Development and validation of a GC–MS method for soybean organ-specific metabolomics

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ABSTRACT

Field mold (FM) can easily deteriorate the preharvest soybean in the field, and Fusarium moniliforme is demonstrated as the dominant pathogenic fungi. Metabolomics is a powerful tool to reveal the resistance mechanism in response to microbial infection. Therefore, in this research, the Design of Experiment (DOE) model was developed to optimize the extraction solvent combinations for metabolomic study of soybean seed and pod based on gas chromatography–mass spectrometry (GC–MS). Combined with the number of extracted peaks and the peak area of common substances, the extraction efficiency of different solvent was analyzed by multivariate statistical analysis. The result showed that isopropanol/water/methanol (1:1:1 and 1:1:4, v/v/v) mixture was highly efficient for metabolites extractions of soybean seed and pod, respectively. Additionally, the potential metabolites and pathways concerned in FM resistance were explored by the optimized extraction solvent system based on metabolomics analysis. Amino acid metabolism in soybean seed was disturbed by F. moniliforme and metabolic pathways related to energy conversion in soybean pod strongly responded to fungal infection. This study constructs a GC–MS-based metabolonic method for soybean metabolites; comparative analysis of organ-specific metabolomics for soybean fruit could be further applied in soybean metabolomics researches.

Facing the inevitable pathogen infection in nature, plants develop their own defense mechanisms, such as triggering systemic acquired resistance (SAR), meanwhile the secretion of effectors, genes related to resistance, enzymes, and secondary metabolism are induced to protect plants against pathogen infection (Zheng & Dong, 2013).

Soybean (Glycine max L. Merrill) contains rich protein, fiber, fat, and other functional components, such as isoflavones, saponins, and tocopherols (Tang & Peng, 2000). However because of its abundant protein and oil, mildew-induced deterioration easily happens to soybean (Vollmann, Fritz, Wagentristl, & Ruckenbauer, 2000). In our previous studies, potential pathogenic fungi isolated from FM-damaged soybean fruits were confirmed as Aspergillus flavus, Aspergillus niger, Fusarium moniliforme, and Penicillium chrysogenum. Moreover F. moniliforme was considered as the main field mold (FM) fungi (Liu, Deng, Yang, Huang, Chang, Zhang, Yong, 2017).

In the era of rapid development of systematic biology, metabolomics technology has become one of the important means in basic research fields such as physiological metabolism and response mechanism of stress. It is a powerful tool that focuses on the study of metabolite components, and their dynamic change process in cell, tissue, or organism could help us to understand plant responses under different conditions and to research the potential metabolite as biomarker (Schauer & Fernie, 2006). The precise and sensitive analysis platform is an important aspect to explore and explain the endogenous metabolic changes of organism caused by the internal and external environmental stimuli in the analysis of metabolomics.

Gas chromatography–mass spectrometry (GC–MS), liquid chromatograph–mass spectrometer (LC–MS), and nuclear magnetic resonance (NMR) are the most common analytical platforms (Zhang, Sun, Wang, Han, & Wang, 2012). Especially, GC–MS has the advantages of high sensitivity, good reproducibility, low cost, and comprehensive metabolic standard atlas database, which are beneficial to qualitative analysis. After data preprocessing, the data are transformed into a data matrix of the pattern recognition for multivariate statistical analysis (Wu, Huang, Lehmann, Zhao, & Xu, 2009). Pattern recognition...
is divided into two methods: unsupervised analysis and supervised analysis. The unsupervised method is used to analyze the pretreatment data without background information, such as principal component analysis (PCA), and the supervised method is to establish different mathematical models to achieve the maximum separation of different classes of samples to predict the unknown metabolic groups, such as partial least squares discrimination analysis (PLS-DA) (Wei & Tolstikov, 2009).

In our current research, the extraction solvent systems were optimized by DOE (the Design of Experiment) method; their extraction efficiency for soybean seed and pod were evaluated by NEP (the number of extracted peaks) and PAC (the peak area of common substances) based on GC–MS. Furthermore, the comparative metabolomics analysis based on the optimized extraction solvent systems was conducted for the soybean fruit infected with F. moniliforme. The potential organ-specific FM resistance mechanism was also briefly discussed based on the selected differential metabolites and pathways.

