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Stress response and health affecting compounds in Brassicaceae

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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 (^1H 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, ^1H 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 content³⁴ 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 biological⁴⁹ 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 organism³⁷ 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 °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 reported³⁷³ 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 ¹H 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 ^1H 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 ^1H 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 ^1H 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 leaves³⁷⁶ and adjust physiologically to changes in resources availability and to extreme changes in the internal nutrient balance.³⁷⁷

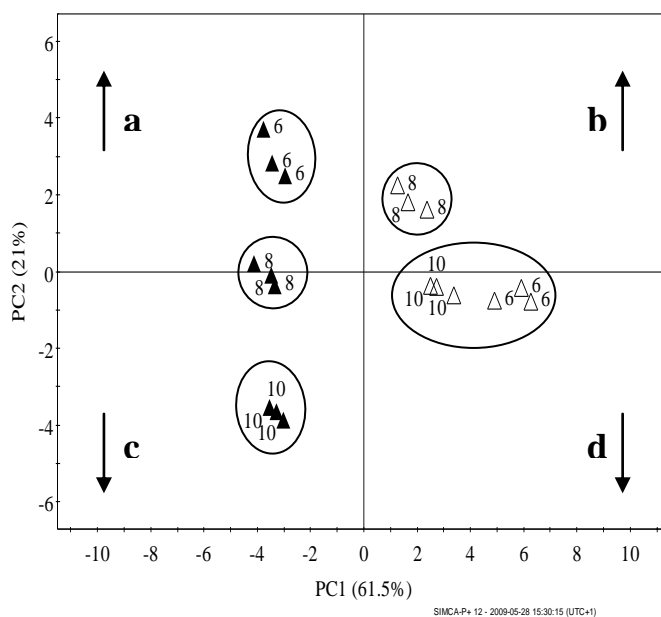


Figure 1A. Score plot of PCA for leaves of *Brassica rapa* (var. raapstelen) and *Raphanus sativus* (radish), based on whole range of ^1H NMR signals (δ 0.3 – δ 10.0, bucketed data) and HPLC (quantitative) data. Radish, 6 weeks (6▲); 8 weeks (8▲); 10 weeks (10▲) old plants. *Brassica rapa*, 6 weeks (6Δ); 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**).

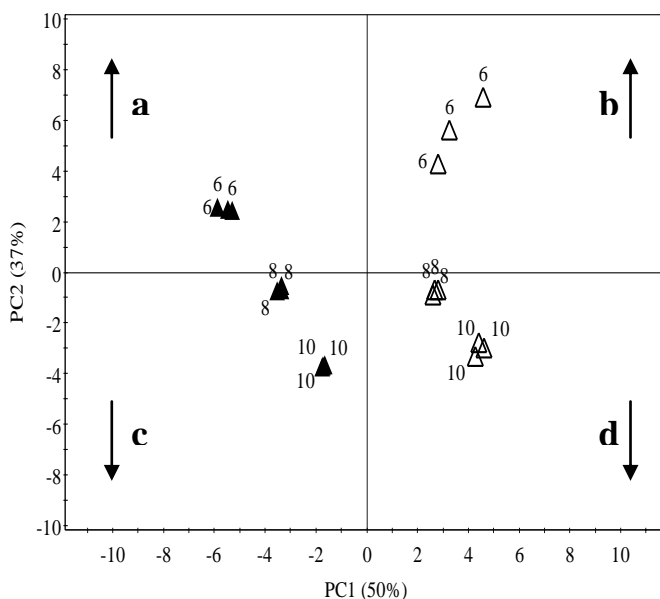


Figure 1B. Score plot of PCA for roots of *Brassica rapa* (var. raapstelen) and *Raphanus sativus* (radish), based on whole range of ^1H NMR signals (δ 0.3 – δ 10.0, bucketed data) and HPLC (quantitative) data. Radish, 6 weeks (6▲); 8 weeks (8▲); 10 weeks (10▲) old plants. *Brassica rapa*, 6 weeks (6Δ); 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.

