MetaLine – Establishing an extraction, screening and formulation pipeline for bioactive metabolites with anticarcinogenic and antifungal potential from plants and fungi in heavy-metal plant communities

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- **Industry partner**
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Im Fokus dieses Projektes stehen Sekundärmetaboliten endopyhtischer Pilze, isoliert aus Wurzeln Schwermetall-resistenter Pflanzen. Diese biobasierten Wirkstoffe werden hinsichtlich ihrer biologischen Aktivität (zytotoxisch, antifungal, antibakteriell) untersucht, um diese entweder im Pflanzenschutz als Fungizide, oder aber als Bestandteile von Kosmetika sowie als pharmazeutisch aktive Substanzen in der Therapie von Krebserkrankungen einzusetzen. Gewonnene pilzliche Reinisolate werden im Zuge des Projektes funktionell sowie taxonomisch charakterisiert und in einem gerichteten, biologischen Screening hinsichtlich ihrer wirksamen Sekundärmetaboliten untersucht.

I. Project parameters

1. Topic, open questions and purpose

The project aims to evaluate bioactive metabolites isolated from endophytic fungi (EF) invading root tissue of heavy metal tolerant plants for cosmetic, pharmacological and agricultural applications. In this context, extracts generated from pure fungal isolates were screened for their bioactivity (antifungal, antibacterial, esterase blocker) combining the screening platforms of both institutes. Subsequently, chemical compositions of active extracts were determined to isolate effective substances or substance groups.

2. Interdisciplinarity of the project

Within the project, different sectors of sciences have to cooperate: Molecular and microbiology expertises are needed for the isolation, taxonomical and functionally characterization -including species-appropriate handling- of isolated, probable undescribed EF as well as for the bio-assay driven screening of the biological activity of EF and their extracts. For the characterization of biological active SeMe, practical experiences relating non-target analyses is necessary and the possibility for increasing biological effects of isolated SeMe by means of semi synthesis could only realize with great know-how in organic chemistry synthesis.

3. Innovativity of the research

The project enables the acquisition of new knowledge about the handling, specific cultivation and the influencing factors in producing SeMe of EF isolated from heavy-metal contaminated sites. Due to their expected special metabolite spectra, they have a particularly high active-ingredient potential, which has hardly been studied till now. The discovery of unknown bioactive compounds using bioassay-driven screening platform is very likely.

4. Application-oriented research

The first results of research correspond with the statements of literature and show the huge potential of EF in producing bioactive compounds. Further studies concerning scale up experiments in cultivation will show, if they can act as direct producer for bio-based ingredients, used for cosmetic, pharmacological and agricultural applications. Considering the growing risk of pathogen resistance against current pesticides/medicines, the increasing number of cancer diseases and crop losses caused by plant diseases, it is necessary to expand the research focus in this field. In case of positive results, applied research and close cooperation to companies enable their quick implementation for industrial applications.

5. Relevance of the project for the bio economy, the ScienceCampus and the state of Saxony-Anhalt

This project allows investigations relating drug research in extreme habitats located in Saxony-Anhalt and may lay the foundation for new perspectives in regional economy.

II. Theory and methods

Two sampling areas, characterized by dry and nutrient-poor soil with high heavy metal contamination, were chosen for the collection of roots of four plant species (Fig.1) for the isolation of EF.

