

Final report

1. Introduction, aims.

This project aimed to obtain a better understanding of the interactions between the non-pathogenic endophytic fungal community and the plant metabolome (all molecules in the plant system) using an important crop of our region, *Armoracia rusticana* (horseradish), as a model.

The goal was to answer the research questions in two distinct experimental approaches: (A) experiments relying on isolated endophytic and soil fungi and (B) culture-independent (high-throughput) approaches. While approach A was useful to prove variability of the members of the endophytic community and to obtain information about their functions, approach B is the only way to get a global, holistic view on the assembly of the endophytic community in field-grown horseradish roots.

The detailed research plan of the current grant was built around four hypotheses/research directions: (1) characterization of the endophytic fungal microbiome of horseradish, (2) showing the adaptation of endophytes to the plant metabolome, (3) assessing the chemical variability of horseradish varieties and (4) the study of postharvest changes in horseradish roots. After the failure to reproduce our promising preliminary results from hypothesis 4, we focused our efforts on the other three research directions.

2. Key results.

2.1. Studies employing isolated fungi.

A collection of endophytic fungi from horseradish and soil fungi from the same site were used for these studies. The fungi were identified via standard genomic methods.

With isolated endophytic fungi and soil fungi, a VOC (volatile organic constituent) profiling was successfully accomplished using headspace GC-MS with a technique capable of sensitive detection of several typical VOCs (acetone, methyl acetate, methyl formate, ethyl acetate, methyl butanol isomers, styrene, beta-phellandrene), along with sulfur-containing glucosinolate decomposition products, including allyl cyanide and allyl isothiocyanate and other sulfuric compounds - carbon disulfide, dimethyl sulfide. The VOC patterns of fungi belonging to *Setophoma*, *Paraphoma*, *Plectosphaerella*, *Pyrenochaeta*, *Volutella*, *Cadophora*, *Notophoma* and *Curvularia* genera were described for the first time. The VOC pattern was significantly different among the isolates. The ability to accelerate decomposition of sinigrin (putative myrosinase activity) was detected in case of many fungi, supporting our former results showing usage of plant defensive precursors by fungi. On the other hand, endophytes and soil fungi as groups could not be separated by VOC pattern or intensity. The results were published in *Metabolites* (Q2 in Biochemistry, IF 4.9).

Another of our key hypotheses has been proven and published recently in a paper in *Plants* (Q1 in Plant Sciences, IF 4.6), namely, differences in fungal tolerance towards glucosinolate-derived compounds differ among various fungal groups. To our surprise, endophytes and soil fungi could not be distinguished, but taxonomic differences were revealed.

A special system capable of studying extremely volatile antifungal agents was used to assess MIC values for glucosinolate-derived nitriles and a model ITC (isothiocyanate). The results suggest that the difference between the soil microbiome and the endophytic microbiome ("the root effect") can be in part explained by different sensitivities to glucosinolate downstream products. E.g. the higher abundance of *Fusarium* in the vicinity of Brassicaceae roots can also be the result of such taxonomic differences.

What is more, we have also shown that a synergistic interaction exists between glucosinolate downstream products, in particular, nitriles and ITCs. These explain several former questions on this interaction, as detailed in the published paper.

We believe these results will influence how we look at glucosinolate downstream machinery, as they explain some unknowns regarding glucosinolate-derived nitriles, as we recently reviewed.

2.2. Metabolome - microbiome correlations in horseradish roots.

Instead of the originally planned on-gel methods, characterization of the fungal community was carried out using the more advanced high-throughput sequencing, resulting in much more information and the possibility to test correlations between fungal abundance and metabolome features. A key paper of the project was successfully published on metabolome - fungal community correlations in horseradish in *Frontiers in Plant Science* (D1 in Plant Sciences, IF 5.6).

