Physiological and molecular mechanisms of resistance to *Sclerotinia* and *Phomopsis*.

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Introduction

Resistance of sunflower to the major fungal pathogens *Sclerotinia sclerotiorum* and *Diaporthe helianthi* is quantitative. Our understanding of the basis for quantitative disease resistance to necrotrophic phytopathogens is currently limited. *S.sclerotiorum* causes white mold diseases on many crop plants, including three distinct diseases on cultivated sunflower: head rot, basal stalk rot (BSR), and mid-stalk rot (Harveson et al., 2016). The related fungal species *D. helianthi* and *D. gulyae* are the causal agents of Phomopsis stem canker (PSC), a disease that has increased in prevalence in the US over the past decade (Mathew et al., 2015). Resistance to these important sunflower diseases is genetically complex and the mechanisms conferring resistance are poorly understood. Our efforts toward improving sunflower resistance to these important diseases are focused in two main areas: 1) Characterizing mechanisms of sunflower quantitative resistance to *S. sclerotiorum* and Diaporthe spp. and identifying the underlying genes; 2) Investigating the virulence strategies used by *S. sclerotiorum* and *D. helianthi* to successfully colonize sunflower. This paper updates recent progress on projects to identify sunflower lines with high levels of Sclerotinia basal stalk rot resistance and characterize resistance mechanisms, to define mechanisms of resistance to Phomopsis stem canker caused by *D. helianthi* in sunflower germplasm resources, and to evaluate genetic and pathogenic variation among *D. helianthi* isolates.

Materials and Methods

Growth of pathogen and plant materials

Sunflower plants for Sclerotinia BSR inoculation were grown in potting mix (Premier Horticulture Pro-Mix BX) in 32-space deep sheet pots (TO Plastics). Plants were grown under greenhouse conditions at 22 ± 3°C with supplemental lighting to maintain a 16h photoperiod. Sunflower plants for Phomopsis stem inoculations were grown in potting mix (Premier Horticulture Pro-Mix BX) in 2-gallon plastic nursery pots with two plants per pot. These plants were grown under greenhouse conditions at 25 ± 3°C with supplemental lighting to maintain a 16h photoperiod. *S. sclerotiorum* inoculum was produced by growing mycelia of isolate NEB-274 on autoclaved white proso millet seed, followed by drying and storage of millet inoculum at 4°C until use. *D. helianthi* and *D. gulyae* isolates were grown on potato dextrose agar (PDA) plates at 22°C and plugs bearing mycelia were used as inoculum.

Sclerotinia BSR inoculation and evaluation of resistance

Fifteen five-week-old sunflower plants per genotype were inoculated with 0.76g *S. sclerotiorum* NEB-274 millet inoculum by removing the root mass and soil from the pot, placing the millet inoculum in the bottom of the pot, and returning the root-bound plant to place the root-

mass in contact with the mycelial inoculum. Plants were evaluated daily for terminal wilt or whole-plant desiccation indicative of plant death due to BSR for a total of 28 days after inoculation. Days to plant death were recorded. The experiment was conducted three times and data were combined for analysis (n=45 total plants per genotype). Mean days to plant death for each line and significant differences among genotypes at α=.05 were determined using a generalized linear model implemented in SAS v9.4 PROC GLM (SAS Institute, 2013).

Phomopsis stem and leaf inoculations

Eight six-week-old sunflower plants per genotype were inoculated with *D. helianthi* isolate Rothsay-2 or *D. gulyae* isolate N4. Stem inoculations were performed by wounding the plant stem with a scalpel midway between the first and second internode and affixing a 4mm diameter PDA plug carrying mycelia to the wound using Parafilm. Inoculated plants were evaluated at 14 days post inoculation (dpi) and rated on a 0-5 ordinal rating scale described previously (Elverson et al., 2020). Ratings were converted to disease severity index as described (Chiang et al., 2017) and mean disease severity indices were determined and compared to the susceptible control genotype HA 410 (Miller and Gulya, 1999) using SAS PROC GLM and Dunnett's test to identify genotypes exhibiting levels of resistance significantly different from the control. Leaf inoculations were performed by affixing mycelial plugs to the leaf tip with tape and enclosing inoculated leaves in a quart plastic bag with 10ml water. The number of days required for the lesion to reach the petiole and the stem were recorded and these data along with leaf size measurements were used to determine rates of progression of the fungus.

