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Thesis of doctoral (Ph.D.) Dissertation

Identification of xenobiotics produced by *Lactobacillus casei* and metabolic fingerprinting of self-incompatibility of apricot

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Thesis of PhD Dissertation

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INTRODUCTION

The analysis of primary and secondary metabolites under the molecular weight of 1.5 – 2 kDa is covered typically by the field of metabolomics which is becoming more and more popular and hence is developing rapidly. Metabolomics can help us to explore metabolic pathways, looking for biomarkers and understanding biochemical processes by complementing the results of transcriptomics and proteomics, through the analysis of metabolites which greatly contribute to the final phenotype. The applicability of metabolomics is quite wide, therefore I used it in my studies for completing two different tasks.

Part of my work covers the investigation of xenobiotics (xenos is originated from greek; it means stranger) which are artificially synthesized, unnatural materials. The main sources of chemical xenobiotics are the absorption of drugs, food additives and industrial or agricultural contaminations *e.g.* the presence of pesticide residues on the surface of vegetables and fruits. Living organisms may be exposed to their harmful effects. The most obvious way is getting in direct contact with consumed xenobiotics. In addition, the formation of such reactive metabolites which appear in huge amount from the interaction between xenobiotics and the microbiom of the gastro-intestinal system (including *Lactobacilli*) is also significant. Therefore the investigation of new xenobiotics originated from the interaction of the microbiom and pesticides is a highly current topic.

In the second part of my work I investigated the molecular background of sexual incompatibility of rosaceous fruit tree species. However, the pistil and pollen part genes are already identified, their interactions are still unclear. The increasing number of data suggests that there could be other *loci* outside of the *S-locus* which may severely influence the evolution of self-compatible/self-incompatible phenotype. However, in case of fruit trees such modifier *loci* have not been isolated yet. This field of plant science in addition to its economic significance can play an important role to deduce molecular networks and the evolution of plants.

AIMS

First aim:

I investigated *in-vitro* the interaction between *Lactobacillus casei*, a common member of the human digestive system and a worldwide utilized pesticide, fenhexamid. My objective was to develop a method which is capable for the detection of chlorine-containing metabolites by the software. My further aim was to detect and identify the chlorine containing xenobiotics originating from the interaction between bacteria and fenhexamid,

Second aim:

I investigated the sexual incompatibility of the rosaceaus apricot by metabolomic fingerprinting. The mechanism of sexual incompatibility reactions have not been fully deduced yet, consequently my aims were as follows:

- Tracing the processes which undergo within the pistils of self-incompatible and self-compatible apricot flowers after self-pollination by LC-MS metabolomic fingerprinting. With this kind of metabolomic fingerprinting my objective was to distinguish the groups originating from different genotypes and pollination time with statistical methods.
- Searching for biomarkers which can aid the differentiation of self-incompatible and self-compatible groups. After their discovery my aim was to identify them.

MATERIALS AND METHODS

For separation I used an Agilent 1100 binary HPLC instrument (Agilent Technologies, Waldbronn, Germany). As stationary phase I used a Zorbax Eclipse XDB-C18 (3.5 µm, 2.1 mm x 50 mm, Agilent) column. For compound identification during my experiments I used an Agilent 6530 Accurate Mass Q-TOF LC/MS (Agilent Technologies, Palo Alto, CA, USA) mass spectrometer. The liquid chromatography system and mass spectrometer was coupled by an electrospray ion source (ESI), a so called Dual-spray source. Mass accuracy of the instrument was below 3 ppm and mass resolution >10000 (FWHM) at *m/z* 400 which was checked by the analysis of a pesticide standard mixture. I used the same coupled system during both the analysis of xenobiotics of fenhexamid origin produced by *L. casei* and the metabolomic investigation of apricot. Separation was achieved by solvent gradient elution on

the RP C18 stationary phase already mentioned. The settings of the mass spectrometer and the applied data acquisition modes were also the same in both parts: first I used MS^1 detection then I determined the differentiating compounds with the aim of the software and in a subsequent step the compounds of interest were fragmented in Auto MS/MS mode. Mass spectra were recorded in MS^1 mode in the range *m/z* 100-1700 and *m/z* 50-1700 in MS^2 mode.

After the LC/MS analysis of the prepared samples, Molecular Feature Extraction (MFE) a non targeted searching algorithm was applied to find the metabolites. Usage of the algorithm was justified by the high number of the present metabolites coming from the complex biological matrix. According to the manufacturer's instructions I used the so called recursive workflow which ensures that the software to operate more reliably.

