Title: Prevalence and characterization of *Neopestalotiopsis* spp. on strawberry cultivars in the southeastern United States

Final Report: Funded Research Proposal

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Public Abstract:

Neopestalotiopsis fruit rot and leaf spot, caused by *Neopestalotiopsis* species, was first reported to cause damage in strawberry fields in Florida in 2019 and 2020. This disease has since been observed in most southeastern states. This new species is considered very aggressive on leaves and fruit, although it can also infect all plant tissues. The novel species infects nursery transplants, and this is considered the primary source of infection and dissemination to other regions. In 2024, a survey supported by SRSFC was conducted to identify the prevalence of the aggressive *Neopestalotiopsis* in the southeastern US. Strawberry leaf and fruit samples (*n*=40) were received and tested at the Plant Molecular Diagnostic Lab at the UGA-Tifton campus. Samples were shipped from Louisiana, Arkansas, Mississippi, North Carolina and from several Georgia counties. A total of 22 samples (55%) tested positive for the aggressive strain. Samples from all the participating states, except for MS, tested positive for the aggressive *Neopestalotiopsis* species. In response, growers that had positive samples received specific management recommendations from their state specialist (e.g. remove infected plants, apply fungicides,

etc.). In addition, samples from at least eight different nurseries who provided plugs/plants tested positive for the aggressive strain after molecular testing, indicating that this may contribute to spreading the pathogen to other regions. In fact, it was documented recently that strawberry tip suppliers from Prince Edward Island had a large outbreak of *Neopestalotiopsis*, and therefore nurseries who relied on this source of strawberry tips for propagation could not fulfill plug orders. Monitoring the species causing Neopestalotiopsis fruit rot and leaf spot, as well as the nurseries providing the plants, has helped strawberry producers to be informed as to the full extent of the *Neopestalotiopsis* problem. The large number of nurseries providing infected plants last year was surprising, but in light of the issues observed this year, this likely means that many East Coast and Canadian nurseries are now sources of diseased plants.

Objectives:

1. Determine the prevalence of *Neopestalotiopsis rosae* and the virulent *Neopestalotiopsis* sp. on strawberry in the Southeastern United States.
2. Conduct morphological and molecular characterization of *Neopestalotiopsis* spp. to facilitate and enhance accurate disease diagnosis.

Introduction:

Neopestalotiopsis sp. on strawberry is a novel fungal pathogen that has been reported to cause severe outbreaks in the United States (Baggio et al. 2021). Pestalotia leaf spot and fruit rot, caused by *Neopestalotiopsis rosae* (aka *Pestalotiopsis longisetula*), was considered a weak or secondary pathogen of strawberry, often isolated from the roots and crown of poorly established plants. However, in 1972, an outbreak of *N. rosae* was reported in Florida causing significant losses in research plots and commercial fields (Howard and Albregts 1973). In 2017, severe outbreaks were reported once again in commercial fields in Florida, with symptoms observed on leaves and fruit. Significant yield losses were reported, and the fungal pathogen was characterized as *Neopestalotiopsis* sp., which is a closely related species to *N. rosae* (Baggio et al. 2021). The literature is somewhat unclear as to whether this new disease is in fact different than that reported by Howard and Albregts, but for now, we will reference the current disease as Neopestalotiopsis leaf spot and fruit spot. The new species that causes this disease is considered very aggressive on leaves and fruit (**Fig 1**), although it can also infect all plant tissues. It infects nursery transplants, and this is considered

Figure 1. Symptoms and signs of strawberry samples sent to the molecular diagnostic lab at UGA-Tifton campus

the primary source of infection and dissemination to other regions. Since the initial report from 2017, many cases of Neopestalotiopsis leaf spot and fruit spot have been observed in numerous states throughout the southeastern US (Alabama, Georgia, Louisiana, North Carolina, South Carolina and Texas). Indeed, the disease has now threatened strawberry production almost everywhere in the Southeast (Jimenez Madrid et al. 2024; Conner 2022; Hoffmann 2020; Holland 2021; Morgan 2022). Monitoring the species causing Neopestalotiopsis leaf spot and fruit rot is critical for understanding the epidemiology and management of both *Neopestalotiopsis rosae* and the new *Neopestalotiopsis* sp. Although *N. rosae* is considered an opportunistic pathogen, this fungus has been reported to cause severe symptoms on strawberry leaves, roots and crowns in many countries, including Mexico (Rebollar-Alviter et al. 2020). While both species can cause similar symptoms on strawberry, the new species is reported as being the one responsible for the recent outbreaks in the US and was found to be more aggressive than *N. rosae* in Florida (Baggio et al. 2021). Accordingly, it is important to actively monitor the prevalence of this disease in the southeastern US and accurately diagnose the particular pathogen species causing disease on strawberry.

