Study of endophyte-plant interactions with metabolomic techniques in horseradish

Final report

1. Scientific articles and presentations based on the project results.

1.1. Research articles.

The reserach within the project has resulted in the following published / submitted articles:

1., S Gonda et al., Myrosinase Compatible Simultaneous Determination of Glucosinolates and Allyl Isothiocyanate by Capillary Electrophoresis Micellar Electrokinetic Chromatography (CE-MEKC). 2016, *Phytochemical analysis.* [1]

2., R Bertóti et al. Glutathione protects *Candida albicans* against horseradish volatile oil. 2016, *Journal of Basic Microbiology.* [2]

3., S Gonda et al., Efficient biotransformation of non-steroid anti-inflammatory drugs by endophytic and epiphytic fungi from dried leaves of a medicinal plant, *Plantago lanceolata* L. 2016, *International Biodeterioration & Biodegradation*. [3]

4., Zs Szűcs, T Plaszkó, Z Cziáky, A Kiss-Szikszai, T Emri, R Bertóti, L T Sinka, G Vasas, S Gonda: Endophytic fungi from the roots of horseradish (*Armoracia rusticana*) and their interactions with the defensive metabolites of the glucosinolate - myrosinase - isothiocyanate system. 2017, submitted to *BMC Plant biology*, a revised version is under preparation.

1.2. Conference presentations.

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

SUSTAIN: Endophytes for a Growing World. Dublin, 28-29th August 2017.

2. Introduction.

2.1. Horseradish.

Horseradish is a member of the plant family Brassicaceae which contains many edible plants of high economical significance. Horseradish is a cultivated plant, mainly considered a food and a spice, but is also a medicinal plant with high amounts of bioactive natural products. Therapeutic use in ethnobotany has been described with high proportion of natives attributing therapeutic value to the root, as summarized in our recent review [4]. The main bioactive constituents of horseradish roots are the glucosinolates, which undergo enzymatic breakdown during damage of the plant tissues into volatile isothiocyanates, but under certain conditions, corresponding nitriles, or other molecules are also formed [5]. Freshly homogenized horseradish root is a rich source of allyl-, and other isothiocyanates [6].

2.2. Endophytes.

Endophytes are microbial organisms that live inside higher plants, without causing apparent symptoms. Presence of endophytes in plants is considered a mutualistic interaction [7], that generally results in higher tolerance against herbivory, drought stress and diseases in the host, and higher growth rate as well. Endophyte research mostly focuses on ecology, host preference and purification of bioactive natural products from isolated cultures of the endophytes as well as development of field applications to contribute to plant health and yield. However, the interaction at the metabolome level is not researched in-depth, data on the contribution of the endophytes to the plant metabolome are lacking. Before the current grant, we started our studies in this field by describing effects of endophytic fungi on the metabolome of a model medicinal plant, *Plantago lanceolata* (ribwort plantain). We successfully isolated ten plant-associated fungal strains from *P. lanceolata*, and have shown, that the plant-associated microbes can inhibit breakdown of phenylpropanoid glycosides, and add new phenolic compounds to plant natural product matrices [8], and many also have the ability to decompose iridoid glycosides [9].

2.3. Hypothesis, approaches.

The main goal of the current research project was to test the hypothesis stating that endophytes in horseradish have myrosinase activity. In a broader sense, we wanted to show that an activity usually attributed to the plant is also there in the endophytic fungi of the same plant.

To test the above hypothesis, we have chosen horseradish (*Armoracia rusticana*, syn.

Armoracia lapathifolia) as a main model plant, because it contains a very high amount of antifungal secondary metabolites and it is also a significant crop of the Debrecen region.

The most important approach was the following: to test the effects of a few endophytic fungi on the pure plant secondary metabolites or the horseradish extract.

This required isolation and identification of endophytic fungi from horseradish and development of different analytical methods to monitor the changes. Three methods, a GC-MS, an LC-MS and a CE-MEKC method were developed to be able to quantify the isothiocyanates, glucosinolates and myrosinase activity, respectively.

