IN VITRO STUDY OF ANTIFUNGAL COMPONENTS FROM HORSERADISH (*ARMORACIA RUSTICANA*)

PhD thesis

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INTRODUCTION

Horseradish (*Armoracia rusticana* P. Gaertner, B. Meyer & Scherbius) belongs to Brassicaceae. Both for the condimental and medicinal usage of horseradish, it's essential oil (a natural mixture of different isothiocyanates) is responsible. The isothiocyanates (ITCs) are the default hydrolytic breakdown products of the glucosinolates (GLS). Glucosinolates are N-hydroxy-sulfates with a highly variable side chain (R) and a sulfur-linked beta-d-glucopyranose. The myrosinase enzyme (MYR), which catalyzes the hydrolytic reaction, is stored in different compartments, typically in myrosin cells. When demage is done to the plant, MYR reacts with GLSs, resulting in bioactive ITCs, nitriles, tiocyanates, epithionitriles,or in oxazolydins. The main components of horseradish essential oil are allyl isothiocyanate (AITC) and 2-phenylethyl isothiocyanate (PEITC).

Beside of the isothiocyanates anticarcinogenic effect, its antimicrobial – primarily the antibacterial - activity, and the mechanism of action is wide-spread studied. ITCs have also several other biological and possibly medicinal effects, e.g. autism, cardiomyopathia in diabetes, antiplatelet aggregation inhibition, etc.

Horseradish has also been researched for its abundant enzyme horseradish peroxidase. This enzyme is used in several molecular biology methods, e.g. measuring blood glucose and cholesterol levels.

Hairy root cultures are initiated by the infection of plant tissues by *Agrobacterium rhizogenes*. The transfer-DNA of the bacterial Ri (root inducing) plasmid, integrates into the plant genome, causing hairy root disease, which can also result in changes in the secondary metabolite synthesis. The neoplastic hairy root clones (HRCs) are capable of unlimited growth, are genetically stable in hormone free media. They are suitable for production of high levels of secondary metabolites (e.g., pharmaceuticals or flavors). As a consequence of the gene transformation, secondary metabolite biosynthesis differs from that in the native plants.

AIMS

1. **Establishment, compound and enzyme analysis of horseradish (*Armoracia rusticana*) hairy root cultures**

The aim of the Ph.D. Thesis was to establish a set of horseradish HRC lines and study their chemical and enzymatic variability. The HRC lines were assayed for GLSs, myrosinase, and volatile decomposition products of GLSs, as well as for peroxidase. What is more, lines of leaf blade and petiole origins were compared for the above features.

1. The antifungal activity and mechanism of action of horseradish essential oil

We planed to test the antifungal activity of horseradish essential oil (as a naturally occurring and regularly consumed mixture of ITCs) against the human pathogenic mould *Aspergillus fumigatus* (aspergillosis), and yeast *Candida albicans* (candidiasis), as well as against the apatogenic mould *Aspergillus nidulans,* and against the yeast *Saccharomyces cerevisiae,*as model organisms.

Our aim was also to show the antifungal effect both in liquid and volatile phase and to compare the antifungal activity of horseradish volatile oil, AITC and PEITC on *Candida albicans.*

To certify the oxidative stress hypothesis of the horseradish essential oil, our aim was also to test interactions with oxidative stress causing antifungal agents, and with molecules taking part in oxidative stress response. We also planed to measure enzyme activities taking part in oxidative stressresponse.

METHODS

1. Establishment, compound and enzyme analysis of horseradish (*Armoracia rusticana*) hairy root cultures

a) Plant material, in vitro cultures, transformation

Transformation was made by *Agrobacterium rhizogenes* A4 of surfice streilised petiolum and leaf blade, resulting in HRCs. For the experiments the HRCs were cultured in Murashige-Skoog liquid media.

b) Confirmation of the gene transformation by Polimerase Chain Reaction (PCR)

Transformation was confirmed by PCR after DNA isolation from all of the clones, from the mother plant as a negative control and from *A. rhizogenes* A4 bacteria as a positive control. For the PCR reaction, *RolC* primers were used.

c) Biomass production

Biomass production was expressed as a daily growth index (DGI = (final weight/starting weight)/days of culture. To calculate dry material content (%), dry weight was also determined from lyophilized samples. Branching and additious shoot formation were scaled after visual evaluation.