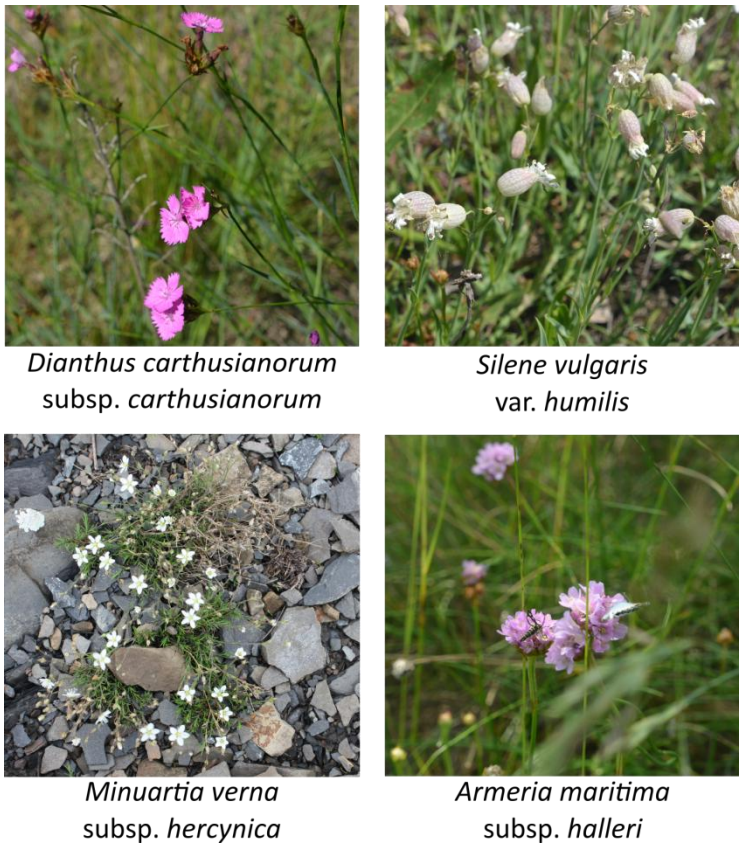


Figure 1: Heavy-metal tolerant plants, collected for the isolation of endophytic fungi (J. Hummel, H. Baltruschat)

By colonizing this ecological niche with its extreme abiotic conditions, EF, invading root tissue of heavy-metal tolerant plants, provide a new spectrum of unknown active metabolites they need for survival. Fungal isolates that showed antagonistic activity against relevant agricultural phytopathogenic fungi in dual-culture assay (DCA, Fig.2) were largely cultivated to produce fungal extracts.

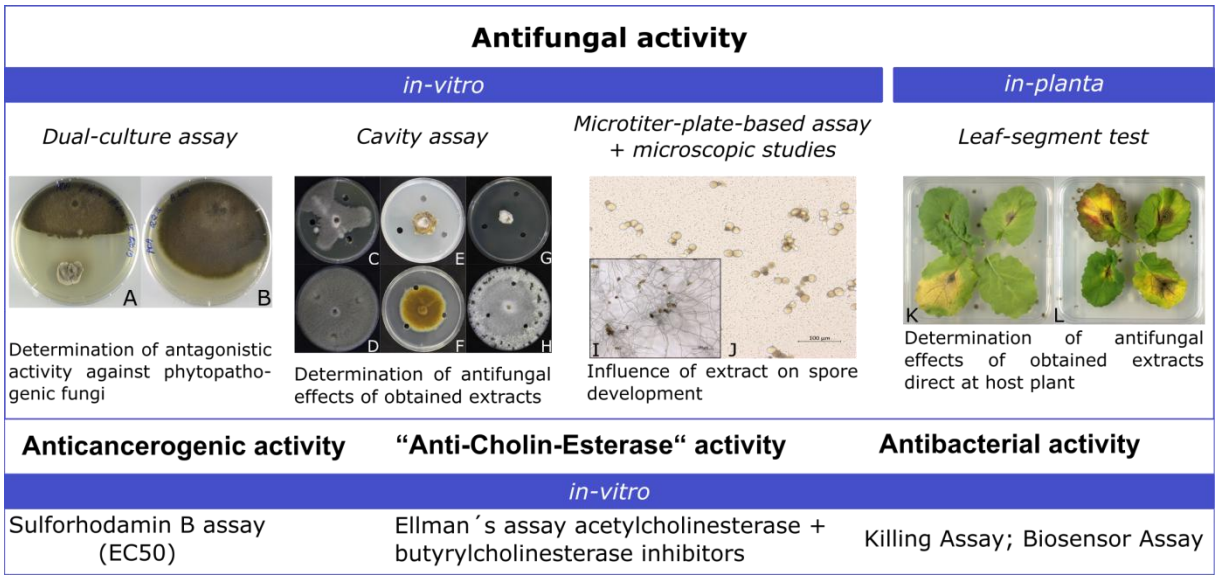


Figure 2: Screening platform for determining the biological activity obtained from endophytic fungi extracts. Dual-culture assay: (A) Antagonistic activity of EF against ALTEBI (B) control plate. Cavity assay: Reduced mycelia growth caused by EF extracts in comparison to control against the phytopathogenic fungi BOTRCI (C+D), ALTEBI (E+F) and SCLESC (G+H). Microtiter-plate-based assay: Development of ALTEBI spores after 48 h (magnification: 400x), treated with fungus extracts (J) in comparison to control (I). Leaf-segment test: Development of ALTEBI spores on rape leafs after seven days, treated with fungal extract (K) or with 2.5% DMSO solution (L).

