Stress response and health affecting compounds in Brassicaceae

Jahangir, M.

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 °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]
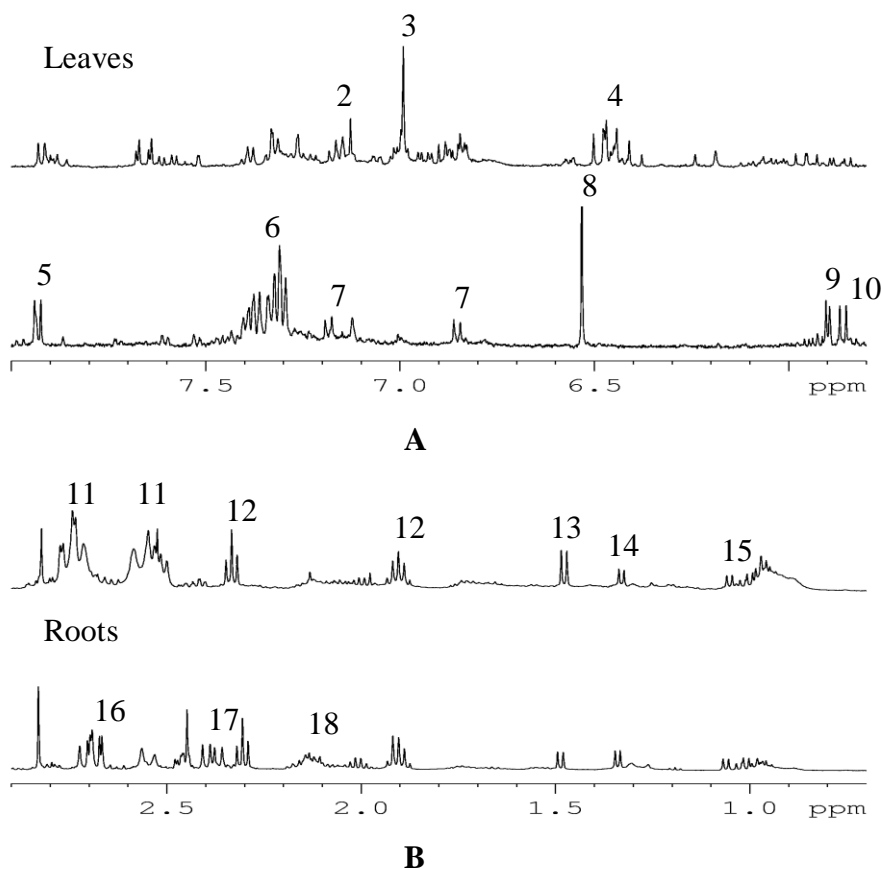
Figure 1 – ^{1}H NMR spectra of *Brassica rapa* leaves and roots in aromatic (A) and amino acid region (B). 1; H-7' of phenylpropanoids, 2; phenolic signal of caffeoyl malate, 3; phenolic signal of sinapoyl malate, 4; H-8' of phenylpropanoids, 5; a cytosine analogue, 6; phenylalanine, 7; tyrosine, 8; 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.
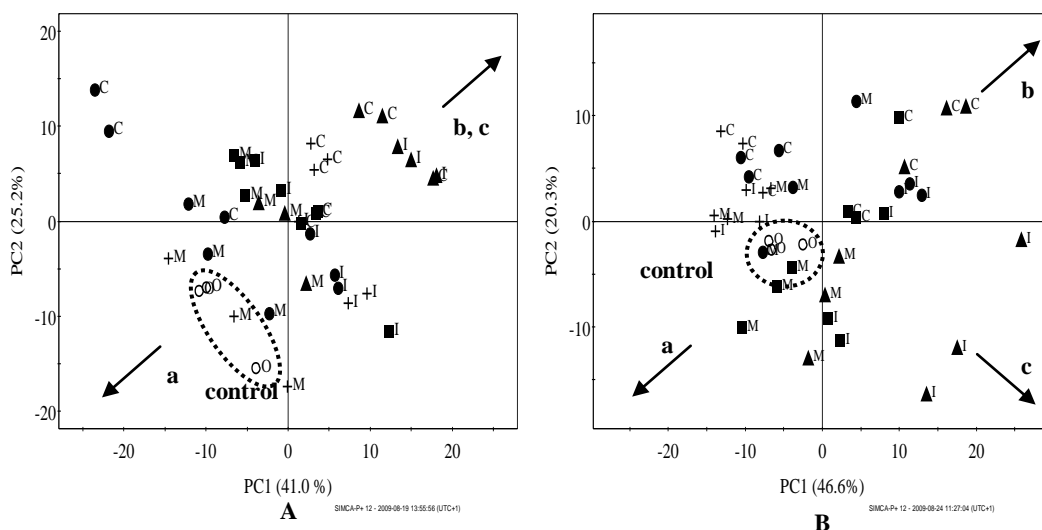