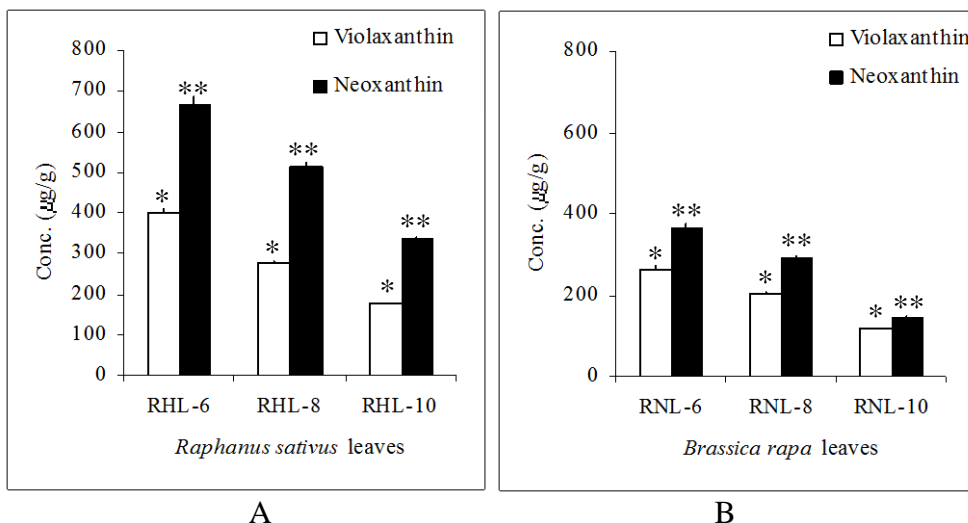


Figure 2 Concentration of violaxanthin and neoxanthin ($\mu\text{g/g}$ of dry weight) and at different developmental stages of *Raphanus sativus* (A) and *Brassica rapa* (var. raapstelen) (B) leaves. RHL = Radish leaves; RHR = Radish roots; RNL = Raapstelen leaves; RNR = Raapstelen roots; 6 weeks old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

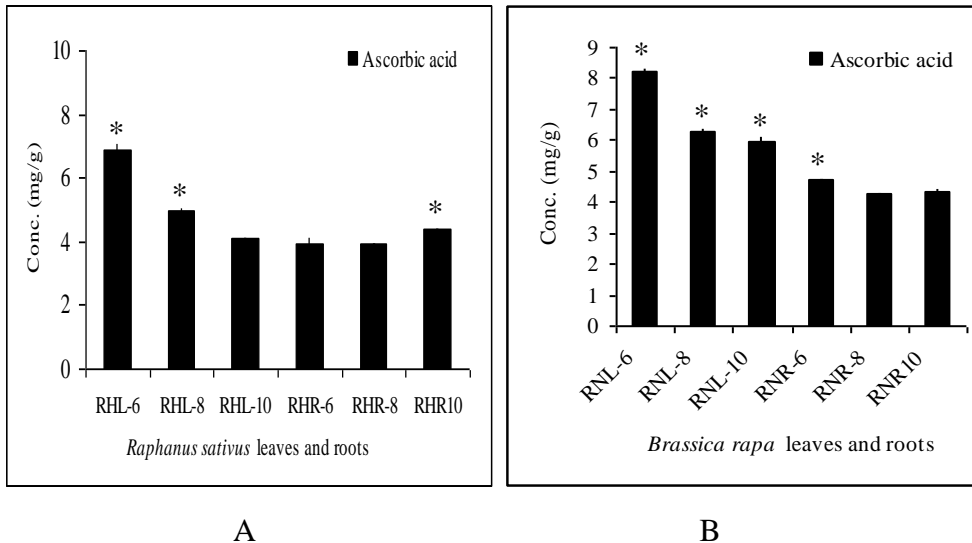


Figure 3 Concentration of ascorbic acid (mg/g) and at different developmental stages of *Raphanus sativus* (A) and *Brassica rapa* (var. raapstelen) (B) leaves. RHL = Radish leaves; RHR = Radish roots; RNL = Raapstelen leaves; RNR = Raapstelen roots; 6 weeks old plant (6); 8 weeks old plants (8); 10 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-hydroxypropylglucosinolate 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.