This paper describes the variability of the horseradish fungal microbiome among accessions with diverse chemistry and suggests which compound classes are important for shaping the plant microbiome. The most abundant endophytic genera were *Plectosphaerella*, *Thanatephorus*, *Fusarium*, *Tetracladium*, *Volutella*, *Neocosmospora*, *Nectria*, *Peziza*, *Paraphoma* and *Dactylonectria*, along with non-resolved genera from Ceratobasidiaceae, Pleosporales, Pezizaceae, Nectriaceae, Didymellaceae and Morosphaericaceae. The abundance of several fungi significantly correlated with the level of one or more chemical features. Overall, about 1/3 of the plant endophytic fungal community was subject to this phenomenon. The most influenced strains included *Brachyphoris*, *Debaryomyces*, *Fusarium Melanoleuca*, *Monosporascus*, *Setophoma*, *Tetracladium* and *Thanatephorus*. Surprisingly, the most influential compound groups were peptides, which included a glutathione - isothiocyanate conjugate (a downstream product of isothiocyanates). The compounds influencing fungal community assembly also included flavonoids, indoles as well as primary metabolites.

What is more, the LC-ESI-MS method and untargeted metabolomics workflow developed for horseradish could be used without major modifications in another matrix, germinating fenugreek. These results have been published in *Food research international* (D1 in Food Sciences, IF 8.1).

The link between plant chemistry and plant microbiome was also tested in a much more sophisticated experiment: a horseradish monoculture used for commercial production in a 4-ha area with various soil types but uniform agrotechnology was sampled for soil and horseradish roots. The sampling was run for 4 consecutive years, at 13 locations within the site. To obtain a holistic

view on the soil chemistry - soil microbiome - horseradish chemistry - horseradish microbiome associations, several statistical methods have been tried. After non-rewarding attempts to get meaningful results through redundancy analyses and pathway analysis (SEM) models, our recent success in network analysis has shown that (1) soil chemistry influences endophytic community assembly in horseradish, but the effects are delivered indirectly, through changes in a specific subset of plant chemical constituents and changes in the soil microbiome; (2) a small subset of the endophytic community is responsible for the interaction with the plant metabolome and the soil microbiome, and the residual part of the community is assembled through secondary, tertiary competitive / cooperative interactions. We believe that these results are especially interesting as this variability exists in a single, 4-ha area, where dispersion and homogenization of the microbial community by wind and agrotechnology acts against formation of gradients, not to mention the major meteorological differences between the years 2019-2022. A paper is currently in preparation from these results.

We also prepared and later published two review papers on the core topic of the grant, namely, the interactions between fungi and the compounds of the glucosinolate - isothiocyanate pathway, in *Phytochemistry* (Q1 in Plant Sciences, IF 3.8) and in *Journal of Fungi* (Q1 in Plant Sciences, IF 4.6). These two papers cover all downstream products of GSLs known to date and their documented interactions with fungi, including but not limited to, antifungal effects, transcriptome-level changes, biotransformation products as well as possible agricultural applications including soil microbiome changes during biofumigation and shelf-life improvement of post-harvest products. To date, these two papers received 29 independent citations (Scopus).

An additional study on the possible application of horseradish essential oil (mainly consisting of allyl isothiocyanate) has also been published in *Plants* (Q1 in Plant Sciences, 4.6). Another paper on the chemical variability of horseradish tissue cultures was published in *Molecules* (Q1 in Pharmaceutical Science, IF 3.0).

3. Publications, dissemination.

In total, 8 articles have been published in association with the research carried out in the proposal, with a total IF of 39.843. In 7 of 8 articles, the PI is last/first author and corresponding author.

The grant results on the interaction between the plant metabolome and the plant microbiome have also enabled significant networking possibilities during the grant period for the PI and other group members.

I visited the Misión Biológica de Galicia (Pontevedra, Spain) in April 2022 and a Department of the University of Aarhus (Flakkebjerg, Denmark) in February 2023. During both visits, I held lectures on horseradish fungal endophytes and metabolome - metagenome correlations. At both institutes, we agreed on starting collaborations on plant - microbe interactions in Brassicaceae crops.

I also presented the main project results regarding microbiome - metabolome correlations as a poster at the Micrope 2022 (Microbe-assisted Crop Production - Opportunities, Challenges & Needs) conference (July 11-14, 2022, Vienna, Austria), in a lecture at the 10th Congress of the International Society of Symbiosis / 3rd International Conference on Holobionts (July 25-29, 2022, Lyon, France) and on a poster at the IS-MPMI congress (July , 2023, Providende, RI, USA). The feedbacks at all events have reinforced my notion that metabolome - metagenome correlations are of considerable scientific interest.

15th Sep 2023

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