Final report

Examination of endophytic fungi and postharvest changes of horseradish roots (PD 124339)

The PD 124339 3-year project aimed to examine various aspects of the microbiome and metabolome (phytochemical constituents) of horseradish. The final report is structured so that the subsection numbering matches the numbering of the original grant workplan.

1. Microbial diversity.

1.1. Isolation and identification of endophytes from horseradish roots.

Identification of endophytic fungi has successfully been carried out, resulting in a set of isolates from several genera. Our diverse set of endophytic fungi from horseradish now includes five *Fusarium spp.* isolates, two *Paraphoma radicina* strains, two *Plectosphaerella sp.* isolates, and one strain from each of the following taxa: *Pyrenochaeta sp.*, *Volutella sp.*, *Phomopsis sp.*, *Cadophora sp.*, *Colletotrichum sp.*, *Macrophomina phaseolina*, *Setophoma terrestris*, *Oidiodendron cerealis*.

The reference set fungi from the soil includes species from *Fusarium sp.*, *Penicillium sp.*, *Aspergillus sp.*, *Paraphoma sp.*, *Notophoma sp.* and *Curvularia sp.*, the first two being most dominant.

Some of these fungi were successfully used to test chemical adaptation phenomena, as published in (Szűcs et al. 2018; Plaszkó et al. 2020) and are currently involved in several studies. Additional information on the horseradish endophytic microbiome was gathered, as detailed in 1.2.

1.2. Pilot studies to assess the diversity of horseradish roots, using DGGE.

To study the endophytic microbiome of horseradish roots, next-gen sequencing on Illumina was used instead of DGGE, because it can deliver much more information for the same price and effort.

The most frequently used primers unfortunately also amplify the horseradish ITS, resulting in <1% of fungal sequence reads. Therefore, we had to develop a fungus-selective primer set to selectively amplify fungal ITS amplicons from field samples of horseradish roots. The reverse transcripts of the ITS region were successfully used to identify several fungal clades from field horseradish samples. These include *Bacillicladium clematidis*, *Tilletiopsis washingtonensis*, *Schizophyllum spp.*, *Sarocladium spp.*, *Lecanicillium spp.*, *Holtermanniella spp.*, *Mortierella spp.*, *Rhinocladiella spp.*, *Phaeophyscia spp.*, and many sequences that are identified as higher taxonomic levels from both Ascomycota and Basidiomycota. What is more, soil-type dependence was observed in the ratio of different fungal groups in horseradish roots. A manuscript from the data is under preparation. The approach is also to be extended to horseradish varieties in the future (see 3.1.).

1.3. Colonization studies with endophytic strains, in in vitro horseradish clones, assessment of colonization pattern.

Selective staining of fungi in colonized roots of *in vitro* horseradish plantlets has been successfully accomplished (Fig. 1.). The established functional groups (fungi that colonize vascular bundles, fungi that do not, fungi that cannot colonize at all) did not correlate with any of the tested parameters so far, and was not significantly different between the endophyte and soil fungus group.