Materials and methods

Plant materials

The germplasm ‘C103’ (Glycine max L.) was used in this study. The plants were grown in pots in the experimental field of the Sichuan Agricultural University at Ya’an in China (103°00’ E, 30°08’ N). Five seeds were sown per pot and these were thinned to two plants per pot 2 weeks after seeding.

Conidial suspension preparation

For 1 L PDA, 10 g yeast extract and 20 g peptone were dissolved in 960 mL water. The solution was sterilized by autoclaving, and then combined with 40 mL of sterile 50% glucose. The F. moniliforme strain was isolated and identified in our previous study (Liu et al., 2017). The strain was grown at 30°C overnight in a 50 mL Erlenmeyer flask with 25 mL PDA medium on a rotary shaker. Then, the culture medium was centrifuged for 10 min at 3000 rpm in a 50 mL centrifuge tube. The resulting supernatant was discarded. The conidia were washed three times with 20 mL each of sterile distilled water. They were counted with a hemocytometer and adjusted to 10^5 cfu mL⁻¹.

Fungal inoculation

Soybean fruits were infected with F. moniliforme at the RS stage (beginning seed) of seed development. The fruits from the middle portion of the healthy plants were punctured using sterile needle and sprayed with conidial suspensions of F. moniliforme (10⁵ cfu mL⁻¹) for inoculation; the fruits sprayed with sterile water as the control. The experiment was conducted under natural conditions (26°C, PPFD: 530 µmol m⁻² s⁻¹). Five biological replicates of the above experiments were performed. Soybean fruits were harvested at the R6 stage (full seed) of seed development. All the inoculated and control fruits were collected; pod and seed were striped, and immediately frozen in liquid nitrogen. Samples were then stored in air-tight tubes at -80°C until further analysis.

Optimization of extraction solvent system

Common extraction solvents, including water, methanol, and isopropanol were selected with different polarities which are 10.2, 6.6, and 4.3, respectively. JMP statistical package (Version 10.0.0, SAS, USA) was used to design experiments for the optimization of extraction solvent system. The experimental design with different solvent formulas was described in Table 1.

Extraction and preparation

All frozen dried soybean seed and pod were extracted using their respective optimum extraction solvent system. Briefly, samples were ultrasonically extracted (40 kHz) for 30 min by adding 1.5 mL extraction solvent (Table 1) and 60 µL ribitol (internal standard). Then, the samples were centrifuged at 14,000g and 4°C for 10 min. The supernatant was collected into a vial and dried in the rotary evaporator.

After addition of 100 µL methoxamine hydrochloride in pyridine (7.5 mg mL⁻¹), the vial was vortexed and ultrasonicated. Methoximation was carried out at 70°C for 1 h. Then, after adding 80 µL N-methyl-N-(trimethylsilyl) trifluoroacetamide and 1% trimethylchlorosilane, vortexing and silylation was carried out at 70°C for 40 min. After centrifuging at 14,000g and 4°C for 10 min, the sample was

Table 1. Experimental design to investigate the extraction efficiency of different solvent formulas.

| Solvent No. | Solvent amount (µL) |
|-------------|---------------------|
|             | IPA     | H₂O     | MeOH   |
| 1           | 250     | 250     | 1000   |
| 2           | 500     | 500     | 500    |
| 3           | 1500    | 0       | 0      |
| 4           | 0       | 1500    | 0      |
| 5           | 750     | 750     | 0      |
| 6           | 250     | 1000    | 250    |
| 7           | 1000    | 250     | 250    |
| 8           | 0       | 750     | 750    |
| 9           | 750     | 0       | 750    |
| 10          | 0       | 0       | 1500   |

Note: IPA: Isopropanol; H₂O: ultrapure water; MeOH: methanol.
transferred to 200-µL glass insert and analyzed using GC-MS system.

