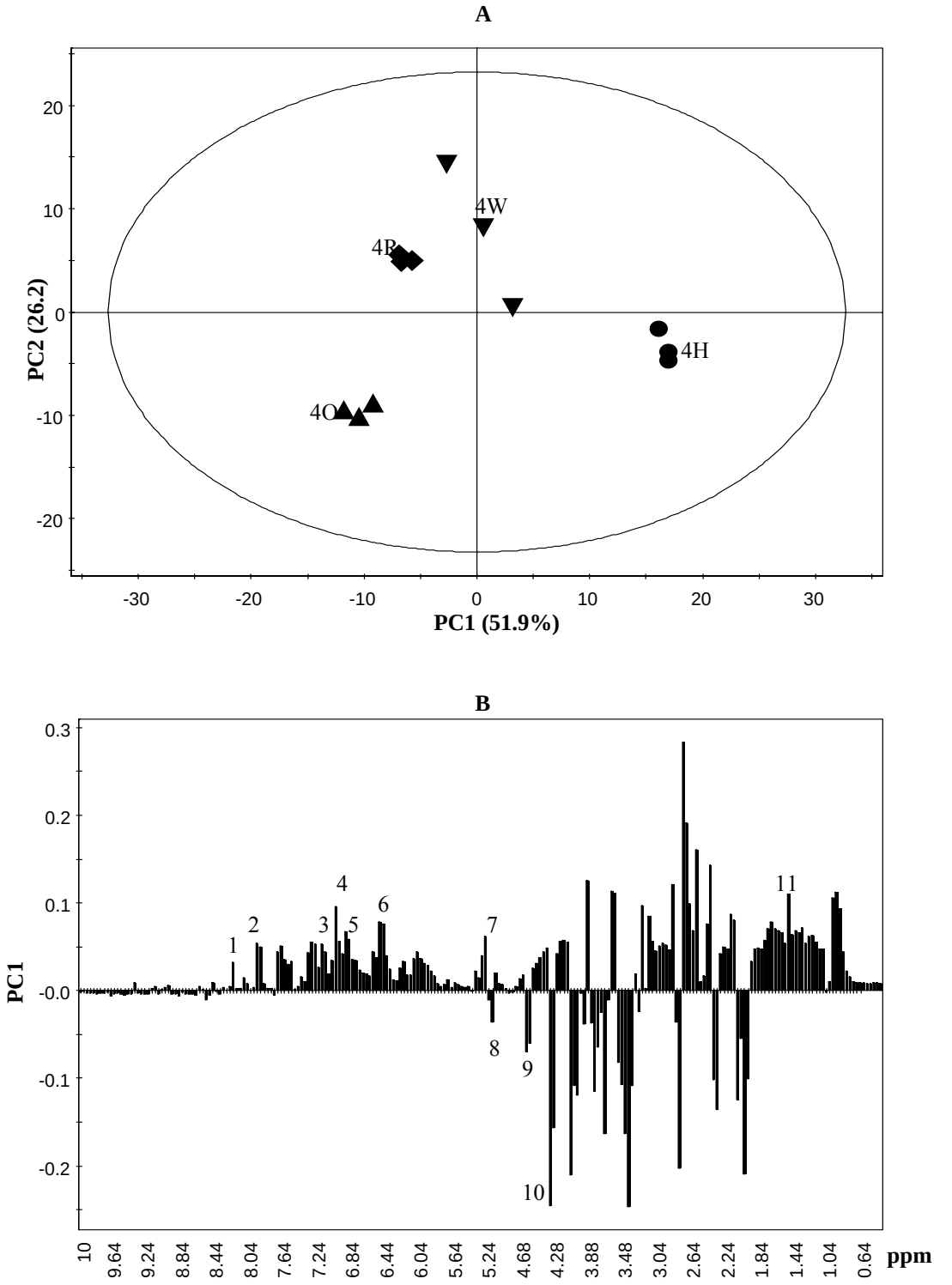

Figure 3.5. Score (PC1 vs. PC2, **A**) and loading (PC1, **B**) plot of PCA results obtained from ^{1}H-NMR spectra of four cultivars of *Brassica rapa* upper leaves of four weeks old. 4H: 4 weeks old Herfstraap (●), 4O: 4 weeks old Oleifera (▲), 4R: 4 weeks old Raapstelen (♦), 4W: 4 weeks old Witte Mei (▼). 1: adenine, 2: coumaroyl malate, 3: neoglucobrassicin, 4: sinapoyl malate, 5: flavonoid glycoside, 6: feruloyl and 5-hydroxyferuloyl malate, 7: progoitrin, 8: α-glucose, 9: β-glucose, 10: malic acid, 11: alanine.