In a targeted biological screening the extracts were tested for their bioactive potential. The screening platform (Fig.2) was built on *in-vitro* and *in-planta* bioassays, including microtiter-plate-based assay (MTPA) and agar-diffusion assay (cavity assay, CA) as well as leaf-segment tests. Last year, the platform was extended to include antibacterial test systems in cooperation with the working group of Prof. Mascher. The bioassay-guided isolation of effective compounds has to be performed with various analytical methods like HPTLC, HPLC, LC-MS and NMR. Cultivation experiments (different media, liquid or solid; added stress factors during the cultivation process like exposure to light, heavy metals and reduced nutrients) aimed to generate extracts firstly with higher yield for further studies and secondly to shift the metabolite spectra in favor of effective compounds. To execute all of these experiments, the sample range was focused onsolely) two isolates by the following criteria:

1. High bioactivity of generated extracts
2. Easy cultivation (stable plate cultivation, no co-culture with bacteria)
3. Storage at -86°C
4. Reproducible results and effects in *in-vitro* cavity assay

Based on the criteria, an *Alternaria* sp. and *Penicillium* sp. species (Tab.1) were chosen for the cultivation experiments (Tab.2). Each change in the cultivation process required a modification in the extraction and analytical step.

Table 1: Fungal isolates selected for further cultivation studies

Fungus	Species	Host	Sampling area
P37.3	*Alternaria* sp.	*Minuartia verna* subsp. *hercynica*	Mansfelder Mulde
P66.9	*Penicillium* sp.	*Armeria maritima* subsp. *halleri*	Harz mountains

Table 2: Cultivation experiments for evaluation of secondary-metabolites production

No.	Experiment	Description
1	Influence of nutrient supply	1 l culture; media: PDM and ½ PDM; light exposure; 3-weeks-cultivation
2	Influence of the scale-up cultivation and extraction	1 l cultures; media: 1/2 PDM; light exposure; 3-weeks-cultivation; extraction of 15-16 l broth
3	Influence of the cultivation duration	1 l cultures; media: 1/2 PDM; light exposure; Stop cultivation after 1, 2 or 3 week(s)
4	Solid rice culture	750 ml; media: rice; light exposure; addition of heavy metal solutions, adapted to the sampling area; 4 to 5-weeks-cultivation

In the DCA, the two fungal isolates showed an antagonistic activity against the phytopathogenic fungi ALTEBI, RHIZSO AG2, FUSACU, SCLESC, BOTRCI and PHYTCP. Thereupon, extracts obtained from broth and mycelia of P37.3 and P66.9, cultivated in different media, were tested against these phytopathogens, whereby PDM-broth extracts had the best effectiveness in cavity assay. These results of the preliminary study were basis for the cultivation experiments.

First came a scale up of the cultivation conditions from 150 ml to 1 l liquid potato-dextrose-media (PDM) cultures to increase the extract yield. Then the aforementioned stress factors were integrated into the cultivation process. After 3 weeks, the broths were extracted with ethyl acetate after evaporation from 1 l to 200 ml. In a second experiment, the influence of heavy metals on secondary-metabolites profile was analyzed.

Next, rice was used as nutritional basis for the cultivation of the fungal isolates. The stress factors light and heavy metal supplement were integrated as well. The extraction took place with ethyl acetate whereby the rice was overlaid for 24 h with the solvent before it was decanted. The concentration of heavy-metal solutions, used for the cultivations, was adapted to the area, the fungus was isolated from (Tab.3).

Table 3: Concentration in ppm of heavy-metal compounds used in cultivation experiments, adapted to the sampling points of isolated fungi P37.3 (*Alternaria* sp.) and P66.9 (*Penicillium* sp.)