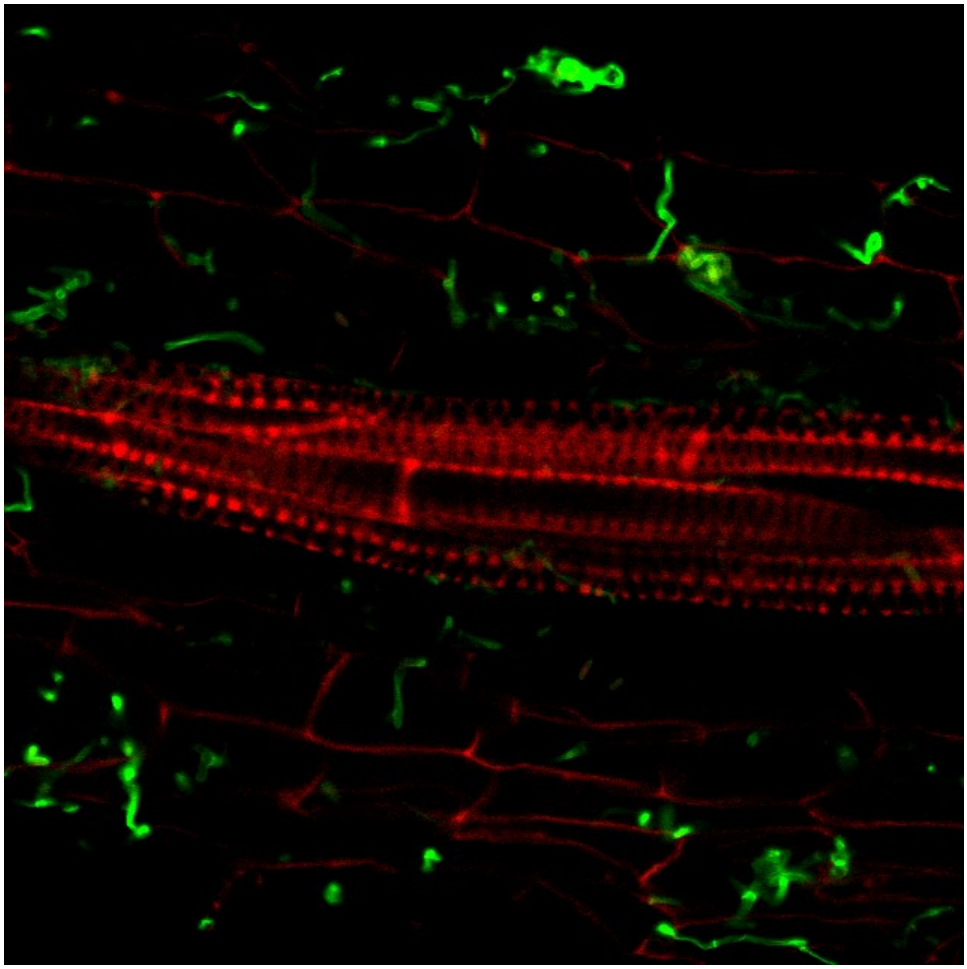

Figure 1. *Plectosphaerella cucumerina* horseradish endophyte colonizing a sterile *in vitro* horseradish root, as visualized by confocal microscopy after selective staining. Green, fungal hyphae stained with Alexa 488 fluorescent labelled wheat-germ agglutinin; red, plant cell walls stained with propidium iodide.

2. Chemical adaptation of the to the plant metabolome.

2.1. Growth of horseradish endophytes in presence of antifungal components of horseradish.

A comparative study was conducted among endophytes and soil fungi from the same soil, with respect to interactions with the glucosinolate – myrosinase – isothiocyanate system. The main results are the following: 1., endophytes tolerate the antifungal compounds of the host better than the soil fungi; 2., endophytes have a higher chance to be able to use the chemical defense compounds of the host as carbon source, than soil fungi; 3., endophytes decompose the chemical defense compounds of the host more efficiently than the soil fungi. These results were included in a published article (Szűcs et al. 2018).

As a follow-up to (Szűcs et al. 2018), the set of endophytic fungi and soil fungi as reference were compared for synergy between isothiocyanates and other, alternative glucosinolate degradation products, regarding antimicrobial activity in 2020. Of 44 tested fungal strains, synergy between phenylethyl isothiocyanate (PEITC) and the corresponding nitrile was proven in 11 cases. A similar phenomenon was found for the PEITC - indol-3-acetonitrile pair, but not PEITC - indoyl-methyl amine pair. Interestingly, raphanasumic acid, diindoyl-methane did not result in sufficient inhibition of the tested strains. Of special interest is the fact that endophytes and soil fungi showed some difference regarding sensitivity to combinations of the compounds. This might be a new possible ecological

function of glucosinolate decomposition products, a manuscript is under preparation from the above observations. This research question is still under examination.

2.2. Biotransformation of horseradish constituents by endophytic fungi.

A VOC (volatile organic constituent) profiling of the endophytes and soil fungi was accomplished using headspace GC-MS by examining the headspace of the fungi growing on horseradish extracts. A new cultivation technique in headspace vials was proposed. The proposed technique enabled 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 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. On the other hand, endophytes and soil fungi as groups could not separated by VOC pattern or intensity. A manuscript (Plaszkó et al. 2020) from these data was successfully published in the special issue "Plant metabolomics" of the journal *Metabolites*.

2.3. Endophyte myrosinase characterization.

A straightforward analytical method was developed by our lab that enables studying of myrosinase (the enzyme of horseradish that biosynthesizes the pungent allyl- and phenylethyl isothiocyanates). The study was published in 2018 (Gonda et al. 2018), it shows an application to detect both plant-, and endophyte-derived myrosinase enzymes.

3. Chemical variability of horseradish.

3.1. Chemical variability of horseradish varieties.

The chemical variability of horseradish is quite high, as shown in our study examining a few samples (Papp et al. 2018). Our studies were extended to the overall metabolome variability, and were conducted on both different varieties in the same soil, and different soils with the same variety as well.

A single-site variability study on 27 horseradish varieties conducted from 2018 showed significant variance among the tested varieties, but year-to-year variability was quite high: the 2019 samples contained much less allyl isothiocyanate and 2-phenylethyl isothiocyanate than the 2018 samples of the same varieties. The obtained data are to be used to test the influence on glucosinolate and isothiocyanate content on microbial composition, in case the non-glucosinolate metabolome shows low variance - which we expect based on the already evaluated soil dataset. This requires untargeted metabolomic measurements that are to be done this year.

Chemical variability and enzymatic variability of a series of plant tissue cultures of horseradish have also been evaluated and the results were published. It was shown that the organ of origin influences glucosinolate composition, and myrosinase isoenzyme expression patterns, which, interestingly also results in alternative breakdown pathways for glucosinolates (Bertóti et al. 2019).