Results and Discussion

Identification of sunflower germplasm with high levels of resistance to Sclerotinia basal stalk rot using an improved greenhouse evaluation method

We recently developed an improved, greenhouse-based method to evaluate sunflower lines for resistance to Sclerotinia BSR (Underwood et al., 2020). The improved method involves root-inoculation of sunflower plants grown in small pots and kinetic analysis of time to plant death from BSR (Figures 1 and 2). This method provides considerably improved resolution over inoculated field trials and is more suitable particularly for identification of sunflower lines with high levels of resistance. We have subsequently employed this improved method to re-evaluate 60 sunflower lines for which prior evidence of resistance based on inoculated or naturally infected field nurseries was available. We determined that 38 of the 60 lines were significantly more resistant than the highly susceptible control PI 650798 (variety Cabure 1004) while 16 lines were significantly more resistant than the moderately susceptible inbred line control HA 89 (Table 1). Importantly, we identified 3 lines that were significantly more resistant than the moderately resistant inbred control line RHA 801, including USDA released inbred line HA 124. These lines will be prioritized for further characterization and genetic mapping efforts. Our observation that 22 of the 60 lines were not significantly different from the highly susceptible control highlights the challenges with evaluation of this disease in field nurseries including the possibility for disease escape and the relatively poor resolution of field-based methods.

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Identification of sunflower lines exhibiting resistance to stem lesion formation by D. helianthi and D. gulyae, causal agents of Phomopsis stem canker

Numerous prior efforts by the Sunflower & Plant Biology Research Unit have been undertaken to screen sunflower germplasm for PSC resistance in the field under conditions of natural infection. To begin characterizing the physiological mechanisms responsible for resistance in lines that have been identified as resistant in field trials, we evaluated 80 sunflower lines for resistance to stem lesion formation in inoculated greenhouse experiments. The lines were selected based on evidence for resistance in prior field screening efforts. Stem wound inoculations were conducted using the highly susceptible inbred line HA 410 as a control (Figure 3). We identified 8 lines exhibiting significantly higher levels of resistance to stem lesioning than HA 410 after inoculation with *D. helianthi* isolate Rothsay-2 and 6 lines exhibiting resistance to *D. gulyae* isolate N4 (Table 2). Importantly, 3 lines were identified with significant levels or resistance to both species of the pathogen. We also observed a significant correlation (Pearson's coefficient 0.474) between response to *D. helianthi* and *D. gulyae*, suggesting at least some overlap in resistance to these two species causing PSC in sunflower. Additionally, we evaluated a subset of lines for resistance to leaf lesion expansion. We identified 6 lines with higher levels of leaf resistance to D. helianthi than the HA 410 control, including the inbred line HA 378 which exhibits resistance to both stem and leaf lesioning and is highly resistant in field trials Table 3). Evaluations to determine if resistant lines are broadly resistant to multiple isolates of *D. helianthi* are currently ongoing.

Figures and Tables

RHA801 HA89

Figure 1. BSR responses of moderately resistant line RHA 801 (left) and moderately susceptible line HA 89 (right) at 11 dpi.

