Outbreak of Leaf Spot and Fruit Rot in Florida Strawberry Caused by *Neopestalotiopsis* spp.

Juliana S. Baggio,¹ Bruna B. Forcelini,² Nan-Yi Wang,¹ Rafaela G. Ruschel,³ James C. Mertely,¹ and Natalia A. Peres^{1,†}

¹ Gulf Coast Research and Education Center, University of Florida, Wimauma, FL 33598, U.S.A.

² Corteva Agriscience, Indianapolis, IN 46268, U.S.A.

³ São Paulo State University, College of Agricultural Sciences, Botucatu, São Paulo 18610-034, Brazil

Abstract

Pestalotiopsis-like species have been reported affecting strawberry worldwide. Recently, severe and unprecedented outbreaks have been reported in Florida commercial fields where leaf, fruit, petiole, crown, and root symptoms were observed, and yield was severely affected. The taxonomic status of the fungus is confusing because it has gone through multiple reclassifications over the years. Morphological characteristics, phylogenetic analyses, and pathogenicity tests were evaluated for strawberry isolates recovered from diseased plants in Florida. Phylogenetic analyses derived from the combined internal transcribed spacer, *β-tub*, and *tef1* regions demonstrated that although there was low genetic diversity among the strawberry isolates, there was a clear separation of the isolates in two groups. The first group included isolates recovered over a period of several years, which was identified as *Neopestalotiopsis rosae*.

Most isolates recovered during the recent outbreaks were genetically different and may belong to a new species. On potato dextrose agar, both groups produced white, circular, and cottony colonies. From the bottom, colonies were white to pale yellow for *Neopestalotiopsis* sp. and pale luteous to orange for *N. rosae*. Spores for both groups were five-celled with three median versicolored cells. Mycelial growth and spore production were higher for the new *Neopestalotiopsis* sp. isolates. Isolates from both groups were pathogenic to strawberry roots and crowns. However, the new *Neopestalotiopsis* sp. proved more aggressive in fruit and leaf inoculation tests, confirming observations from the recent outbreaks in commercial strawberry fields in Florida.

Keywords: epidemiology, fungi, small fruits

The United States is the second largest producer of strawberry (*Fragaria × ananassa* Duch.) in the world with a total production of 1.3 million tons across 20,000 ha in 2018 (FAOSTAT 2020). California is the largest producer of strawberries in the United States, followed by Florida, which is the largest winter producer in the world. In Florida, strawberry plants are usually acquired from nurseries located in the northern United States, California, and Canada and transplanted in late September to mid-October on plastic-mulched raised beds. Fruit production occurs from November to March (Brown 2003; Whitaker et al. 2019).

Strawberry belongs to the family Rosaceae and is affected by numerous pathogens, especially fungi, causing severe disease and crop losses at different production stages, from nursery to postharvest. Among these pathogens, *Pestalotiopsis longisetula* (*Pestaltia longisetula*) was first reported causing strawberry fruit rot in Florida in 1972, with serious losses in some research plots and commercial fields (Howard and Albregts 1973). Subsequently, the pathogen has been described causing root and crown rots in Florida, as well as in Argentina, Bangladesh, Belgium, Egypt, Mexico, and Spain (Ara et al. 2017; Chamorro et al. 2016; Essa et al. 2018; Mertely et al. 2015; Obregón et al. 2018; Van Hemelrijck et al. 2017).

During the 2012–2013 Florida strawberry season, *Pestalotiopsis* spp. were repeatedly isolated from brown necrotic areas in crowns and roots of weak and stunted transplants (Mertely et al. 2015). Belowground symptoms were characterized by darkening of the roots and orange-brown necrosis in the crowns, which contributed to stunting or poor establishment after transplanting. Aboveground symptoms ranged from stunting to wilting and necrosis of older leaves, to eventual collapse and death of the entire plant. Similar symptoms were reported in Spain and Belgium (Chamorro et al. 2016; Van Hemelrijck et al. 2017). In some cases, *Pestalotiopsis* sp. is isolated along with other root and crown pathogens such as *Colletotrichum* spp., *Phytophthora cactorum*, and *Macrophomina phaseolina*. Hence, the fungus has always been considered a secondary pathogen, a weak and opportunistic pathogen that has never been of major concern in the strawberry industry.

