WOLF – Weed control by biological compounds identified in necrotizing plant pathogenic fungi

- **Principal investigators**
  Prof. Dr. Holger B. Deising, Martin Luther University Halle-Wittenberg,
  Dr. Arnold, Norbert: Leibniz Institute of Plant Biochemistry,
  Prof. Dr. Csuk, René, Martin Luther University Halle-Wittenberg
- **Name of PhD students:** Renata Amorim, Lea M. Schmitz
- **Project duration:** 3 years
- **Funding amount:** 245.000 EUR
- **Industry partner:** Bayer Weed Control, Frankfurt, Germany

Ziel des Projektes WOLF ist die Identifizierung von sekundären Pilzmetaboliten aus *Colletotrichum graminicola*, die herbizide Eigenschaften aufweisen und sich für den Einsatz in der biologischen Unkrautbekämpfung eignen.

I. Topic, open questions and purpose

The enormous structural diversity of fungal secondary metabolites (SMs) and, more importantly, their high potency make fungal phytotoxins attractive as a new source of herbicides (Duke, Dayan *et al*., 2000; Varejão, Demuner *et al*., 2013). In addition, new fungal metabolites may have novel modes of action that are not covered by synthetic herbicides. Furthermore, the fungal metabolites may address several targets simultaneously, which decreases the risk of resistance development (Duke, Dayan *et al*., 2000, Triolet, Guillemin *et al*., 2019). Based on this rich reservoir of putative herbicides it is surprising that there is no fungal molecule-based product on the market so far (Triolet, Guillemin *et al*., 2019). One reason might be that secondary metabolism gene clusters are silent under standard laboratory conditions and, consequently, no compounds have been identified (Brakhage and Schroeckh, 2011; Derntl *et al*., 2017).
This project aims at obtaining deletion mutants of genes controlling secondary metabolism in *Colletotrichum graminicola,* enabling the expression of silent gene clusters and the secretion of SMs. Subsequently, natural fungal SMs of *C. graminicola* exhibiting herbicidal activity to be used in bio-compatible weed control strategies will be identified. Furthermore, understanding of regulatory aspects of secondary metabolism and production of necrotizing SMs will be improved.

II. Theory and methods

1) Work flow

Extraction

Cultures of *Colletotrichum graminicola* provided by the Deising lab were investigated for their herbicidal activity. Mycelium and culture broth were separated and independently investigated according to Figure 1. All extracts were tested for their phytotoxic activity.

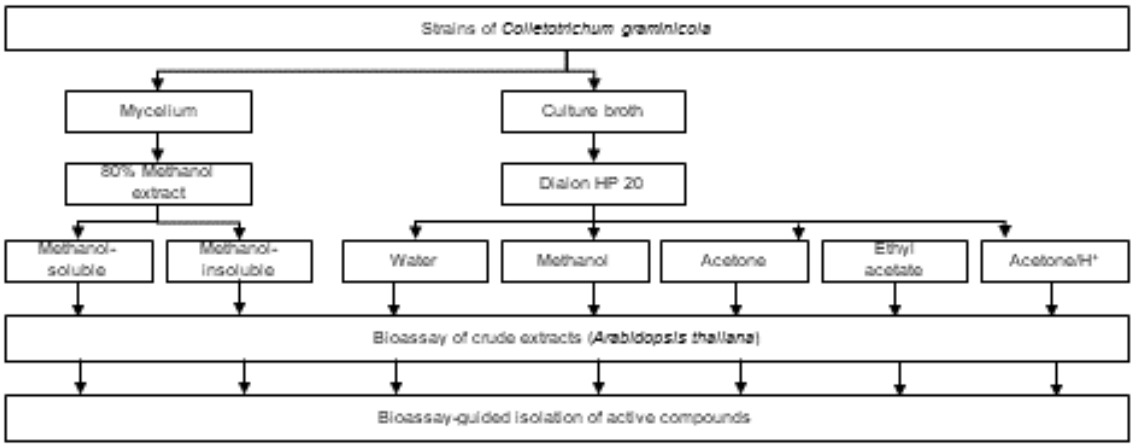


Figure 1: *Extraction process (Lea Schmitz)*

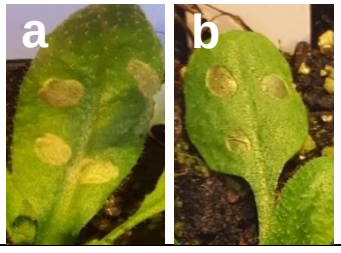
Leaf-Spot Assay (Dicotyledones)