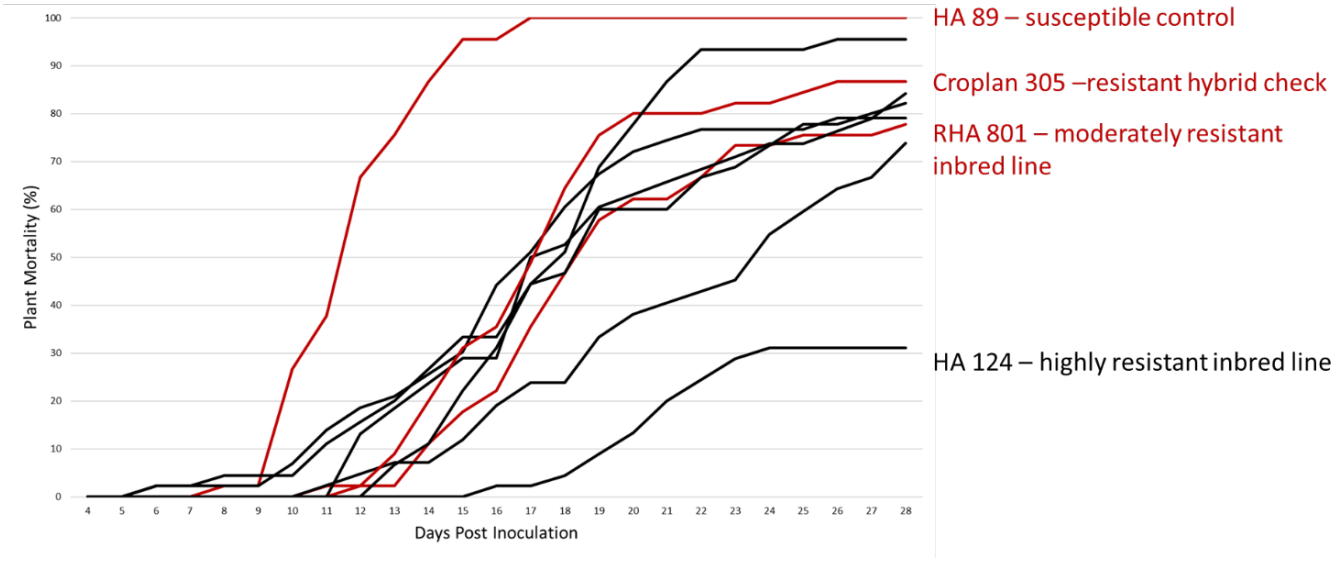

Figure 2. Kinetic evaluation of BSR response for selected susceptible, moderately resistant, and highly resistant sunflower genotypes. Forty-five plants per genotype were evaluated daily for death due to BSR for 28 dpi.

Accession Name	PI	Greenhouse Avg Days to Death	Statistical Grouping
A-1499	413050	20.6	a
HA 124	599775	20.2	a
No. 9121	175733	18.9	ab
Short Russian	650379	17.1	abc
Olea	650369	16.9	abc
Zelenka	650831	16.8	abc
FS-a-3	480471	16.6	abcd
HA 61	599771	16.5	abcd
Voshod Elite 7	650458	16.4	abcde
RHA 408	603989	16.4	abcde
VIR 160	497250	16.3	abcde
CMG-3	650400	16.3	abcde
Romsun V3355 AC	650498	16.0	bcdef
A-1405	380562	15.9	bcdefg
Lengyel A	531366	15.8	bcdefg
PO 6/4-2	431560	15.5	bcdefg
HZ.SM 27.208	531359	15.4	bcdefgh
VK-53	650468	15.4	bcdefgh
RHA 801	**599768**	**15.2**	**cdefghi**
Pioner Sibiri	497933	15.0	cdefghij
VIR 117	650485	14.2	cdefghijk
Polstar	650372	12.8	defghijk
VK-10	650464	12.4	defghijkl
Guaran	650810	12.3	efghijkl
HA 410	603991	12.2	fghijkl
D-75-11	431543	12.0	ghijkl
HA 89	**599773**	**11.8**	**hijkl**
Ostonne	650371	11.6	ijkl
Ames 102	490282	11.4	jkl
VIR 110	650536	10.5	kl
Cabure 1004	**650798**	**8.8**	**l**

Table 1. BSR responses for a subset of 60 sunflower genotypes evaluated for resistance using a high-resolution, greenhouse-based method. Susceptible control Cabure 1004 (PI 650798) and moderately susceptible HA 89 are indicated in red and moderately resistant control RHA 801 is indicated in green. Lines indicated by the same letter are not significantly different (α=0.05).

Figure 3. Response of PI 650675 (left), with resistance to PSC stem lesioning, compared to the susceptible control line HA 410 (right).