3. Results.

3.1. Endophytic fungi.

3.1.1. Identification of endophytic fungi.

Seven endophytic fungal strains were successfully isolated from surface-sterilized horseradish [10] and successfully maintained on Malt Extract Agar, Potato Dextrose Agar or horseradish extract media. It is interesting that fungi harbor such a chemically unfriendly environment. The strains were identified using relevant DNA sequences as described earlier [8] (ITS, β-tubulin, α-actin and translation elongation factor 1α). The strains will be referred to as bold italic numbers throughout the rest of the report.

The strains belong to the following species:

1. *Fusarium oxysporum*

2. *Macrophomina phaseolina*

3. *Fusarium oxysporum*

4. *Setophoma terrestris*

5. *Paraphoma radicina*

6. *Paraphoma radicina*

7. *Oidiodendron cerealis*

F. oxysporum was shown to have endophytic strains in a relative plant species, *Brassica napus* [11]. The members of the other group belong to the related clades *Pleosporales* (***4-6***) and *Botryosphaeriales* (***2***) [12]. These species have also been described as endophytes so far [13,14]. The case of

Oidiodendron cerealis (*7*) is somewhat unusual, as it is a typical Ericaceous ("ericoid") endophyte [15]. This identification is part of a manuscript under review in *BMC Plant Biology*.

3.1.2. *In vivo* recolonization study.

The parameters of maintaining a horseradish whole plant tissue culture have been successfully optimized. Horseradish clones were regenerated from a stable tissue culture maintained on liquid Murashige Skoog medium (MSM) with 2% sucrose and indole-butyric acid. The plantlets were transfered to hormone-free MSM with 2% sucrose for rooting. After transfer to inorganic MSM (no sucrose), the plants' roots were inoculated with the viable endophytic fungi. After two weeks, the roots were stained with lactophenol blue and examined in light microscope to assess colonization with endophytes. All tested fungi were present in the plant tissues. Most fungi did not cause any symptom during this session, meaning that they are "true" endophytes and not latent pathogens. One exception was *Macrophomina phaseolina* which sometimes turned pathogenic.

3.2. Analytical method development.

3.2.1. Capillary electrophoresis method development.

A capillary electrophoresis (CE) method for simultaneous quantification of glucosinolates (sinigrin, gluconasturtiin) and isothiocyanates (allyl isothiocyanate (AITC), phenethyl isothiocyanate) was successfully developed. Development steps included background electrolyte optimization, optimization of derivatization reaction to be able to sensitively detect isothiocyanates (pH; testing of different derivatization agents; concentration of the chosen derivatization agent, mercaptoacetic acid; compatibility with enzyme activator ascorbic acid) as well as compatibility tests with the myrosinase reaction.

The method allows quantification of the two major glucosinolates of horseradish (sinigrin, gluonasturtiin) and allyl isothiocyanate after myrosinase compatible derivatisation in-vial by mercaptoacetic acid. The chromatograhpic separation takes 2.5 min (short-end injection) or 15 min (long-end injection). For the tested vegetables, measured myrosinase activity was between 0.960–27.694 and 0.461–26.322 µmol/min/mg protein. Horseradish showed the highest values, by far. The glucosinolate content was between 0–2291.8 and 0–248.5 µg/g fresh weight for sinigrin and gluconastrutiin, respectively. Horseradish contained the highest glucosinolate content of all vegetables tested. The possible specificity of plants to different glucosinolates was also shown. Allyl isothiocyanate

release rate was different in different vegetables (73.13–102.13%). The method could also be used for quantification of allyl isothiocyanate from food products.

The presented capillary electrophoresis method requires a minimal amount of sample and contains only a few sample preparation steps, and can be used in several applications (glucosinolate determination, myrosinase activity measurement, isothiocyanate release estimation).

The method was published as a research article in *Phytochemical Analysis* [1].