In case of the investigation of xenobiotics produced by *Lactobacillus casei* the used *L. casei* Shirota species originated from the National Collection of Agricultural and Industrial Microorganisms (NCAIM, Budapest). The interaction between bacteria and the pesticide was analysed *in-vitro* in MRS broths and the concentration of fenhexamid was set to 5, 40 and 100 µg/ml. Broths were kept at 37 °C for 72 hours and as control samples cell-free solutions have also been prepared containing fenhexamid in a concentration of 100 µg/ml. Samples were collected 24, 48 and 72 hours after starting fermentation. Supernatant was separated from each sample by centrifugation and ten times dilution was performed to set the final ACN concentration to 5 % v/v. Samples were pipetted into glass HPLC vials and filtered through a disposable 0.45 µm PTFE syringe filter before injection to the LC-MS system. In a subsequent step samples were enriched with C18 solid phase extraction (SPE) cartridges in order to enhance detection of xenobiotics present in low concentration. During MFE compound search I focused on chlorine containing ones among all compounds and differentiating ones were filtered out after the comparison of control and experimental samples with the following statistical methods: filter by frequency, t-test, and fold change analysis.

During metabolic fingerprinting experiments of apricot self-incompatibility two apricot species were used: 'Ceglédi Óriás' (S_8S_9) and 'Pannónia' (S_CS_C) as self-incompatible and self-compatible species, respectively. After the branches that had relatively high number of flower buds on them were collected, they were transported into the laboratory and kept in tap water. In a subsequent step, the flowers were emasculated (anthers were removed leaving only pistils in the flowers) and controlled hand-pollination of the pistils was performed by collecting pollen grains from their own removed anthers. Pistils of both species without pollination were also collected, and then the other ones were harvested 3, 24 and 96 hours after pollination.

This was done to be able to trace temporal evolution of pollen tubes. Collected pistils were immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Thereafter they were disrupted in a mortar and from the gained powder 100±1 mg portions were weighed on an analytical balance into sterile 1.5 mL Eppendorf tubes. Three biological replicates were made from both species in every pollination stage and a total of 24 samples were prepared. Then 990 µl of 80:20% v/v methanol:deionised water mixture as extraction solvent and 10 µl tebuconazole in ACN as internal standard was added to the samples. Extraction was performed by thorough vortexing and shaking in a thermomixer at 25 °C for 15 minutes. Insoluble plant material was separated from the extracts by centrifugation. Ten fold dilutions with deionised water were performed on the separated supernatants and samples were filtered through disposable syringe filters of 0.45 µm pore size before injection into the LC-MS system. After compounds were found by MFE algorithm statistical analysis was performed in order to filter the differentiating ones. Statistical differential expression analysis consisted of the following methods: 1, filtering by frequency; 2, retaining entities that monotonously increase or decrease with time; 3, t-test; 4, finally a heatmap containing the differential compounds was created and PCA was performed in order to visualize how the groups are differentiated and to determine the most significant metabolites.

RESULTS

Searching for xenobiotics of fenhexamid origin produced by *Lactobacillus casei*

Cell death could not be observed in cell cultures with the three fenhexamid levels applied. On the other hand, higher fenhexamid concentration could not be justified since 100 µg/ml is already 2.5 times higher than the highest allowed MRL value for any fruit or vegetable commodity, therefore only the samples with 100 µg/ml fenhexamid addition were analyzed in further experiments. Based on the observation that initial TIC chromatograms were rather “crowded” with peaks I modified the program of the initial gradient elution. However, baseline separation of every chosen compound could still not be achieved; it was not investigated further as this was not the goal of this work. Separation of several thousand other compounds could also not be achieved since it could not be a realistic expectation with the available instrumentation.

Contrary to the generally observable decrease of full width at half maximum (FWHM), and theoretical plates (N) values, considering the aim of the work the increase of signal-to-noise and chromatographic resolution (R_s) justified the previously mentioned change.

In a subsequent step towards getting a particular feedback about the reliability of the non-targeted searching algorithm I considered testing it on a known pesticide standard mixture necessary. Based on the observations I could make certain of the capability of the software to properly predict the isotopologue pattern of compounds. Through modification of the settings of the algorithm my aim was to find all of the pesticides and to decrease the number of false hits. For this purpose I used a standard stock mixture solution containing 17 known pesticides in the concentration of 10 ng/ml. Additionally in order to prove the applicability of the algorithm for the detection of chlorine containing compounds in a biological matrix I added the previously applied pesticides into MRS broth. Due to the proper settings the software was also able to recognize all pesticides (17/17) with at least 4 isotopologues per compound (contrary to the default settings). Based on these experiences the optimized settings were applied for further experiments. However, despite the settings optimization it is still possible that the software makes mistakes thus manual supervision and correction cannot be avoided, this way quality of molecule recognition could be significantly improved.