Although the epidemiology of the new *Neopestalotiopsis* sp. is not well-understood, it has been hypothesized that infected nursery transplants are spreading the pathogen to new regions. However, the pathogen can also be easily introduced through wind and rain splash from alternative host plants. It is important to note that environmental conditions play an important role in disease development, and even if infected strawberry plants are transplanted into a field, it may not develop symptoms under dry conditions (Peres 2023).

Figure 2. A. Colony morphology and black acervuli of Neopestalotiopsis sp. on potato dextrose agar after 14 days of incubation at 25°C. B. Conidia morphology observed under the microscope (x40) (Jimenez Madrid et al. 2024)

To reiterate, there are several other *Neopestalotiopsis* species, such as *N. rosae*, that may cause similar symptoms on strawberry (Rebollar-Alviter et al. 2020), but symptoms can vary quite a bit, and it is highly recommended that producers send samples to a diagnostic lab or specialist for confirmation. An accurate and reliable identification of the species associated with the disease can significantly help to properly manage it. Morphological features among species cannot be used for definitive identification, since symptoms and spore types are very similar (**Fig 2**). Therefore, a molecular tool based on polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) has been developed for the identification of the aggressive *Neopestalotiopsis* sp. from strawberry (Kaur et al. 2023), and this method can also determine whether nonaggressive secondary strains, such as *N. rosae*, are present instead **(Fig 3)**. A second aggressive strain also plays a role in recent outbreaks (Peres, *personal communication*); however, to date, a sequencing analysis for this strain has not been released, and further testing must be conducted to ensure an accurate diagnosis between species/strains. Molecular detection assays can be expensive, but such assays provide the only means to accurately confirm the aggressive form of *Neopestalotiopsis*. Monitoring the species associated with Neopestalotiopsis leaf spot and fruit rot is critical in order to establish an understanding of pathogen distribution and to incorporate immediate management strategies to mitigate total crop loss.

Materials and Methods:

Collection and isolation of *Neopestalotiopsis* spp. A field survey was coordinated with Extension Specialists from Georgia, Louisiana, Arkansas, Mississippi, and North Carolina. Symptomatic strawberry plants from fields with significant suspect damage were collected and sent to the Plant Molecular Diagnostic Laboratory at the University of Georgia -Tifton Campus. The client was required to submit a sample submission form along with the sample to obtain information such as, disease incidence, cultivar, nursery providing the plugs/plants and sample location. Symptomatic plants were carefully examined to document symptoms and/or signs (e.g., dark fruiting bodies, mycelium). Fungal isolations were conducted using standard isolation methods on potato dextrose agar amended with chloramphenicol (75 ppm; cPDA). Isolates were incubated at 25°C for 5-6 days. Morphological differences between isolates were noted.

Morphological and molecular characterization of *Neopestalotiopsis* species. Colony morphology was characterized on cPDA. Features such as culture color, growth pattern, texture, as well as conidia (spore) morphology including shape, color, and size was documented for each fungal isolate. Genomic DNA extraction from pure fungal cultures was performed with fungal DNA extraction kits according to the manufacturer's instructions. The molecular identification of *Neopestalotiopsis* spp. was conducted following a polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) method as described by Kaur et al. 2023 (**Figure 3**). A previously confirmed *Neopestalotiopsis* sp. isolate (AJ07-2023) and a *Neopestalotiopsis rosae* isolate from Georgia were used as positive controls for the molecular assay (Jimenez Madrid et al. 2024).