*d) **Liquid Chromatography – Electrosray ionisation – Mass spectrometry*** (LC-ESI-MS/MS)

LC-ESI-MS/MS was used to analyse GLS molecules in HRCs. Qualitative and quantitative analyses were made from water extracts of 4 week old lyophilized HRC samples. Results were concerned to dry weight content.

RP-HPLC: an Agilent 1100 HPLC system was used; column: Zorbax SB-C18 (150× 3.0 mm; i.d. 3.5 μm), maintained at 30 °C; eluents: A: 0.1% formic acid, B: methanol;gradient: 0–30 min from 10% to 40% B, 30–31 min from 40% to 100% B, 31–37 min100% B, 37–38 min from 100% to 10% B, flow rate: 0.3 mL/min; injection volume: 5μl.

ESI-MS/MS: an Agilent 6410 Triple Quadrupole Electrospray ion source was used in negative ion mode.

e) Gas Chromatography - Mass Spectrometry (GC-MS)

GC-MS was used to analyse volatile ITCs and nitriles of fresh four-week-old HRCs. Qualitative and quantitative analyses were made from HRC acetone extracts. Results were concerned to dry weight content.

Injection was carried out in split mode (15:1 split ratio), injection volume was 2 μl. The GC-MS analysis was performed on an Agilent 6890GC, equipped with a 5973N mass selective detector, and Chrom Card Server Ver.1.2. software. Capillarycolumn: 30 m × 0.25 mm × 0.25 μm, SLB-5ms 5% phenyl-methyl syloxane. Carriergas: He. Rate of flow: 1.6 ml/min. Temperature program: 50°C (3 min); by 15 °C/min to 200 °C (2 min); by 40 °C/min to 280 °C (1 min). Analysis: 18 minutes. MS conditions: 70 eV ionisation energy, 40–500m/z mass range (scan mode). Peak identification: based on standards, retention times, comparing the mass spectra in NIST05 library and comparing the data to that in the literature. The major components were integrated from TIC (total ion chromatogram). The integration of minor components was made in SIM (selected ion monitoring) mode. Four-point calibration curves were made from AITC and PEITC, in the 0.00976 μg/ml to 1.25 μg/ml range

f) MYR activity measurements with PAGE gelelectrophoresis

Native PAGE gels were used. Measurements zwere concerned to protein units.

g) Peroxidase content and activity

PAGE gelelectrophoresis on native gel, and spectrophotometry was used. Measurements were made both with guaiacol and pyrogallol substrates, concerned to protein units.

h) Statistical analysis

Multivariable statistical analysis was made based on all examined HRC features. Correlations and anticorrelations were studied by principal component analysis, heatmap and hierarhical clustering.

2. The antifungal activity and mechanism of action of horseradish essential oil

a) ITCs used in the antifungal experiments

Horseradish essential oil: KELET PRODUKT Co., AITC: Sigma, PEITC: Sigma.

b) Fungal strain used in the antifungal experiments

Aspergillus fumigatus AF293, *A. nidulans* FGSCA4, *Candida albicans* SC5314, *Saccharomyces cerevisiae* S288C (strains were obtained from Department of Microbial Biotechnology and Cell biology, University of Debrecen).

c) Analysis of the antifungal activity of horseradish essential oil

The antifungal activity of horseradish volatile oil was tested ont he four fungi strains, both in volatile (in Petri dish) and liquid phase (counting IC_{50} after MTT assay).

d) „Time-kill assay"

„Time-kill assay" was made to investigate the fungicid or fungistatic effect of horseradish volatile oil. Erlenmeyer flasks containing 20ml YPD broth were inoculated with *C. albicans* and were incubated at 37°C and 140 rpm until the OD_{640} reached the 0.6–0.7 value (the starting OD_{640} was 0.1). These cultures were treated with 0–25 μl horseradish essential oil-YPD solution/ml medium and were further incubated at 37°C and 140rpm for 24h. Samples were taken regularly (counting from the treatment at 0, 3, 6, 9, 12 and 24 hours) and after appropriate dilution they were spread out onto YPD agar plates. Plates were incubated at 37°C for 1 day and the formed colonies were counted.

e) Analytical examinations with molecular biology methods

C. albicans was cultured at 37°C and 140 rpm until the OD_{640} reached the 0.6–0.7 value. Cultures were treated with 0–2.5 μl/ml medium horseradish essentioal oil-, or AITC- or PEITC-YPD instead. Samples were taken at 3 hours after treatment. Superoxide content of the cells were measured after adding dihydroethidium to the cultures, ethidium content of the cells were detected.

