IDENTIFICATION OF HOST-SELECTIVE TOXINS IN *CORYNESPORA CASSIICOLA* CAUSING TARGET SPOT OF COTTON, SOYBEAN AND TOMATO

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Abstract

Host-selective toxins (HSTs) are metabolites produced by pathogenic fungal necrotrophs that serve as important determinants of virulence, pathogenesis and are also associated in host-specificity. *Corynespora cassiicola* (Berk & M.A. Curtis) C. T. Wei, the causal agent of the target spot epidemics on cotton, tomato and soybean in the southeastern United States (U.S.), is one of the fungal species known to produce secondary metabolites or HSTs. Specifically, it produces *cassiicolin*, a well-characterized phytotoxin encoded by six different *cas* gene variants. Southeastern U.S. isolates of *C. cassiicola* collected from different host plants have also been found to show host-specialization. Additionally, these isolates were also discovered to contain putative genes of metabolites or HSTs (e.g. T-toxin cluster; cas variants) in their genomes via an *in-silico* approach. Nonetheless, the function of these HSTs or metabolites in pathogenesis and host-specificity is yet to be determined. Hence, this study is being conducted to identify the specific metabolites or HSTs produced by *C. cassiicola* and to delineate their interactions with various inoculated plants (i.e. cotton, tomato and soybean). Twenty-four isolates of *C. cassiicola* (eight from each host: cotton, soybean and tomato) were used in toxin production. Crude extracts were obtained by a 2-step filtration process and the resulting fungal filtrates were infiltrated onto host plants (cotton, soybean and tomato). Necrotic area and visual symptoms were assessed at 5, 10 and 21 days post infiltration. Moreover, the filtrates were treated with Proteinase K, thereafter also infiltrated onto plants. Separation of fungal extracts into components was conducted via a bio-assay mediated fractionation. The fractions were also infiltrated onto plants. Results show that the fungal filtrates were more virulent on the host of origin. The measured necrotic area on the leaves were statistically significant for fungal filtrates infiltrated onto their original hosts. Furthermore, non-proteinaceous compounds causing plant toxicity are also present on the fungal filtrates. Identification of metabolites will be conducted by liquid chromatography-mass spectrometry (LC-MS).

Introduction

Target spot has been cited as an emerging disease across economically important crops in the southeastern U.S. such as cotton (*Gossypium hirsutum*), soybean (*Glycine max*) and tomato (*Solanum lycopersicum*). It has been responsible for yield losses and to early defoliation in cotton plants in Georgia in 2008. Target spot in cotton has also been expanding in geographical range across Alabama, Louisiana, North Carolina, South Carolina, Florida, Arkansas, Virginia, Tennessee and Mississippi. Yield losses due to cotton target spot has been reported to reach about 5-40%. In tomato plants, this disease has been regarded as the most damaging fruit and foliar disease in Florida. Conversely, target spot in soybean is a minor concern in the United States. Nonetheless, recent reports were accounted in South Carolina in 2004 and Mississippi in 2016 (Sumabat et al. 2018).

The pathogen instigating target spot disease in cotton, soybean and tomato, *Corynespora cassiicola*, is an asexual fungi known to have a diverse host range of about 300 plants grown in the subtropical to tropical regions including agricultural crops such as cotton, tomato, cucumber, soybean, cowpea, rubber tree and cocoa (Barthe et al. 2006). It taxonomically belongs to Order *Pleosporales*, the fungal taxa known for producing secondary metabolites or host-selective toxins. As a necrotroph, *C. cassiicola* is capable of killing and growing on host tissues prior to or during colonization, eventually leading to cell necrosis by means of releasing phytotoxic metabolites, reactive oxygen species and peptides (Wang et al. 2004). Invasion of host tissues by the fungal pathogen through direct penetration of the abaxial epidermis also involves extracellular fibrillary sheaths coming from the germ tubes. These fibrillary sheaths

have been associated with penetration and mycelial adhesion to host tissues. Ultimately, this action leads to collapse of the epidermis and cell disorientation which manifests as loss of starchy grains and nuclear disintegration (Breton et al. 2000).