All extracts/fractions were tested for their herbicidal activity in a modified leaf-spot assay (Evidente 1995). A droplet (5 µL) of a methanol/water (1:1) containing 500 µg of crude extract was placed on the surface of a leaf from *Arabidopsis thaliana* Col-0 grown in the greenhouse. The plants were incubated for 24 h in the greenhouse and formation of necrosis was monitored.

III. Results and perspectives

Chemical investigations

Methanolic crude extracts of the mycelia caused necrosis on the leaf surface, whereas the methanol-insoluble fraction exhibited no activity. In case of the crude extracts of the media, only methanol soluble fractions showed activity and only under certain growth conditions. Importantly, the strongest necrosis were caused by extracts of *C. graminicola* grown in a medium containing a nitrogen source and in the absence of glucose. Results of the bioassay are given in Table 1-3.

active		Extract colorless (a) or B (b); necrotic areas slightly yellow-brown

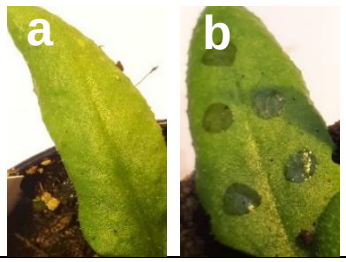
inactive		Extract colorless (a) or brown (b); no necrotic cells observable

Table 1: Criteria for activity (Lea Schmitz)

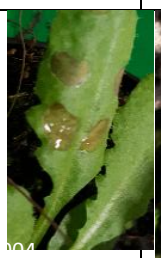
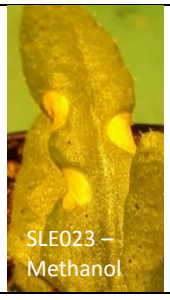
Condition / Strain	+ N + Glc	+ N - Glc	- N + Glc	HMG
Wild type				
→ Activity	++	+++	+++	+++
Δ*KMT6*				
	++	++	++	Not available
Δ*KMT5*				
→ Activity	+	++	++	(+)

Table 1: Bioactivity of crude extracts (methanol soluble) from mycelia (Lea Schmitz)

Condition	+ N	+ N	- N	HMG

Strain	+ Glc	- Glc	+ Glc	
Wild type				
→ Activity	+	+++	+	-
Δ*KMT6*				
→ Activity	+++	-	-	Not available

Table 3: Bioactivity of crude extracts (methanol soluble) from media (Lea Schmitz)

The first step aims at finding a suitable SM-inducing medium for *C. graminicola.* Therefore, four different media were tested. Methanolic crude extracts of the mycelia and methanol soluble fractions of the media were analyzed via HPLC-DAD to characterize the effect of the cultivation medium on formed metabolites.

Mutagenesis

The *XPP1* gene, previously described as a repressor of secondary metabolism (Derntl *et al*., 2015; Derntl *et al*., 2017), is thought to represent a global regulator of primary and secondary metabolism and was therefore deleted in *C. graminicola* via targeted mutagenesis. Through homologous recombination, the *XPP1* gene was exchanged by the hygromycin phosphotransferase gene (*HPH*), allowing the selection of the mutants in medium containing hygromycin. Southern blot analysis confirmed successful deletion of the gene and single insertion of the deletion cassette (Figure 2). Selected deletion mutants and ectopic strains will proceed to the next steps of cultivation and chemical analysis or SMs produced under the conditions described above.

Previously generated *C. graminicola* deletion mutants of the H3K27 methyltransferase Kmt6 and of the histone H4K20 methyltransferase Kmt5, establishing repressive histone modification marks (Connolly *et al*., 2013) are also being cultivated. Preliminary results of plant necrotizing assays comparing the crude extract from mutants and wild type strains are given in Table 2 and 3.

