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METABOLOMICS - USEFUL TOOL FOR STUDY OF PLANT RESPONSES TO ABIOTIC STRESSES

Abstract: Abiotic stresses are produced by inappropriate levels of physical components of the environment and cause plant injury through unique mechanisms that result in specific responses. Metabolomics is a relatively new approach aimed at improved understanding of metabolic networks and the subsequent biochemical composition of plants and other biological organisms. The paper is focused on the use of metabolomics, metabolic profiling and metabolic fingerprinting to study plant responses to some environmental stresses (e.g., elevated temperature, chilling and freezing, drought, high salinity, UV radiation, high ozone levels, nutrient deficiency, oxidative stress, herbicides and heavy metals). Attention is also devoted to the effects of some environmental factors on plants such as high or low levels of CO_{2} or different levels of irradiance. Alterations of plants metabolites due to multiple abiotic stresses (drought-heat, drought-salinity, elevated CO_{2}-salinity) are analysed as well. In addition, metabolomic approach to study plant responses to some artificial abiotic stresses, mechanical stress or pulsed electric field-induced stress is discussed. The most important analytical methods applied in metabolomics are presented and perspectives of metabolomics exploitation in the future are outlined, too.

Keywords: abiotic stress factors, environmental stress, metabolic fingerprinting, metabolic profiling, metabolomics

Introduction to metabolomics

Metabolites are the end products of cellular regulatory processes and their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes. The set of metabolites synthesized by a biological system constitute its “metabolome” [1]. The metabolome has been defined as the qualitative and quantitative collection of all low molecular weight molecules (metabolites) present in a cell that are participants in general metabolic reactions and that are required for the maintenance, growth and normal function of a cell [2].

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Metabolomics is a relatively new approach aimed at improved understanding of metabolic networks and the subsequent biochemical composition of plants and other biological organisms. It represents a non-biased identification and quantification of all metabolites in a biological system, and consequently the selectivity and sensitivity of the analytical technique must be high [2]. Analytical tools within metabolomics including mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy can determine the impact of time, stress, nutritional status and environmental perturbation on hundreds of metabolites simultaneously resulting in massive, complex data sets [3]. The most widely used methods for plant metabolite analysis are gas chromatography coupled to mass spectrometry (GC-MS) [4, 5] and liquid chromatography coupled to mass spectrometry (LC-MS) [6-8], further important analytical techniques include liquid chromatography (photodiode array detection) coupled to mass spectrometry (LC-PDA/MS) [9], capillary electrophoresis coupled to mass spectrometry (CE-MS) [10-12], Fourier transform - ion cyclotron resonance mass spectroscopy (FT-ICR/MS) [13] and NMR spectroscopy [14-16].

The term “metabolic profiling” is used for identification and quantification of a selected number of pre-defined metabolites, generally related to a specific metabolic pathway(s). On the other hand, metabolic fingerprinting represents a high-throughput, rapid, global analysis of samples to provide sample classification, in which quantification and metabolic identification are generally not employed, but such screening enables to discriminate between samples of different biological status or origin. Qualitative and quantitative analysis of one or a few metabolites related to a specific metabolic reaction employed when low limits of detection are required is called metabolite target analysis (MTA) [2].

The plant kingdom has been estimated to produce up to 200,000 metabolites in total [1, 17]. The plant metabolome contains organic species - such as amino acids, fatty acids, carbohydrates, organic acids and lipids and elemental and inorganic species. The differences in the concentration of components in the metabolome are estimated to vary from picomol to milimol [2]. For example, leaves of one of the most studied plants Arabidopsis thaliana have been estimated to contain approximately 5000 different primary and secondary metabolites [18].

Plant biologists routinely use comprehensive analyses of plant metabolites to discover new responses to genetic or environmental perturbation or to validate initial hypotheses on the function and in vivo action of gene products. Fiehn et al [19] suggested a minimum of parameters to be reported in order to define details of experimental study designs in plant metabolomics studies. PlantMetabolomics.org (PM) is a web portal and database for exploring, visualizing and downloading plant metabolomics data. PM integrates metabolomics data generated from different analytical platforms from multiple laboratories along with the key visualization tools such as ratio and error plots. Each metabolite is linked to relevant experimental data and information from various annotation databases (for details see Bais et al [20]).

**Analytical methods used in plant metabolomics**

Plants contain a wide spectrum of chemically diverse metabolites which are usually present in a large range of concentrations, and so for extracting and detecting all metabolites no single analytical method is sufficient. For large scale analysis and comparison of
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metabolites in plant extracts several methods are suitable, such as GC-MS [4, 21, 22], direct flow injection mass spectrometry [23], LC-MS [24, 25], CE-MS [10] and NMR [14, 26].

Many semi-polar compounds in plants (involved or not involved in the primary metabolism), including key secondary metabolite groups, can be best separated and detected by LC-MS approaches [3, 24, 25, 27].

LC preceding MS enables the detection of isomeric compounds, and LC in combination with high resolution mass spectrometry (HRMS), eg TOF-MS, FT-ICR-MS, Orbitrap, enables the detection of several hundreds of compounds in a single crude plant extract [8, 25]. Further improvement of the potential to identify metabolites and to provide an even more detailed metabolite profile of plant extracts could be obtained by combining LC with ultra-HRMS such as FT-ICR-MS [28, 29] or LC-NMR-MS [30, 31] or ultra performance LC (UPLC) coupled to MS [32, 33].

GC coupled to electron-impact time-of-flight (TOF)-MS can be applied for a large variety of non-volatile metabolites, mainly those involved in primary metabolism (organic and amino acids, sugars, sugar alcohols, phosphorylated intermediates occurring in the polar fraction of extracts, as well as lipophilic compounds such as fatty acids and sterols which are situated in the apolar fraction) [21].

A suitable method for analysing semi-polar metabolites is LC-MS with a soft-ionisation technique, such as electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI), resulting in protonated (in the positive mode) or deprotonated (in the negative mode) molecular masses. Using this method plant secondary metabolites such as alkaloids, saponins, phenolic acids, phenylpropanoids, flavonoids, glucosinolates, polyamines and all kinds of derivatives thereof could be detected [24, 25, 34].

Different metabolomics approaches can be applied when using NMR. Using this method high-throughput metabolite analyses could be performed. Sample preparation is relatively simple, the analysis is non-destructive, and spectra can be recorded in vivo from cultured cells and tissues and even whole plants [35, 36]. On the other hand, the sensitivity of NMR is relatively low, about 106-109-fold less than that of chromatography-coupled MS [37]. However, at high metabolite concentrations NMR-based metabolic fingerprinting is usually effective, as it can provide both structural and quantitative data and does not require much pre-analytical preparation as mass spectrometry needs.

For most bioorganic compounds, the 1D 1H NMR spectrum is not sufficient for full structural elucidation. Two-dimensional (2D) NMR takes advantage of interactions between detectable nuclei within a molecule and can be used to increase the spectral resolution. Homonuclear NMR examines correlations between nuclei of the same type (usually protons), whereas heteronuclear NMR reveals correlations between two different nuclei (eg 1H and 13C). A variation of 2D NMR used in metabolomics is J-resolved spectroscopy providing 1D spectra simplified by the absence of proton-proton coupling [38].

Homonuclear 1H-2D spectra, eg correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) are very informative about the 3D position of the protons in a molecule, and heteronuclear 2D spectra can be acquired for detecting direct 1H–13C bonds by heteronuclear multiple quantum coherence spectroscopy (HMQC) or, over a longer range, by heteronuclear multiple bond correlation spectroscopy (HMBC). Heteronuclear 2D-NMR data acquisition is extremely useful for identification of unknown molecules [39].
One of the characteristics of metabolomics data which ensures that the dataset is inherently complex is multidimensionality. Widely applied supervised and unsupervised tests in metabolomics include principal component analysis (PCA), hierarchical cluster analysis (HCA), and partial least squares (PLS) and discriminant analysis (DA) [40-42]. These methods not only simplify the data by reducing dimensionality but also provide visual representation of the data. The development of bioinformatics tools will facilitate the management of large amounts of data, help to integrate different datasets by sieving the metabolite information from the instrumental chromatographs and spectra and improve the description of metabolic networks and cellular phenomena in general [42].