3.2.2. GC-MS and SPME-GC-MS method development.

A GC-MS method was successfully developed that is able to sensitively identify major and minor isothiocyanates from horseradish (allyl, phenetyl, butyl, pentenyl and butenyl isothiocyanates). The method was developed further to be able to quantify nitriles as well. In this case, the adsorbent (activated charcoal) is placed in the air of the Petri dish to capture the allyl isothiocyanate released by the fungi growing on the solidified horseradish extract. Thereafter, the sample is eluted with methyl acetate and analyzed by GC-MS on a DB5-MS column. In a new SPME-GC-MS approach, the method was applied to show generation of volatile allyl isothiocyanate by *Paraphoma radicina* endophytic strains. The method is part of the article under review *BMC Plant Biology*.

3.2.3. LC-MS method development. Metabolomics.

Horseradish cooked extracts were used to develop an LC-MS method capable of detection of glucosinolates. The method uses a H_2O / MeCN gradient with 0.1% formic acid on a XB-C_{18} column. It was used for screening on an HPLC - LTQ XL mass spectrometer and successfully transferred to an UPLC – Orbitrap for studies using the metabolomics approach. Negative ion mode allowed detection of ten glucosinolates from horseradish extracts, as well as detection of a decomposition product. The detected glucosinolates include sinigrin, gluconapin, glucocochlearin, glucobrassicanapin, glucoiberin, glucoibarin, glucotropaeolin, gluconasturtiin, glucobrassicin, and 4-methoxyglucobrassicin. The same method is positive ion mode is capable of separation and detection of desulfo-glucosinolates (obtained by a sulfatase treatment [16]) but these were not produced by the fungi.

The data were evaluated using the untargeted “metabolomics” approach, the XCMS Online [17] parameters were those suggested for the instrument. Later on, targeted search for glucosinolates [18,19], desulfoglucosinolates and non-volatile glucosinolate metabolites have been carried out, data were further processed in in R [20], using ggplot2 [21]. The used LC-MS protocol was also used to cross-

validate the CE-MEKC method [1].

The method is also used as the main analytical technique in the article under review in *BMC Plant Biology*.

Based on our experience with derivatization of isothiocyanates for CE, the development of a method for sensitive detection of ITCs by LC-MS is running with promising results. Several derivatization reagents were compared for sensitivity.

Scripts and data vizualisation approaches for metabolomic data were those used in [22] with minor additional changes.

3.3. Myrosinase activity of fungal endophytes – decomposition of the plant defensive glucosinolates

We found that testing in standard microbiological media (Saboraud glucose broth) supplemented with sinigrin results in several false negatives for myrosinase activity. In this case, only two of six active strains appeared to have myrosinase activity. It is likely that the "original" plant-like metabolome has a different effect on the gene expression in endophytes. Therefore, horseradish extract had to be used as growth medium. Horseradish roots were extracted with MeOH, evaporated to dryness, resuspended in water and filtered sterile and this liquid was used as the medium for endophytes. The medium contains the horseradish constituents at approximately original concentration, and besides the main glucosinolates (sinigrin, gluconasturtiin) it also contains several minor ones.

The endophytes were inoculated into the horseradish extract aliqouts and incubated for 16 days. For pre-screening of the sampling time, the CE-MEKC method was used. The glucosinolate content of the extract was quantified by LC-MS, after five-point calibration with sinigrin, gluconasturtiin, glucoiberin and glucobrassicin. Thus, all four chemical subclasses were assayed.

LC-MS/MS examination showed that a 250 µL aliquot contained 1.42 µmol sinigrin (2.37 mg mL^{-1}), 0.077 µmol gluconasturtiin (2-phenylethyl glucosinolate) (129 µg mL^{-1}) and several, less abundant minor GLs. In controls, the concentrations of most glucosinolates did not change significantly during the 16 day incubation period ($p > 0.05$). Most of the tested endophytic fungi successfully decomposed most or all glucosinolates (Fig.1.). By the end of incubation, six of seven strains significantly decreased the amount of the major glucosinolate, sinigrin. However, strain *7* (*O. cerealis*) could not decompose any glucosinolates. After calculating the slope of concentration decrease for sinigrin and gluconasturtiin, a wide range of decomposition capacities could be observed among the the strains: values spanned the range 0.606 – 1.476 mM sinigrin / day, 0.018 – 0.057 mM gluconasturtiin /