**GC-MS analysis**

Metabolite profiling was performed by using a Shimadzu QP2010. About 1.0 µL of each sample was applied to the capillary column (Rtx-5Ms, 30 m × 0.25 mm × 0.25 µm). The following chromatographic conditions were used: initial temperature, 80°C; final temperature, 310°C; and injection temperature, 300°C. The column temperature was set at 80°C for 2 min at first, then increased to 310°C at the heating rate of 5°C min⁻¹ and was maintained for 12 min. Helium gas was used as the carrier gas at the low rate of 36.8 cm s⁻¹. The ionization potential of the mass-selective detector was 70 eV and the mass spectrometry was operated at 200°C. The splitting ratio was 15:1, and the full-scan mode was from m/z 50 to 650.

**Data preprocessing and statistical analysis**

Data preprocessing was performed by the automated mass spectrometry deconvolution and identification system, including peak detection, deconvolution, chromatographic alignment, and normalization. Then the data transformed into a metabolite table with retention time (RT) and mass-to-charge ratio (m/z), and imported to GC-MS solution (Shimadzu) software for peak area integration. Data were corrected by calculating peak area ratios of the peak and the internal standard. The sum normalized data were subjected to metabolomics analyses using MetaboAnalyst 3.0: [http://www.metaboanalyst.ca/](http://www.metaboanalyst.ca/); PLS-DA was employed to summarize the systematic alteration of samples using SIMCA-P 13.0 software (Umetrics, MKS Instruments Inc., Umea, Sweden).

**Results**

**Metabolite identification and variation analysis**

In this study, three kinds of extracting solvents, including isopropanol, water, and methanol were selected as the extraction solvents; the considered 10 different combinations were designed by JMP software. Based on the mass spectral matching, 10 common compounds extracted using different solvent systems for soybean seed, including myo-Inositol, d-pinitol, allo-inositol, catechin, epicatechin, galactose, sucrose, palmitic acid, octadecanoic acid, and linoleic acid were identified. These metabolites can be divided into four categories: phenol, alcohol, sugar, and fatty acid. Variation analysis results indicated that there was considerable variability among different extract solvent systems, especially to galactose, palmitic acid, and linoleic acid, with the variation coefficients of 64.691%, 58.719%, and 54.218%, respectively (Table S1). For soybean pod, 13 common extracted metabolites, including malic acid, fumaric acid, phosphoric acid, glyoxylic acid, glycerol, myo-Inositol, d-pinitol, arabitol, mannitol, galactose, lyxose, palmitic acid, octadecanoic acid that were classified into fatty acid, sugar, alcohol, and organic acid. The differences of extraction efficiency of 10 solvent systems are focused on lyxose and glycerol with the variation coefficients of 135.479% and 113.853%, respectively (Table S2).

**Optimization of extraction solvent system**

After peak deconvolution, identification and matching, NEP, and PAC were combined to synthetically investigate the extraction effect of different solvent systems. The total ion chromatograms of 10 different solvent systems acquired from GC-MS were shown in Figure S1. There were 91 to 177 peaks extracted from soybean seeds with the relative PACs ranging from 2.779 to 9.299. Similarly, 113 to 153 peaks were extracted from soybean pods with the relative PACs ranging from 11.014 to 24.749 (Figure S2). To visualize the comparison among the extraction efficiency of different solvent systems, a two-dimensional score plot was established. As shown in Figure 1, the color and size of the bubble are determined by NEP and PAC, respectively. It was indicated that No. 2 solvent (IPA/H₂O/MeOH = 1/1/1) and No. 1 solvent (IPA/H₂O/MeOH = 1/1/4) seemed to be the best solvent system for the metabolites extracting of soybean seed and pod, respectively. (Figure 1). Then two indexes, NEP and PAC performed in membership function, showing that the max additive comprehensive value for seed and pod were 1.734 and 1.931 which belong to solvent 2 and solvent 1, respectively. The formula was established as follows:

\[
U_{ij} = \frac{X_{ij} - X_{jmin}}{X_{jmax} - X_{jmin}}
\]

Note: \(U_{ij}\) represents solvent i of extraction efficiency subordinate function values based on \(j\) index; \(X_{ij}\) represents \(j\) index of solvent \(i\); \(X_{jmin}\) represents the minimum value of \(j\) index of all solvents; \(X_{jmax}\) represents the maximum value of \(j\) index of all solvents.