**Abiotic stresses**

Environmental stresses arise from conditions that are unfavourable for the optimal growth and development of organisms [43, 44]. Abiotic stresses are produced by inappropriate levels of physical components of the environment and cause injury through unique mechanisms that result in specific responses [43, 45]. Global metabolite profiling analysis permits simultaneous monitoring of precursors, intermediates and products of metabolic pathways. Stress responses of plants at the metabolite and metabolome levels were studied by several researchers, eg [46, 47]. Plant responses to abiotic stresses were comprehensively summarised in review papers of Shulaev et al [48] and Genga et al [49]. This paper is aimed at the use of metabolomics, metabolic profiling and metabolic fingerprinting to study plant responses to environmental stresses such as elevated temperature, chilling and freezing, drought, high salinity, UV radiation, high ozone levels, nutrient deficiency, oxidative stress, herbicides and heavy metals. The article also deals with some environmental factors such as high or low levels of CO$_2$ or different irradiance levels as well as multiple stresses, eg drought-heat, drought-salinity, elevated CO$_2$-salinity. In addition, metabolomic approach to study of plant responses to some artificial abiotic stresses, mechanical stress or pulsed electric field-induced stress is discussed.

**Temperature stress**

Temperature stresses experienced by plants can be classified into three types: those occurring at (a) temperatures below freezing, (b) low temperatures above freezing and (c) high temperatures [50]. Temperature stress can have a devastating effect on plant metabolism, disrupting cellular homeostasis and uncoupling major physiological processes. Cellular homeostasis is achieved by a delicate balance between multiple pathways that reside in different organelles. However, it may be disrupted by temperature stress, because different pathways within cells have a different temperature optimum [51]. Higher plants exposed to excess heat, at least 5°C above their optimal growing conditions, exhibit a characteristic set of cellular and metabolic responses required for the plants to survive under the high-temperature conditions [44]. One of the consequences of high temperature in plants is oxidative damage caused by the heat-induced imbalance of photosynthesis and respiration [52]. Due to temperature acclimation changes in membrane structure and function, tissue water content, global gene expression, protein, lipid and primary and secondary metabolite composition occur [43, 45].

Several metabolites that could functionally contribute to induced stress tolerance have been associated with stress responses and therefore recent metabolite-profiling studies have
refocused attention on these and other potentially important components found in the “temperature-stress metabolome”. Such metabolomic studies have demonstrated that active reconfiguration of the metabolome is regulated in part by changes in gene expression initiated by temperature-stress-activated signalling and stress-related transcription factors and central carbohydrate metabolism seems to be a major feature of the reprogramming of the metabolome during temperature stress [53].

Changes in membrane lipid composition play multiple roles in plant adaptation and survival in the face of chilling and freezing damage. Wang et al [54] used an ESI-tandem MS (MS/MS)-based approach to quantitatively profile membrane lipid molecular species in plant response to low temperatures, and the profiling analysis revealed significant and distinct lipid changes during cold acclimation and freezing. Welti et al [55] profiled membrane lipid molecular species in Arabidopsis undergoing cold and freezing stresses using ESI-MS/MS and found that freezing at a sublethal temperature induced a decline in many molecular species of phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol but induced an increase in phosphatidic acid and lysophospholipids, what resulted in destabilizing membrane bilayer structure.

Gray et al [56] examined the effects of cold acclimation on the Arabidopsis metabolome using a non-targeted metabolic fingerprinting approach and found that a global reprogramming of metabolism occurs as a result of cold acclimation. They determined a comprehensive, unbiased assessment of metabolic processes relative to cold acclimation by measuring an entire spectrum of putative metabolites based on mass-to-charge (m/z) ratios, vs an individual or group of metabolite(s). The leaves shifted to low temperature presented metabolic profiles that were constantly changing but leaves developed at low temperature demonstrated a stable complement of components.

Kaplan et al [45] explored the temperature-stress metabolome of Arabidopsis and found that cold shock influenced metabolism far more profoundly than heat shock. The steady-state pool sizes of 143 and 311 metabolites or mass spectral tags were altered in response to heat and cold shock, respectively, and comparison of heat- and cold-shock response patterns revealed that the majority of heat-shock responses were shared with cold-shock responses. Coordinate increases in the pool sizes of amino acids derived from pyruvate and oxaloacetate, polyamine precursors and compatible solutes were observed during both heat and cold shocks. However, it could be highlighted that many of the metabolites that showed increases in response to both heat and cold shocks were previously unlinked with temperature stress. Similar results were obtained by Korn et al [57] who used metabolic profiling to discover combinations of metabolites that predict freezing tolerance and its heterosis in Arabidopsis thaliana. They identified compatible solutes and, in particular, the pathway leading to raffinose as crucial statistical predictors for freezing tolerance and its heterosis, while some tricarboxylic acid (TCA) cycle intermediates contributed only to prediction of the heterotic phenotype.

Davey et al [58] used seeds of Arabidopsis lyrata ssp. petraea obtained from populations along a latitudinal gradient, namely Wales, Sweden and Iceland and grown in a controlled cabinet environment to study whether geographically separated marginal populations of A. lyrata have distinct metabolic phenotypes associated with exposure to cold temperatures. Mannose, glucose, fructose, sucrose and raffinose concentrations were different between cold treatments and populations, especially in the Welsh population, but polyhydric alcohol concentrations were not. Free amino acid compositions were
population-specific, with fold differences in most amino acids, especially in the Icelandic populations, with gross changes in amino acids, particularly those associated with glutamine metabolism.

Salt stress

High salinity caused by either natural (e.g., climatic changes) or anthropogenic factors (e.g., agriculture) is a widespread environmental stressor that can affect development and growth of salt-sensitive plants, leading to water deficit, the inhibition of intake of essential ions and metabolic disorders. Application of NMR-based metabolomics to the investigation of salt stress in 2, 4 and 6 days old maize (*Zea mays*) plants exposed to 0, 50 or 150 mmol/dm$^3$ saline solution was performed by Gavaghan et al [59]. The shoot and root extracts were analysed and a clear dose-dependent effect, superimposed on the growth effect, was observed for saline treated shoot and root extracts. This was correlated with increased levels of alanine, glutamate, asparagine, glycine-betaine and sucrose and decreased levels of malic acid, *trans*-aconitic acid and glucose in shoots. Correlation with salt-load shown in roots included elevated levels of alanine, *γ*-amino-*N*-butyric acid (GABA), malic acid, succinate and sucrose and depleted levels of acetoacetate and glucose. The metabolic effect of high salinity was predominantly consistent with osmotic stress and was found to be stronger in the shoots than in the roots.

Zhang et al [60] analyzed the metabonome (the complete set of metabolically regulated elements in cells) of tobacco plants and its dynamic responses to salt treatments using NMR spectroscopy in combination with multivariate data analysis in order to understand the dosage and duration dependence of salinity effects on plant metabolisms. The tobacco metabonome was dominated by 40 metabolites including organic acids/bases, amino acids, carbohydrates and choline, pyrimidine and purine metabolites. A dynamic trajectory was clearly observable for the tobacco metabonomic responses to the dosage of salinity. Short-term low-dose salt stress (50 mmol/dm$^3$ NaCl, 1 day) caused metabolic shifts toward gluconeogenesis with depletion of pyrimidine and purine metabolites. Prolonged salinity with high-dose salt (500 mmol/dm$^3$ NaCl) induced progressive accumulation of osmolytes, such as proline and myo-inositol as well as changes in GABA shunt. Such treatments also promoted the shikimate-mediated secondary metabolisms with enhanced biosynthesis of aromatic amino acids. Salinity caused systems alterations in widespread metabolic networks involving transamination, TCA cycle, gluconeogenesis/glycolysis, glutamate-mediated proline biosynthesis, shikimate-mediated secondary metabolisms and the metabolisms of choline, pyrimidine and purine.