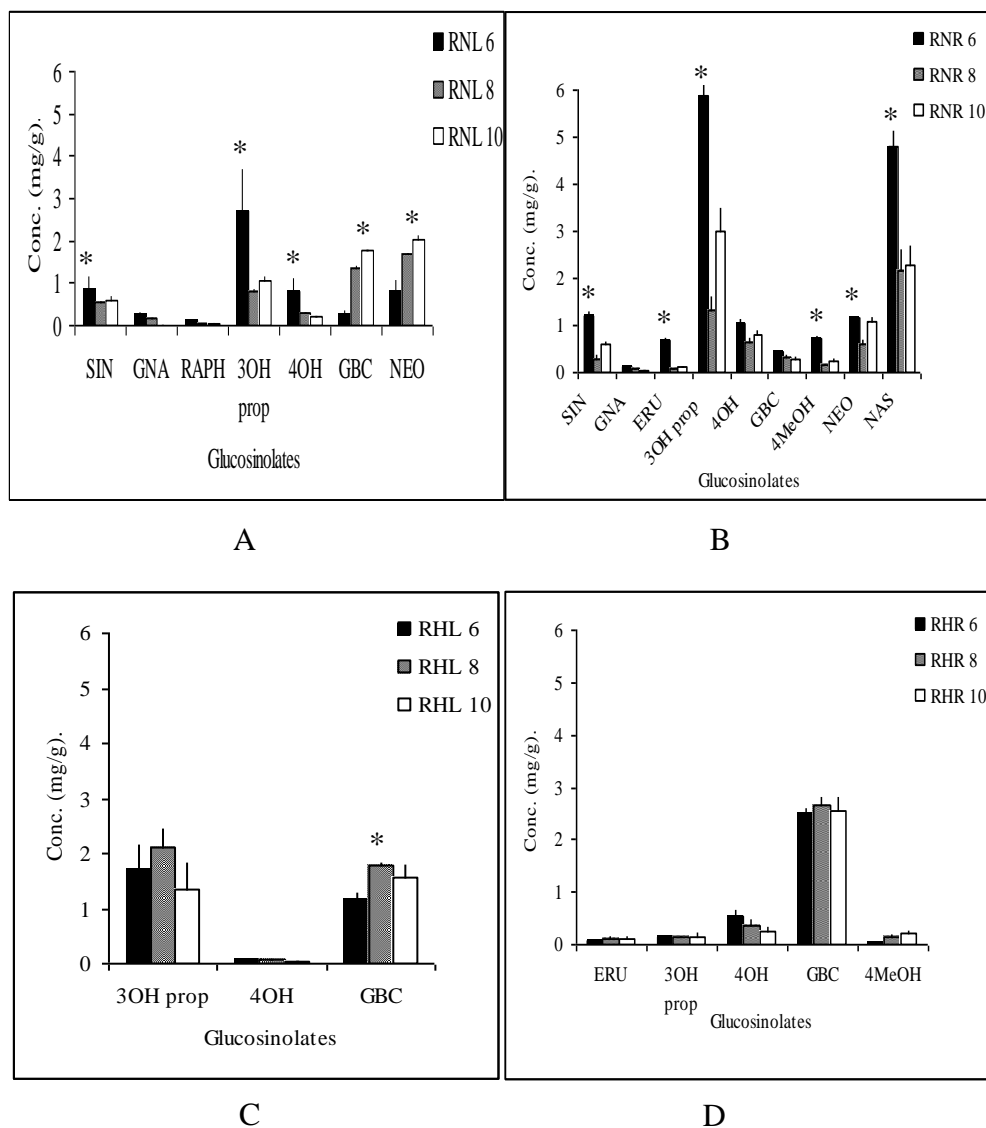


Figure 4. Concentration of glucosinolates (mg/g of dry weight) at different developmental stages of *Brassica rapa* (var. Raapstelen) and *Raphanus sativus* leaves. RNL = Raapstelen leaves (A); RNR = Raapstelen roots (B); RHL = Radish leaves (C); RHR = Radish roots (D); 6 weeks old plant (6); 8 weeks 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-hydroxypropylglucosinolate (3OH prop), Sinigrin (SIN). * = The value shows a significant difference ($P < 0.001$).

