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Toxins associated with *Phomopsis*

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Outline

- Research background
- Rationale
- Research Objective
- Materials and Methods
- Results and Summary

Research Background

- ✓ Species of *Phomopsis* produce toxins (Patwardhan et al. 1985)

- ✓ Cytological observations revealed that *Phomopsis helianthi* kills the host cells before colonizing tissues.
  - ✓ This could be because of phytotoxic compounds secreted by the fungus (Desanlis et al. 2012).

Rationale

Strong correlation between host resistance to phytotoxins and host resistance to pathogens in few pathosystems (Lamari and Bernier 1989; Brooks 2007).

Limited information on the Phomopsis-sunflower system.

Eg:
***Pyrenophora tritici-repentis* and wheat**
***Rhizoctonia solani* and rice**

Research Objective

To identify the potential role of toxins and/or metabolites produced by U.S. isolates of *Phomopsis helianthi* in the development of Phomopsis stem canker

Greenhouse study

- Two experiments – different inoculation methods
  - **Stem wound inoculation**
  - **Culture free filtrate inoculation**
- Completely randomized design
- *Phomopsis*-susceptible variety N4HM354 (Nuseed genetics)
- Experiment conducted two times
- Six plants (replication) for each treatment

Isolates

- Ten isolates of *P. helianthi*

Name of the Isolate	Location
ROTH 2	Wilkin, MN
L1	Burleigh, ND
AK7	Potter, SD
K2	Cass, ND
GLY3	Clay, MN
I9	Cass, ND
H45	Polk, MN
STAP6	Todd, MN
AG1	Walworth, SD
J10	Cass, ND

Inoculum preparation

Stem wound inoculation

Potato Dextrose Agar

Incubate at 23° C for three weeks in diffused light

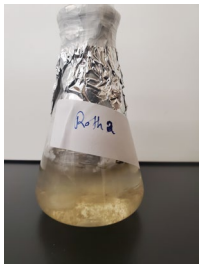
6 mm mycelial plugs

Culture free filtrate inoculation

10 mm mycelial plugs (10 days old)

Potato Dextrose Broth

Incubate as stationary cultures at 23 C for 3 weeks in diffused light



Whatman no. #1

Crude culture free filtrate

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Inoculation

- V4 to V6 growth stage
- Third internode of the plants
  - Stem wound inoculation – placed mycelial plugs after wounding using a 20-gauge, hypodermic needle and secured with parafilm
  - Culture-free filtrate inoculation – 500 µl filtrate delivered using 20-gauge, hypodermic needle

Greenhouse conditions

- Wound secured with Parafilm
- Temperature 23±2°C
- Light conditions (16 h photoperiod)
- Pots watered to saturation every alternate day

Observation

- After inoculation plants examined daily for necrosis

  Necrosis refers to dead tissue visible after 3 to 4 days post-inoculation surrounding small, brown to black colored spots (Lamari and Bernier 1989)

- Necrotic symptoms visible four days post-inoculation on all plants

- Observations were taken 10 days post-inoculation

Observation

- Fungus reaction - Disease rating scale (0 to 5) (Mathew et al. 2015)

0: No discoloration

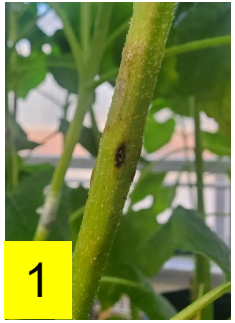
1: low level discoloration

3: necrotic lesions 2–5 mm, leaf wilting and twisting

5: very severe necrosis and lesions, or plant death

- Toxin reaction : (+)= sensitive; and (-) = insensitive [sensitivity and insensitivity are used to describe the reaction of the host to the toxin].

Stem wound Inoculation



Culture filtrate Inoculation

ROTH2

K2

L1

H45

AK7

STAP6

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Results

Name of the Isolate	Reaction	
	Fungus	Toxin
ROTH 2	3	+
L1	1	+
AK7	1	+
K2	5	+
GLY3	2	+
I9	1	+
H45	1	+
STAP6	2	+
AG1	1	+
J10	2	+

Approaches

To characterize the toxins and/or metabolites produced by *P. helianthi*, we adopted two methods:

- ✓Molecular assay
- ✓Mass spectroscopy

Molecular assay

- ✓ Polyketides are secondary metabolites associated with pathogen virulence
- ✓ Genome of the highly aggressive isolate of *P. helianthi* was analyzed for putative polyketide synthase (PKS) genes
  - ✓ > 40 PKS genes, more than those reported in other fungi such as *Penicillium*, *Aspergillus*, *Gibberella,* and *Cochliobolus*.
- ✓ Among the PKS genes, *DhPKS1* was fully characterized
  - ✓ Present in virulent French and Yugoslavian isolates
  - ✓ No information about U.S. isolates

(Ruocco et al. 2018)

Methodology

- ✓ DNA extraction of the 10 isolates
- ✓ CTAB method (Doyle and Doyle 1987)
- ✓ Primers designing using NCBI Primer-BLAST
- ✓ Primer sequences of *DhPKS1 gene*:

  Forward primer - 5' AAGCGCGGTGTATCTTGACA 3'

  Reverse primer - 5' CCAACTTTCCCTCGGCTTCA 3'

PCR Conditions:

94° C - 3 min

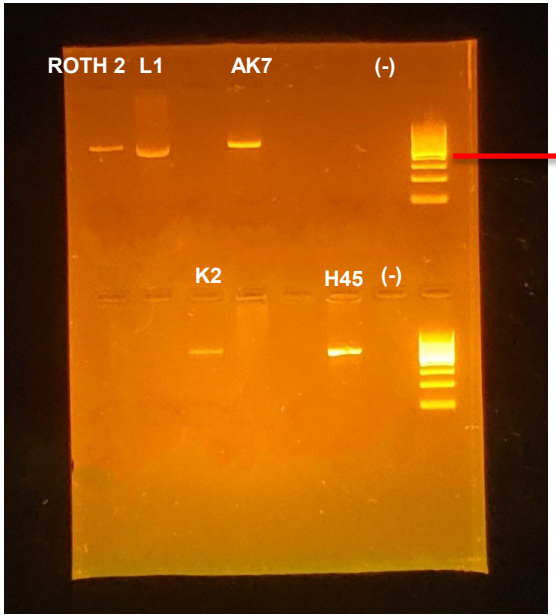
94° C - 30 s

59° C - 30 s } **39 cycles**

72° C - 1 min

72° C - 5 min

Results



500 bp

- ✓ Six out of the 10 isolates were amplified
- ✓ *DhPKS1* gene is presumably present in the six isolates, and responsible for pathogen virulence and toxin production

Results

Name of the Isolate	Reaction		Amplification of *DhPKS1* gene
	Fungus	Toxin	
ROTH 2	3	+	Amplified
L1	1	+	Amplified
AK7	1	+	Amplified
K2	5	+	Amplified
GLY3	2	+	Not amplified
I9	1	+	Not amplified
H45	1	+	Amplified
STAP6	2	+	Amplified
AG1	1	+	Not amplified
J10	2	+	Not amplified

Non *PKS* (putative) producers also caused necrosis suggesting possible role of other metabolites in disease development

Mass spectroscopy

- ✓ Identifies and quantifies unknown compounds via molecular weight determination.
- ✓ Three compounds produced by *P. helianthi* have been identified:
  - ✓ Phomozin from French isolates of *P. helianthi* (Mazars et al. 1990)
  - ✓ Cis-4,6 – dihydroxymellein and Trans-4,6-dihydroxymellein from French and Italian *P. helianthi* isolates (Avantaggiato et al 1999)