Ghosh et al [61] investigated the responses of two rice varieties (Nonabokra and Pokkali) to salinity in terms of some physiological and biochemical attributes. During the exposure to salinity (200 mmol/dm$^3$ NaCl for 24, 48 and 72 h), a significant increase in sodium was recorded which was also concomitant with the changes of other metabolic profiles like proline, phenol, polyamine, etc. The protein oxidation was significantly increased and also varied between the two cultivars, and the changes in activities of antioxidative enzymes under stress were significantly different from the control. The detrimental effects of salinity were also evident in terms of lipid peroxidation, chlorophyll content, protein profiles and generation of free radicals, and these were more pronounced in Pokkali than in Nonabokra.
Lugan et al [62] studied the relationships between stress tolerance, the metabolome, water homeostasis and growth performance using two species - a halophyte *Thellungiella salsuginea* and *Arabidopsis thaliana* which were exposed to osmotic stress. They analysed a broad range of metabolites by metabolic fingerprinting and profiling and the results showed that, despite a few notable differences in raffinose and secondary metabolites, the same metabolic pathways were regulated by salt stress in both species. However, *Thellungiella* had much higher levels of most metabolites than *Arabidopsis* whatever the treatment. Comprehensive quantification of organic and mineral solutes showed a relative stability of the total solute content regardless of the species or treatment, meaning that little or no osmotic adjustment occurred under stress, and the reduction in osmotic potential observed in plants under stress was found to result from a passive loss of water. Differences were observed in shoot water content of studied plant species. *Thellungiella* shoots contained less water than *Arabidopsis* shoots and had the ability to lose more water, which could contribute to maintenance of a water potential gradient between soil and the plant. Osmotic stress was also found to impact the metabolome properties in both species, increasing the overall polarity. In general, the *Thellungiella* metabolome appears to be more compatible with dehydration. Under salt stress *Thellungiella* plants accumulated lower amounts of Cl⁻ ions than *Arabidopsis* but exhibited a similar proline response [63]. Under these conditions abietic acid and jasmonic acid levels increased in *Arabidopsis*, whereas minimal changes in both hormone concentrations were recorded in *Thellungiella*. It was found that the impact of salt stress on metabolite profiles was higher in *Thellungiella* than in *Arabidopsis*, and obtained data indicated that physiological responses in *Arabidopsis* are induced after stress imposition through hormonal regulation whereas *Thellungiella* has a basal metabolic configuration better prepared to endure environmental cues.

**Water stress**

Drought, the most prominent threat to agricultural production worldwide, accelerates leaf senescence, leading to a decrease in crops size, loss in photosynthesis and reduced yields, and it is the most serious environmental factor limiting the productivity of agricultural crops worldwide with devastating economical and sociological impact [64].

Water deficits and nutrient deficiencies promote greater relative allocation of photosynthate to root growth, ultimately resulting in plants that have higher root:shoot ratios and greater capacity to absorb water and minerals relative to the shoots that must be supported. Plants experience drought stress when water supply to roots is decreased and transpiration rate is higher than water uptake. Water deficit causes many adverse effects on plants, eg disruption of water potential gradients, loss of turgor, denaturation of proteins or disruption of membranes [65]. Drought is one of the major factors that limit crop production. Plants respond to drought stress at the molecular, cellular and physiological levels, whereby the response depends on the species and genotype, the length and severity of water loss, the age and stage of development, the organ and cell type and the sub-cellular compartment [66].

Sziderics et al [67] observed that drought reduced yield of pepper plants (*Capsicum annuum* L) cultivated in a greenhouse which were stressed by withholding water for 1 week. This indicated that plants adapted to the decreasing water content of the soil by adjustment of their osmotic potential in root tissue. As a consequence of drought, strong accumulation of raffinose, glucose, galactinol and proline as well as reduced concentration of polyamines
in the roots was determined. On the other hand, in leaves of pepper plants the water deficit led to increased levels of fructose, sucrose, galactinol, cadaverine, putrescine, spermidine and spermine. Sensitive cultivars of barley accumulated more proline, glycine betaine and other compatible solutes than tolerant cultivars [68, 69].

The accumulation of small molecules which function as compatible solutes has long been implicated in the acquisition of drought tolerance. Sanchez et al [70] used a non-targeted metabolomic approach to explore plastic systems responses to non-lethal drought in model and forage legume species of the genus *Lotus*. In *Lotus japonicus* increased water stress caused gradual increases of most of the soluble small molecules profiled, reflecting a global and progressive reprogramming of metabolic pathways. The comparative metabolomic approach between *Lotus* species revealed conserved and unique metabolic responses to drought stress. As a general metabolic trend in response to water deficit, significant increases of organic acids, sugars and polyols were determined. Organic acids included the TCA cycle intermediates succinic and malic acid, while fructose, glucose, galactose and maltose and arabitol, ononitol and galactitol were amongst the most accumulated sugars and polyols, respectively. The concentration of glutamic, aspartic and phosphoric acids decreased during drought. On the other hand, amino acids showed a variable response: the content of proline, leucine and isoleucine increased, while serine, glycine and threonine decreased and some amino acids (eg asparagine, lysine and valine) showed no significant change. In general, tolerant plants accumulate higher levels of compatible solute and the level of accumulated solutes and primary metabolites may reflect a gradual global metabolic rearrangement which appears to be adjusted to the stress-dose perceived by the plants [71].

Warren et al [72] used GC-MS metabolite profiling to examine the response of leaf metabolites to a long (2 months) and severe water stress in two species of the perennial trees of the genus *Eucalyptus* (the mesic *Eucalyptus pauciflora* and the semi-arid *Eucalyptus dumosa*). Water stress led to more negative osmotic potential and increased total osmotically active solutes in leaves of both species, whereby it affected around 30–40% of measured metabolites in *E. dumosa* and 10–15% in *E. pauciflora*. There were many metabolites that were affected in *E. dumosa* but not in *E. pauciflora*. For example, in *E. dumosa* there were increases in five acyclic sugar alcohols and four low abundance carbohydrates that were unaffected by water stress in *E. pauciflora*. In *E. dumosa* water stress increased the levels of galactose, fructose, glucose, monosaccharides, sugar acids and acyclic sugar alcohols derived from galactose, fructose, glucose, however reduction of compounds that are part of the raffinose/stachyose biosynthetic pathway, galactinol (threefold decrease), myo-inositol (1.7-fold decrease) and raffinose (2.5-fold decrease), was determined. However, notable increase was shown by cyclohexanepentols: vibo-quercitol (23-fold increase) and proto-quercitol (1.5-fold increase).

Charlton et al [73] profiled leaf metabolome of *Pisum sativum* L (pea) using 1D and 2D NMR spectroscopy, to monitor the changes induced by drought-stress, under both glasshouse and simulated field conditions. They attributed significant changes in resonances to a range of compounds, identified as both primary and secondary metabolites, highlighting metabolic pathways that are stress-responsive. The metabolites that were present at significantly higher concentrations in drought-stressed plants under all growth conditions included proline, valine, threonine, homoserine, myo-inositol, GABA and trigonelline (nicotinic acid betaine).
The increase of the content of greenhouse gases in the atmosphere due to anthropogenic activity is connected with the increase in global temperature, altered precipitation and increased CO$_2$ concentrations [74, 75]. Productive and sustainable agriculture requires growing plants (crops) in arid and semiarid regions with less input of precious resources such as fresh water. Shao et al [76] in their review paper discuss physiological and molecular insights and effects on basic plant metabolism, drought tolerance strategies under drought conditions in higher plants for sustainable agriculture and ecoenvironment in arid and semiarid areas of the world.

**Flooding**

Flooding imposes a severe selection pressure on plants principally because excess water in their surroundings can deprive them of certain basic needs, notably of oxygen and of CO$_2$ and light for photosynthesis [77]. Flooding is a serious problem for soybeans because it reduces growth and grain yield. Proteomic and metabolomic techniques were used to examine whether mitochondrial function is altered in soybeans by flooding stress [78]. Proteins and metabolites related to the TCA cycle and GABA shunt were up-regulated, the amounts of NADH and NAD were increased, but ATP was significantly decreased by flooding stress.

**UV light**

Because of ever-increasing environmental deterioration it is likely that the influx of ultraviolet B (UV-B) radiation ($\lambda = 280\text{-}320$ nm) will increase as a result of the depletion of stratospheric ozone. The UV-B radiation impacts on the levels of a broad range of metabolites, including phenolic, terpenoid and alkaloid compounds. The levels of some of these metabolites increase following UV-B exposure, while those of others decrease, change transiently or are differently affected by low and high UV-doses [79]. Kusano et al [80] compared the metabolic responses of wild-type *Arabidopsis* with that of mutants impaired in flavonoid or sinapoyl-malate biosynthesis, exposed to a short (24 h) or a longer (96 h) exposure to photo-oxidative stress using UV-B light. From evaluation of the dynamic response of metabolites including flavonoids, sinapoyl-malate precursors and ascorbate (which are well known to play a role in cellular protection from UV-B stress) as well as a broader range of primary metabolites it could be concluded that short-term responses occur only at the level of primary metabolites, suggesting that these effectively prime the cell to facilitate the later production of UV-B-absorbing secondary metabolites.

By means of *in vivo* NMR spectroscopy Marangoni et al [81] determined the metabolic profile of living *Fabrea salina* cells exposed to visible light and to polychromatic UV-B + UV-A + VIS radiation for several different exposure times and discovered some metabolites whose concentration changed specifically upon UV exposure and in a dose-dependent manner. In the first step the concentration of formate, acetate and saturated fatty-acid metabolites was altered, whereas the osmoprotection modified the activity of betaine moieties and other functionally related metabolites. In the latter pathway, alanine, proline and sugars suggested a possible incipient protein synthesis as defence and/or degeneration mechanisms.