**Overview of FM-induced metabolic responses**

The quantification data for all the above-identified metabolites in soybean seed and pod, which performed by comparing the integral of ribitol, were presented as the relative peak areas in Tables S3 and S4. To compare the
metabolic differences between FM-infected and corresponding control samples, the normalized concentration value of common metabolites and their cluster analysis were shown in the visualization heat map (Figure 2). The visualized concentration values of seed and pod were extracted using the optimized extraction solvents of No. 2 (IPA/H$_2$O/MeOH = 1/1/1) and No. 1 (IPA/H$_2$O/MeOH = 1/1/4), respectively. According to the heat map, the inoculated group and the control group can be clearly distinguished by the metabolite concentration, indicating that metabolites in soybean fruits were indeed response to biotic stimulus (Figure 2). Detailed analysis of soybean seed, compared with the control group, metabolic changes mainly focus on the decrease of amino acid (i.e. serine, proline, glycine, glutamic acid, and pyroglutamic acid) and amide compounds (i.e. butyro-1,4-lactam and asparagine), whereas the content of sugar alcohols (i.e. myo-inositol, D-pinitol) and organic acids (i.e. phosphoric acid, citric acid, malic acid, 1-pyrroline-3-hydroxy-5-carboxylic-acid, lyxonic acid, glyoxylic acid), which were distinctly decreased after FM-inoculation, whereas galactose, allo-inositol, fumaric acid, and sorbose were increased (Figure 2(b)).

**Organ-specific metabolomics analysis**

To obtain more detailed metabolic information about organ-specific responses to FM in soybean fruit, a multivariate statistical analysis PLS-DA was applied. Specifically, for soybean seed, its parameters of the cross-validation plot, i.e. $R^2(X) = 0.687$, $R^2(Y) = 0.998$, and $Q^2 = 0.981$, and the parameters of soybean pod were 0.639, 0.979, and 0.912, respectively, which demonstrated that the models were credible. As shown in Figure 3, the FM-inoculated and control groups of soybean seed (Figure 3(a)) and pod (Figure 3(d)) were both clearly separated based on the first principal component in PLS-DA score plots. Their corresponding loadings scatter plots indicated that the

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**Figure 1.** Two-dimensional score plots of different solvent systems for soybean seed (a) and pod (b); the color and size of the bubble are determined by NEP and PAC, respectively.
Contents of several metabolites were higher in the FM-inoculated seed, including kestose, epicatechin, octadecanoic acid, etc., and other metabolites were higher in the FM-inoculated pod, including allo-inositol, fumaric acid, sorbose, etc. The characteristics of PLS-DA loadings scatter plot were predominantly consistent with the result of cluster analysis in the heat map of Figure 2. Furthermore, the most important metabolites that contributed to the PLS-DA separation were selected by variable importance on projection (VIP) scores. The calculated VIP scores of seed and pod are presented in Figure 3(c) and (f), respectively. Metabolites with a VIP score greater than 1 were...
Figure 3. PLS-DA score plots (a and d), corresponding loadings scatter plots (b and e) and variable importance in projection (VIP) plots (c and f) of soybean seed (a–c) and pod (d–f), respectively; metabolites with a VIP score >1 are enclosed in quadrangles with red broken lines. Ck-1–Ck-5 and Tt-1–Tt-5 indicate control and FM-inoculated biological replicates, respectively (a and d). The metabolites that show higher levels in FM-inoculated seeds and pods than in controls are on the left side (b and e). PME: Phosphoric acid monomethyl ester; PHC: 1-pyrroline-3-hydroxy-5-carboxylate-acid.
considered important to the PLS-DA model. Detailedly, in the VIP-identified plot of soybean seeds, 13 metabolites were selected as the most influential, including 5 amino acids, 2 sugars, 2 fatty acids, etc.; contents of these compounds altered remarkably in response to FM (Figure 3(c), Table S3). A total of 10 metabolites, including 4 organic acids, 3 sugars, 2 sugar alcohols, etc., were considered important to the PLS-DA model of soybean pods (Figure 3(f), Table S4).