Accession Name	PI	Disease Severity Index	More Resistant Than Control
HA 410	603991	91.7	
HA-R3	650754	91.7	
AMM 683	526261	91.7	
Kisvardai	531365	91.7	
ZFA 3225	494857	89.6	
Penyigei E	531383	87.5	
Tournesol	181769	87.5	
Taiyo	650839	87.5	
V8883 4/1-1	431567	85.4	
ZM/A 5199	505653	83.4	
RHA 801	599768	83.3	
L1585U		82.1	
3100399	507896	79.2	
Abadsens	250085	77.1	
Rannespely		77.1	
Zelenka	650831	70.8	
CO-PB 48	650681	68.8	
Nyiregyhazi A	531377	66.7	
TA-4181-8		66.7	
Giza	433862	65.5	
CM 214		64.6	
HA 323	664232	60.4	
3100397	507894	58.3	***
Slovenska siva	531389	58.3	***
AMM 608	526254	58.3	***
CO-PB 84	650699	56.2	***
CO-PB 90	650703	56.2	***
HA 378	561918	52.1	***
CO-PB 39	650675	50.0	***
CM 198		47.0	***
HA 821	599984	41.7	***

D. helianthi isolate Rothsay-2

Accession Name	PI	Disease Severity Index	More Resistant Than Control
HA 410	603991	91.7	
CM 214		91.7	
Rannespely		91.7	
Taiyo	650839	91.7	
Penyigei E	531383	91.7	
ZFA 3476	494862	91.7	
3100399	507896	89.6	
Abadsens	250085	89.6	
TA-4181-8		87.5	
Nyiregyhazi A	531377	87.5	
Tournesol	181769	87.5	
L1585U		86.9	
ZM/A 5199	505653	86.9	
HA-R3	650754	83.3	
Zelenka	650831	81.3	
Kisvardai	531365	81.3	
HA 323	664232	79.2	
AMM 608	526254	79.2	
3100397	507894	75.0	
Ames 10101	650657	75.0	
CM 198		75.0	
Giza	433862	68.7	
Slovenska siva	531389	68.7	
HA 421	618725	67.9	
Ames 101	490281	65.5	
Giza	433862	63.9	***
Ames 102	490282	63.9	***
RHA 354	509064	61.1	***
CO-PB 39	650675	60.4	***
CO-PB 84	650699	58.3	***
HA 378	561918	36.1	***

D. gulyae isolate N4

Table 2. PSC stem lesion response of selected sunflower genotypes evaluated for resistance to *D. helianthi* (left) and *D. gulyae* (right). Asterisks indicate lines with significantly higher levels of resistance than the susceptible control HA 410.

Accession Name	PI	Leaf Lesion Progression (mm/hr)	More Resistant Than Control	Leaf Score (days)	More Resistant Than Control
3100397	507894	0.6489		8.4	
HA-R3	650754	0.5916		7.2	
HA 410	603991	0.5901		7.5	
RHA 486	690019	0.5681		6.7	
Portugal E	531385	0.5681		8.0	
CM 198	531383	0.5332		9.7	
Zelenka	650831	0.5153		8.8	
Taiyo	650839	0.5039		8.7	
AMM 608	526254	0.5027		10.3	***
AMM 683	526261	0.4965		10.3	***
Slovenska siva	531389	0.4901		8.8	
Nyiregyhazi A	531377	0.4866		9.0	
Giza	433862	0.4850		10.0	***
ZM/A 5199	505653	0.4831		11.0	***
CO-PB 39	650675	0.4824		8.3	
HA 821	599984	0.4802		7.8	
HA 61	599771	0.4620		9.7	
RHA 801	599768	0.4552		8.3	
RHA 274	599759	0.4298		8.0	
HA-R4	650755	0.4236		10.2	***
Bodroghalmi	531340	0.4211	***	11.8	***
CO-PB 90	650703	0.4089	***	9.8	
Ames 10101	650657	0.4088	***	10.8	***
HA 378	561918	0.3875	***	11.2	***
RHA 354	509064	0.3751	***	9.3	
HA 421	618725	0.3586	***	10.3	***

Table 3. PSC leaf lesion response of selected sunflower genotypes evaluated for resistance to *D. helianthi* isolate Rothsay-2. Asterisks indicate lines with significantly higher levels of resistance than the susceptible control HA 410.

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