Global metabolic profiling of 'Granny Smith' apple peel was employed for evaluating metabolomic alterations resulting from prestorage irradiation by UV-white light. The profile, including more than 200 components, 78 of which were identified, revealed changes
in the metabolome provoked by UV-white light irradiation and cold storage, distinct temporal changes, before and after cold storage, related to prestorage irradiation in a diverse set of primary and secondary metabolic pathways. The results demonstrated that metabolic pathways associated with ethylene synthesis, acid metabolism, flavonoid pigment synthesis and fruit texture were altered by prestorage irradiation, whereby many of the alterations were detectable after 6 months of cold storage in air [82].

**Excessive or low irradiance**

The different cultivation methods affect tea quality by altering the basic metabolite profiles. The metabolomic approaches could be applied in the study of the effect of cultivation methods on chemical composition in plants and the relationship with antioxidant activity.

Ku et al [83] investigated the metabolome changes in green tea and shade cultured green tea (tencha) by LC-MS and GC-MS coupled with a multivariate data set and found that green tea clearly showed higher levels of galloylquinic acid, epigallocatechin, epicatechin, succinic acid and fructose together with lower levels of gallocatechin, strictinin, apigenin glucosyl arabinoside, quercetin \( p \)-coumaroylglucosylrhamnosyl-galactoside, kaempferol \( p \)-coumaroylglucosylrhamnosylgalactoside, malic acid and pyroglutamic acid than tencha. In addition, green tea showed stronger antioxidant activity than tencha, indicating that the antioxidant activity of green tea samples were significantly correlated with their total phenol and total flavonoid contents.

Miyagi et al [84] analyzed the metabolic pathway of *Rumex obtusifolius* L, destructive weed worldwide, which accumulates major organic acids such as oxalate in leaves and citrate in stems, in relation to major environmental factors (light and temperature). Light or dark experiments showed that in the case of the oxalate accumulation, the major or the most dominated pathway was found to be the citrate-isocitrate-oxalate shunt. Furthermore, under the dark and/or low temperature (5°C) leaves showed sustainable growth with normal accumulation of TCA metabolites but there was a different pattern of metabolite accumulation in stems and other metabolites (eg amino acids) also showed the organ specific alterations under the different ambient environments.

**Ozone**

Ozone \( (O_3) \), a serious air pollutant, is known to significantly reduce photosynthesis, growth and yield and to cause foliar injury and senescence. D’Haese et al [85] reported that 2-day \( O_3 \) treatment significantly upregulated synthesis of flavonoid (anthocyanin), thioredoxin, salicylic acid, auxin and gibberellin in *Arabidopsis thaliana* whereas synthesis of alkyl cinnamates, ethylene and jasmonate was downregulated. Cho et al [86] applied integrated transcriptomics, proteomics and metabolomics approaches to investigate the molecular responses to \( O_3 \) in the leaves of 2-week-old rice seedlings exposed to 0.2 ppm \( O_3 \) for a period of 24 h. Capillary electrophoresis-mass spectrometry-based metabolomic profiling revealed accumulation of amino acids, \( \gamma \)-aminobutyric acid and glutathione in \( O_3 \) exposed leaves until 24 h over control. The results showed that ozone triggers a chain reaction of altered gene, protein and metabolite expressions involved in multiple cellular processes in rice.

Metabolomics studies focused on the effects of ozone pollution on plant tissues, specifically on white birch (*Betula pendula*) in field conditions showed that 98 from out of
339 metabolites determined by GC-MS and HPLC-UV were associated with ozone treatments. The main ozone-caused changes included increases in quercetin-phenolic compounds and compounds related to leaf cuticular wax layer, whereas several compounds related to carbohydrate metabolism and function of chloroplast membranes and pigments (such as chlorophyll-related phytol derivatives) were decreasing [87].

Ossipov et al [88] performed an experiment with two birch genotypes (GT 2 and GT 5) that have been grown over seven years on the two open fields and for the study of biochemical responses of the GT 2 to elevated (1.5 x ambient) concentration of ozone they used metabolomics based on the analyses of lipophilic and polar compounds of birch leaves. The metabolome database included 331 chemical traits and was analyzed with descriptive and multivariate statistics. Application of cluster and principle component analyses clearly discriminated genetically different birch trees whereby biochemical discrimination between phenotypes of control and ozone-treated birch trees of GT 2 was found. Elevated concentration of ozone increased levels of some lipophilic compounds such as 1-dotriacontanol, squalene, octadecanoic acid derivative and pentacyclic triterpenoid 20,24-epoxy-dammaran-3-ol and decreased levels of tetramethyl-2-hexadecen-1-ol. In contrast, levels of polar compounds, such as organic acids (malic and quinic) and carbohydrates (arabinose, fructose and galactose) were decreased in response to ozone exposure. It was confirmed that treatment of birch trees by ozone increased the levels of chlorogenic acid and of three flavonoid-glycosides. However, the total content of flavonoid-glycosides and the content of chlorogenic acid were 1.4-fold higher in the leaves of the genotype sensitive to ozone (GT 5) than in the leaves of the ozone-tolerant genotype GT 2.  

**Pulsed electric field-induced stress**

Metabolite profiling was used to characterize stress responses of potato tissue subjected to reversible electroporation, using pulsed electric fields (PEF), which is an artificial stress. Wounded potato tissue was subjected to field strengths ranging from 200 to 400 V/cm, with a single rectangular pulse of 1 ms. Electroporation was demonstrated by propidium iodide staining of the cell nuclei. Metabolic profiling of data obtained through GC-(TOF)-MS and UPLC-(TOF)-MS complemented with orthogonal projections to latent structures clustering analysis showed that 24 h after the application of pulsed electric fields potato (PEF)-specific responses in the potato metabolism were characterized by the changes in the hexose pool that may involve starch and ascorbic acid degradation [89].

**Mechanical stress**

Ceoldo et al [90] applied a metabolomic approach followed by principal components and partial least square analysis for investigating the effect of environmental factors on two Daucus carota L cv. Flakkese cell lines (R3M and R4G), which had ability to produce anthocyanins in the light and the dark, respectively. A positive correlation between total anthocyanin, hydroxycinnamic and hydroxybenzoic acid accumulation was found in both lines. Moreover, mechanical stress (obtained by increasing flask agitation) induced overproduction of all anthocyanins, hydroxycinnamic and hydroxybenzoic acids except sinapic acid derivatives, whose accumulation was inhibited.

Strazzer et al [91] used a red basil (Ocimum basilicum) cell line (T2b) rich in rosmarinic acid for the stable production of anthocyanins in the dark. They subjected cell suspension cultures to mechanical stress through increased agitation (switch from 90
to 150 rpm) and analyzed the cell extracts by HPLC and LC-MS. It was found that mechanical stress increased the total anthocyanin and rosmarinic acid contents, but reduced biomass accumulation. Whereas many metabolites were induced by mechanical stress, including rosmarinic acid and some of its derivatives, most anthocyanins, hydroxycinnamic acids and flavonoids, the abundance of some rosmarinic acid dimers was reduced.

**High and low levels of CO₂**

Renberg et al [92] used GC-MS-TOF technique to determine major metabolite changes during induction of the carbon-concentrating mechanism in the unicellular green alga *Chlamydomonas reinhardtii*. In total, 128 metabolites with significant differences between high- and low-CO₂-grown cells were detected, of which 82 were wholly or partially identified, including amino acids, lipids and carbohydrates. It was shown that in a 24h time course experiment the concentration of amino acids serine and phenylalanine increased transiently, while the content of aspartate and glutamate decreased after transfer to low CO₂. Metabolomic examination comparing low-CO₂ treatment to high-CO₂ control that was performed at the 3h time point showed that the levels of five metabolites involved in photorespiration, 11 amino acids and one lipid increased, while the levels of six amino acids and, interestingly, 21 lipids were significantly lower. The results showed that the metabolic pattern during early induction of the carbon-concentrating mechanism fitted a model where photorespiration was increasing.

Miyagi et al [93] studied conversion of fixed carbon to oxalate using “new leaves”, ie, leaves removed from 2-month-old *Rumex obtusifolius* L plants grown under different environmental conditions. The results showed the mobilization of metabolites from stems to new leaves, where active TCA cycle and oxalate pathways occurred. These authors found significant changes in metabolite accumulation in *R. obtusifolius* plants exposed to high carbon dioxide level (1000 ppm) and nutrients (Hoagland’s formulation). The accumulation of most abundant metabolite oxalate in leaves was affected by both high CO₂ as the carbon source and nutrients and the results indicate that the common weed *R. obtusifolius* may proliferate in cultivated lands under high CO₂ level, a potential cause of global warming.