**Difference analysis of biomarkers**

To further validate the reliability of biomarker candidates, difference analyses were conducted for these 23 metabolites separately. One-way ANOVA and non-parametric Kruskal–Wallis test were performed. The results indicated that all the candidate metabolites were significantly different between FM-inoculated and control samples. Detailed analyses for seed, all of the 13 metabolites were significantly alerted after fungal infection (Figure 4(a)). Five amino acid, glutamic acid, proline, glycine, serine, pyroglutamic acid, and linoleic acid showed dramatical reduction, whereas the content of kestose, octadecanoic acid, PME (phosphoric acid monomethyl ester) extremely increased. Similarly, difference analysis results of the 10 metabolites in pod were consistent with the VIP score plot (Figure 4(b)). The content of lyxose, glyoxylic acid, PME (phosphoric acid monomethyl ester) were dramatically reduced, whereas the content of 4-amino-Butanoic acid, epicatechin, 1-Pyrrole-3-hydroxy-5-carboxylic acid, and PME were significantly increased.

**Figure 4.** Box and whisker plots of the significantly different metabolites identified from the VIP analyses of soybean seed (a) and pod (b). The red and blue box represent the control group and FM-inoculation group, respectively. *p < 0.05; **p < 0.001.
ester), and 1-pyrroline-3-hydroxy-5-carboxylic-acid in FM-inoculated pods were greatly lower than control, whereas sorbose, fumaric acid, and allo-inositol were dramatically increased after fungi inoculation. After selecting the key metabolites responded to mildew, the difference analysis shown in box plots further verifies these metabolites are potential biomarkers associated with plant biotic tolerance.

Pathway analysis

Pathway enrichment and metabolite topology analyses based on the specific hypergeometric test could provide a platform to interpret omics data in a biochemically meaningful manner. To facilitate data exploration, an overview of the interactive visualization pathway analysis based on the metabolites selected from PLS-DA was performed under the comparison with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database of Arabidopsis thaliana as shown in Figure 5.

Comparing the control and FM-inoculated samples of soybean seed, metabolite differences were enriched primarily in several metabolic pathways, including glycine, serine and threonine metabolism, alanine, aspartate and glutamate metabolism, arginine and proline metabolism, methane metabolism, aminoacyl-tRNA biosynthesis, glutathione metabolism, etc. (Figure 5(a)). Almost all the responding metabolic pathways between the control and FM-inoculated samples significantly affected by FM belong to amino acid metabolism. However, the pathway analysis revealed in soybean pod the responses of several pathways of carbohydrate metabolism, including galactose metabolism,
glyoxylate and dicarboxylate metabolism, citrate cycle (TCA cycle) were responded strongly to FM (Figure 5(b)).

Discussion

Metabolomics have been widely used in the plant chemical ecology studies (Das, Rushton, & Rohila, 2017; Harrigan et al., 2015; Lima et al., 2014; Song et al., 2014; Yang et al., 2017; Yun et al., 2016). The acquisition of ‘global’ chemical information is essential for non-targeted metabolomics analysis, in which sampling procedure is more critical. DOE, a powerful assist tool to establish experimental schemes, can help researchers to mine data effectively and optimize experimental parameters (Hecht, Oberg, & Muddiman, 2016). DOE model has been widely applied to many aspects of biology researches, especially to the mass spectrometry analyses optimizing and metabolomics studies (Eliasson et al., 2012; Zheng, Clausen, Dalsgaard, Mortensen, & Bertram, 2013). Nowadays, various analytical platforms were widely used in the study of soybean metabolism and corresponding molecular mechanism elucidation (Chai et al., 2012; Lee et al., 2017; Silvente, Sobolev, & Lara, 2012; Sun, Feng, & Ort, 2014; Wu et al., 2008). However, metabolomics researches on soybean mainly focus on seed and leaf rather than pod, which was demonstrated as an important organ in the FM resistance (Liu et al., 2016). Single solvent is widely used in different plant tissue and organs (Benkeblia, Shinano, & Osaki, 2007; Chebroul et al., 2016). However, for untargeted metabolomics, it is necessary to choose proper solvent for extracting metabolites as much as possible. Single solvent may not achieve it. The extraction solvent systems composed of isopropanol/methanol/water have special advantages in metabolomics studies based on GC–MS system, especially targeted for the high polar metabolites, such as organic acids and amino acid (Swenson, Jenkins, Bowen, & Northen, 2015). Mix of isopropanol/methanol/water with different polarities, 3.9/5.1/10.2, respectively could cover more metabolites detection.