Their specific catalase, superoxide dismutase (SOD), glutathione reductase (GR), glutathione-S-transferase (GST), and glutathione peroxidase (GPx) activities were measured 3 hours after the volatile oil treatments.

Glutathione (GSH) and oxidized glutathione (GSSG) content of the samples were determined by the NADPH-GR-DTNB assay.

f) In vitro reaction experiments between horseradish essential oil and GSH, GSSG, or glutathione reductase

GSH (50mM), GSSG (50mM), or GR (35 U/ml) was incubated in 0.1 M Na-phosphate buffer (pH 7.5) with or without 1µl/ml horseradish essential oil for 0.5 h at room temperature. After incubation, the GSH and GSSG content as well as the GR activity of the samples were determined by the

NADPH-GR-DTNB assay.

g) Interactions between horseradish essential oil and diamide or MSB or CDNB

Aliquots (1ml) of YPD broth were supplemented with 0, 0.13, or 0.25 µl/ml horseradish essential oil-YPD and/or 9.6 or 16mM MSB, 6 or 10mM diamide, 0.03 or 0.06mM CDNB and were inoculated with 1 x 10^4 *C. albicans* cells. All cultures were incubated at 37°C for 1 day. Growth was characterized with the increase of optical density measured at 640 nm. Interaction ratio (IR) was calculated according to the Abbott formula: IR¼Io/Ie, where Io is the measured and Ie is the expected percentage growth inhibition caused by the tested two compounds together. Ie = $X_1+X_2-(X_1X_2/100)$, where $X_{1,2}$ represents the percentage growths inhibited caused by the tested two compounds alone. IR > 1.5, 1.5 > IR > 0.5, and IR < 0.5 are indicative of synergistic, additive, and antagonistic interactions, respectively.

RESULTS

1. Establishment, compound and enzyme analysis of horseradish (*Armoracia rusticana*) hairy root cultures

From the 50 isolated HRC lines, 21 were found to be viable; 10 from the inoculation of petiole (ArP) and 11 from the leaf blade (ArLB). All of the viable HRC lines have been created with the infection of *A. rhizogenes* A4. Strains

Presence of the bacterial *RolC* gene was confirmed by PCR analysis in each clone, using the DNA of *A. rhizogenes* A4 as a positive, and the DNA of mother plant as negative controls.

Based on their characteristic fragments, several GLSs were identified from the water extract of HRCs. sinigrin, glucoiberverin, glucoibarin, glucobrassicin, gluconasturtiin, 4-methoxy- or neoglucobrassicin, glucoarabishirsutain. In all of the HRCs, Gluconasturtiin was the main component. Results of quantitative measurements were used formultivariable statistical analysis.

Although seven GLSs were detected, from their breakdown products only three ITCs (AITC, 3-(methyltio)propyl ITC, PEITC), and two nitriles could be identified, from which 3-phenylpropionitrile is the nitrile hydrolysis product of the main GLS, gluconasturtiin. Results of quantitative measurements were used for multivariable statistical analysis.

The differring isoenzyme pattern of HRCs could be account for the various patterns in volatile bioactive compounds. ArP and ArLB HRC groups showed visible variability in MYR pattern. The 2nd MYR isoenzyme was present almost in all clones. Some hairy root clone contained three isoenzymes (ArLB113, ArLB116, ArP23).

By peroxidase analysis, the two tested methods (gel analysis, and spectrophotometric assay) gave comparable results with both substrates (pyrogallol, guaiacol), correlation values were above 0.70 ($p < 0.001$). On the gels, five isoenzymes were visible.

Through the principal component analysis, we manifested that the inoculated plant organ results in different HRC features. Box-plot analysis demonstrated several significant differences of examined features between HRC group ArLB and ArP, e.g. DGI, sinigrin, 3-(phenyl)propionitrile, peroxidase activity, etc.) In all cases HRCs from ArLB group had higher values. MyrB2 isoenzyme showed significant positive correlation with 3-(phenyl)propionitrile, altough it demonstrated anticorrelation with 3-(methyltio)propil ITC.

2. The antifungal activity and mechanism of action of horseradish essential oil

Horseradish volatile oil showed remarkable antifungal activity against *S. cerevisiae* and *A. nidulans*, as well as against the human pathogenic *C.albicans* and *A.fumigatus* when it was applied in the volatile phase. As itwas expected, the pure volatile oil was more efficient than its YPD broth solution containing the same amount of volatile oil. It also showed strong antifungal activity when it was added to fluid cultures. The IC_{50} values were approximately 5, 6, 10, and 13 μl horseradish volatile oil-YPD/ml media, in case of *S. cerevisiae*, *C. albicans*, *A. nidulans*, and *A. fumigatus*, respectively.