Heavy-metal compound	P37.3	P66.9
$ZnSO_4 \times 7H_2O$	17,5 ppm	2,5 ppm
$CuSO_4 \times 5H_2O$	4,0 ppm	0,2 ppm
$Pb(CH_3COO)_2 \times 3H_2O$	7,0 ppm	1,2 ppm
$FeCl_3 \times 6H_2O$	20,0 ppm	16,2 ppm

The extracts, obtained from the cultivation experiments, were first transferred to the screening platform to evaluate their biological activity. Positive results were followed by analytical investigation.

Studies about the increase of bioavailability and cytotoxicity of secondary metabolites by semi synthesis were performed using the triterpenoic acid oleanolic acid. Previous investigations have shown that the concentration of heavy metals in soil influences the concentration of triterpenoic acids in plants.

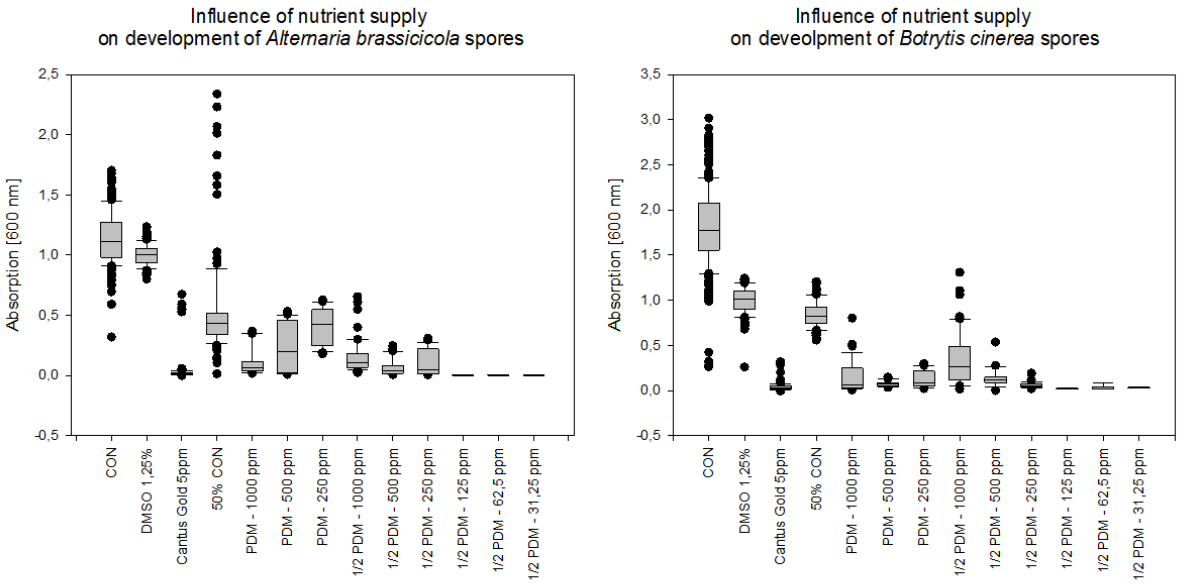
III. Results and perspectives

Previous studies have shown that extracts obtained from PDM broths reach the highest antifungal activity in cavity assay. Thereupon, scale-up cultivation (from 150 ml to 1 l cultures) took place, followed by microtiter-plate based assay (MTPA) confirming their bioactive effect.

Discussing the cultivation experiments, all antifungal activity data of obtained extracts is based on MTPA. The statistic evaluation was performed with the One Way RM ANOVA and the post-hoc Holm-Sidak method ($P<0,050$).

Extracts obtained from fungal isolate P37.3 (*Alternaria* sp.), cultivated with reduced nutrient supply (1/2 PDM) and at light exposure show no significance in spore development in the test systems ALTEBI and BOTRCI to the fungicide control group (Cantus Gold 5 ppm + Bellis 10 ppm) even at concentrations as low as 31.25 ppm (Fig.3A).

A) P37.3 - *Alternaria* sp.



B) P66.9 – *Penicillium* sp.