In addition, *C. cassiicola* as a necrotroph, is capable of producing the host-selective toxin cassiicolin. This compound was chemically characterized as a peptide consisting of 27 amino acids, with 6 cysteine in disulfide bonds, a N-terminal pyroglutamic acid and has a second residue lycosylation. This protein has a molecular mass of 2884.96 Da (De Lamotte et al. 2007). One of its gene variants, cassiicolin-encoding gene (*Cas1),* was found to be present during the initial stage of the infection process (Deon et al. 2012). Furthermore, this toxin was also used in bio-assay studies, specifically in *Corynespora* leaf fall disease in rubber (CLFD) wherein results demonstrated that crude filtrates are capable of producing disease symptoms on inoculated host plants (De Lamotte et l. 2007). Symptoms produced by the culture filtrates containing cassiicolin were found to be identical to the symptoms due to fungal inoculation, such as brown discoloration and necrosis. The ability of the toxin to diffuse away from site of infection was also demonstrated as evidenced by cell death of host tissues distal from site of infiltration (Breton et al.2000).

As secondary metabolites, HSTs are known as compatibility factors (Barthe et al. 2006) and are implicated in host-specificity, virulence and pathogenicity such that these toxins are most toxic on the original host of the fungus producing them. Some of these host-specific necrotrophs and their HSTs are *Cochliobolus carbonum* producing HC-toxin (causing northern corn leaf spot), *Pyrenophora triciti-repentis* producing Ptr-toxins A and B (causing tan spot of wheat), *Cochlionolus victoriae* producing victorin, *Alternaria alternate* f. sp. *lycopersici* producing AAL-toxin, *Cohliobolus heterostrophus* producing T-toxin, among others (Wang et al. 2004; De Lamotte et al. 2007).

This study generally aims to identify HSTs or other secondary metabolites produced by *Corynespora cassiicola* causing target spot disease on cotton, soybean and tomato and to determine the interactions among the toxins or secondary metabolites with inoculated host plants. This was done by a series of greenhouse assays using crude filtrates from *C. cassiicola* isolates collected from target spot infected cotton, soybean and tomato. Moreover, these filtrates will be subjected to a bio-assay guided fractionation to further narrow down identification.

Materials and Methods

Extraction of fungal filtrates from selected isolates of *Corynespora cassiicola*

Twenty-four isolates of *C. cassiicola* (eight from each host: cotton, soybean and tomato) collected from target spot infected cotton, soybean and tomato plants were selected. These isolates were cultured in Potato Dextrose Agar (PDA) medium for 7 days at 23°C in 12 h dark/light condition. Toxin production was done following the procedures of De Lamotte et al. (2007) with modifications: Three mycelial plugs (5 mm diameter) from 7 day-old cultures were obtained and transferred to 500 mL Erlenmeyer flasks containing 100 mL of Czapeck's modified medium which consisted of the following: 30 g/L of Saccharose, 2.2 g/L of glutamic acid, 1 g/L of K_2HPO_4, 0.5 g/L of KCl, 0.5 g/L of $MgSO_4 \cdot 7 H_2O$, 36 µM $FeSO_4 \cdot 7H_2O$, 35 µM $ZnSO_4 \cdot 7 H_2O$, 40 µM $CuSO_4 \cdot 5 H_2O$. These liquid media were incubated at 23°C in 12 h dark/light condition without agitation for 14-20 days. Extraction was then done to remove the mycelial fragments by filtering the liquid medium through sterile Whatman filter paper #1, followed by filtering through 0.22 µm Millipore membranes.

Infiltration onto host plants (cotton, soybean and tomato)

Fungal crude filtrates from selected cotton, soybean and tomato *C. cassiicola* isolates were infiltrated onto susceptible host plants of cotton (Phy499 WRF), soybean (Asgrow) and tomato (Bonny Best). Plants were grown in the greenhouse for 30 days or up to the 3-true leaf stage. Three leaves per plant were infiltrated with the crude filtrates. Replication per plant was done thrice per crude filtrate. About 20 µL of filtrates were placed on syringe-wounded portion of the leaves using a 1 mL syringe. The site of infiltration on the leaves were marked. An untreated Czapeck's modified medium was also infiltrated onto leaves as control. Agar mycelial plugs of selected *C. casiicola* isolates from cotton, soybean and tomato were placed on leaves as comparison. Visual symptoms of chlorosis, necrosis or wilting and lesion area were assessed at 5, 10 and 21 days post infiltration. The bioassay was repeated twice. Statistical analysis was conducted using JMP 14 (SAS Institute, Inc.). Tukey's HSD was done to determine if significant differences exist among mean lesion areas.