Figure 2 – Score plot (PC1 vs PC2) of PCA for *Brassica rapa* leaves (A) and roots (B), based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0). Control (O), Cu (C), Fe (I), Mn (M); Control (black-open circle); 50 mmol (●); 100 mmol (▲); 250mmol (+); 500 mmol (■). **a** = glucose, sucrose; **b** = 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.
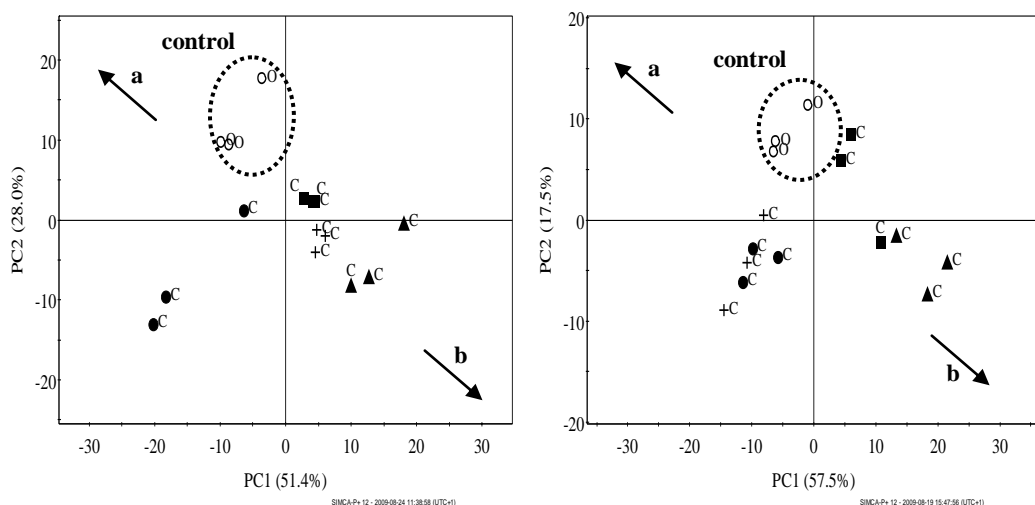
Figure 3 – Score plot (PC1 vs PC2) of PCA for *Brassica rapa* leaves (A) and roots (B), based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0). Control (O), Cu (C); Control (black-open circle); 50 mmol (●); 100 mmol (▲); 250mmol (+); 500 mmol (■). **a** = glucose, sucrose; **b** = progoitrin, gluconapoleiferin, glucobrassicanapin, fumarate, serine; **c** = alanine, threonine, valine, glutamate, glutamine, feruloyl malate, sinapoyl malate, 5-hydroxyferuloyl malate, caffeoyl malate.
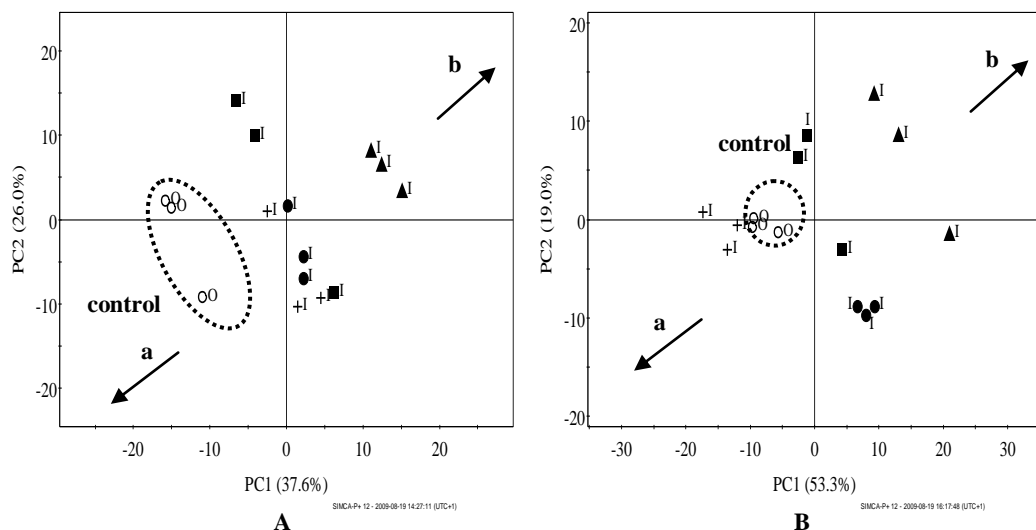