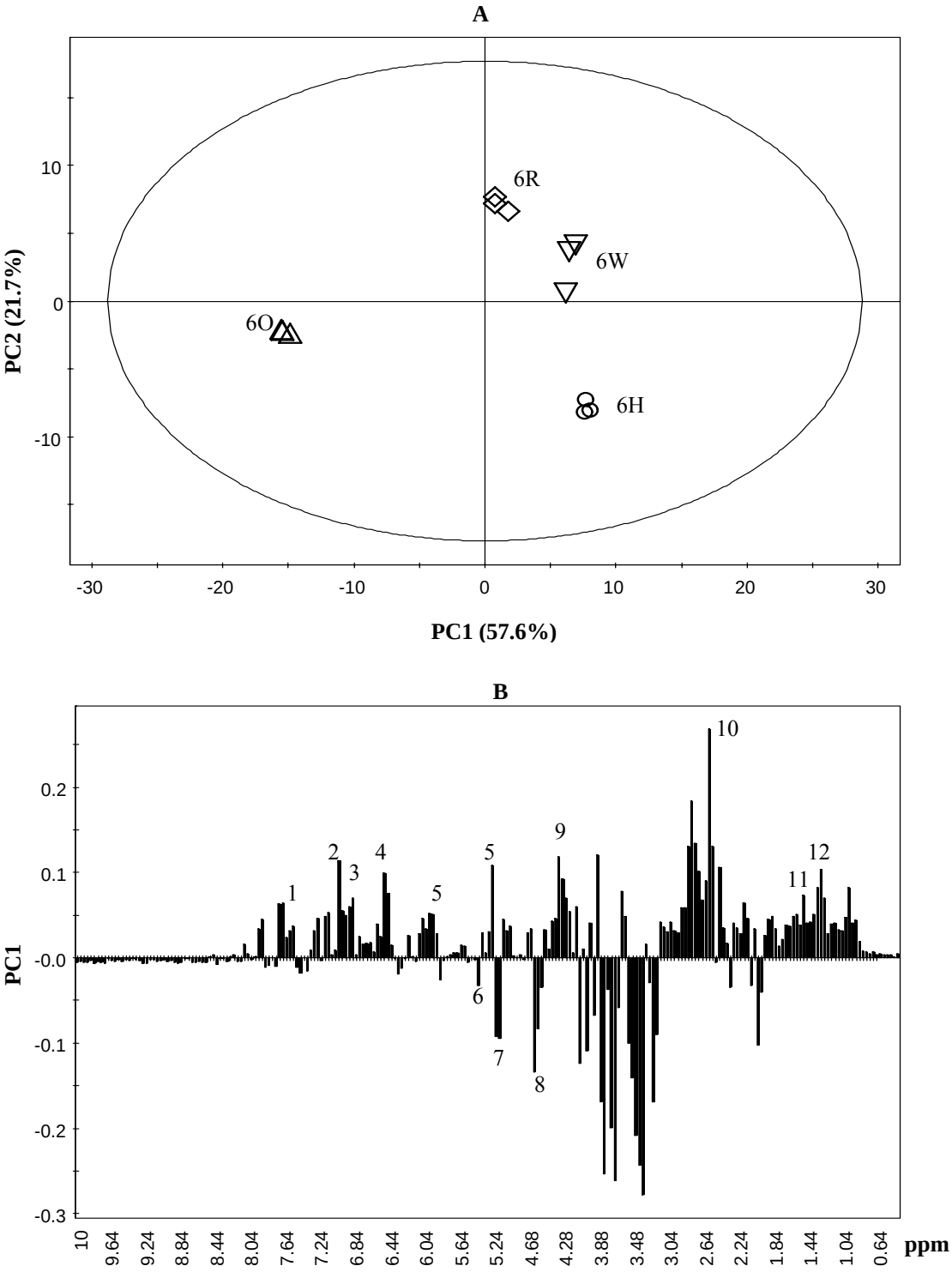

Figure 3.6. Score (PC1 vs. PC2, **A**) and loading (PC1, **B**) of PCA results obtained from ^{1}H-NMR spectra of four cultivars of *Brassica rapa* upper leaves of six weeks old. 6H: 6 weeks old Herfstraap (○), 6O: 6 weeks old Oleifera (Δ), 6R: 6 weeks old Raapstelen (◊), 6W: 6 weeks old Witte Mei (∇). **B**: 1: coumaroyl malate, 2: sinapoyl malate, 3: flavonoid glycoside, 4: feruloyl and 5-hydroxyferuloyl malate, 5: progoitrin, 6: sucrose, 7: α-glucose, 8: β-glucose, 9: malic acid, 10: succinic acid, 11: alanine, 12: threonine.