Culture filtrate treatment and fractionation

Culture filtrates from one cotton (isolate CGA-3) and one tomato (isolate 1551) isolate of *C. cassiicola* causing target spot which showed the most virulent response to infiltrated host plants in prior bioassays were selected and were treated with (recombinant) Proteinase K (Thermo Fisher Scientific). The resulting solutions were infiltrated onto cotton and tomato plants to determine if non-proteinaceous components are involved in plant toxicity. Three marked leaves per cotton or tomato plants were infiltrated. Replication was done thrice. Likewise, the control liquid medium was also treated with Proteinase K and were infiltrated to plants for comparison. Visual symptoms and lesion area were assessed at 5 and 10 days post infiltration.

Moreover, these culture filtrates were separated into fractions by chromatography using a glass column with C18 (odS SPE Bulk Sorbent, Agilent Technologies) as stationary phase. C18 was washed with HPLC grade methanol (Fischer Scientific) prior to fractionation. The filtrates were eluted using methanol and were separated into 11 fractions (0% to 100%). The resulting fractions were infiltrated to cotton plants. Three marked leaves per plant, replicated thrice, were infiltrated with each of the fractions. Visual symptoms and lesion area were assessed at 5 and 10 days post infiltration. Tukey's HSD was done, and analysis was conducted using JMP 14.

Results and Discussion

At 5 days post-infiltration (dpi), visual symptoms on the infiltrated plant materials were observed. These symptoms became more apparent at 10 dpi. Fungal culture filtrates also demonstrated host-specificity. Visual symptoms of necrosis and yellowing were more evident when fungal crude extracts were infiltrated on their host of origin. Culture filtrates from isolates of *C. cassiicola* collected from target spot infected cotton plants were more toxic on cotton as compared to culture filtrates of fungal isolates collected from target-spot infected soybean and tomato when infiltrated onto cotton plants (Figure 1). Similar visual responses were noted when the culture filtrates from *C. cassiicola* isolates collected from target spot infected tomato were infiltrated onto tomato plants (Figure 2). Conversely, variation in symptoms produced was not evident on infiltrated soybean plants (Figure 3).

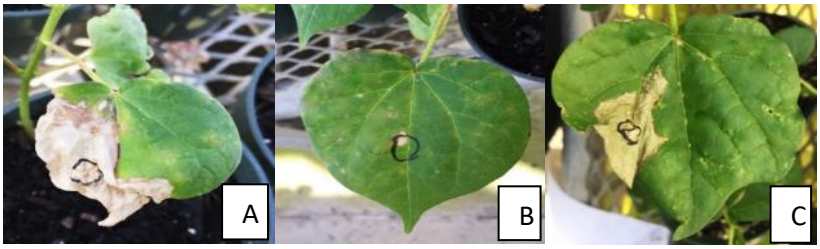

Figure 1. Cotton (Phy 499 WRF) infiltrated with fungal crude extracts from *C. cassiicola* isolates of cotton (A), soybean (B) and tomato (C), 5 dpi.

Figure 2. Tomato (Bonny Best) infiltrated with fungal crude extracts from *C. cassiicola* isolates of cotton (A), soybean (B) and tomato (C), 5 dpi.

Figure 3. Soybean (Asgrow) infiltrated with fungal crude extracts from *C. cassiicola* isolates of cotton (A), tomato (B) and soybean (C), 5 dpi.

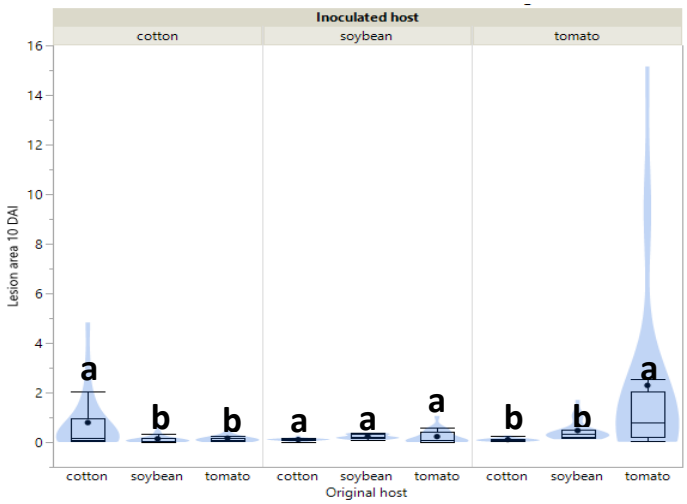

Figure 4. Mean lesion areas (cm^2) produced by cotton and tomato fungal culture filtrates on their host of origin (cotton and tomato) were statistically different, 10 dpi.