Levine et al [94] employed non-targeted and targeted metabolite profiling by GC-MS and LC-MS to examine primary and secondary metabolites in wheat (*Triticum aestivum*, cv Yocoro rojo) continuously exposed to suboptimal (400 ppm), near-optimal (1500 ppm) and supra-optimal (10,000 ppm) atmospheric CO₂ levels for 14, 21 and 28 days. They found that metabolite profile was altered by both CO₂ and physiological age. Plants grown under high CO₂ exhibited a metabolite profile characteristic of older plants under ambient CO₂, and elevated CO₂ resulted in higher levels of phosphorylated sugar intermediates. Whereas the percentage increase of starch content resulting from CO₂ enrichment declined as plants developed, elevated CO₂ promoted the accumulation of secondary metabolites (flavonoids) progressively to a greater extent as plants became mature.

Pears (*Pyrus communis* L cv. Conference) when stored under low oxygen or elevated carbon dioxide conditions may develop brown spots due to oxidation of phenolic compounds and eventually, cavities in the centre of the fruit. Based on metabolic profiling using GC-(EI-TOF)-MS it was found that brown tissue was clearly characterized by a distinctive pattern in changes which included a decrease of malic acid and an increase in fumaric acid and GABA, which indicated a reduced metabolic activity at the level of the Krebs cycle and a putative block of the GABA shunt pathway [95]. On the other hand,
increased gluconic acid concentration might be related to ascorbic acid degradation due to insufficient reducing equivalents or to an impaired pentose phosphate pathway. Similarly, the concentrations of other compounds which are believed to be related to hypoxic stress response (eg trehalose and putrescine) were also considerably higher in brown tissue than in sound tissue. The increased concentration of some sugars found in xyloglucans during brown development possibly indicates cell wall breakdown due to enzymatic processes or chemical reactions of hydroxyl radicals.

**Oxidative stress**

Baxter et al [96] investigated the metabolic response of heterotrophic *Arabidopsis thaliana* cells treated with menadione to oxidative stress. The stress had a profound effect on the central metabolic pathways and caused extensive metabolic inhibition of the tricarboxylic acid cycle. Oxidative stress also led to decreases in amino acids such as glycine, serine and alanine that are not directly connected to the TCA cycle.

Lehmann et al [97] investigated the response of *Arabidopsis* roots to menadione treatment, analyzing the transcriptome, metabolome and key metabolic fluxes with focus on both primary and secondary metabolism. It was also shown that menadione (used for induction of oxidative stress) caused redox perturbation, not just in the mitochondrion but also in the cytosol and plastids of roots, and in the first 30 min of treatment there was a decrease in metabolites of the TCA cycle and amino acid biosynthesis. After 2 and 6 h of treatment metabolite levels in the root did not remain depressed, but instead recovered and, in the case of pyruvate, some amino acids and aliphatic glucosinolates showed a steady increase above control levels. However, no major changes in fluxes of central carbon metabolism were observed. These results suggested that root tissues can recover metabolic activity after oxidative inhibition and highlighted potentially important roles of glycolysis and the oxidative pentose phosphate pathway.

**Nutrient deficiency**

Mineral nutrients are essential for plant growth and development because they affect and regulate fundamental processes of plant physiology and biochemistry, such as photosynthesis and respiration. Depending on how great the growth requirement for a given nutrient, the nutrient is referred to as either macronutrient (eg nitrogen, phosphorus, potassium, sulphur, magnesium) or micronutrient (eg iron, zinc, etc). Limited supply of nutrients is unfavourable for plant growth and development and such abiotic stress can significantly affect plant metabolism [98].

**Nitrogen**

Plants can assimilate inorganic nitrogen sources to organic nitrogen such as amino acids. Nitrogen is the most important of the mineral nutrients required by plants and its metabolism is tightly coordinated with carbon metabolism in the fundamental processes that permit plant growth. Metabolomics is a biochemical approach useful for study of nitrogen metabolism, because metabolites not only reflect the ultimate phenotypes (traits), but can mediate transcript levels as well as protein levels directly and/or indirectly under different nitrogen conditions [99].
Lubbe et al [100] investigated the effect of fertilizer on galanthamine production in *Narcissus* bulbs in a field study using a $^1$H NMR metabolite profiling approach. Galanthamine was quantitated and major metabolites in the bulbs were identified. The application of standard fertilization levels of nitrogen and potassium caused a significant increase in galanthamine as compared with a control. Multivariate data analysis of the $^1$H NMR data revealed that applying double the standard level of nitrogen fertilizer resulted in production of more amino acids and citric acid cycle intermediates, but not more galanthamine.

Hirai et al [101] integrated metabolomics and transcriptomics in investigation of gene-to-metabolite networks regulating sulphur and nitrogen nutrition and secondary metabolism in *Arabidopsis*. Mathematical analyses, including principal component analysis and batch-learning self-organizing map analysis of transcriptome and metabolome data suggested that beside general responses to sulphur and nitrogen deficiencies, specific responses to either sulphur or nitrogen deficiency occurred in several metabolic pathways: in particular, the genes and metabolites involved in glucosinolate metabolism were shown to be coordinately modulated.

Rice plants grown in paddy fields preferentially use ammonium as a source of inorganic nitrogen. The conversion of ammonium to glutamine is catalysed by glutamine synthetase (GS). Kusano et al [102] performed quantitative comparative analyses between the metabolite profiles of a rice mutant lacking OsGS1;1 gene (gene encoding cytosolic GS in rice which is critical for normal growth and grain filling) and its wild type. They observed severe retardation of shoot growth of the mutant in the presence of ammonium (in comparison to the wild type) and overaccumulation of free ammonium in the leaf sheath and roots. The mutant plants exhibited severe retardation of shoot growth in the presence of ammonium compared with the wild type. Overaccumulation of free ammonium in the leaf sheath and roots of the mutant indicated the importance of OsGS1;1 for ammonium assimilation in both organs. The metabolite profiles of the mutant line revealed: (1) an imbalance in levels of sugars, amino acids and metabolites in the tricarboxylic acid cycle and (2) overaccumulation of secondary metabolites, particularly in the roots under a continuous supply of ammonium. Metabolite-to-metabolite correlation analysis revealed the presence of mutant-specific networks between tryptamine and other primary metabolites in the roots.

**Sulphur**

Sulphur assimilation by plants plays a key role in the sulphur cycle in nature, and metabolism of the assimilated sulphur provides various compounds that are useful for animals and human population [103]. At the time trajectory of sulphur stress response, two system states can be distinguished. The first state of short-term responses is characterized by the development of enhanced lateral roots exploring the space in search for the lacking nutrient. When this physiological reaction cannot be accomplished by bringing the system back to the initial state of sulphur sufficiency, a new program is toggled aiming at saving the organism resources for vital seed production [104]. NMR metabolite profiling revealed significant effects of suboptimal nitrogen or sulphur supply in leaves but not in developing grains. The analysis of amino acid pools in the grain and leaves revealed a strategy whereby amino acid biosynthesis switches to the production of glutamine during grain filling. Glutamine accumulated in the first 7 days of grain development, prior to conversion to other
amino acids and protein in the subsequent 21 days. Nitrogen deficiency resulted in much slower accumulation of grain nitrogen and sulphur and lower final concentrations, indicating that vegetative tissue nitrogen has a greater control of the timing and extent of nutrient remobilization than sulphur [105].

**Phosphorus**

Phosphorus is an important determinant of productivity of C-3 and C-4 plants. Ghannoum et al [106] examined dynamic response to phosphorus deficiency in tropical grasses (*Panicum laxum* (C-3) and *Panicum coloratum, Cenchrus ciliaris* and *Panicum maximum* belonging to the C-4 subtypes) which were grown under contrasting phosphorus supplies, including phosphorus withdrawal from the growing medium. They compared changes in photosynthesis and growth with leaf carbohydrate contents and metabolic fingerprints obtained using $^1$H NMR. The response of CO$_2$ assimilation rates to leaf contents of inorganic phosphate was linear in the C-3 grass, but asymptotic for the three C-4 grasses. Principal component analysis of the $^1$H NMR spectra revealed distinctive profiles of carbohydrates and amino acids for the four species, and photosynthesis of the three C-4 grasses had a higher phosphate use efficiency and lower inorganic phosphate requirement. Although each of the four grass species showed distinctive $^1$H NMR fingerprints, there were no differences in response that could be attributed to the C-4 subtypes.