day. The decomposition of minor glucosinolates were slower (below 0.01 mM / day). Within-species differences were remarkable for strains ***1 - 3*** (*F. oxysporum, **1*** was about 2-fold faster) and ***5 - 6*** (*P. radicina, **6*** was about 2-fold faster for gluconasturtiin, but not sinigrin). The indolic GLs were shown to be less prone to fungal decomposition (Fig.1.d.), even though they were present at much lower concentrations than the main compounds. The other classes (aliphatic, methythiolalkyl and aromatic) were decomposed with more or less similar efficacy.

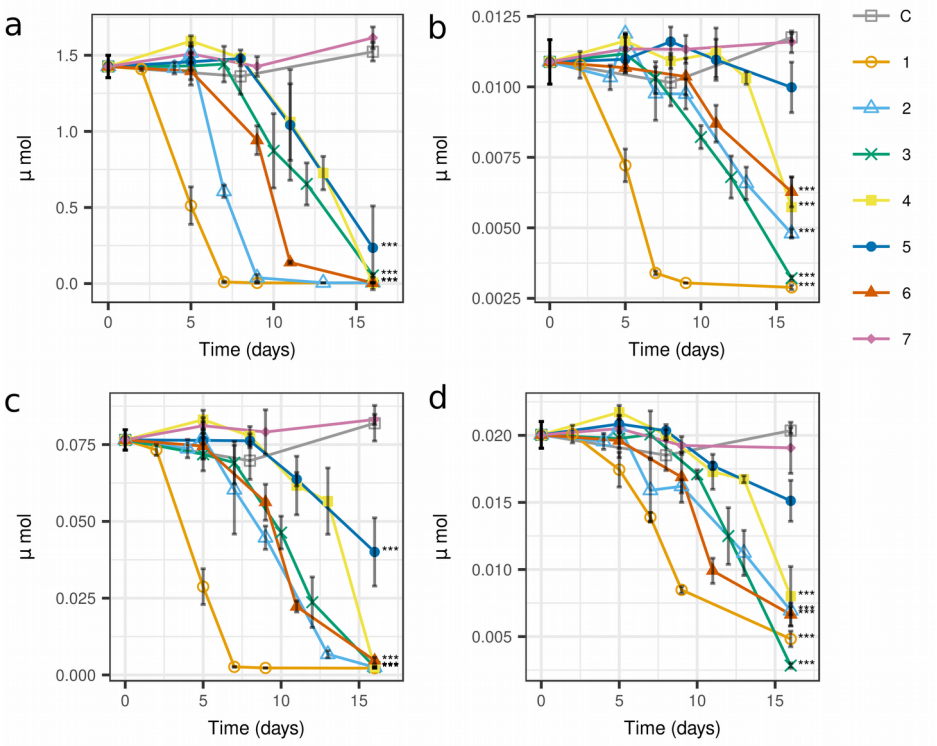

Fig. 1. Decomposition of sinigrin and minor glucosinolates in horseradish extract inoculated by endophytic fungi from horseradish. Subplots: a., sinigrin; b.,glucoiberin; c., gluconasturtiin; d., glucobrassicin. Endophytic fungi: ***1***, *Fusarium oxysporum*; ***2***, *Macrophomina phaseolina*; ***3***, *Fusarium oxysporum*; ***4***, *Setophoma terrestris*; ***5***, *Paraphoma radicina*; ***6***, *Paraphoma radicina*; ***7***, *Oidiodendron cerealis*; ***C***, control (not inoculated). Statistical test: Dunnett's test, endtime samples compared to end-time control (n = 3, ***, $p < 10^{-5}$; **, $p < 10^{-4}$ *, $p < 5*10^{-4}$).