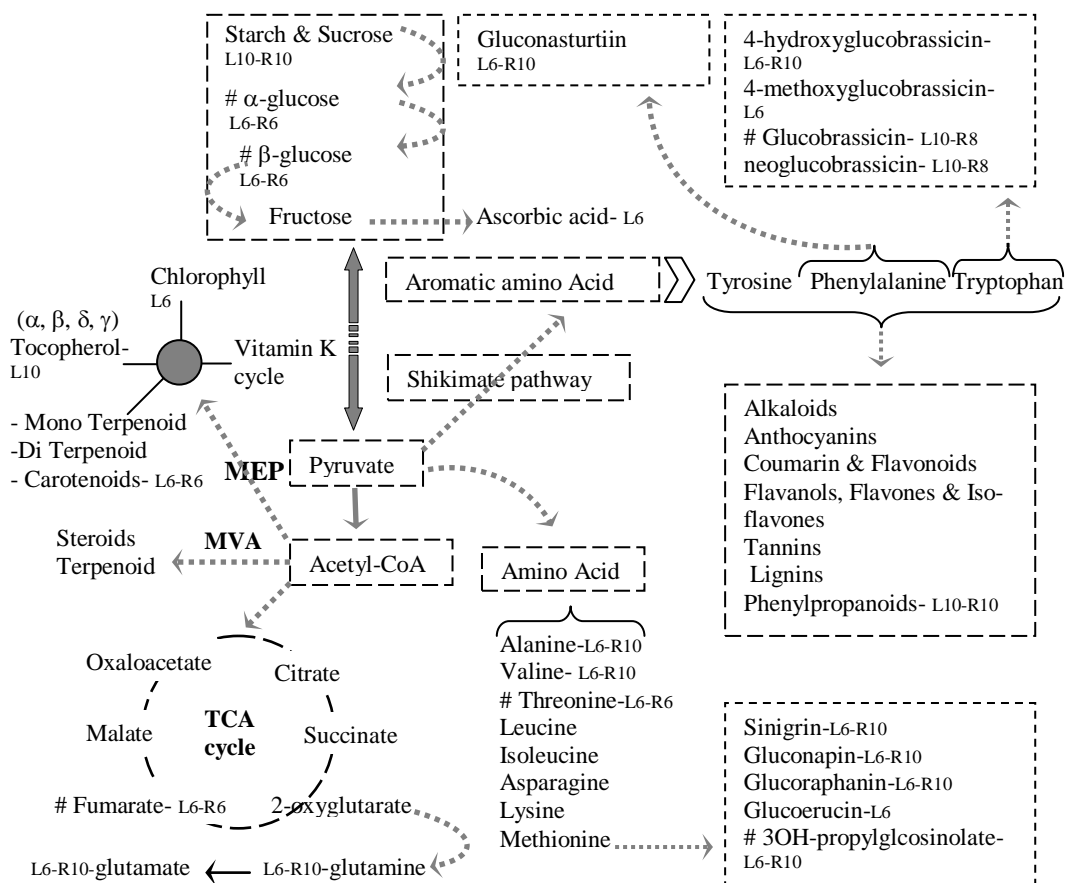


Figure 5. Change in metabolite quantities during plant growth. L6 = maximum in 6 weeks old plants leaves; L10 = maximum in 10 weeks old plant leaves; R6= maximum in 6 weeks old plant roots; R8 = maximum in 8 week old plant roots; R10 = maximum in 10 weeks old plant roots. # = The exception of the fumarate, threonine and glucose are higher in 8 weeks old roots in case of *Brassica rapa* while glucobrassicin and 3-hydroxypropylglucosinolate 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.