Horseradish volatile oil similarly to its major components (AITC and PEITC) inhibited largely the growth of *C. albicans* in these cultures. Although the antifungal activity of horseradish volatile oil, AITC and PEITC were generally similar, the growth reduction effect of horseradish volatile oil was significantly higher than that of PEITC or AITC had.

AITC had stronger inhibitory effect than PEITC had Time-kill assays demonstrated that horseradish volatile oil had fungicide (fungitoxic) rather than fungistatic effect against *C. albicans* and it killed the cells within few hours. In some cases, a few cells survived even the 25 μl horseradish volatile oil-YPD/ml medium treatment in our experiments, altough these

survivors were detected only in old cultures. only few cells (less than the detection limit) remained alive, and after the evaporation of horseradish volatile oil from the cultures they could start to grow and their density reached the detection limit only in old cultures.

In vitro interaction of horseradish volatile oil with GSH, GSSG, and GR. Horseradish volatile oil (1μl/ml), during a 0.5h incubation period, decreased the starting 50mM GSH concentration of the samples by 84,5% and the starting 35U/ml GR (from *S. cerevisiae)* activity by 42,7%. However, in case of GSSG (50mM starting concentration) no significant decrease was observed.

Horseradish volatile oil generated oxidative stress in *C. albicans* even at moderate (0.25 μl horseradish essential oil-YPD/ml medium) concentration. This oxidative stress was characterized by elevated superoxide content of the cells, induced specific GR,GPx, catalase and SOD activities,and surprisingly did not alter the GSH or GSSG contents, it reduced, however, the growth of the culture At higher concentration (2.5 μl horseradish essential oil-YPD/ml medium), short (3h) exposure depleted the GSH pool, increased the formation of superoxide heavily, and killed the cells rapidly prior the induction of antioxidant enzymes.

The antifungal effect of horseradish volatile showed antagonism with MSB and diamide and also showed synergism with CDNB. The antagonism can be explained with the induction of antioxidative enzymes and/or GSH production. Synergism is most likely the consequence of the GSH pool depletion effect of CDNB.

CONCLUSION, THESIS

We manifested that the GLS pattern of HRCs differs from the pattern of the native horseradish root. In HRCs, the aromatic GLN was dominating, instead of the aliphatic SIN.

We determined that Agrobacterial inoculation of different horseradish plant organs results in different feature (enzymatic and compound) pattern.

Based on our experiments it is likely that the different MYR isoenzyme activities are in part responsible for the significantly different aromatic nitrile concentrations between the ArP and ArLB groups.

The antifungal effect of horseradish volatile oil was manifested both on yeast and mould, on human parthogen and apathogen, and both in liquide and volatile phase.

Based on our experiments, the growth inhibitory effect of horseradish essential oil was stronger than its main components (AITC, PEITC) alone.

Horseradish volatile oil was fungicide at high concentration, and fungistatic in low concentrations.

We confirmed the oxidative stress generating hypothesis of horseradish essential oil. We determined that glutathione protects *Candida albicans* against horseradish volatile oil. The cells are surviving, untill it has enough GSH.

OWN PUBLICATIONS

Own publications, which the Ph.D. Thesis is based on

Bertóti R, Böszörményi A, Alberti Á, Béni S, Szőke É, Vasas G, Gonda S. (2019) Variability of Bioactive Glucosinolates, Isothiocyanates and Enzyme Patterns in Horseradish Hairy Root Cultures Initiated from Different Organs. Molecules, 24(15): 2828.

Bertóti R, Vasas G, Gonda S, Nguyen N. M, Szőke É, Jakab Á, Pócsi I, Emri, T. (2016) Glutathione protects *Candida albicans* against horseradish volatile oil. J basic microbiol, 56(10): 1071-1079.

A publication, which is independent from the Ph.D. Thesis

Szűcs Z, Plaszkó T, Cziáky Z, Kiss-Szikszai A, Emri T, Bertóti R, Sinka L.T, Vasas G, Gonda S. (2018) Endophytic fungi from the roots of horseradish (*Armoracia rusticana*) and their interactions with the defensive metabolites of the glükosinolate-myrosinase-isothiocyanate system. BMC Plant Biol. 18: 85.