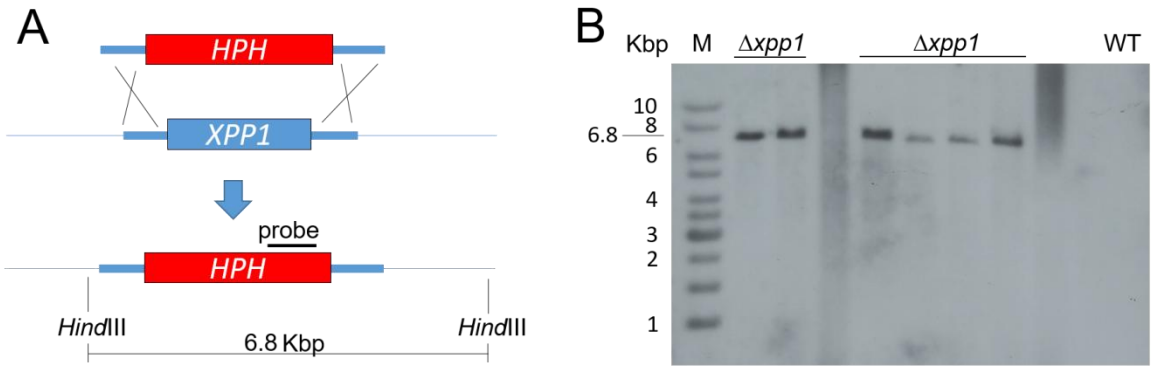


Figure 2: Southern blot analysis of C. graminicola strains. A. Scheme of XPP1 deletion. Successful deletion is indicated by homologous integration of the HPH gene, residing on a 6.8 Kbp DNA fragment. B. After digestion of chromosomal DNA with HindIII and hybridization with a DNA probe directed against a 6,8 bp fragment harboring the HPH gene six Δxpp1 mutants were identified (Renata Amorim)

Growth conditions/method cultivation

The manipulation of regulatory genes that govern secondary metabolism combined with the selection of best inductive conditions can result in activation of SMs production. Based on that, five different media were tested until present. Erlenmeyer flasks (1 L) containing 4 g of cotton wool and 200 mL media were inoculated with two mycelium plugs of 1 cm^2 and incubated at 23°C, without agitation for 13 days. Preliminary results indicate that larger yields of culture broth and mycelia are required for complete chemical characterization and plant necrotizing assays.

Index

Brakhage AA, Schroeckh V (2011) Fungal secondary metabolites – strategies to activate silent gene clusters. Fungal Genet. Biol. 48: 35-48.

Connolly LR, Smith KM, Freitag M (2013) The *Fusarium graminearum* histone H3 K27 methyltransferase KMT6 regulates development and expression of secondary metabolite gene clusters. PLOS ONE 9: 1-18.

Derntl C, Rassinger A, Srebotnik E, Mach RL, Mach-Aigner AR (2015) - Xpp1 regulates the expression of xylanases, but not of cellulases in *Trichoderma reesei*. Biotechnol. Biofuels 8:112

Derntl C, Kluger B, Bueschl C, Schuhmacher R, Mach RL, Mach-Aigner AR (2017) Transcription factor Xpp1 is a switch between primary and secondary fungal metabolism. Proc. Natl. Acad. Sci. 10: E560-E569.

Duke SO, Dayan FE, Romagni JG, Rimando AM (2000) Natural products as sources of herbicides: current status and future trends. Weed Res. 40: 99-111.

Evidente A (1995) Putaminoxin, a phytotoxic nonenolide from *Phoma putaminum*. Phytochemistry 40: 1637-1641.

Triolet M, Guillemin JP, Andre O, Steinberg C, Hatcher P (2019) Fungal-based bioherbicides for weed control: a myth or a reality? Weed Res. 60: 60-77.

Varejão EVV, Demuner AJ, Barbosa LCA, Barreto RW (2013) The search for new natural herbicides – Strategic approaches for discovering fungal phytotoxins. Crop Prot. 48: 41-50.