Thioglucosidase / myrosinase enzymes of fungal origin have already been described before [23–26]. Nevertheless, the fact that almost all endophytic strains could decompose most glucosinolates is striking: This suggests that this enzymatic activity is perhaps more widespread than previously expected. The presented potential glucosinolate decomposing ability is a fine example of how the microbial community can modify the plant metabolome in various ways. The presence of this ability is one of the possible reasons of the high abundance of e.g. *Fusarium* sp. (like ***E1***, ***E3***) in the microbiome associated with Brassicaceae plants [27]. The results also highlight the importance of within-species

variability when studying plant – microbe interactions, as also shown by [28].

The above phenomenon can be a mechanism by which the endophytes control the release of the fungitoxic plant chemical constituents. Endophytes usually penetrate root cells intracellulary during root colonization [29]. When non-myrosin cells (cells with glucosinolates) are intracellularly penetrated by endophytes, the fungal myrosinase enzymes can decompose the glucosinolates therein, instead of arming the plant's "chemical bomb".

As we show in the next section, the decomposition is not a "side product" of growth, the glucosinolates (sinigrin, in particular) are used by the fungi as nutrients.

Altogether, we think that the myrosinase activity of these endophytic fungi is now proven.

These results were presented on the Sustain conference in 2017, and are core parts of the manuscript under review in *BMC Plant Biology*.

3.4. The plant defensive metabolite sinigrin as the sole carbon source

The fungal growth was tested in media where sinigrin was the sole carbon source. Czapek-Dox medium (containing inorganic salts) was supplied with sinigrin as the carbon source, an amount equimolar to 2 % glucose. As control, the medium containing glucose was used. Water served as negative control. The experiment was run in 96-well plates for 12 days. The growth was monitored by a plate reader and confirmed by visual investigation.

Surprisingly, four of seven horseradish endophytes (***1-4***) accepted sinigrin as the sole carbon source (Fig. 2.). The two most efficient sinigrin decomposers performed well on both sinigrin and glucose as carbon sources (***1-2***). Less efficient decomposers showed growth but apparently sinigrin provided less favorable conditions than glucose (***3-4***). This is another good example of within-species variability (***1*** vs ***3***).

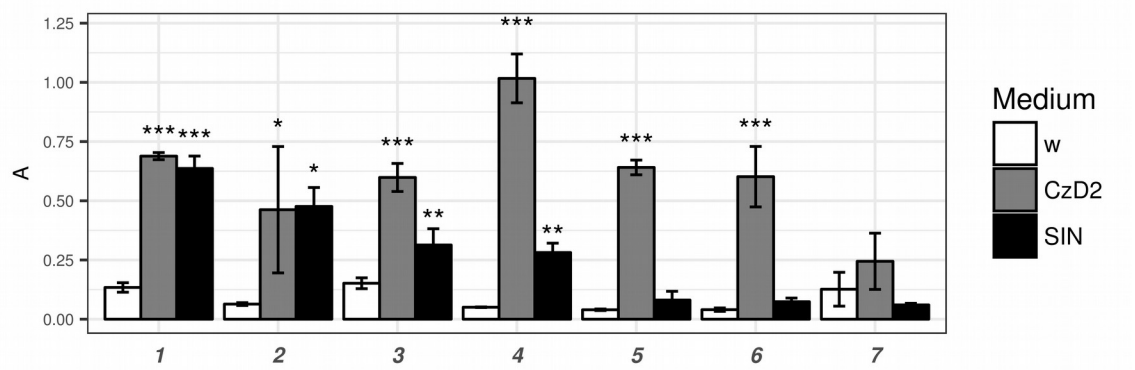

Fig.2. Growth of horseradish endophytes in Czapek Dox media with different carbon sources: 2% (CzD2) glucose or equimolar amount of sinigrin (SIN). Control is pure water (w). Absorbance (A) values were measured by a plate reader at 800 nm. Fungi: ***1***, *Fusarium oxysporum*; ***2***, *Macrophomina phaseolina*; ***3***, *Fusarium oxysporum*; ***4***, *Setophoma terrestris*; **5**, *Paraphoma radicina*; ***6***, *Paraphoma radicina*; **7**, *Oidiodendron cerealis.* Statistical test: Dunnett's test, end-time samples were compared to end-time controls, for each fungus separately (n = 3, ***, $p < 10^{-3}$; **, $p < 10^{-2}$ *, $p < 0.05$).

