Identification and Characterization of Phytotoxins produced by *Neopestalotiopsis* sp. on Strawberry

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Summary

We discovered 18 potential phytotoxins in aggressive *Neopestalotiopsis* strains and diverse gene clusters encoding phytotoxin-related proteins. Highly expressed unique genes and two upregulated genes could be linked to their aggressiveness, but further research on the gene-editing effect on pathogenicity is necessary. Moreover, each of the potential phytotoxins will need to be further investigated to determine their role(s) in the disease infection mechanisms.

Methods

Objective 1: Identify potential phytotoxins related to the aggressive strains of *Neopestalotiopsis* sp.
In the previously funded proposal, we used only one representative isolate of the aggressive *Neopestalotiopsis* sp. and *N. rosae* to carry out plant inoculation to collect symptomatic tissue samples. In this proposal, a total of 11 isolates, with 5 *Neopestalotiopsis* sp. (aggressive) and 6 *N. rosae* (non-aggressive) were selected for inoculation. Non-inoculated plants were used as controls. Strawberry transplants of 'Florida Brilliance' were inoculated using a spore suspension from each isolate. Symptomatic tissues were collected 7 days after, and UHPLC-Q/Orbitrap MS/MS was applied combined with an untargeted metabolomics workflow to "fish out" and identify the potential phytotoxins. Moreover, we created a self-built research database to refine our data comparisons and the likelihood of identifying whether only one or more than one phytotoxin is being produced by *Neopestalotiopsis* spp.

Objective 2: Identify phytotoxin-related gene clusters in the new *Neopestalotiopsis* sp.
One isolate of *Neopestatoliopsis* sp. and one from *N. rosae* were selected for whole genome sequencing (WGS). A hybrid assembly was used to combine Illumina and Oxford Nanopore Technologies. The same two isolates were grown on culture media amended with strawberry leaves for sample collection to perform RNA extractions. RNA sequencing results were used in combination with the whole genome assemblies for read mapping and quantification of gene expression from both aggressive and non-aggressive *Neopestalotiopsis* strains. Moreover, we used a comparative genomic approach to identifying potential secondary metabolite production and characterizing phytotoxin-related gene clusters.

Results

Objective 1
Only plants inoculated with the aggressive strain of *Neopestalotiopsis* sp. developed symptoms, whereas those inoculated with *N. rosae* had no visible symptoms (Figure 1). Data analysis using mathematical-statistical modeling for differential metabolite screening, followed by the systematic metabolite identification strategy based on high-resolution mass spectrometry (HRMS), indicated 18 different compounds consistently produced in higher concentrations on aggressive isolates than on non-aggressive isolates. Comparing the chemical structure of these 18 potential phytotoxins with our self-built database, we found that most of them had been reported as toxins produced by other fungal plant pathogens, such as species within *Pestalotiopsis*,

Alternaria, *Magnaporte*, *Phomopsis,* and *Pyrenochaeta.* Regrettably, some of these compounds are not readily available and can only be synthesized by specific companies, which makes the next step for this project quite expensive (i.e., approximately over USD 2,000 / milligram). Thus, we intend to submit a proposal to a federal agency to secure additional funding and continue this research.

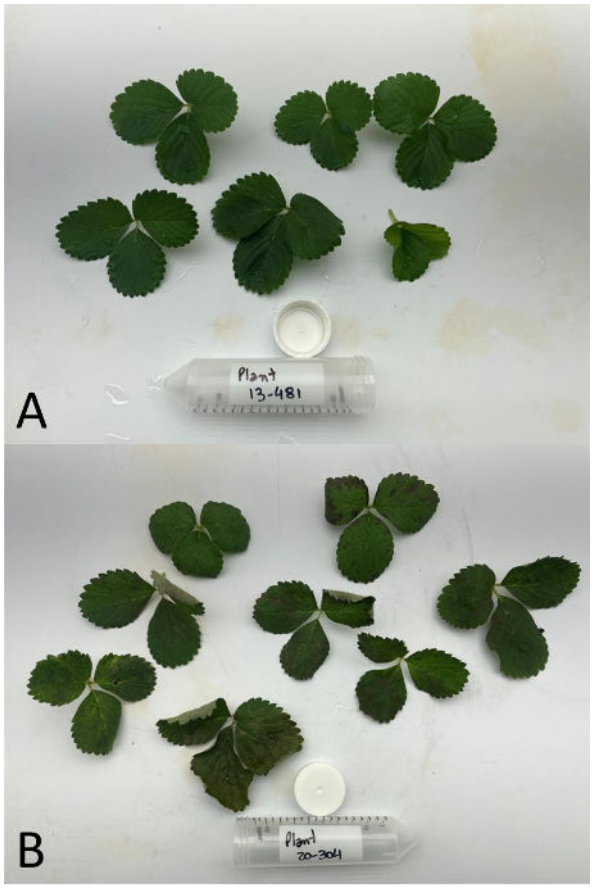

Figure 1. Strawberry leaves inoculated with *N. rosae* had no symptoms after 7 days (A), whereas several leaf spots were observed when the aggressive *Neopestalotiopsis* strain was used (B).

Objective 2

Utilizing a comparative genomic approach, we identified gene clusters associated with phytotoxins, including non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs), exhibiting variability between aggressive and non-aggressive *Neopestalotiopsis* strains. Moreover, we identified 50 unique genes highly expressed in the aggressive strain using the RNA sequencing data with no corresponding ones in *N. rosae*. In addition, we found two genes that were upregulated in the aggressive strain and downregulated in *N. rosae*. All these genes could confer aggressiveness to *Neopestalotiopsis* sp. However, further studies with a large collection of isolates are needed to narrow down some genes and study the effect of gene editing (gene knockouts) on the pathogenicity of modified strains of strawberry.

Takeaways

We discovered several potential phytotoxins with reported chemical structures and biofunctions, plus potential genes related to the aggressiveness of the new strains of *Neopestalotiopsis*. Although more studies are needed, these findings will contribute to the understanding of the infection mechanisms of aggressive strains and their arsenal to cause the severe disease outbreaks experienced in strawberry nurseries and fruit production fields. We are currently seeking funds to continue these quite costly studies. If successful, we expect to substantially contribute to the development of rapid screening of strawberry seedlings for resistance to *Neopestalotiopsis* in the breeding program. We could also potentially identify chemicals/enzymes to inactivate phytotoxins as an alternative disease management strategy. Moreover, we could possibly manipulate the genes to improve the resistance of current or future cultivars.

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