Wild-type cells of *C. reinhardtii* strain CC-125 subjected to nitrogen-, phosphorus-, sulphur-, or iron-depleted growth conditions develop highly distinctive metabolite profiles. Individual metabolites undergone marked changes in their steady-state levels. Compared with control conditions, sulphur-depleted cells accumulated 4-hydroxyproline more than 50-fold, whereas the amount of 2-ketovaline was reduced to 2% of control levels and phosphorus-depleted conditions induced a deficiency syndrome quite different from the response to nitrogen, sulphur, or iron starvation [107].

**Zinc**

Citrus Huanglongbing (HLB) is considered the most destructive citrus disease worldwide, however, symptoms-based detection of HLB is difficult due to similarities with zinc deficiency. Cevallos-Cevallos et al [108] used GC-MS methods for untargeted metabolite analysis in extracts from healthy, zinc deficient or HLB-infected sweet orange leaves. Significant ($p < 0.05$) differences in oxo-butanedioic acid, arabitol and neo-inositol were exclusively detected in samples from plants with zinc deficiency.

**Iron**

Jiménez et al [109] evaluated the metabolic changes of three *Prunus* rootstocks submitted to iron chlorosis and their different responses for tolerance using measurements of metabolites and enzymatic activities. Sugar, organic and amino acid concentrations of root tips were determined after two weeks of iron shortage by proton NMR spectroscopy of extracts; complementary analyses of organic acids were performed by LC-MS. The major soluble sugars found were glucose and sucrose, the major organic acids were malic and citric acids, and the major amino acid was asparagine. After two weeks of iron deficiency root sucrose, total organic and amino acid concentrations and *phosphoenolpyruvate carboxylase* (PEPC) activity increased; the malic, citric and succinic acid concentrations
increased in three rootstocks. Whereas the tolerant rootstock Adesoto showed higher total organic and amino acid concentrations, the susceptible rootstock Barrier showed lower total amino acid concentration and PEPC activity values. From these results it could be concluded that the induction of this enzyme activity under iron deficiency indicates the tolerance level of rootstocks to iron chlorosis.

Rellan-Alvarez et al [110] investigated changes in the proteomic and metabolic profiles of Beta vulgaris root tips in response to iron deficiency and resupply. Iron deficiency and/or resupply caused significant changes (p < 0.05) in the levels of 62 out of 77 identified metabolites and in the response ratios of 26 metabolites. Large (> 4-fold) increases were found for some organic acids (citric and aconitic acid), some sugars (sucrose, myo-inositol and those of the raffinose family of oligosaccharides (RFO), namely galactinol and raffinose), nicotianamine and 2-aminoadipic acid. The response ratio of oxalic acid decreased markedly in iron deficient conditions, whereas those of other amino acids, nitrogen compounds, lipid metabolites and others did not show large (> 4-fold) changes when compared with the iron-sufficient controls. A major change in carbohydrate metabolism was the large increase in RFO compounds (34- to 16-fold changes in the response ratios of galactinol and raffinose, respectively) that occurred in roots with Fe deficiency. This increase was higher than that found for sucrose (5-fold). RFOs have diverse roles in plants, including transport and storage of carbon [111] and acting as compatible solutes for protection in abiotic stresses [112, 113]. Other explanation for the large increase in the relative amounts of RFOs could be the ability to function as antioxidants. Metabolite studies revealed large increases in organic acids, including a 20-fold citric acid increase. These increases in TCA cycle organic acids with Fe deficiency are coupled with increases in glycolysis [114] and root carbon fixation by PEPC.

**Herbicides**

In regards to the study of agroecosystems, metabolomics enables monitoring of metabolic changes in association with biotic or abiotic agents such as agrochemicals. Focusing on crop protection chemicals, a great effort has been given towards the development of crop protection agents safer for consumers and the environment and more efficient than the existing ones. Metabolomics has been so far a valuable tool for high-throughput screening of bioactive substances in order to discover those with high selectivity, unique modes of action and acceptable ecotoxicological/toxicological profiles [115].

Rapid classification and identification of the mode of action of bioactive compounds applied to plants can be achieved by a robust and easy-to-use metabolic-profiling method. This method uses artificial neural network analysis of 1D $^1$H NMR spectra of aqueous plant extracts to classify rapidly changes in the total metabolic profile caused by application of crop protection chemicals [116].

Sauter et al [117], using GC, quantified a large variety of common plant metabolites in a single chromatogram and compared peak intensities in the profiles obtained with barley seedlings treated with various herbicides (at sublethal doses) and with bioregulators with those from untreated plant profiles. Computer assisted evaluation of the resulting data revealed that, generally, the treatments give reproducible “response patterns” characteristic of the respective treatment and showed that metabolic profiling is useful in classifying
compounds of known or unknown modes of action on the basis of the characteristic response patterns.

Aliferis and Chrysayi-Tokousbalides [118] investigated the biochemical mode of action of (5S, 8R, 13S, 16R)-(-)-pyrenophorol isolated from a Drechslera avenueae pathotype using metabolic fingerprinting. For rapid screening of phytotoxic substances for metabolic effects the researchers compared $^1$H NMR spectra of crude leaf extracts from untreated Avena sterilis seedlings and A. sterilis seedlings treated with pyrenophorol with those obtained from treatment with the herbicides diuron, glyphosate, mesotrione, norflurazon, oxadiazon and paraquat. The results of multivariate analysis revealed that none of the herbicide treatments fitted the pyrenophorol model and indicated that the effect of the phytotoxin on A. sterilis differs from the effects caused by glyphosate, mesotrione, norflurazon, oxadiazon, paraquat and diuron, which inhibit 5-enolpyruvylshikimate-3-phosphate synthase, 4-hydroxyphenyl-pyruvate-dioxygenase, phytoene desaturase, protoporphyrinogen oxidase, photosystem I and photosystem II, respectively.

Lange et al [119] used isolated peppermint (Mentha piperita) oil gland secretory cells as an experimental model system to study the effects of the herbicides fosmido-mycin, phosphonothrixin, methyl viologen, benzyl viologen, clomazone, 2-(dimethylamino)ethyl diphosphate, alendronate and pamidronate on the pools of metabolites related to monoterpene biosynthesis via the mevalonate-independent pathway and found that newly developed isolation protocol for polar metabolites together with an improved separation and detection method based on liquid chromatography-mass spectrometry have allowed assessment of the enzyme targets for a number of these herbicides.

Saflufenacil (Kixor™) is a protoporphyrinogen IX oxidase (PPO)-inhibiting, new peroxidizing herbicide of the pyrimidinedione chemical class for preplant burndown and selective preemergence dicot weed control in multiple crops, including corn. With the use of treated duckweed plants Grossmann et al [120] performed metabolite profiling based on quantification of metabolite changes, relative to untreated controls. Physiological and metabolite profiling suggested a mode of action similar to inhibitors of PPO in tetrapyrrrole biosynthetic pathway. Saflufenacil inhibited PPO enzyme activity in vitro with 50% inhibition of 0.4 nmol/dm$^3$ for the enzymes isolated from black nightshade, velvetleaf and corn, while PPO inhibition by saflufenacil caused accumulations of protoporphyrin IX (Proto) and hydrogen peroxide (H$_2$O$_2$) in leaf tissue of black nightshade and velvetleaf.

Ott et al [121] subjected crude isolates from maize (Zea mays) plants treated with various herbicides such as imazethapyr, glyphosate, sethoxydim and diuron, which represent various biochemical modes of action (MOA) such as inhibition of specific enzymes (acetohydroxy acid synthase, PPO, 5-enolpyruvylshikimate-3-phosphate synthase, acetyl CoA carboxylase, etc), or protein complexes (photosystems I and II), or major biological processes such as oxidative phosphorylation, auxin transport, microtubule growth and mitosis to $^1$H NMR spectroscopy, and the spectra were classified by artificial neural network analysis to discriminate the herbicide modes of action. For the study 27 herbicidal compounds representing inhibitors for 19 different MOAs were used. The authors showed the feasibility of $^1$H NMR spectroscopy of plant extracts in combination with artificial neural network analysis to distinguish treated samples from untreated (control) samples and discriminate, with high reliability, the modes of action of many different, commercially important herbicides. NMR was shown to be sensitive enough to produce fingerprint information that enables the researcher to discern between related MOAs, and about twenty
MOA classes have been discerned by the automated pattern recognition approach. Compounds affecting the same target enzyme are classified by their metabolic profile to the corresponding MOA, even if only one reference compound is used to create the signature for that MOA. Detailed analysis also highlighted differences between compounds of a series that affect the same target but that are being metabolized differently.