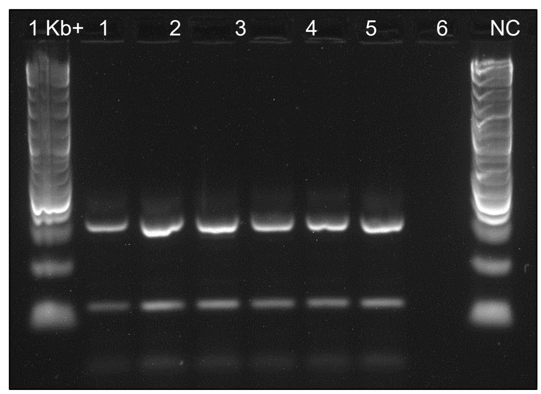

Figure 3. Gel electrophoresis analysis of the β-tubulin (β-tub) gene fragments amplified with primers Bt2a and Bt2b and digested with restriction enzyme BsaWI. Lane 1 to 4 is positive for the virulent Neopestalotiopsis sp. Line 5: positive control (Jimenez Madrid et al. 2024)

Four *Neopestalotiopsis* spp. strains isolated from GA were sent for sequencing to target the β-tubulin (β-tub), and transcription elongation factor EF-1 alpha (tef1) regions. Sequencing analysis was conducted to determine any differences between the aggressive, non-aggressive and the second lineage of aggressive strains observed in FL (Peres, *personal communication*)

Results

Collection and isolation of *Neopestalotiopsis* spp. In total, 40 strawberry samples from AR (n=5), GA (n=21), LA (n=10), MS (n=1) and NC (n=3) were processed. Most of the samples exhibited characteristic symptoms and signs on leaves and/or fruit (**Fig 1**). Crown and root discoloration was also observed on severely infected samples, and observation of plant death in the field was generally reported by the clients who submitted the samples. Reported disease incidence ranged from >1% to 40%. Foliar symptoms differed by cultivar, but general symptoms included: foliar, irregularly distributed, and different-sized spots (dark brown with light brown centers) and dark brown V-shaped necrotic areas starting at the leaf edge. In some cultivar (e.g. Brillance) leaves were reddish with necrotic margins and dry/brown margins. Red discoloration was observed in dissected crowns, as well as necrotic roots. Signs of the pathogen (mycelium, acervuli) were observed in severely infected leaf and fruit samples. Samples were received from January to November 2024, with most of samples coming from GA. Fungal isolation was conducted from either leaves, fruit, crown and/or roots. For most of the samples, at least four fungal isolates were recovered. A total of 123 pure cultures were recovered from all samples (**Table 1**).

Morphological characterization of *Neopestalotiopsis* species. A slight difference in colony morphology was noted between the aggressive *Neopestalotiopsis* sp. and non-aggressive strains. A dense white/cottony growth and black acervuli developing in the center of the colony was very consistent for the aggressive strain (Fig 4A). In contrast, non-aggressive strains showed a less dense pattern, brownish coloration in the center of the plate and black acervuli formation (Fig 4B).

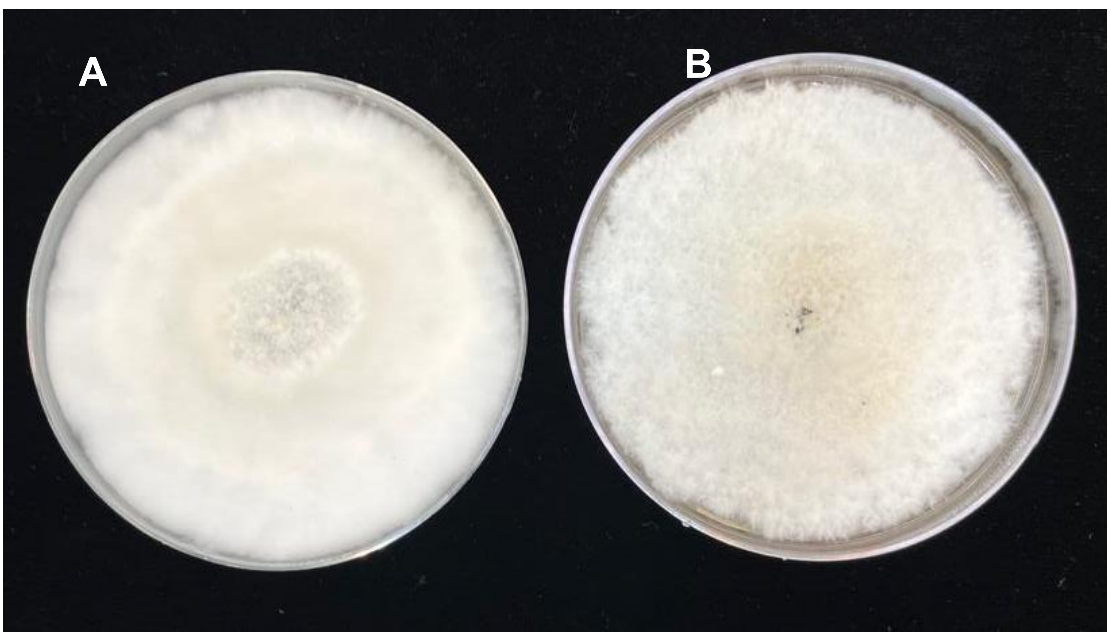

Figure 4. Colony morphology on cPDA of the aggressive strain (A) and non-aggressive strain(B) of Neopestalotiopsis after 10 days of incubation at 25C.