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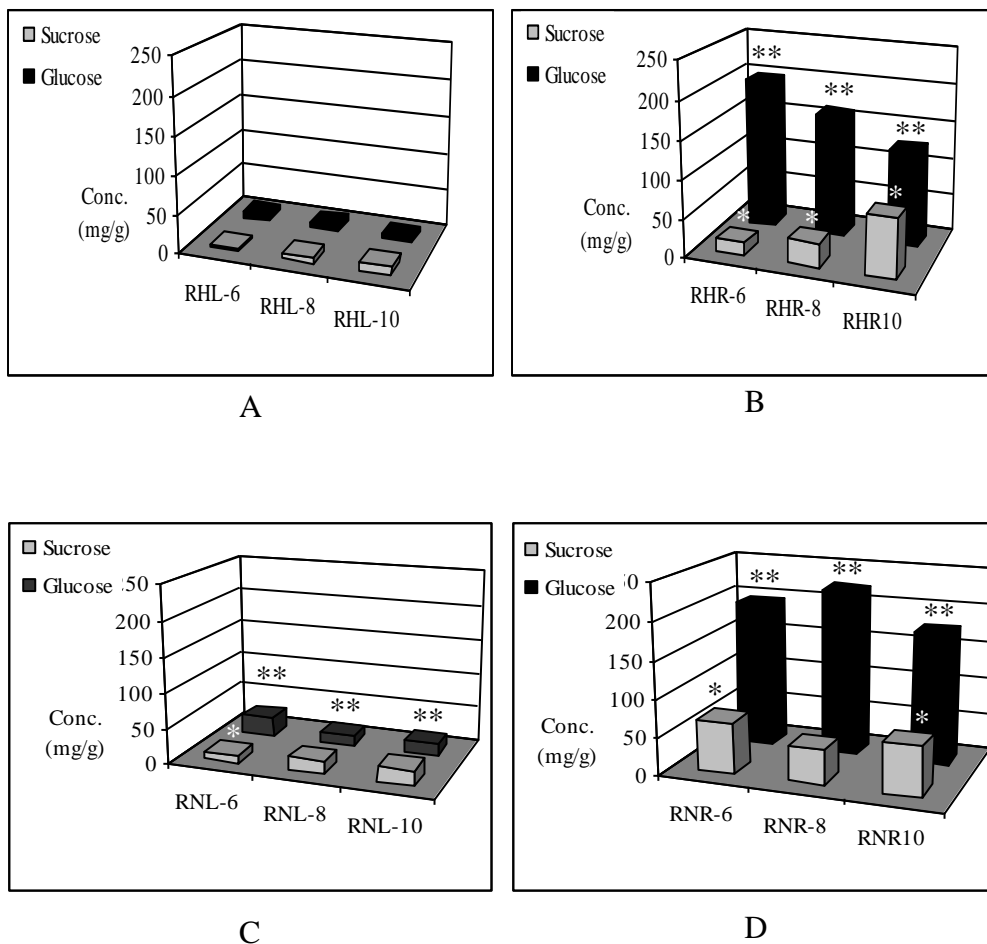


Figure 6. Concentration of sucrose and glucose (mg/g of dry weight) in radish leaves (A), radish roots (B), raapstelen leaves (C) and raapstelen roots (D) at different developmental stages. RHL = Radish leaves; RHR = Radish roots; RNL = Raapstelen leaves; RNR = Raapstelen roots; 6 weeks old plant (6);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