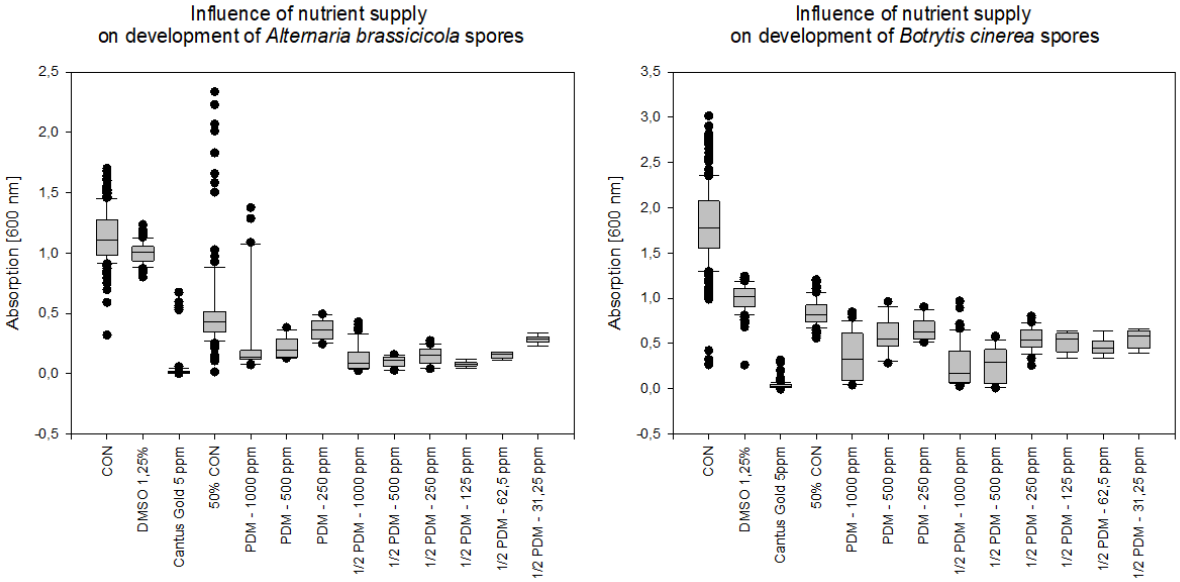
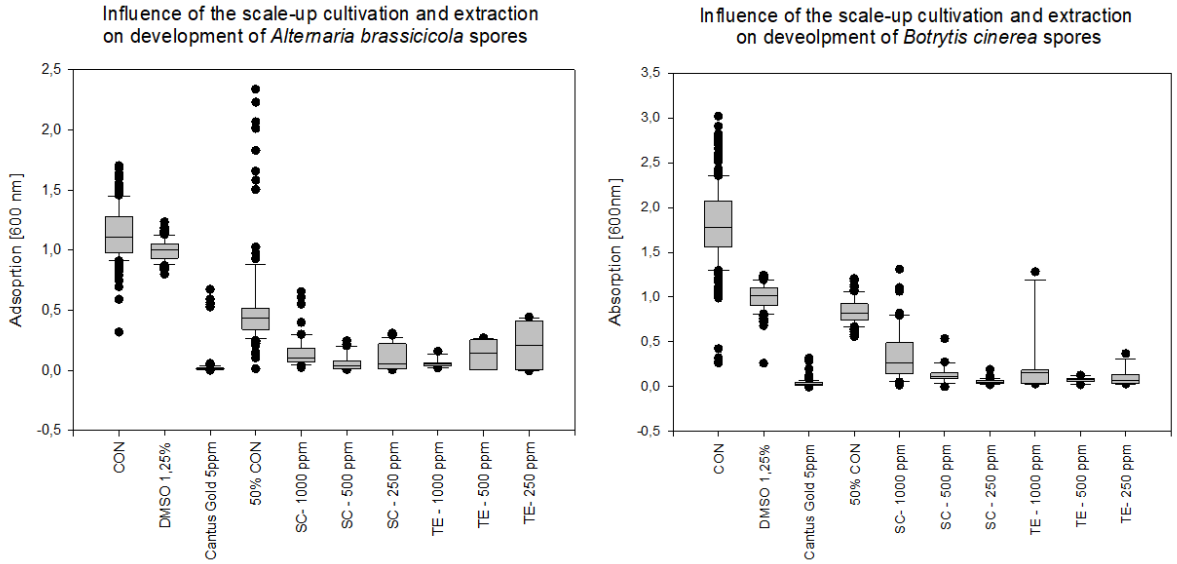