Molecular characterization of *Neopestalotiopsis* species. All 123 pure cultures were tested using the polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) method. Amplified DNA was digested with the restriction enzyme BsaWI. After gel electrophoresis (2%), if two bands were clearly visualized (~130 bp and ~290 bp), that indicated the samples were positive for *Neopestalotiopsis* sp. (aggressive strain; **Fig 5**). From the 123 isolates, 55 were confirmed as the aggressive strain, and 64 as non-aggressive strain(s). In addition, four were confirmed as the second aggressive strain, which was tested at UF (Peres Lab). Interestingly, these four strains didn't amplify with the PCR/RFLP method. However, due to the severity of symptoms and disease reported, samples were further tested for a second aggressive strain that has been found in FL, as it is also contributing to outbreaks. These four strains were also sequenced to target the β-tubulin (β-tub), and transcription elongation factor EF-1 alpha (tef1) genes. No nucleotide differences were observed on any of those genes between the second aggressive strain (second lineage) and non-aggressive strains (*N. rosae*).

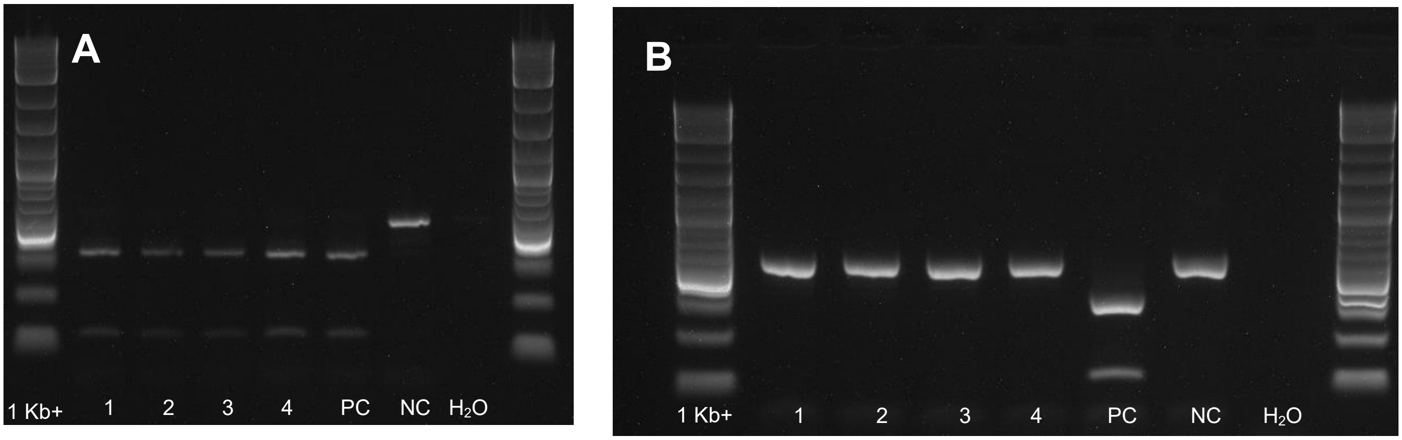

Figure 5. Amplification of the β-tubulin (β-tub) gene fragments and digested with restriction enzyme BsaWI for the aggressive (A) and non-aggressive isolates (B) of Neopestalotiopsis spp. PC: aggressive control. NC: nonaggressive control.