Figure 4 – Score plot (PC1 vs PC2) of PCA for *Brassica rapa* leaves (A) and roots (B), based on whole range of ^{1}H NMR signals (δ 0.3 – δ 10.0). Control (O), Fe (I); Control (black-open circle); 50 mmol (●); 100 mmol (▲); 250mmol (+); 500 mmol (■). **a** = glucose, sucrose; **b** = alanine, threonine,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

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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.
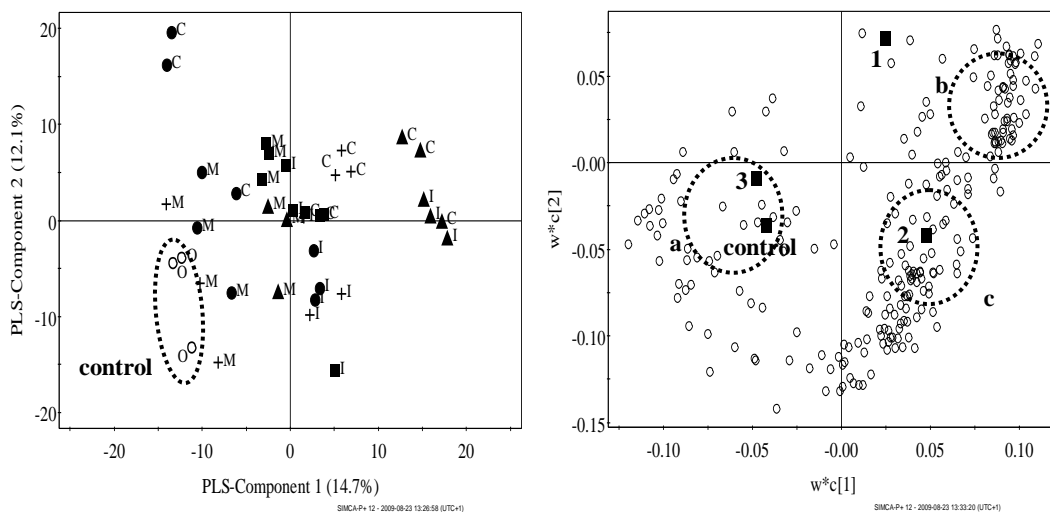
Figure 5 – Score plot (PLS-Component 1 vs PLS-Component 2) (A) (Control – **O**; Cu – **C**; Fe – **I**; Mn – **M**); Control (black-open circle); 50 mmol (●); 100 mmol (▲); 250mmol (+); 500 mmol (■); and loading plot (B) (Control, Cu – **1**; Fe – **2**; Mn – **3**) of PLS-DA for *Brassica rapa* leaves, 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.
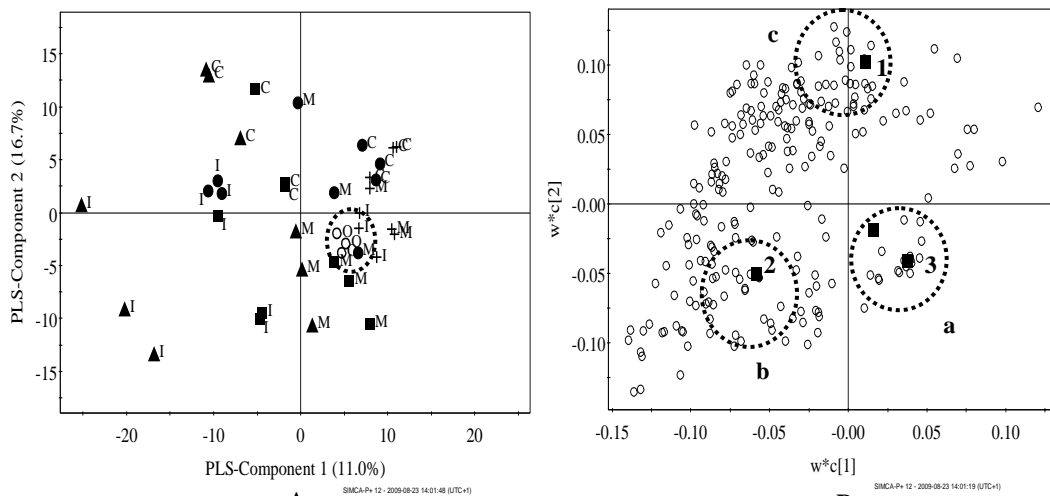

Figure 6 – Score plot PLS-Component 1 vs PLS-Component 2) (A) (Control – **O**; Cu – **C**; Fe – **I**; Mn – **M**); Control (black-open circle); 50 mmol (●); 100 mmol (▲); 250mmol (+); 500 mmol (■); and loading plot (B) (Control, Cu – **1**; Fe – **2**; Mn – **3**) of PLS-DA for *Brassica rapa* roots, 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.

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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.