Here we chose several typical solvents to investigate their extraction efficiency for the metabolites of soybean seed and pod; the solvent systems were optimized and applied for the extraction of polar metabolites in soybean fruit based on GC–MS. Experiments turn out isopropanol/water/methanol (1:1:1 and 1:1:4, v/v/v) were the optimal extraction solvent systems for soybean seed and pod respectively on account of NEP and PAC.

Plants have evolved intricate mechanisms to protect themselves from harm caused by biotic and abiotic stresses (Jones & Dangl, 2006; Tippmann, Schlüter, Collinge, & Teixeira, 2006). According to the results of metabolomics analysis, either seed or pod, the different pathways were mainly focused on primary metabolic and signaling responses. Compared to control, glutamic acid content in the leaves of sunflower fell by 35% after fungal infection and researchers believe that those rapidly disappeared amino acids were converted to fungal metabolites (Dulermo, Bligny, Gout, & Cotton, 2009). The situation resembled what we observed in seed. Amino acid metabolism was strongly responded to FM and the content of glutamic acid, proline, glycine, serine, pyroglutamic acid, and fructose with fungal infection decreased; therefore, we considered fungal assimilation happened in soybean after inoculating F. moniliforme. Glutathione metabolism could repair the redox imbalance triggered by biotic stress from two aspects, directly scavenging and ascorbate–glutathione cycle (Zechmann, 2014). Researchers found out a bunch of transcription of defense genes encoding enzymes of phytoalexin and lignin biosynthesis, including PAL and CHS transcripts were induced by exogenous glutathione (Wingate, Lawton, & Lamb, 1988). Glutathione metabolism was strongly provoked in soybean seed after inoculating FM and may play a role in detoxification of ROS and activation of downstream defense-related reactions. In contrast, pathways induced by FM in soybean pod were involved with energy metabolism, including galactose metabolism, glyoxylate and dicarboxylate metabolism, citrate cycle (TCA cycle), which may perform its function in energy conversion in the process of protection against fungal infection.

Conclusion

In the present study, isopropanol/water/methanol (1:1:1 and 1:1:4, v/v/v) were selected as the optimal extracting solvent systems for soybean seed and pod, respectively. GC–MS metabolomics analysis was performed in soybean fruit infected with F. moniliforme. We combined DOE and GC–MS to build the most suitable solvent systems for extracting polar metabolites of soybean. Moreover organ-specific metabolite analyses of soybean seed and pod infected by FM were performed to explore the possible resistance mechanisms. Our results reflected that metabolomics methods based on specific organ have adequate sensitivity and foreseeability to distinguish the fungi-infected plant from controls. This study laid the methodological foundation for the future researches of soybean metabolic regulation and chemical ecology.

Disclosure statement

No potential conflict of interest was reported by the authors.
Funding
This study was financially supported by the National Natural Science Foundation of China [Grant Number 31401329] and the China Postdoctoral Science Foundation [Grant Numbers 2014M560724 and 2017T100707].

References
Benkeblia, N., Shinano, T., & Osaki, M. (2007). Metabolite profiling and assessment of metabolome compartmentation of soybean leaves using non-aqueous fractionation and GC-MS analysis. Metabolomics, 3, 297–305.

Chai, C., Ju, H. K., Kim, S. C., Park, J. H., Lim, J., Kwon, S. W., & Lee, J. (2012). Determination of bioactive components in fermented soybean products using GC/MS and further investigation of correlation of their bioactivities. Journal of Chromatography B Analytical Technologies in the Biomedical & Life Sciences, 880, 42–49.

Chebrolu, K. K., Fritschi, F. B., Ye, S., Krishnan, H. B., Smith, J. R., & Gillman, J. D. (2016). Impact of heat stress during seed development on soybean seed metabolism. Metabolomics, 12, 28.

Das, A., Rushton, P., & Rohila, J. (2017). Metabolomic Profiling of Soybeans (Glycine max L.). Reveals the Importance of Sugar and Nitrogen Metabolism under Drought and Heat Stress. Plants, 6, 21.