Since I was looking for degradation and detoxication products originating from fenhexamid, I used the information about the previously determined compounds. Namely, the chlorine containing hydroxy-phenyl group which is part of fenhexamid does not degrade and one from the two chlorine atoms at positions 2 or 3 stays in the detoxication product for sure. Since Cl^{37} is a relatively frequent isotope the A+2 isotope is remarkably more intensive than organic compounds comprising of only CHON elements and eventually phosphorus or sulphur. This reason they are recognizable even manually or by software. Recognition by the software was performed as follows: based on the isotopologue pattern of the compounds assigned by the non-targeted MFE algorithm I determined the molecular formulae. This molecular formula generation was done by the software and at least one obligatory chlorine atom had to be considered. Consequently good quality molecular formulae could only be observed in the case of compounds that had a great chance to contain chlorine. However, the predicted chlorine containing compounds were also supervised manually, it seemed to be completed easily because of the significantly decreased number of potential candidates. Statistical differentiation could be started after the whole list of compounds was finished and the grouping of samples was also done.

Control and experimental samples were first analyzed without enrichment but except impurities of fenhexamid originating from its synthesis no other chlorine containing compounds were assigned. Consequently the metabolites of interest can be present in lower concentration in the cell cultures. Based on the remaining chlorine containing hydroxy-phenyl ring of all known bio and photo degradation products of fenhexamid I supposed that they can be retained due to their hydrophobic character on a reversed phase C18 SPE cartridge, therefore I enriched the samples this way. The most intensive chlorine containing metabolite (463.1176 Da) after enrichment could also be observed in positive and negative ion mode. One more quite intensive compound was present in negative ion mode (505.1279 Da). Accurate mass and molecular formula of the most intensive compound were the same as those of the glucose conjugate of fenhexamid, fenhexamid-*O*-glucoside which is one of the already identified detoxication products in plants. Fortunately, fenhexamid-*O*-glucoside standard originating from individual synthesis was available. By injecting it into the same LC-MS system the identity of fenhexamid-*O*-glucoside was proved by the same retention time values, MS^1 and MS^2 mass spectra with those of the compound of interest. It was also proved that the latter compound was also a fenhexamid derivative because fenhexamid was observed in its mass spectrum as in-source fragment. Unforunately the intensity of MS^2 fragments and the observed mass accuracy was not sufficient for unambiguous identification. Since there is no previously identified fenhexamid derivative having a monoisotopic mass of 505.1279 Da, no attempts were done for the identification of this compound. Even though, glycosilation is an already known way of solubilization and stabilization of compounds, neither fenhexamid-*O*-glucoside and to the best of our knowledge nor any other pesticide-glucosides were identified so far in bacteria. Based on the observations, production of the more hydrophilic fenhexamid-*O*-glucoside and the supposed glucoside conjugate by lactic acid bacteria can serve as useful information in the evaluation of the MRL value of fenhexamid and its absorption in the gut. Nevertheless, it surely has to be proved by further *in-vivo/in-vitro* experiments if these can occur in the gut.

Metabolic fingerprinting of self-incompatibility reactions in apricot

Due to the applied methanolic extraction a great number of compounds were present in the extracts. Compound search was performed also in a non-targeted way with the distinction to the previous case that no common property could be determined that could narrow the number of metabolites of interest. By running the algorithm I was looking for the most effective operation therefore the resulted settings of the optimization by the pesticide standard

solution were also applied in this case. However no chlorine containing compounds were expected, modification of the settings were not justified since they are not chlorine specific. They aimed the proper recognition of isotopologue pattern and based on the results the settings of MFE were proved to be appropriate. Similarity of parallel samples was checked by PCA which is an appropriate tool to recognize outlier samples. In case of apricot samples no outliers were observed, groups consisted of three replicates and they showed good similarity to each other. This observation was a good feedback about the reliability of the software in compound assignment. Statistical analysis revealed 72 and 61 differentiating compound in positive and negative ion modes, respectively. The two lists were merged then checked manually to remove false features and redundancy coming from appearance of the same metabolite in both ionization modes. Finally I found 15 significant, differentiating compounds. PCA and heatmap performed based on the final list samples could be clearly differentiated from each other both by pollination time and compatibility. Species and consequently self-incompatible/self-compatible reactions were separated by F2 principal component while F1 was responsible for the separation of samples by pollination time. Based on all these observations I concluded that under recent circumstances the most significantly contributing metabolites to F2 could play role in the evolution of self-incompatibility reactions. The most intensive and most significant differentiating compound showed monotonous temporal intensity increase in the self-incompatible 'Ceglédi óriás' species, detected in positive ion mode (also found as differentiating in negative ion mode). At the retention time of the compound, 10.1 minutes several in-source fragments and adducts were observed. Their common origin was confirmed by the pattern of their EIC chromatograms in retention time which showed good similarity. The monoisotopic protonated form was determined in the following way: molecular ions detected in MS^1 mode were selected for CID fragmentation. The MS^2 spectrum of that in which the actual in-source fragments were present was considered as the protonated form, being observed at *m/z* 549.3266. Presence of in-source fragments were mainly useful because full isotopologue pattern of them was observable which supported the more accurate determination of their molecular formula (isotopologue pattern was not accurate in MS^2 mode). Molecular formulae were finally determined with the aim of molecular formula generator feature of the used software and $C_{27}H_{47}O_{10}$ proved to be the right one among the possible candidates. In a subsequent step databases were searched for the molecular formula and DNP and Scifinder gave the most useful information. Based on the observations Peloruside A seemed to be the most promising candidate. Among the hits Peloruside A had 10 isomers among the total hits of 27 in the list of