However, in 2017 a *Pestalotiopsis*-like fungus was identified from spotted fruit and leaves in a commercial field near Dover, Florida (Fig. 1). Another outbreak of leaf spot and fruit rot occurred across three commercial fields in 2018. In one case, a grower was forced to destroy 35 ha midseason and stopped harvesting another 15 ha before the season ended. Another grower elected not to harvest an 8-ha block because of fruit spotting and weak plant growth caused by loss of infected leaves. A more severe outbreak happened in the 2019–2020 season, affecting about 18 commercial farms. Symptoms were more severe in organic production fields, and some growers have reported destroying the most affected parts of their fields to avoid pathogen dissemination and contamination of other areas. It is estimated that at least 80 ha of strawberry fields was destroyed. Most of the commercial strawberry cultivars grown in Florida were affected by the disease, and the common link between these outbreaks was the nursery source for the plants. Fruit and leaf symptoms caused by *Pestalotiopsis* sp. have also been reported in other strawberry-producing areas such as Brazil, China, Egypt, India, Iran, Israel, Mexico, and Morocco (Ayoubi and Soleimani 2016; Camili et al. 2002; Embaby 2007; Kenneth et al. 1968; Mahapatra et al. 2018; Mouden et al. 2014; Rebollar-Alviter et al. 2020; Zhao 2016).

[†]Corresponding author: N. A. Peres; nperes@ufl.edu

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Symptoms on strawberry fruit are lesions 2 to 4 mm in diameter, dry, light tan, slightly sunken, and irregular in shape in the early stages (Fig. 1A). Lesions grow and eventually are covered by numerous acervuli oozing shiny black droplets of matricial liquid containing spores (Fig. 1B). Under humid conditions, dense white mycelium may also form on the lesion. Eventually, the entire fruit is affected and mummified (Howard and Albregts 1973; Kenneth et al. 1968; Maas 1998). On leaves, light to dark brown spots of different sizes are irregularly distributed (Fig. 1C), and in more advanced stages, black acervuli can develop in older necrotic tissue (Fig. 1D; Ayoubi and Soleimani 2016; Rodrigues et al. 2014). In recent outbreaks in Florida, mass spotting often produced blight-like necrosis of broad areas of the lamella, eventually killing the leaf. The fungus produces spores on the surface of infected tissues that are dispersed by water, and disease outbreaks are favored during periods of heavy rains and warm temperatures. When conditions are highly conducive to disease development and plants are severely affected, the disease also contributes to poor development of the plants, which become stunted and produce small leaves (Fig. 1E).

Pestalotiopsis is an asexual genus in the family Sporocadaceae (Liu et al. 2019) and is common in tropical and temperate areas. Various species cause leaf blights or spots, shoot dieback, fruit rots, and postharvest diseases on a wide range of hosts (Maharachchikumbura et al. 2012; Xu et al. 1999). They can also be isolated as endophytes or occur as saprobes and are known to produce a broad range of important secondary metabolites (Maharachchikumbura et al. 2011).

In strawberry, the fungus was originally reported as *Pestalotia longisetula* (Howard and Albregts 1973; Kenneth et al. 1968), following the description of Guba (1961), who isolated the fungus from canes of *Rosa* sp. Guba (1961) divided *Pestalotia* into three groups with species having four-, five-, or six-celled spores. However, Steyaert (1949, 1955) separated *Pestalotia* into three genera: *Truncatella*, *Pestalotiopsis*, and *Pestalotia*, accommodating species with four-, five-, and six-celled spores, respectively. Morphological characteristics, especially spore morphology, were considered to be stable characters for the classification of these fungi, which have been organized in genera according to the concolorous and versicolorous color patterns of median cells in the spores (Guba 1961; Jeewon et al. 2003; Steyaert 1949). Recent morphological analysis and molecular taxonomic techniques have resulted in the reclassification of *Pestalotiopsis* species into three major clades: *Neopestalotiopsis*, *Pestalotiopsis*, and *Pseudopestalotiopsis* (Maharachchikumbura et al. 2014). Species presenting versicolorous median cells were grouped in the genus *Neopestalotiopsis*, and several *Pestalotiopsis* species were transferred to this genus (Maharachchikumbura et al. 2014). *Pestalotiopsis* and *Pseudopestalotiopsis* are distinguished from each other by sequence data and the generally darker-colored concolorous median cells of the latter (Maharachchikumbura et al. 2014).

Because of the recent and unprecedented outbreaks caused by *Pestalotiopsis*-like fungi in Florida and the severe impact the disease has had on the strawberry industry, our objective was to study the morphological, molecular, and pathogenic characteristics of the pathogen.