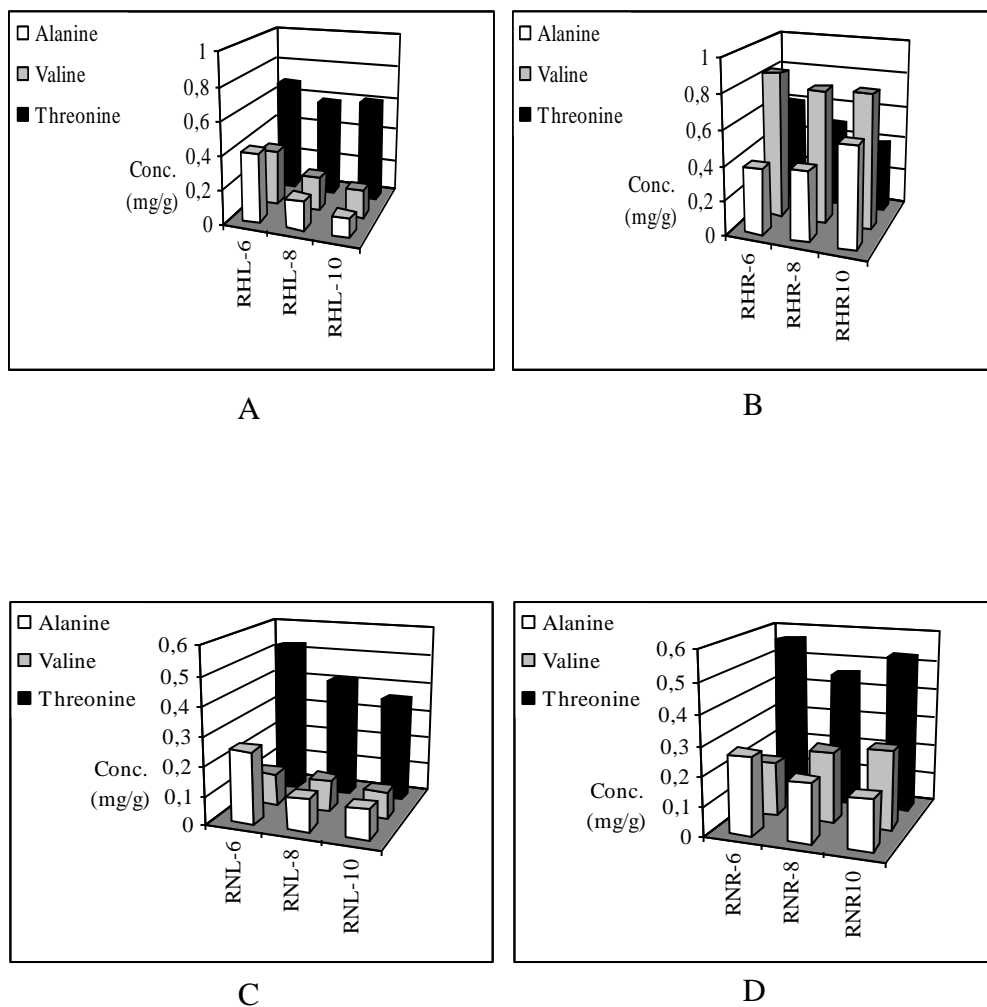
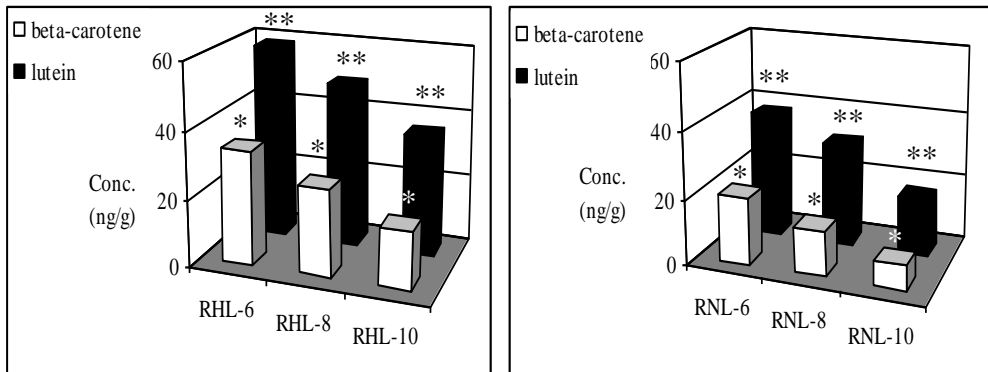


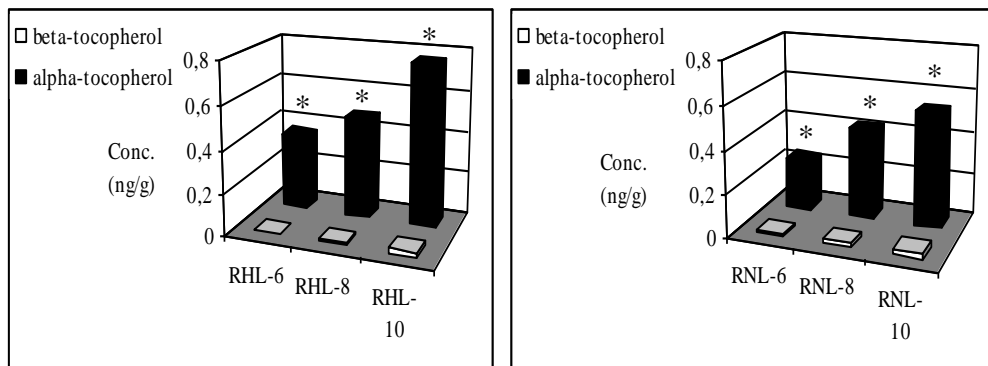
Figure 7. Concentration of alanine, valine and threonine (mg/g of dry weight) in radish leaves (A), radish roots (B), raapstelen leaves (C) and raapstelen roots (D) at different developmental stages. RHL = Radish leaves; RHR = Radish roots; RNL = Raapstelen leaves; RNR = Raapstelen roots; 6 weeks old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



A

B

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 ($P < 0.001$).



A

B

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 ($P < 0.001$).