SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Metabolomic case studies of horseradish (*Armoratia rusticana*) and large-leaved linden (*Tilia platyphyllos* Scop.)

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The Examination takes place on online web client (Zoom), 19th of January, 2021

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The PhD Defense takes place on online web client (Zoom) at 1 p.m., 19th of January, 2021.
If you wish to participate in the discussion, please send an e-mail to szucs.zsolt@science.unideb.hu by 20:00 on the day before the discussion (18th of January, 2021). After the deadline, for technical reasons, it is no longer possible to connect to the defense.

Review and aims of the dissertation

Introduction

Medical plants have recently occupied an important place in the world of healing. Consumption of herbal products has intensified in recent years, so the study of these plants is very important.

Due to new herbal medicines and food supplements, phytochemical testing of plants is important, which in a broader sense means not only the bioactive substance produced by the plant, but also the study of microorganisms in contact with the plant.

The studies, were so-called metabolomic methods, which has the advantage that we can extract orders of magnitude more data from the instrumental measurement than in the case of a classical measurement with standards.

Metabolomic studies may be targeted or untargeted. For the first, a compound or family of compounds is tested, while for the second, we want to test as many metabolites as possible in the matrix. Metabolomics also play a significant role in the quality assurance of herbs.

The aim of the dissertation is to investigate the metabolomes of two different plants according to the two different approaches.

One of our model plants was *Armoratia rusticana* (horseradish). This plant is primarily an edible plant, but also has a number of pharmaceutically relevant bioactivities. These effects are mainly due to the isothiocyanates contained in them, which are formed from their precursors at the end of an enzymatic process. In this case, a study of a family of compounds in the plant was performed to examine the interaction between non-pathogenic, so-called endophytic fungi and the compounds by a targeted metabolomic approach.

Our other model plant was *Tilia platyphyllos* (large-leaved linden), which is a very valuable raw material from a medicinal point of view. Its therapeutic significance is very high, its traditional use is widespread throughout Europe, but many scientific

results also support the efficacy of the bioactive compounds it contains. The metabolome of a special organ of this plant, the bract, which is part of the inflorescence, was monitored for more than a hundred days, as this organ is present much longer than the botanical flower with which it is collected. For this study, we used a non-targeted metabolomic approach.

Its studies were performed using well-developed instrumental analytical methodologies.

Our two studied plants are very different at the level of both organ and metabolome, so these studies are also very different in terms of their metabolomic approach.

Aims of current PhD research

In the course of our work we sought answers to the following questions:

Armoratia rusticana:

- Are endophytes and soil fungi able to degrade GLS compounds?
- If they are able to break down the compounds, to what extent is this breakdown?
- The compound family has several different members. Can fungi break them all down or discriminate on the basis of chemical structure?
- How are the fungicidal effects of isothiocyanates (ITCs) released during the decomposition of GLCs tolerated by fungi?
- How can we monitor the breakdown of GLCs by fungi, and how can we detect the released, volatile ITCs?
- Further interactions between fungi and the compound family.
- Based on the experiments discussed above, do horseradish-derived endophytes differ from soil fungi?

Tilia platyphyllos:

- Is the metabolite pattern of the bract that makes up a significant part of the inflorescence affected by the harvest time?
- If the amount of metabolites is affected by the time of collection, how does the amount of these metabolites change over time?
- Can compounds be grouped over time?
- Is there a group of compounds that are not the most abundant during flowering, during the normal harvest period?
- Are there any groups of compounds that do not change significantly during the entire collection period?
- What are the possible consequences of changes in compound levels?

We used the methodology of metabolomics to answer the questions.

Applied methods

Parameters of instrumental measurements

LC-ESI-MS/MS: Glucosinolate degradation by endophytes and soil fungi and metabolite analysis of *Tilia platyphyllos* bract were determined qualitatively and quantitatively in LC-ESI-MS. The UHPLC system used (Dionex Ultimate 3000RS) was connected to a Thermo Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, USA) with an electrospray ionization source (ESI). The raw data were processed using the XCMS Online platform. We continued to work in R with the data obtained by the platform.