Trenkamp et al [122] conducted a comprehensive metabolic phenotyping of primary metabolism of photosynthetic tissue of Arabidopsis thaliana following spray treatment with a number of commercially used herbicides (glufosinate, sulcotrione, foramsulfuron, benfuresate, AE944 [N²-(1-ethyl-3-phenylpropyl)-6-(1-fluoro-1-methylethyl)-1,3,5-triazine-2,4-diamine] and glyphosate) using a well established GC-MS profiling method. They identified and quantified in excess of 80 polar metabolites and a similar number of lipophilic components and determined causal changes in the metabolite profiles by following their time-dependent changes using a serial sampling strategy. The resultant profiles were compared both by looking at the largest changes in a metabolite by metabolite manner and by performance of statistical analyses.

Zou et al [123] reported a novel approach to detection, analysis and characterization of low-abundant metabolites using herbicide clomazone. Their methodology derived from the predictive multiple reaction monitoring (pMRM) mode available on triple-quadrupole linear ion trap (QqQ-LIT)-MS systems offered the highest sensitivity among various acquisition modes for studying trace levels of metabolites of the herbicide clomazone in plants and allowed the identification of positional isomers of metabolites. Unknown metabolites were further identified and validated by obtaining accurate masses and isotopic ratios using selected ion monitoring (SIM) and data-dependent MS/MS scans on LC-HRMS.

Plant glutathione transferases (GSTs) comprise an enzyme superfamily tightly connected with crop herbicide tolerance. The overexpression of GmGSTU4 isoenzyme from transgenic tobacco plants confers increased tolerance to the chloroacetanilide herbicide alachlor. Kissoudis et al [124] studied the effect of alachlor treatment (3 mg/dm³) on the metabolome of in vitro grown wild type and GmGSTU4 overexpressing tobacco plants of cv. Basmas, and for identification of metabolites gas chromatography/mass spectrometry (GC/MS) was used. Alachlor treatment had a significant effect on the level of several metabolites in the wild-type plants compared with the control; significant decrease was observed mainly in the relative concentration of pyruvate, succinate, maltose, melibiose and tryptophan. On the other hand, mannitol, hydroquinone and proline levels were significantly increased. Comparing the metabolome of wild type and transgenic plants treated with alachlor, several metabolites were identified as differentially altered. Transgenic plants exhibited significantly increased levels of acetyl-serine (a precursor in the biosynthetic pathway of glutathione, a major determinant of alachlor detoxification); an increase was also observed in tryptophan, proline, maltose, melibiose and hydroquinone levels. On the other hand, compared to wild-type plants, a significantly decreased level of metabolites was determined for glucose, fructose and mannitol. The results indicated that increased tolerance to alachlor of transgenic tobacco plants is an outcome of sustained metabolic homeostasis and enhanced detoxification potential that leads to the continuation of growth in the presence of the herbicide.

The response pattern to an herbicide can be viewed as the end result of changes induced in the molecular and biochemical process chain and should be diagnostic of its
physiological mode of action. The results can be interpreted directly, or a fingerprint database for all known modes of action can be screened for analogy [125].

**Heavy metals**

Since the beginning of the industrial revolution, pollution of the biosphere with toxic metals has accelerated dramatically [126], and contamination of agricultural soil by heavy metals has become a critical environmental concern due to their potential adverse ecological effects. Plants grown in soil containing high levels of heavy metals show visible symptoms of injury reflected in terms of chlorosis, growth inhibition, browning of root tips, hindered transfer of micronutrients from root to shoot, generation of oxidative stress and reactive oxygen species (ROS), alterations in the germination process, disruption of biomembrane lipids as well as in deleterious effects on plant physiological processes such as photosynthesis, water relations and mineral nutrition [127].

Bailey et al [128] used high field $^1$H NMR spectroscopy in conjunction with chemometric analysis to obtain information regarding fluctuations in endogenous metabolic profiles for *Crotalaria cobalticola* plant cells following exposure to cobalt chloride. Branched chain amino acids, succinate and secondary metabolite precursors phenylalanine and tyrosine were all higher in the control samples, whilst choline, glutamate, alanine and lactate were higher in the dosed samples. Li et al [129] exposed *Brassica carinata* seedlings to increasing concentrations of the non-physiological ion lithium and found significant effects on the germination rate, root length, chlorophyll content and fresh weight in brown-seeded and yellow-seeded near-isogenic lines. Metal content analysis and phytochemical profiling indicated that lithium was hyper-accumulated, and the lipid and phenolic composition dramatically changed in brown-seeded seedlings. Here, sinapic acid esters and chloroplast lipids were replaced by benzoate derivatives, resveratrol and oxylipins after lithium exposure. In contrast, the yellow-seeded plants maintained the same phenolic and lipid composition before and after exposure to lithium and did not tolerate the high metal concentrations tolerated by the brown-seeded line.

The exposure of tomato plants to various cadmium concentrations resulted in significant changes in primary metabolism compounds, especially in the accumulation of some amino and organic acids involved in cellular compartmentation and detoxification of cadmium [130]. Roessner et al [131] used a metabolomics approach to compare metabolite profiles in root and leaf tissues of an intolerant, commercial cultivar (cv Clipper) and a boron-tolerant *Algerian landrace* (cv Sahara) exposed to elevated boron concentrations (200 and 1000 $\mu$mol/dm$^3$). They found that the number and amplitude of metabolite changes in roots was greater in Clipper than in Sahara. On the other hand, leaf metabolites of both cultivars responded only after treatment with higher boron concentration, at which boron toxicity symptoms (necrosis) were visible. Overall, there were always greater differences between tissue types (roots and leaves) than between the two cultivars.

Metabolite profiling based on GC-MS was used to study the nickel-rich latex of the hyperaccumulating tree *Sebertia acuminata* [132]. More than 120 compounds were detected, 57 of them were subsequently identified. A methylated aldaric acid (2,4,5-trihydroxy-3-methoxy-1,6-hexan-dioic acid) was identified for the first time in biological extracts, and its structure was confirmed by 1D and 2D NMR spectroscopy. After citric acid it appears to be one of the most abundant small organic molecules present in the latex studied. Nickel(II) complexes of stoichiometry Ni(II):acid = 1:2 were detected for
these two acids as well as for malic, itaconic, erythronic, galacturonic, tartaric, aconitic and saccharic acids. These results provide further evidence that organic acids may play an important role in the transport and possibly in the storage of metal ions in hyperaccumulating plants.

The response of tomato plants to long-term cadmium exposure was evaluated after a 90-days long culture in hydroponic conditions (0, 20 and 100 µmol/dm$^3$ CdCl$_2$) [133]. Cadmium preferentially accumulated in roots and to a lower extent in upper parts of plants. Absolute quantification of 28 metabolites was obtained through $^1$H NMR, HPLC-PDA and colorimetric methods. The principal component analysis showed a clear separation between the control and cadmium treated samples. Proline and total ascorbate amounts were reduced in cadmium-treated leaves, whereas $\alpha$-tocopherol, asparagine and tyrosine accumulation increased, principally in 100 µmol/dm$^3$ cadmium-treated leaves. Carotenoid and chlorophyll contents decreased only in 100 µmol/dm$^3$ cadmium-mature leaves, which correlates with a reduced expression of genes essential for isoprenoid and carotenoid accumulations. The results of Hediji et al [133] showed that tomato plants acclimatized during long-term exposure to 20 µmol/dm$^3$ cadmium. On the contrary, 100 µmol/dm$^3$ cadmium treatment resulted in drastic physiological and metabolic perturbations leading to plant growth limitation and fruit set abortion.

The metabolic responses of Arabidopsis thaliana to cadmium exposure was characterized by Sun et al [134] who cultivated the A. thaliana plants in the medium contaminated with different cadmium concentrations (0, 5 and 50 µmol/dm$^3$, respectively) for 2 weeks. Metabolite analyses were performed using GC-MS. More than 80 metabolites characterized by retention time indices and specific mass fragments were identified. The levels of carbohydrates, organic acids, amino acids and other stress-responsive metabolites changed under cadmium stress. Treated plants showed increased levels of alanine, β-alanine, proline, serine, putrescine, sucrone and other metabolites with compatible solute-like properties, notably 4-aminobutyric acid, glycerol, raffinose and trehalose, compared with the control (untreated) plants. Studies indicated that concentrations of antioxidants ($\alpha$-tocopherol, campesterol, $\beta$-sitosterol and isoflavone) also increased significantly. These results confirm the important role of antioxidant defences in the mechanisms of plant resistance to cadmium stress and suggested that metabolic profiling is a powerful tool that can help to rapidly classify environmentally modified plants and simplify the process of cadmium stress responses.