Moreover, mean comparison analyses (Tukey's HSD at α=0.05) of lesion areas were statistically significant for crude extracts from cotton *C. cassiicola* isolates when infiltrated onto cotton plants and for crude extracts from tomato *C. cassiicola* isolates when infiltrated onto tomato plants. Measured mean lesion sizes on cotton plants infiltrated with crude extracts from tomato and soybean *C. cassiicola* isolates were not statistically different from each other. Likewise, same results were observed for the mean lesion sizes on tomato plants infiltrated with crude extracts from cotton and soybean *C. cassiicola* isolates. Interestingly, measured mean lesion sizes from infiltrated soybean plants with crude extracts from soybean, cotton and tomato *C. cassiicola* isolates were not statistically different from each other (Figure 4). From these findings, it can be inferred that metabolites from the filtrates of cotton and tomato *C. cassiicola* isolates were manifesting host-specificity.

Fungal culture filtrates from cotton *C. cassicola* isolate CGA-3 treated with Proteinase K were still able to show symptoms of toxicity and necrosis on infiltrated cotton plants. Fractionation of selected fungal filtrate from cotton isolate of *C. cassiicola* into 11 components was also done. These fractions also showed varying degrees of symptoms of toxicity when infiltrated onto cotton plants. Mean comparison analysis using Tukey's HSD at α=0.05 also showed that two lesion sizes measured from two fractions (10% and 20% methanol) of the filtrate were statistically significant (Figure 5). The succeeding step for this study will be to sub-fractionate these two fractions showing the most toxic response on infiltrated cotton plants.

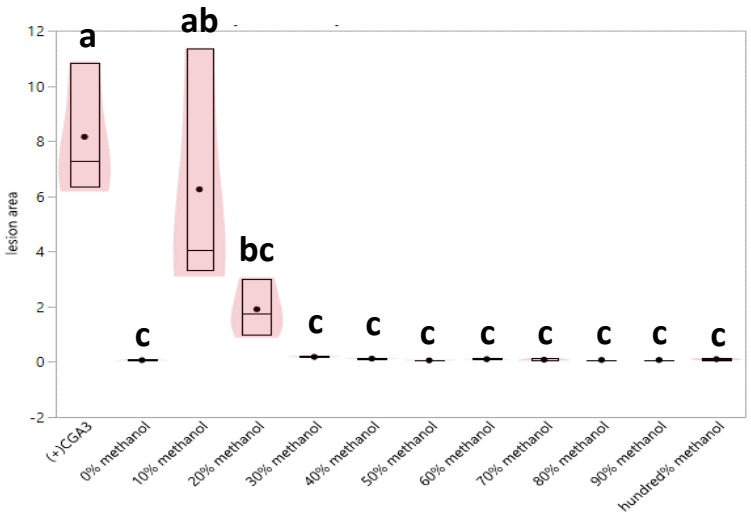

Figure 5. Mean lesion areas (cm^2) produced by the 11 eluted fractions (0% to 100% methanol) of the fungal culture filtrates from the cotton *C. cassiicola* isolates CGA-3 upon infiltration to cotton plants (Phy 499 WRF), 5 dpi.

Summary

Host-selective toxins or secondary metabolites are being produced by the necrotrophic fungal pathogen, *C. cassiicola* causing target spot disease on cotton, soybean and tomato. Previous studies (Sumabat et al. 2018) have shown that the southeastern U.S. populations of *C. cassiicola* are exhibiting host-specialization. These HSTs were implicated to be involved in the host-specialization yet still needs to be established. The main objective of this research is to identify the specific HSTs or metabolites being produced by this pathogen causing target spot disease on cotton, soybean and tomato and to determine their role in host-specificity. Fungal filtrates from selected *C. cassiicola* isolates were extracted and were infiltrated to susceptible plant materials of cotton, soybean and tomato. Result showed that these filtrates exhibit host-specificity. Moreover, selected fungal filtrates from cotton and tomato isolates of *C. cassiicola* were treated with Proteinase K and subjected to fractionation to narrow down identification of the metabolites present. Results from this research can be used in screening for resistant cultivars to target spot disease and for future breeding programs.

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