SPME-GC-MS: An SPME-GC-MS method was used to measure glucosinolate degradation products (isothiocyanates and nitriles) that are volatile. The material released into the atmosphere from the medium could be adsorbed with 5 mg of activated carbon. The measurement parameters were as follows: split ratio: 10:1; the inlet temperature was 150 °C and then a temperature gradient was used for the measurement. An HP-5MS 5% (30 m × 0.25 mm × 0.25 μm) phenylmethylsiloxane column was used. The carrier gas is He; flow rate was 1 ml/min.

Preparation of fungal inoculums

To perform the experiments, we had to prepare a suitable, administrable fungal inoculum. By culturing the strains on an appropriate liquid medium, the mycelium was dispersed to a nearly homogeneous consistency under sterile conditions, and the inoculum thus prepared was stored at 4 °C. The amount of inoculum required for the experiments was standardized based on the dry matter content of the fungus.

Incubation of horseradish extract with fungi

The fungi were incubated on HRE medium. A fungi suspension corresponding to a dry weight of 40 µg/ml was used in Eppendorf tubes. The control was uninoculated medium. Samples were lyophilized and taken up in 250 µL of 10% 1-propanol for further processing.

Collection of plant material of *Tilia platyphyllos*, sample preparation

Bract were collected from four adjacent *Tilia plathyphyllos* Scop. Trees from the campus of the University of Debrecen, from the appearance of the organ to the senescence (112 days). The samples were air dried at a temperature not exceeding 40 °C, after collection and lyophilized overnight. The dried samples were homogenized and then 25 mg was extracted with 1 mL of MeOH for 30 minutes at 75 °C. After extraction, the supernatant was stored at -24 °C.

Evaluation of chromatograms and identification of compounds

Chromatograms were evaluated using XCMS online software (https://xcmsonline.scripps.edu/) using the following parameters: (I) compound detection: centWave method, min. and max. peak width = 5 and 20, S / N threshold = 10, mzdiff = 0.01, integration method = 1, peak prefiltration = 3, prefiltration intensity = 5000, noise filtering = 1000; (II) Retention time correction: Obiwarp method, profStep = 1; (III) Alignment: mzwid = 0.015, minfrac = 0.5, bw = 5, max = 100, minsamp = 1; (IV) Statistical test: ANOVA. Approximate identification of all components was performed by LC-ESI-MS/MS.

Identifications were based on MS / MS spectra based on a fragmentation pattern from the database (https://metlin.scripps.edu/), references from the literature, and comparison with fragmentation of available standards.

New scientific results of the research

Armoratia rusticana

Glucosinolate decomposition by horseradish endophytes and soil fungi.

The different fungal strains were grown on horseradish extract, and then we quanitfication by LC-MS / MS which samples contained how much synigrin and other glucosinolates. Our experiments show that endophytes generally degraded these compounds more efficiently than soil fungi. The rate of degradation depended to some extent on the side chain of glucosinolates.

Various glucosinolates have been identified from horseradish extract. The minor glucosinolates detected belonged to the aliphatic (gluconapine, glucococheirin), thiomethylalkyl (glucoberberin) and indole (glucobrasicin) groups. Considering the variability in the detection sensitivity of LC-ESI-MS, the signal ratio of several of the detected GSLs was 0.73–1.72 relative to synigrin. Consequently, the smaller GSLs were present in approximately 0.7–40.6 nmol (each) in this extract.

Concentrations of most glucosinolates in controls did not change significantly over the 16-day incubation period ($p > 0.05$). Six of the seven endophytic fungi successfully decrease the amount of most or all their glucosinolates ($p > 0.05$). By the end of the incubation, six of the seven strains significantly decrease the amount of the major glucosinolate, sinigrin ($p < 0.001$). Strain E7 (*O. cerealis*), on the other hand, was unable to break anything down. The ability of synigrin to degrade was also tested on the same fungi. This suggests that the presence of large amounts of GSL alone is not sufficient to induce GLS-degrading enzymes. This justified the importance of work in HRE.