Figure 3: Determination of the spore development after treatment with extracts from cultivation experiment No. 1. Scan at 600 nm. CON: no treatment; DMSO 1.25%: treatment with 1.25% DMSO solution; Cantus Gold 5ppm: fungicide ALTEBI; Bellis 10ppm: fungicide BOTRCI; 50% CON: treatment with *Rheum* sp. extract (250 ppm); PDM: full medium broth; 1/2 PDM: reduced medium broth

Thus the effects of the raw extracts are comparable to commercial fungicides. The extracts of the fungus P66.9 (*Penicillium* sp.) are overall less effective, however at the concentration level 31.25 ppm the spore development of ALTEBI and BOTRCI is still significantly reduced to the control group (Fig.3B; DMSO 1.25%, $P<0,001$). As there are no significant differences for both fungi between the sample groups PDM (cultivation in full media + light exposure) and 1/2 PDM, a cost reduction especially in view of potential commercialization is possible.

Due to low extract yields of this cultivation and extraction method, the focus was on a scale-up modification. Therefore, 15-16 fold of broth were extracted. The average extract yield of about 100 mg (1l culture) could be increased to 4-5 g (40-50 fold). But unfortunately, the extracts show a lower antifungal effect against ALTEBI and BOTRCI (Fig.4A+B).

A) P37.3 - *Alternaria* sp.



B) P66.9 – *Penicillium* sp.

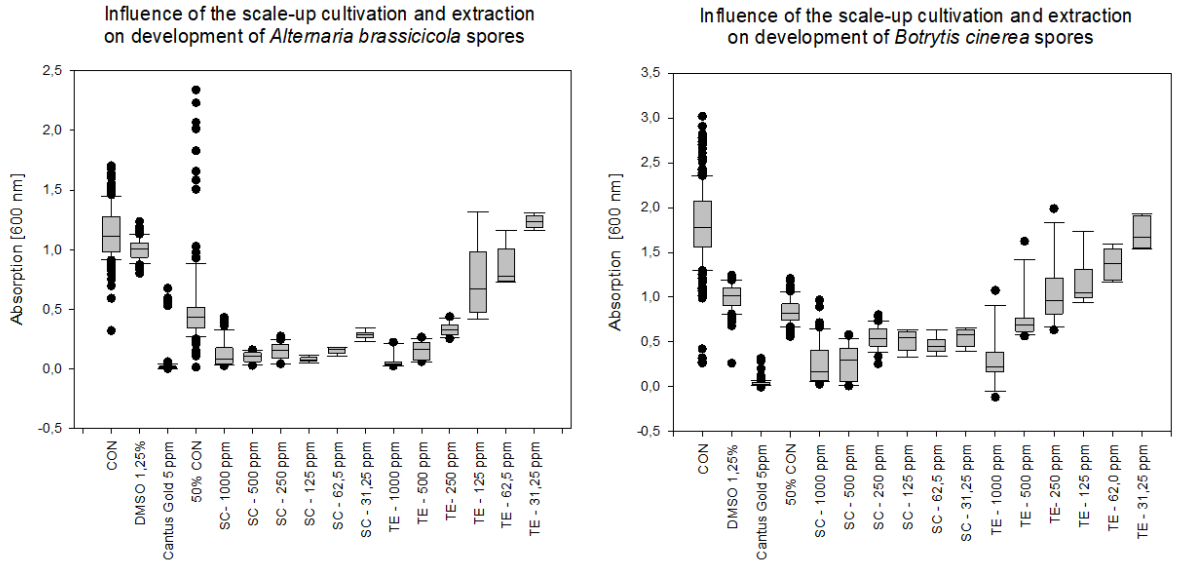
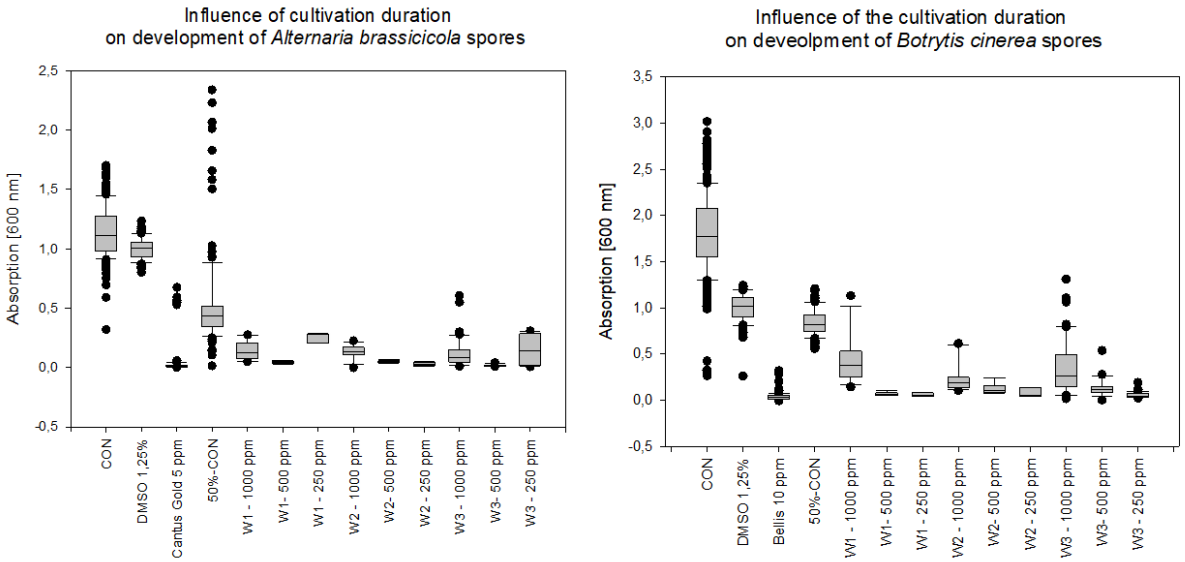