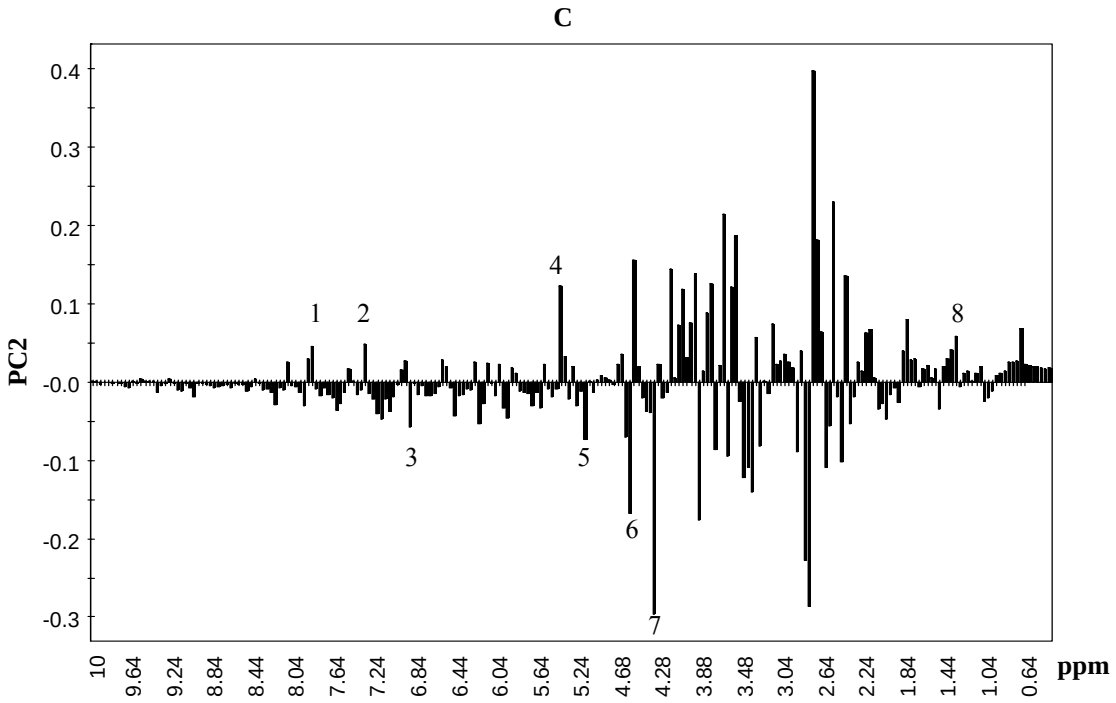

Figure 3.6C. Loading column plot of PC2 of PCA results obtained from ^{1}H-NMR spectra of four cultivars of *Brassica rapa* upper leaves of six weeks old. 1: *cis* caffeoyl and feruloyl malate, 2: neoglucobrassicin, 3: *trans*-feruloyl malate, 4: sucrose, 5: α-glucose, 6: β-glucose, 7: malic acid, 8: threonine.

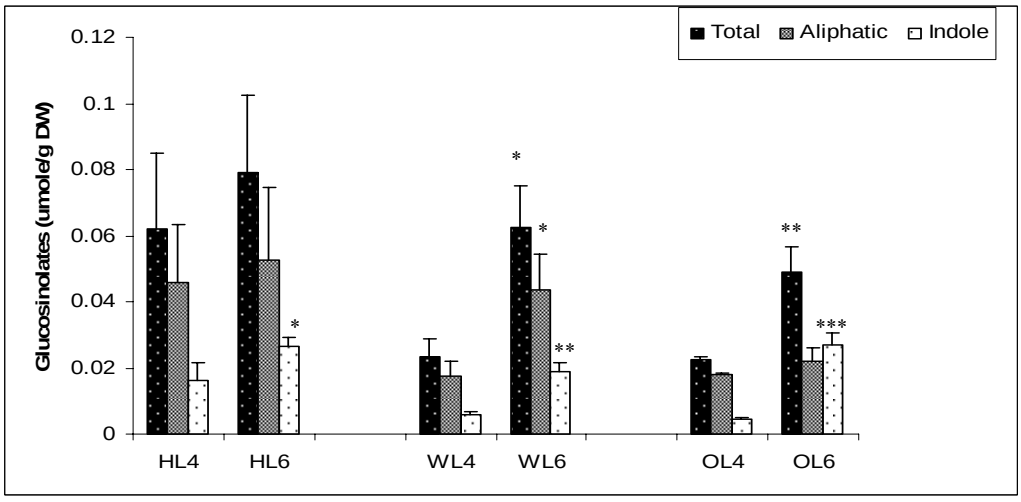

Figure 3.7. Total, aliphatic and indole glucosinolate content in different cultivars of four and six-week-old leaves of *Brassica rapa* (HL: Herfstraap leaves, WL: Witte Mei leaves and OL: Oleifera leaves), the number after the cultivars refers to the age of the plants (4: four week-old and 6: six week-old leaves).
*=significant p< 0.05, **=highly significant p< 0.01 and ***=very highly significant p< 0.001.

3.4 Conclusion

This study shows that cultivars and development stage of the plant are important factors for the metabolome. The trends for development are similar for the four cultivars and in part might be due to changes in channeling of precursors. The results show that when comparing cultivars of the same age a distinction can be made. However, when all materials of 4-and 6-weeks old plants are analyzed in one set, the differences related to age seem to be more important than those related to cultivars. A further detailed time course of the secondary metabolite production would be of interest to learn more about this phenomenon which is for example of interest in connection with the time of harvesting and the quality of the vegetable, e.g. in terms of taste and the presence of health promoting compounds.

The pattern of metabolic variation among cultivars may provide an opportunity to select those suitable for further studies including plant-fungi or plant-insect interaction. The six-week-old plants characterized with higher amounts of phenylpropanoids and glucosinolates than four-week-old plants. Six week-old plants will be used in further studies of plant-fungal interaction.

3.5 Acknowledgements

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