From the slope of the concentration decrease of synigrin and gluconasturcin, the rate of degradation was calculated to be in the range of 0.606–1.476 mM / day and 0.018–0.057 mM / day. Intra-species differences are well observed for strains E1-E3 (*F. oxysporum*) and E5-E6 (*P. radicina*). While E1 rapidly degraded most glucosinolates (1.125 mM / day synigrin; 0.057 mM / day gluconasturcin) during its growth phase,

E3 showed much lower activity, and even after its growth stopped (0.606 mM / day synigrin). 0.030 mM / day gluconasturcin). The same strains differed much less in the degradation of indole glucosinolates. The differences between strains E5 and E6 were less pronounced. The difference was much more pronounced for methylthioalkyl glucosinolates and aromatic glucosinolates. By the end of the experiment, E6 only reduced the concentration of thiomethylalkyl glucosinolates, but this decrease was not significant ($p > 0.05$) compared to E5 ($p < 0.001$). On the other hand, E6 degraded gluconasturcin at a rate of 1.72-fold compared to E5. Strain E2 was much more effective in degrading aliphatic GSLs than slow-growing strains E4–6.

It is clear from the data that the rate of degradation of different glucosinolate groups is different. Indole GSLs were least degraded by fungi. The other classes (aliphatic, methithiolalkyl, and aromatic) were decomposed with more or less similar efficiencies.

Glucosinolates were less degraded by soil fungi, which were isolated. After measurement, we found that while some soil fungi were active, their overall efficacy was lower than that of endophytons. Four decomposed all synigrins by the end of the incubation period (S1–2, S9–10); two were only partially (S4, S6), while four were unable to metabolize synigrin (S3, S5, S7–8).

Based on our results, this enzymatic activity appears to be quite widespread. The potential glucosinolate degradation ability presented above is an excellent example of how the microbial community can alter plant metabolism in a variety of ways. The results highlight the importance of intra-species variability in the study of plant-microbiome interactions.

Detection of decomposition products

Using SPME-GC-MS, allyl isothiocyanate from synigrin was successfully detected from the vapor space above the endophytes growing on solid HRE, and the glutathione conjugate was successfully detected from the medium by LC-MS.

We also attempted to detect ITCs or nitriles from the vapor space. Surprisingly, only for E5 and E6 was AITC detected in the air space of the Petri dish. Approximately 1.25% of the total amount of ITC was released and entered the vapor space on day 6 for E6 in 24 hours. For the other four synigrin-degrading species (E1 – E4), we could only detect traces of AITC from the vapor space. Allyl cyanide could not be detected in any of the samples. However, ITCs can only be detected in free form by the GC-MS technique because the conjugates are not volatile.

We specifically searched for possible non-volatile GSL metabolites (CysGly-AITC, Cys-AITC, glutathione-AITC) in LC-MS measurements. A compound has been found which may be a GSH (glutathione) -AITC conjugate based on its mass and fragmentation. The medium of fungi that did not contain AITC in the vapor space was measured in large amounts of GSH-AITC adduct.

Compared to zero time control, media E1 and E2 contained 8.77-fold and 6.07-fold more GSH-AITC adducts, respectively. The difference is much larger for E4, E5, and E6 (29.14, 14.05, and 43.27-fold increase, $p < 0.01$). The presence of the GSH-AITC adduct suggests that ITCs are present in fungi at least in part as GSL conjugates. AITC spontaneously conjugates to a thiol pool of fungi (mainly GSH), some of which is eliminated by the fungus in the medium. ITC toxicity increases when the GSH stock of fungi is depleted, enhancing the attack on protein -SH groups.

Overall, thioglucosidase activity lags behind the GSL-degrading ability already observed. The product was ITC, in free or conjugated form. These microbial enzymes are likely to have a broad spectrum: they accept most glucosinolates as substrates, but are able to degrade different GSLs at different rates.

Further experiments on endophytic fungi

By growing mushrooms exclusively on CzD medium containing synigrin as a carbon source, we have shown that synigrin can be used by many fungal strains, although there is a greater difference between soil and endophytic fungi.

Of the seven horseradish endophytes, only four were able to use synigrin as the sole carbon source. The most effective fungus used synigrin as efficiently as glucose (E1). In the case of soil fungi, only three were able to use synigrin. This type of experiment appears to be more specific than the decomposition of GSLs to separate endophytes and soil fungi.