Figure 4: Determination of the spore development after treatment with extracts from cultivation experiment No. 2. Scan at 600 nm. CON: no treatment; DMSO 1.25%: treatment with 1.25% DMSO solution; Cantus Gold 5 ppm: fungicide ALTEBI; Bellis 10 ppm: fungicide BOTRCI; 50% CON: treatment with *Rheum* sp. extract (250 ppm); SC: single-culture extracts; TE: total-culture extract, scale-up cultivation and extraction

To reduce time for the extract generation, studies regarding the cultivation duration were executed. In the case of fungal isolate P37.3, an active extract against the spore development of ALTEBI and BOTRCI could already be generated after two weeks of

cultivation (Fig 5A), whereas fungus P66.9 had to be cultivated for three weeks in nutrient-reduced media under light exposition (Fig.5B).

A) P37.3 - *Alternaria* sp.



B) P66.9 – *Penicillium* sp.

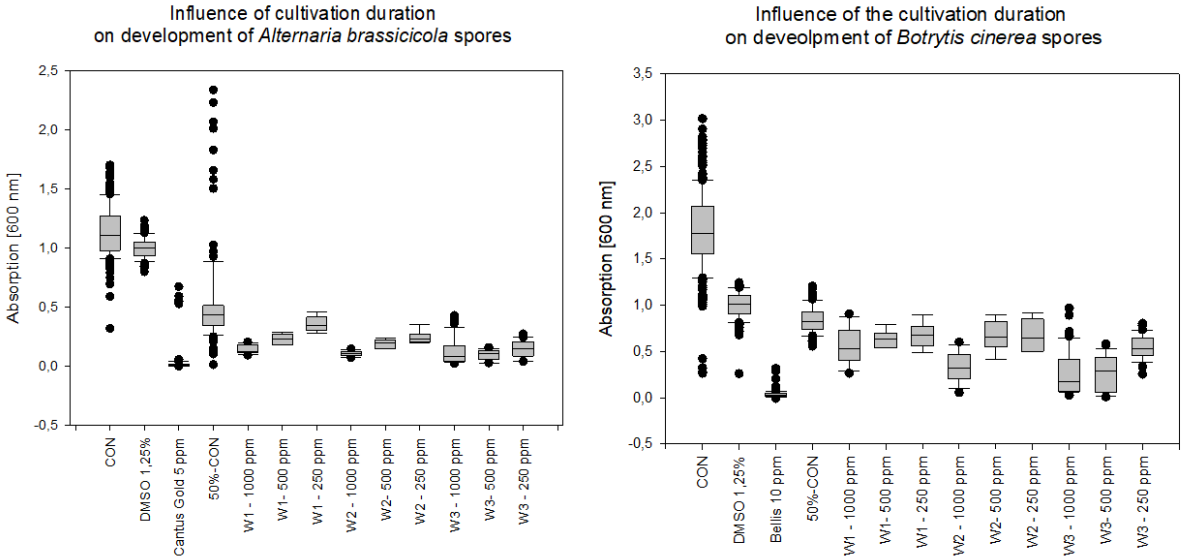


Figure 5: Determination of the spore development after treatment with extracts from cultivation experiment No. 3. Scan at 600 nm. CON: no treatment; DMSO 1.25%: treatment with 1.25% DMSO solution; Cantus Gold 5 ppm: fungicide ALTEBI; Bellis 10 ppm: fungicide BOTRCI; 50% CON: treatment with *Rheum* sp. extract (250 ppm); W1: week 1; W2: week 2; W3: week 3

The extracts from the rice culture of fungi P37.3 and P66.9 have been tested already tested with MTPA. The first results were promising, repetitions still needed for higher validity.
In upcoming studies, the effect of heavy-metal concentrations during the cultivation process on the compounds spectra and therefore the antifungal effect will be investigated.
The screening of antibacterial effects showed that the extracts of fungi P37.3 and P66.9 have bactericidal effect especially to gram positive bacteria. P66.9 extracts even killed gram

negative bacteria at concentration of 50 ppm. The determination of the mode of action is in progress.

Analytical methods for HPTLC and HPLC have been developed. However the wide sample range with different compounds spectra require specific methods for each sample. Therefore method development should only focus on active extracts determined by antifungal testing.