Scifinder. Several other facts supported the hypothesis that the compound of interest could be Peloruside A. Namely, that in the list of differentiating compounds an other significantly differentiating one was also present the molecular formula of which was found to be $C_{26}H_{47}O_{11}$ ($(M+H)^+$). It is the same as that of Peloruside B, a natural congener of Peloruside A (differs from the latter in one methyl group). Peloruside B was also present in the list of DNP database and was also significantly over expressed in the self-incompatible species showing increase of intensity in time. As it was previously determined, evolution of pollen tubes is hindered in the lower quarter of pistils that prevent the pollen to reach the ovary and thus prevents fertilization. This phenomenon might be coupled with the microtubule stabilizing effects of Peloruside A and its congeners. The effective antitumor activity of Peloruside A was proved previously on human ovarian carcinoma cells and murine T cells. Peloruside A standards from both sources were injected to the same chromatographic system and retention time, MS^1 and MS^2 spectra were compared with those of the searched compound. Contrary to the same accurate mass, isotopologue distribution, molecular formula, dominant sodium adduct and the known microtubule stabilizing effect of Peloruside A, based on the comparison it became obvious that the compound is not Peloruside A. However, it seemed to be a profound assumption. At the same time the numerous similarities suggested that the two mentioned compounds belong to the compound class of polyketide-based macrolides and have similar effects to Peloruside congeners. I tried to identify one compound among the other differentiating ones after purchasing a standard but I did not succeed. Further attempts to identify other differentiating compounds were not performed by MS^2 experiments mainly because of their low intensity and thus inaccurate isotopologue distribution causing unreliable determination of molecular formula.

My aim through the metabolic investigation of apricot pistils after self-incompatible and self-compatible pollinations was to shed light on the less known aspects of pollen tube growth and molecules in downstream reactions of the self/non-self recognition. The study successfully revealed putative metabolites that significantly contribute to the sexual compatibility reactions and hence besides genetic and proteomic studies my observations can help to get closer to the understanding of the underlying physiological reactions. However, further experiments would be required for answering the question just as the unambiguous structural identification of such compounds and the verification of their role in sexual incompatibility induced pollen tube arrest.

NOVEL SCIENTIFIC RESULTS

1. During the search for xenobiotics of fenhexamid origin produced by *Lactobacillus casei* I created an algorithm and optimized its settings to be able to recognize chlorine containing compounds. The applicability of the created algorithm was proved by the analysis of 17 pesticides in a biological matrix.

2. With the aim of proper settings of the algorithm I managed to detect chlorine containing compounds in the supernatants of bacteria. Due to unambiguous identification of fenhexamid-*O*-glucoside I managed to prove that detoxication through glycosylation - as already known in plants - is also possible in case of *L. casei*. In addition to the identified fenhexamid-*O*-glucoside one more metabolite is supposed to originate from fenhexamid which could be another glycoconjugate.

3. I investigated sexual incompatibility reactions of apricot flowers by metabolomic fingerprinting. In the experiment design the sample groups to be differentiated were appointed according to self-compatibility/self-incompatibility and pollination time. As a result I succeeded to clearly differentiate sample groups by genotype and also by pollination time which proves that metabolic investigation of self-incompatibility is relevant and significantly contributes to the comprehensive understanding of the reaction.

4. With the aim of LC-MS based metabolomic fingerprinting 15 metabolites significantly contributing to self-incompatibility reactions were determined. Among them, the most significant compound is assumed to belong to the compound class of polyketide-based macrolides. This putative identification of the type of this compound was supported by the known microtubule stabilizing effect of Peloruside A and its similarity to the previously covered physiological aspects of self-incompatibility.

LIST OF PUBLICATIONS

National and international conference abstracts:

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