When evaluating the experiment, it can be concluded that endophytons were more able to use metabolites for control as nutrients than soil fungi.

The relatively low amount of ITC is able to successfully inhibit the growth of our fungus in liquid medium. Here, too, there is a difference between the two groups: endophytons were more tolerant of ITCs. Most of the fungi tolerated AITC better than PeITC: the mean difference between IC_{50} values was 2.30-fold. Interestingly, endophyton fungi are almost devoid of species specifically susceptible to AITC.

Tilia platyphyllos

Seasonal variability of bract of *T. platyphyllos*

The metabolome shows the most significant change before flowering, including 0–14. between days when growth is very intense. In the young organ, its metabolome is very different from that of the flowering and later developmental stages. It is relatively stable during flowering, but later we can see a change during yield growth.

After statistical corrections (n = 504), 241 compounds were found to change significantly ($p < 9.92E-5$) over time (47.82% of compounds). 202 (40.07%) of these had a high level of significance ($p < 1.98E-6$).

Diversity and clustering of metabolites

In our study, we considered not only the metabolite change but also the metabolome diversity, for which we used the Shannon index. Based on this, the initial metabolome is very diverse, which decreases with advancing organ development and reaches its

lowest point immediately before flowering. Initial diversity is reached again only after fruit development.

The data were arranged into 6 clusters, of which 4 large groups could be set up based on whether there is a change in the level of the metabolite and, if so, whether it follows a decreasing or increasing trend.

Metabolites with no apparent or insignificant seasonal variation

Several compounds showed no significant seasonal variability (p> 9.92E-5). Most of these are in Cluster 2, 4 and 5a.

Cluster 2 contains primary metabolites, no secondary metabolites were identified from this cluster. Levels in this group show a temporary decrease during bract growth and then decrease again during the aging phase.

A similar groups are 4-5a. clusters. Concentrations of metabolites in these groups are in the same range throughout the lifetime of the organ, with no significant changes (p> 9.92E-5). A 4-5a. clusters contain flavonoid glycosides such as kempferol diglycosides, quercetein glycosides and a coumarin derivative.

Metabolites showing an increasing trend during development

Cluster 1 is a compact group of compounds. The compounds of this compact group are present in low amounts in young tissue, but their concentration increases significantly during gravel leaf growth and remains unchanged thereafter, although for some metabolites the change in concentration continues after flowering is complete. For most metabolites, the change was statistically significant (p <9.92E-5). This cluster is a diverse group in terms of biosynthetic pathway as it contains a quinic acid derivative, coumarin and other phenylpropanoids.

Metabolites showing a decreasing trend during development

5b.-6a. clusters are responsible for a significant portion of the sudden change in metabolite during the first 14 days of bract development, and 5b. cluster compounds

determine the characteristics of young gravel leaf compounds. The concentration of compounds is very high in the early stage, which decreases to later stages of development. Their maximum concentration was on day 0, followed by a rapid, statistically significant decrease within 7–14 days ($p < 9.92E-5$). For example, (-) - epicatechin concentrations decreased to about one-tenth in the first two weeks. From day 14, concentrations of these compounds remained virtually constant throughout the life of the bract. Members of this group were dominated by flavonoids, such as kempferol pentoside. This group contains many catechins and derivatives, as well as chlorogenic acid.

Metabolites showing a transient increase during the growth of the bract

Figures 3 and 6b. clusters form this group in which the compounds are initially present in low concentrations or absent. Thereafter, the concentration of the compounds increased temporarily, and then no clear trend was shown from the beginning of flowering. This trend is exactly the opposite of that observed in cluster 1, where there was a low amount before flowering, hence a strong negative correlation was observed between the two groups.

A 6b. The maximum of a subgroup is 7-21. days, while in the case of cluster 3 it fell to 21-32 days. This group showed a great diversity of flavonoids and quinic acid derivatives, primarily as glycosides. The compound containing all diglycoside flavonoids and almost all deoxyhexosides sugars is shown in Figure 6b. such as luteolin deoxyhexoside pentoside, succinic acid hexoside. Accumulation in the gravel leaf before the opening of the flavonoid-rich flower may indicate that these compounds are later transported to the flower, so only the compounds are produced and stored here. The concentration of some compounds is reduced to about one-fifth, the purification of which should definitely be preceded by a sampling time optimization.