These results were presented on the Sustain conference in 2017, and are core parts of the manuscript under review in *BMC Plant Biology*.

3.5. Fungal metabolites of the horseradish glucosinolates.

3.5.1. Volatile metabolites.

The plant enzyme myrosinase decomposes the glucosinolates to yield isothiocyanates in the absence of specifier proteins [30]. These isothiocyanates are responsible for the antifungal, insect repellant etc. activities [4]. In the presence of special proteins, the spontaneous rearrangement of the glucosinolate aglycon is driven towards formation of less toxic products. Their role is less clear than that of the isothiocyanates.

It would seem logical if the fungi would have the ability to avoid formation of isothiocyanates to prevent toxicity, as shown in the case of an *Aspergillus* strain [24]. As detection of isothiocyanates from normal SPME vials failed, a new method was developed: SPME was conducted using a whole Petri dish containing solidified horseradish extract.

Only endophytes ***5*** and ***6*** (*Paraphoma radicina*) generated detectable amount of AITC. Allyl nitrile was not detected at all. About 4.4% of the total amount of SIN was released into the air within 24 hours. Interestingly, these fungi could not use sinigrin as the carbon source. The fungi successfully growing on sinigrin (***1-4***) did not emit any detectable amount of AITC. No significant decomposition products of gluconasturtiin could be detected either, but this could be the attributed to their much lower volatility.

The possibly extracellular production of ITCs by ***E5-6*** can give these strains a competitive advantage over more sensitive species. However, volatile products are not always detectable. Despite that the liquid medium was directly extracted with organic solvent and analyzed by GC-MS, volatile products could not be detected during the rapid decomposition of sinigrin by *Citrobacter* [31].

These results were presented on the Sustain conference in 2017, and are core parts of the manuscript under review in *BMC Plant Biology*.

3.5.2. Non-volatile metabolites

No nitrile or AITC was found from the rapid decomposition of sinigrin in case of *F. oxysporum* and *M. phaseolina*. Therefore, non-volatile decomposition products were sought.

The sulfatase activity was not shown in the fungi, as shown by the lack of desulfo-glucosinolates in the treated horseradish extracts (shown by LC-MS).

Hierarchical clustering of the metabolites has revealed compounds whose concentration increases during decomposition of glucosinolates in the late stationary phase of fungal growth. Also, expectable allyl isothiocyanate metabolites were sought based on exact mass. These revealed a metabolite of the allyl isothiocyanate, namely: glutathione – allyl isothiocyanate adduct (GSH – AITC). The compound was synthesized from GSH and AITC under conditions similar to that in our CE-MEKC method [1], the two compounds were found to be identical.

The concentration of GSH – AITC significantly rose in the medium of strains ***4-6*** when the sinigrin decomposition took place. Some increase was also detected in case of ***1-2***. GSH is the major antioxidant in fungi, the allyl isothiocyanate generated from sinigrin were found to spontaneously conjugate with thiols in the fungi.

Altogether we think that the fungal myrosinase is an aspecific enzyme capable of decomposing glucosinolates with different side chains, allyl isothiocyanate being the major product. As free glutathione was also detected from the medium, it seems likely that the fungi avoid toxicity of the generated isothiocyanates by actively recycling their glutathione pool – this can be the mechanism of adaptation to the horseradish chemical environment.

These results were presented on the Sustain conference in 2017, and are core parts of the manuscript under review in *BMC Plant Biology*.