Dulermo, T., Bligny, R., Gout, E., & Cotton, P. (2009). Amino acid changes during sunflower infestation by the necrotrophic fungus B cinerea. Plant Signaling & Behavior, 4, 859–861.

Eliasson, M., Rannar, S., Madsen, R., Donten, M. A., Marsden-Jones, J. D., & Dangl, J. L. (2014). Metabolite adjustments in drought tolerant and sensitive soybean genotypes in response to water stress. Plos One, 7, e38554.

Song, H. H., Ryu, H. W., Lee, K. J., Jeong, I. Y., Dong, S. K., & Oh, S. R. (2014). Metabolomics investigation of flavonoid synthesis in soybean leaves depending on the growth stage. Metabolomics, 10, 833–841.

Sun, J., Feng, Z., & Ort, D. R. (2014). Impacts of rising tropospheric ozone on photosynthesis and metabolite levels on field grown soybean. Plant Science, 226, 147–161.

Swenson, T. L., Jenkins, S., Bowen, B. P., & Northen, T. R. (2015). Untargeted soil metabolomics methods for analysis of extractable organic matter. Soil Biology and Biochemistry, 80, 189–198.

Tang, C. H., & Peng, Z. Y. (2000). Recent development of Soybean functional components. China Oils & Fats, 4, 44–48.

Tippmann, H. F., Schlüter, U., Collinge, D. B., & Teixeira, D. S. J. A. (2006). Common themes in biotic and abiotic stress signalling in plants. Floriculture Ornamental & Plant Biotechnology, 3, 52–67.

Vollmann, J., Fritz, C. N., Wagentristl, H., & Ruckenbauer, P. (2000). Environmental and genetic variation of soybean seed protein content under Central European growing conditions. Journal of the Science of Food & Agriculture, 80, 1300–1306.

Wei, Z., & Tolstikov, V. V. (2009). Pattern recognition and pathway analysis with genetic algorithms in mass spectrometry based metabolomics. Algorithms, 123, 979–996.

Wingate, V. P. M., Lawton, M. A., & Lamb, C. J. (1988). Glutathione causes a massive and selective induction of plant defense genes. Plant Physiology, 87, 206–210.

Wu, W., Zhang, Q., Zhu, Y., Lam, H. M., Cai, Z., & Guo, D. (2008). Comparative metabolic profiling reveals secondary metabolites correlated with soybean salt tolerance. Journal of Agricultural & Food Chemistry, 56, 11132–11138.

Wu, Z., Huang, Z., Lehmann, R., Zhao, C., & Xu, G. (2009). The application of chromatography-mass spectrometry: methods to metabonomics. Chromatographia, 69, 23–32.

Yang, C., Iqbal, N., Hu, B., Zhang, Q., Wu, H., Liu, X., … Liu, J. (2017). Targeted metabolomics analysis of fatty acids in soybean seeds using GC-MS to reveal the metabolic manipulation of shading in the intercropping system. Analytical Methods, 9, 2144–2152.

Yun, D.-Y., Kang, Y.-G., Yun, B., Kim, E.-H., Kim, M., Park, J. S., … Hong, Y.-S. (2016). Distinctive metabolism of flavonoid between cultivated and semiwild soybean unveiled through metabolomics approach. Journal of Agricultural and Food Chemistry, 64, 5773–5783.

Zechmann, B. (2014). Compartment-specific importance of glutathione during abiotic and biotic stress. Frontiers in Plant Science, 5, 566.

Zhang, A., Sun, H., Wang, P., Han, Y., & Wang, X. (2012). Modern analytical techniques in metabolomics analysis. Analyst, 137, 293.

Zheng, H., Clausen, M. R., Dalsgaard, T. K., Mortensen, G., & Bertram, H. C. (2013). Time-saving design of experiment protocol for optimization of LC-MS data processing in metabolomic approaches. Analytical Chemistry, 85, 7109–7116.

Zheng, Q. F., & Dong, X. (2013). Systemic acquired resistance: Turning local infection into global defense. Annual Review of Plant Biology, 64, 839–863.

Silvente, S., Sobolev, A. P., & Lara, M. (2012). Metabolite profiling in plants.