For the studies about the increase of bioavailability and cytotoxicity of secondary metabolites by semi synthesis, extraction of plant material (whole plant) of Gypsophila repellens grown in heavy metal contaminated soil took place. The extract revealed a 10-20 fold higher concentration of oleanolic acid and to a minor extend elevated levels of ursolic acid. In a sulforhodamine B assay guided screening, the fractions rich on these two triterpenoic acids were cytotoxic to a variety of different human tumor cell lines while being less cytotoxic for non-malignant fibroblasts (NIH 3T3). To increase the bioavailability and the cytotoxicity of oleanolic acid another hydroxyl group was introduced in ring A (thus leading to the formation of augustic acid and maslinic acid, respectively). Di-acetylation of these compounds and introduction of a lipophilic amide gave compounds of highly increased cytotoxicity and selectivity. The latter strongly depend both on the triterpenoid skeleton as well on the substituents and the substitution pattern. Finally, attachment of a piperazinyl spacer and a rhodamine B moiety onto a di-acetylated maslinic acid skeleton led to a compound being cytotoxic to human tumor cells even in a nano-molar concentration but being 60 times less cytotoxic for non-malignant fibroblasts. As far as the biosynthesis of the triterpenoids is concerned, several key-enzymes were identified.

IV. Index

Publications

J. Wiemann, S. Sommerwerk, R. Kluge, D. Ströhl, R. Csuk (2019) Epimerization, Claisen and Vorländer reaction starting from methyl platanoate. J. Mol. Struct. 1177: 249-254

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Poster presentations

“Antifungal activity of endopyhtic fungi and their secondary metabolites”, 19. Nachwuchswissenschaftlerkonferenz, 5./6.06.2018, HS-Anhalt Köthen

“Antifungale Wirkung Endophytischer Pilze und deren Sekundärmetaboliten“, ANAKON, 25.-28.03.2019, WWU Münster

Talks

„Antifungale Wirkung Endophytischer Pilze und deren Sekundärmetaboliten“, 27. wissenschaftliche Arbeitstagung „Ökophysiologie des Wurzelraumes“, 16.08.2017, Bergakademie Freiberg

„Biological Activity of Endophytic Fungi and their Secondary Metabolites”, Sitzung des Leitmarktarbeitskreises für Gesundheit und Medizin im Ministerium für Wirtschaft, Wissenschaft und Digitalisierung, 20.06.2017, Magdeburg