3.6. Glutathione and allyl isothiocyanate.

The central role of glutathione in the protection against isothiocyanates was also shown in a separate study in a model system *Candida albicans* in liquid cultures. Horseradish essential oil (about 90% allyl isothiocyanate and 5% phenethyl isothiocyanate) had an antifungal effect that was more significant than those of its main components alone. Horseradish essential oil, at sublethal concentration, induced oxidative stress which was characterized by elevated superoxide content and up-regulated specific glutathione reductase, glutathione peroxidase, catalase and superoxide dismutase activities. Induction of specific glutathione S-transferase activities as marker of glutathione (GSH) dependent detoxification was also observed. At higher concentration, horseradish essential oil depleted the GSH pool, increased heavily the superoxide production and killed the cells rapidly. Horseradish essential oil and the GSH pool depleting agent, 1-chlore-2,4-dinitrobenzene showed strong synergism when they were applied together to kill *C. albicans* cells. Based on all these, we assume that GSH metabolism protects fungi against isothiocyanates.

The case of the endophytes of horseradish is likely to be very similar.

These results were published in the *Journal of Basic Microbiology* [2].

3.7. A possible application of the chemical adaptation phenomenon.

In a broader sense, the above suggest that the endophytes show some specific adaptation to the metabolome of their host.

A paper on using endophytes and plant-associated fungi for decomposition / modification of NSAIDs (non-steroid inflammatory drugs) was also published in *International Biodeterioration & Biodegradation* [3]. This article highlights a potential application of the found phenomenon: the fungi can selectively transform metabolites similar to that *in planta*, which was successfully used in detoxification of organic compounds as well as selective biotransformation applications.

In the study, decomposition of diclofenac, diflunisal, ibuprofen, mefenamic acid and piroxicam was tested using nine identified strains of endophytic and epiphytic fungi (from Ascomycota) adapted to natural products resembling the pharmaceuticals. Metabolites were tentatively identified by liquid chromatography - tandem mass spectrometry ($LC\text{-}MS^3$).

Eighteen of the 45 combinations resulted in significant decrease of the concentration of the NSAIDs in model solutions. The most active strains were *Aspergillus nidulans* and *Bipolaris tetramera*, while *Epicoccum nigrum* and *Aspergillus niger* showed somewhat less potency. Piroxicam and

diclofenac were most resistant to biotransformation, while ibuprofen and mefenamic acid were efficiently metabolized by most strains. Ten metabolites could be tentatively identified, including hydroxy-metabolites of all tested NSAIDs, and a dihydroxy-metabolite of piroxicam. This biotransformation is likely to modify the toxicity and bioaccumulation potential of these pharmaceuticals.

The results highlight the applicability of plant materials as an excellent source of fungi with high biotransforming potential.

These results were published in an article in *International Biodeterioration & Biodegradation* [3].

4. Conclusions.

The main aim of the current research was to characterize the effects of the endophytes on the host plant metabolome in horseradish root, focusing on the glucosinolates – a group of metabolites that give rise to isothiocyanates with antifungal effects as well as beneficial impact on human health.

The myrosinase activity was found in most endophytes from horseradish roots, despite these fungi were taxonomically diverse. It is interesting that fungi harbor such a chemically unfriendly environment: allyl isothiocyanate is potent antifungal agent. It is also interesting that an enzymatic activity usually attributed to the plant is present in most of the endophytes of the host with myrosinase.

The main detected decomposition product was allyl isothiocyanate, which was either present as is or as a conjugate of glutathione. The latter explains the tolerance of the endophytes towards the chemical environment: rapid recycling of glutathione can alleviate the toxicity of the generated
This also explains the ability to use sinigrin as the sole carbon source, which was detected for many endophytic fungi. This is a phenomenon that suggests very specific adaptation to the plant metabolome.

5. Perspective.

The endophytes' myrosinase activity raises several questions that should be examined in further studies. First, the *in vivo* contribution of fungi to the overall myrosinase activity remains to be investigated. It is also unclear that these endophytes only “avoid” the plant's toxicity with this capability, or are active contributors to the plant defense system against pathogenic fungi, etc. With the analytical tools and the *in vitro* systems in hand, the quantification of the chemical adaptation phenomenon is likely to be the next step of this research.

6. References.

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