Rice root metabolome analysis was also carried out to relate differential transcriptome data to biological processes affected by Cr(VI) stress in rice. The findings clearly suggested that a complex network of regulatory pathways modulates chromium-response of rice. The integrated matrix of both transcriptome and metabolome data after suitable normalization and initial calculations provided a visual picture of the correlations between components. Predominance of different motifs in the subsets of genes suggested the involvement of motif-specific transcription modulating proteins in chromium stress [135].

In order to investigate the metabolomic changes induced by metal ions in Brassica, plants were subjected to concentrations 50, 100, 250 and 500 µmol/dm$^3$ of copper, iron and manganese in separate treatments. $^1$H NMR and 2D $^1$H NMR spectra coupled with principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were applied to investigate the metabolic change in Brassica rapa (var. Raapstelen) [136].
$^{1}$H NMR analysis followed by the application of chemometric methods revealed a number of metabolic consequences. Among the metabolites that showed variation, glucosinolates and hydroxycinnamic acids conjugated with malates were found to be the discriminating metabolites as were primary metabolites like carbohydrates and amino acids. This study shows that the effects of copper and iron on plant metabolism were larger than those of manganese and that the metabolomic changes varied not only according to the type of metal but also according to its concentration.

Le Lay et al [137] investigated the incorporation and localisation of $^{133}$Cs in a plant cellular model, and the metabolic response induced was analysed as a function of external potassium concentration. The cellular response to the caesium stress was analysed using metabolic profiling. $^{13}$C and $^{31}$P NMR analysis of acid extracts showed that the metabolome impact of the caesium stress was a function of potassium nutrition. These analyses suggested that sugar metabolism and glycolytic fluxes were affected in a way depending upon the medium content in $K^+$ ions.

**Multiple stresses**

Usually one kind of stress is accompanied or followed by another stress. For example, heat stress can be accompanied by drought stress due to physical loss of water, and cold stress can be followed by drought stress due to physiological unavailability of water. When two or more stresses co-occur, their effects are sometimes additive, while in other cases the influence of one stress has priority. The effects of a combination of stress-factors on crops might be more severe than the effects of the same stresses applied separately. Whereas the effects of different single-stress factors to plants were thoroughly investigated for a long time, the bioinformatics tools for analyzing the complex data generated by experiments under multiple stressing conditions was developed only recently [138, 139].

**Drought-heat stress**

The combination of drought and heat stress represents an excellent example of two different abiotic stress conditions that occur in the field simultaneously. The combination of drought and heat stress results in significantly greater detrimental effect on the growth and productivity of these crops compared with each of the different stresses applied individually [140, 141]. The response of *Arabidopsis* plants to a combination of drought and heat stress was found to be distinct from that of plants subjected to drought or heat stress [47]. Metabolic profiling of plants subjected to drought or heat stress or a combination of drought and heat stresses revealed that plants subjected to a combination of drought and heat stress accumulated sucrose and other sugars such as maltose and glucose. In contrast, proline accumulated in plants subjected to drought did not accumulate in plants exposed to a combination of drought and heat stress. Heat stress was found to ameliorate the toxicity of proline to cells, suggesting that during the exposure to a combination of drought and heat stress sucrose replaces proline in plants as the major osmoprotectant.

**Drought-salinity stress**

Rice (*Oryza sativa* L) is one of the most important crops in the world, and its growth is influenced by several environmental stresses, such as drought and high salinity. Fumagalli et al. [142] investigated the metabolic profile in shoots and roots of two rice cultivars (Arborio
and Nipponbare) using $^{1}$H-high-resolution magic angle spinning (HR MAS) and liquid-state NMR experiments. They found that shoot and root growth of Arborio seedling was more sensitive to drought and salt stress than those of Nipponbare. They determined a significant accumulation of amino acids and sugars in shoots and roots under stress conditions with clear differences between the two analysed rice cultivars, whereby Arborio seedlings accumulated a higher concentration of amino acids and sugars than Nipponbare seedlings.

Cramer et al [143] performed metabolite profiling to define metabolic pathways in Vitis vinifera cv. Cabernet Sauvignon exposed to a gradually applied and long-term (16 days) water-deficit stress and equivalent salinity stress. They found that water deficit caused more rapid and greater inhibition of shoot growth than did salinity at equivalent stem water potentials. Metabolite profiling revealed reduction of sucrose, aspartic, succinic and fumaric acids and the accumulation of proline, asparagine, malic acid and fructose under salt stress, but there were higher concentrations of glucose, malate and proline in water-deficit-treated plants as compared with salinized plants. The metabolite differences were linked to differences in transcript abundance of many genes involved in energy metabolism and nitrogen assimilation, particularly photosynthesis, gluconeogenesis and photorespiration. Water-deficit-treated plants appear to have a higher demand than salinized plants to adjust osmotically, detoxify free radicals (ROS) and cope with photoinhibition.

**Elevated CO$_{2}$-salinity**

Kanani et al [144] investigated the combined effect of elevated CO$_{2}$ conditions and salt (NaCl) stress on metabolic physiology of A. thaliana hydroponic cultures over the first 30 hours of continuous perturbation. It was found that net holistic effect of the salinity stress was stronger at metabolic levels than that of the elevated CO$_{2}$ conditions, the combined effect of the elevated CO$_{2}$ conditions and salinity stress on the metabolic physiology of the plants was milder than that of the salinity stress alone. Differences in the acute and the longer-term responses of the plants to any of the stresses during the first 30 h of treatment were observed as well. Mitigation of the oxidative stress induced by increased salinity through the application of elevated CO$_{2}$ was determined also with two barley cultivars [145], and the protective role of elevated CO$_{2}$ was confirmed also in salt-stressed alfalfa [146], pine and oak [147].

**Integrated “omics” approaches in the study of plant responses to abiotic stress**

To understand the organization principle of cellular functions at different levels, an integrative approach to large-scale experiments, so called “omics” data, including genomics, transcriptomics, proteomics and metabolomics, is needed. Integration of metabolite profiling with other multiple “omics” data (eg transcript profiling) is required to reconstruct complex networks that characterize the phenotypes in the cell. However, metabolomics data are different from transcriptomics and proteomics data, in which correlated metabolites are not always likely to be associated with a common biological function. Although comprehensive and systematic comparison of the metabolomic correlations across different species, tissues, genotypes and stress treatments is required, a combination of transcriptomics data and metabolomic correlations is a promising concept for understanding
of the underlying biochemical systems in cellular metabolism and their regulations. The in silico analysis of genes and metabolites using publicly available data can further construct gene-to-metabolite networks. Such meta-analysis toward systems biology can accelerate the studies required to fill in the missing blank spots in our knowledge of cellular processes. However, due to technical limitations of metabolome detecting, the set of publicly available metabolome data is generally smaller than that of transcriptome data [148]. Transcriptomics, metabolomics, bioinformatics and high-through-put DNA sequencing have enabled active analyses of regulatory networks that control abiotic stress responses. Such analyses have markedly increased our understanding of global plant systems in responses and adaptation to stress conditions. Integrated “omics” analyses are necessary to identify the broad function of metabolite regulatory networks during responses to abiotic stresses [149]. The continued evolution of agrochemistry depends upon the adoption of novel methods to target discovery, mode of action and lead compound identification. The use of “omics” technologies is a logical approach to expanding the arsenal of tools available in this important industry [150].

Conclusion

At present the mankind needs a “second” green revolution to improve the yield of crops grown in infertile soils by farmers with little access to fertiliser. Just as the green revolution was based on crops responsive to high soil fertility, the “second” green revolution will be based on crops tolerant of low soil fertility. Metabolomics offers us the opportunity to gain deeper insights into and have better control of the fundamental biochemical basis of food. It can significantly contribute to design modified breeding programmes aimed at better quality production, optimised food processing strategies and, ultimately, improved (micro)nutrient bioavailability and bioefficacy as well as to better understanding of the pathways responsible for biosynthesis of nutritionally relevant metabolites. Such large-scale analyses enable obtaining information that can explain and identify the differences between certain sets of organisms (eg differences in genotypes) or elucidate factors that influence biochemical events. The wider application of available metabolite-profiling technologies is likely to increase our understanding of metabolic networks by identifying (often unexpected) correlations and links between different metabolites. Metabolomics is suitable for examining the effects of organism exposure to xenobiotic or environmental stressors, and plant responses to different abiotic stresses reflected in altered metabolomes can be useful for better understanding of physiological and biochemical processes in stressed plants. Comprehensive metabolic control analysis can contribute to expansion of metabolic engineering and to generation of predictive models of plant metabolism.

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References

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Słowa kluczowe: stresowe czynniki abiotyczne, stres środowiskowy, metaboliczne odciski palców, profilowanie metaboliczne, metabolomika