3.2. Phytochemical responses to cultivation parameters.

After a succesful pilot in 2018, a large study was conducted on effects of soil type (and parameters) on isothiocyanate content, glucosinolate pattern, peroxidase enzyme activity, myrosinase enzyme activity, metabolome and inorganic constituents in horseradish roots, by examining samples taken from the same crop production site in November 2019 and November 2020. A 65-sample set from 13 sampling points of different soils were obtained each year. The points were selected based on a drone photo of the approximately 4-ha area. Soil sample analysis has proven that a substantial variability has been sampled; some highlighted parameter ranges were as follows: Arany index 25-43, humic acid 0.2-

2.32 w/w%, Mg 18-169 mg/kg; S, 1-6.9 mg/kg; K_2O, 36-145 mg/kg. Many tested parameters of the roots showed significant correlations with soil type, or, more importantly, several of the measured soil parameters. Significantly affected parameters include several glucosinolates (Fig. 2.) including sinigrin, allyl isothiocyanate content, but not phenyl-ethyl isothiocyanate content. Untargeted metabolomics has also been successesfully used to cluster samples according to the soil they've grown on. In other words, a fingerprint-like chemical pattern is observed, that enables identification of the soil the sample is taken from. The 2020 sample set showed a significantly higher glucosinolate and isothiocyanate content, but some the soil effects seem to be preserved across the two years. These samples will serve as the basis of metabolome - microbiome correlation data-mining in the near future, though sampling of the same points in 2021 is also likely. The data are planned to be used as basis of at least one publication.

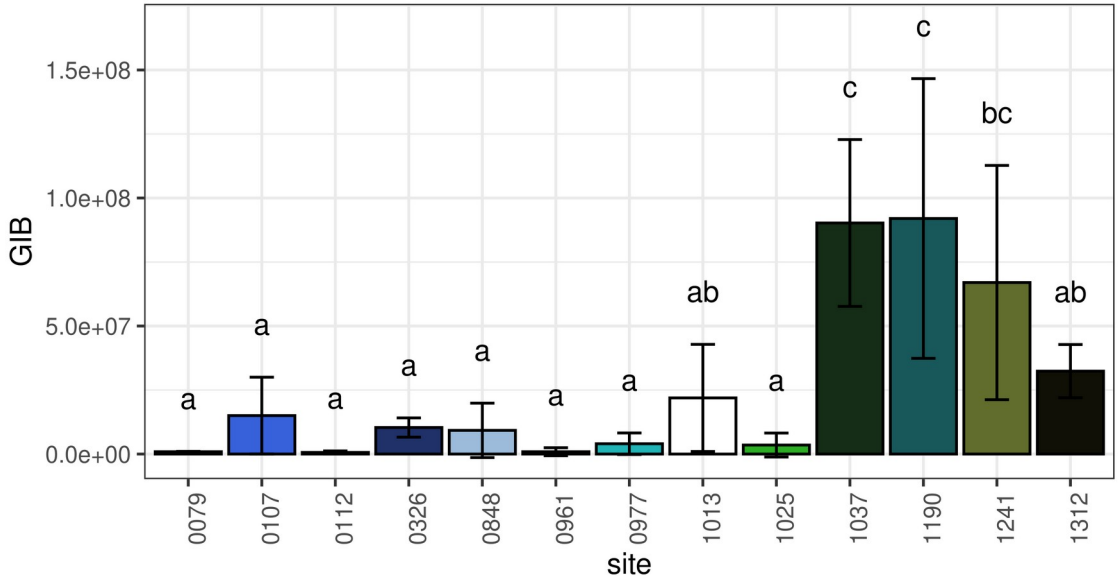

Figure 2. Abundance of the glucosinolate glucoiberin in horseradish roots from different soil types, quantified by LC-MS. The samples were gathered from a 4-ha commercial horseradish production area with various different soil types, in 2019 November.

4. Elicitation.

Despite the initial successes in 2017, no reproducible elicitation phenomenon has been found during post-harvest elicitation of horseradish roots. Various agents have been tested, including methyl salicylate, methyl jasmonate as well as dryout. No significant increase in isothiocyanate content was shown to be reproducible. Therefore, our efforts were focused to the previous (1-3.) hypotheses with much more promising phenomena.

Published articles / references

Bertóti, Regina, Andrea Böszörményi, Ágnes Alberti, Szabolcs Béni, Márta M-Hamvas, Éva Szőke, Gábor Vasas, and Sándor Gonda. 2019. “Variability of Bioactive Glucosinolates, Isothiocyanates and Enzyme Patterns in Horseradish Hairy Root Cultures Initiated from Different Organs.” *Molecules* 24 (15): 2828. https://doi.org/10.3390/molecules24152828.

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