Implications for possible applications

As the metabolome differs significantly at different time points, it can be assumed that this also affects the bioactivity of the drug as well as other absorption and distribution kinetics. For example, coumarin-related bioactivities are likely to be better in later phenological stages, while quinic acid derivatives are present in the highest concentrations before flowering.

Flavonoids are always present in some form in the bract. Shortly before flowering and shortly after flowering, the gravel leaf metabolome bears an extraordinary similarity to the condition at flowering, which is the main harvest period.

The presented data show that gravel leaves may have high bioactivity outside the flowering period.

Summary

Our work has successfully applied the metabolomic approach to answer the questions. In the present study, we performed *Armoratia rusticana* and *Tilia platyphyllos* with a targeted and untargeted metabolomic approach, and both the metabolite-endophyte fungal interaction and the observation of the chemical pattern change in the bract were found to be very useful. The main results are as follows.

In the case of *Armoratia rusticana*, our main conclusion was that endophyte fungi are actively interacting in the glucosinolate-myrosinase isothiocyanate (GLC-MYR-ITC) system. Based on the data, it is likely that endophytes have been shown to adapt to antifungal secondary metabolites of the host plant. This adaptability distinguished endophyton fungi to some extent from soil fungi found in the same soil.

It is not a negligible fact that endophytic fungi that can colonize horseradish can displace other microorganisms that otherwise function as pathogens, thereby also protecting the plant itself. Knowing this fact, the plant itself is much more protected and healthier. Assuming that a similar mechanism occurs in other medicinal plants, this will make the plant healthier, which may even lead to higher drug yields. In this respect, the selection and examination of endophytic fungi of plants cannot be neglected.

In the case of *Tilia platyphyllos*, we found in our present work that bract can be a rich source of bioactive phenolic compounds both before and after flowering, although the metabolite pattern may differ significantly at different developmental stages. In particular, the early developmental stage of bract is characterized by an extraordinary variety of polyphenolic compounds, large amounts of catechin derivatives and flavonoid glycosides. Because of these, bract can be of potential therapeutic value both during flowering and in the early stages of development or late phenostasis of fruit growth.

Because the patterns of the compounds differ at different stages, the biological effects may also depend on the phenological stage. Based on these, one can feel the need to

optimize the harvest time of this herbal drug if we want to focus on different bioactive components. Even a week can greatly affect the pattern of secondary metabolites in a plant.

In the conclusion of the study, we saw the importance of the metabolomic approach, according to which it is acceptable that either a plant metabolite-endophyton fungal interaction or the time course of a metabolome cannot be judged by examining a single parameter.

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List of publications related to the dissertation

1. **Szűcs, Z.**, Cziáky, Z., Kiss-Szikszai, A., Sinka, L. T., Vasas, G., Gonda, S.: Comparative metabolomics of Tilia platyphyllos Scop. bracts during phenological development. *Phytochemistry. 167*, 1-11, 2019.
   DOI: http://dx.doi.org/10.1016/j.phytochem.2019.112084
   IF: 3.044

2. **Szűcs, Z.**, Plaszkó, T., Cziáky, Z., Kiss-Szikszai, A., Emri, T., Bertóti, R., Sinka, L. T., Vasas, G., Gonda, S.: Endophytic fungi from the roots of horseradish (Armoracia rusticana) and their interactions with the defensive metabolites of the glucosinolate - myrosinase - isothiocyanate system.
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List of other publications

3. Gonda, S., **Szűcs, Z.**, Plaszkó, T., Cziáky, Z., Kiss-Szikszai, A., Vasas, G., Mikóné Hamvas, M.: A Simple Method for On-Gel Detection of Myrosinase Activity.
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Phytochemistry. 106, 44-54, 2014.
DOI: http://dx.doi.org/10.1016/j.phytochem.2014.07.002
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