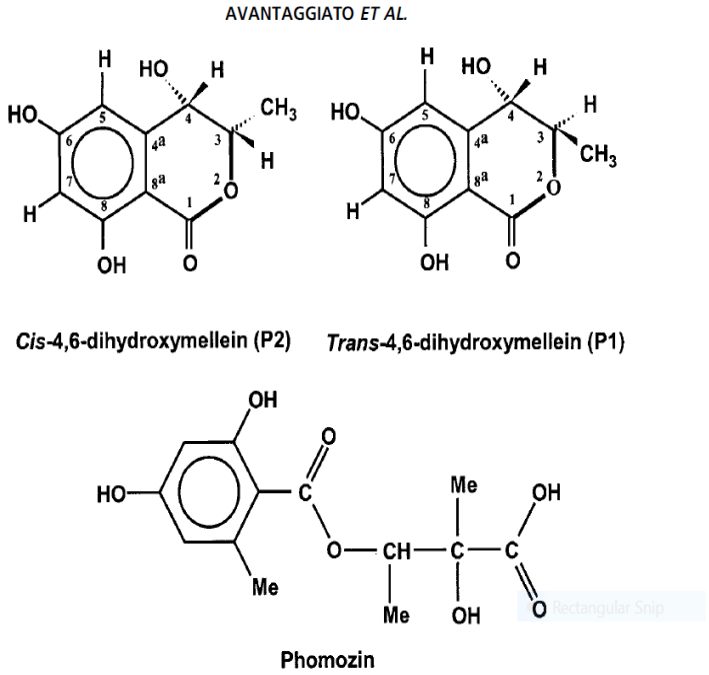
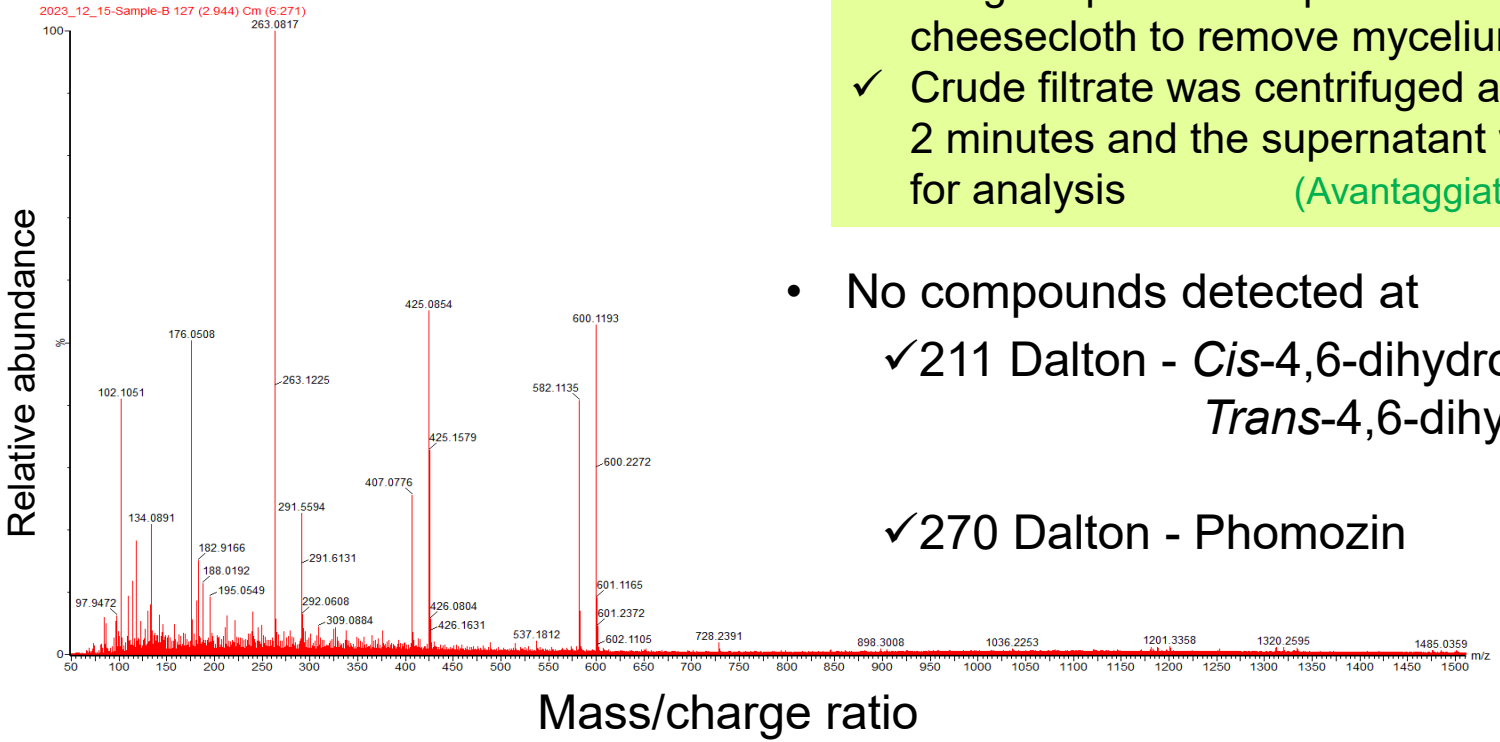


Figure 1. Chemical structure of phytotoxic compounds produced by *Phomopsis helianthi*

Mass spectroscopy

- ✓ Phomozin provokes necrotic lesions on sunflower leaves (Mazars et al. 1990)
- ✓ Mellein and its derivatives affect photosynthesis by causing a decrease in CO_2 absorption (Bousquet et al.1977)
- ✓ The specific mechanism underlying the destruction and/or disruption of host cells by *Phomopsis* is unknown.

Results



Mass/charge ratio

Sample preparation:

- ✓ Fungal liquid culture passed through cheesecloth to remove mycelium
- ✓ Crude filtrate was centrifuged at 8000 g for 2 minutes and the supernatant was used for analysis (Avantaggiato et al 1999)

- No compounds detected at
  - ✓211 Dalton - *Cis*-4,6-dihydroxymellein
    Trans-4,6-dihydroxymellein
  - ✓270 Dalton - Phomozin

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Results

Name of the Isolate	Reaction		Amplification of *DhPKS1* gene	Mass spec results	
	Fungus	Toxin			
ROTH 2	3	+	Amplified	Not detected	Putative *PKS*
I9	1	+	Not amplified	Not detected	Not putative *PKS*

Summary

- In the greenhouse, necrosis was observed on the plants inoculated with the filtrate of *P. helianthi* culture at 10 days post-inoculation
  - Indicates the role of potential toxins (or other metabolites) in disease development

- Six isolates were identified to possibly have the *DhPKS1* gene associated with pathogen virulence and toxin production.

Summary

- Preliminary results suggest that multiple compounds may be associated with *P. helianthi* infection which needs further investigation
  - Demonstrated in *Phomopsis* sp. infecting millet (Patwardhan et al. 1974) and fennel (Evidente et al. 2011)

- In the coming months, characterize toxins and/or other metabolites, and refine the greenhouse inoculation method to screen sunflower accessions for *Phomopsis* resistance

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THANK YOU