Table 1. Sample characteristics in association with the species found via molecular testing in 2024

Sample Origin (State)	Nursery Reported	Cultivar	Tissue Processed	Species Found	Total # isolates tested	Total # Aggressive strains
AR	--	Sensation	Leaf, fruit and crown	Non-aggressive	3	0
AR	--	Ruby June	Leaf, fruit and crown	Non-aggressive	3	0
AR	--	X08	Leaf, fruit and crown	Non-aggressive	4	0
AR	--	--	Leaf and crown	Neo sp.	3	3
AR	--	--	Leaf, fruit and crown	Non-aggressive	1	0
GA	Balamore and Cottle	Camarosa	Leaf	Non-aggressive	4	0
GA	Fresh-Pik Produce	Sensation	Leaf	Neo sp.	4	3
GA	Cottle	Ruby June, Festival	Leaf and stem	Second aggressive	5	2
GA	--	Ruby June, Camarosa, and Camino Real	Leaf and crown	Non-aggressive	3	0
GA	--	Camarosa	Leaf	Non-aggressive	3	0

GA	--	Camino Real	Leaf and crown	Non-aggressive	2	0
GA	Washington Farms	Ruby June	Leaf	Non-aggressive	2	0
GA	--	Ruby June	Leaf	Non-aggressive	4	0
GA	--	--	Leaf and crown	Non-aggressive	3	0
GA	North Carolina	Mevced	Leaf and fruit	Non-aggressive	5	0
GA	Cedar Point Nursery California	Roy's Royce	Leaf	Neo sp.	1	1
GA	--	Camarosa	Leaf	Non-aggressive	2	0
GA	--	Chandler	Leaf	Neo sp.	4	4
GA	--	Rubby June	Leaf	Neo sp.	4	4
GA	Balamore Farm Nursery	Albion	Fruit and leaf	Neo sp.	4	3
GA	--	Albion	Leaf	Neo sp.	4	3
GA	--	Ruby June	Leaf	Neo sp.	1	1
GA	--	Camarosa	Leaf	Non-aggressive	2	0
GA	--	Camarosa	Leaf	Non-aggressive	2	0
GA	Merck Farms	Ruby June	Leaf	Second aggressive	3	2
GA	--	Merced	Leaf	Non-aggressive	4	0
LA	Lassen Canyon	Festival	Fruit and leaf	Non-aggressive	3	0
LA		Camino Real	Fruit	Neo sp.	3	3
LA	Lareault	Victor	Leaf	Neo sp.	3	3
LA	Crown Nursery	Camino Real	Leaf	Neo sp.	4	4
LA	Crown Nursery	Camino Real	Leaf	Neo sp.	3	3
LA	Lereault	Florida Brilliance	Leaf	Neo sp.	5	4
LA	Crown Nursery	Festival	Leaf	Neo sp.	3	3
LA	--	Camino Real	Crown and leaf	Neo sp.	4	4
LA	--	Fronteras	Crown and leaf	Neo sp.	3	1
LA	--	--	Fruit	Neo sp.	2	1
MS	--	--	Fruit	Non-aggressive	3	0
NC	Ezgro Ontario	Brillance	Leaf	Neo sp.	4	4
NC	--	Albion	Leaf	Neo sp.	2	2

NC	--	Chandler	Leaf	Neo sp.	1	1

-- information not provided by submitter

Discussion:

The results from these survey and characterization efforts provided immediate evidence of the prevalence and distribution of *Neopestalotiopsis* sp. causing infection on strawberry plants in the southeastern United States. The aggressive form of *Neopestalotiopsis* was detected in 55% of the samples tested in 2024 and from every state participating in this effort, except for MS (only one sample tested). The aggressive strain was detected in plants coming from the field, with incidence reported from 1% - 40% by the time of symptom observation, but also from plugs still in greenhouses or nursery facilities. Molecular detection tools have facilitated accurate diagnosis for this particular issue. However, a new observation of a second lineage (second aggressive strain) in FL (and also confirmed in GA) may play a role in the recent outbreaks reported in the Southeast. The current diagnostics capabilities are not able to detect the second lineage of aggressive strain, and therefore, caution is required with a negative diagnostic result. Testing of the second aggressive strain is being facilitated, on a limited basis, by UF. Research is ongoing to create a new molecular test for the detection of the first and second aggressive strain and differentiate it from non-aggressive strains.

The results from this study also provided insights into the potential primary sources of infection (e.g., nursery) contributing to disease dispersal. Samples from at least eight different nurseries who provided plugs/plants proved positive for the aggressive strain. Symptoms of Neopestalotiopsis may not developed until environmental conditions are suitable for disease development. In addition, pathogen dispersal could occur via rain splash and wind. Nevertheless, infected plants/plugs are contributing to the widespread dissemination of the pathogen and subsequent disease. Finally, conducting an accurate and rapid diagnosis is a key component of implementing appropriate management strategies to prevent further spread or establishment within a region. Molecular detection tools are reliable methods used to detect plant pathogens in a timely manner, and they can help to prevent production losses for strawberry growers.

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