Materials and Methods

Fungal isolates. Between 1997 and 2020, 67 *Pestalotiopsis*-like isolates from Florida strawberry production fields were collected from commercial farms or from samples submitted to the University of Florida's Gulf Coast Research and Education Center (UF GCREC) diagnostic clinic. In addition, 10 *Pestalotiopsis*-like isolates were collected from other hosts (two from *Smilax* sp., two from *Quercus* sp., four from *Rhododendron* sp., one from *Limonium* sp., and one from *Kalmia* sp.). Fungi were isolated from symptomatic lesions by direct transfer of spores from lesions or pieces of tissues disinfested with sodium hypochlorite and ethanol and plated on general isolation medium (12 g/liter of potato dextrose broth and 17 g/liter of agar amended with streptomycin at 0.1 g/liter and ampicillin at 0.25 g/liter). Single-spore isolates were transferred to potato dextrose agar (PDA) and stored on filter paper or in glycerol at -20 and -80°C , respectively.

Molecular characterization. Sixty-seven isolates from strawberry and 10 from other hosts were grown on PDA for 7 days. Mycelium and spores were harvested to extract genomic DNA by using a DNA isolation kit according to the manufacturer's protocol. DNA of a *Pestalotia longisetula* strawberry isolate from Israel (CBS 638.68), considered a representative specimen of the pathogen in strawberry, was acquired from the culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, in the Netherlands, and included in this study.

The partial internal transcribed spacer (ITS), β -tubulin (β -*tub*), and elongation factor (*tefl*) regions were amplified with PCR assays. The ITS, β -*tub*, and *tefl* regions were amplified with primers ITS1 and ITS4 described by White et al. (1990), Bt2a and Bt2b by Glass and Donaldson (1995), and EF1-526F and EF1-1567R by Rehner (2001), respectively. PCR mixtures contained 2 mM of MgCl_2 , 200 μM dNTP, 0.5 μM of each primer, and 1.5 units of Taq polymerase (GoTaq G2 Hot Start Polymerase, Promega Corp.). For the ITS region, reactions were programmed with an initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 45 s, 52°C for 45 s, and 72°C for 1 min, and a final extension of 72°C for 5 min. For the β -*tub* and *tefl* genes, PCRs were performed under the same conditions, except for the annealing temperatures of 57 and 53°C , respectively, and 30 cycles were used. PCR products were visualized under UV light in a 1% agarose gel in $1\times$ Tris-acetate-EDTA buffer stained with GelRed^{TM} (Biotium) and sent for purification and sequencing in both directions at Genewiz Inc. Sequences generated in this study were compared with those obtained from the NCBI GenBank database based on BLASTn searches and literature (Ayoubi and Soleimani 2016; Chamorro et al. 2016; Maharachchikumbura et al. 2014). Nucleotide sequences of the three regions (ITS, β -*tub*, and *tefl*) were assembled in Geneious (version 11.1.4), and multiple sequence alignments were generated with MAFFT version 7.309 (Katoh et al. 2002; Katoh and Standley 2013). Because of the sequence similarity within the same group of isolates, sequences of ITS, β -*tub*, and *tefl* regions of representative isolates 97-49F, 14-691R, 16-337C, and 17-43L were deposited in the GenBank (Table 1).

Given the possibility that each gene might be shaped by different evolutionary events, the dataset was examined for congruence via the partition homogeneity test (equivalent to the incongruence length difference test) implemented in PAUP* version 4.0a167 (Farris et al. 1994; Swofford 2003). The test was performed with 1,000 sample replicates and a heuristic maximum parsimony search with 100 replicates under random addition sequence and subtree pruning and regrafting branch swapping.

Subsequently, partitioned and unpartitioned maximum likelihood and Bayesian inference analyses of the combined regions were conducted in MrBayes version 3.2.7 (Ronquist et al. 2012) and GARLI version 2.01 (Bazin et al. 2014), respectively. The best-fit substitution model was determined for each partition, and the combined, unpartitioned dataset using the automated model selection function implemented in PAUP* and selected based on the corrected Akaike information criterion scores as follows: general time reversible (GTR) + G (γ distribution, four categories) for ITS and *tefl*, K80 (Kimura two-parameter) + G for β -*tub*, and GTR + G + I (invariable sites) for the combined dataset. Maximum likelihood analyses were run with default settings, except for the maximum program memory usage (availablememory = 10,000), the number of generations without topology improvement required for termination (genthreshfortopterm = 20,000), and the maximum number of generations to run and maximum search time (stopgen = 5,000,000, stoptime = 5,000,000). A total of 20 independent searches were performed, 10 starting from stepwise addition trees (streefname = stepwise, searchreps = 10) and 10 from randomly generated trees (streefname = random). The exhaustiveness of the global search was evaluated by comparison of the final maximum likelihood values between the 20 independent searches. Topology support was evaluated via 100 bootstrap replicates (bootstrapreps = 100) under the same parameters for finding the best tree. Bayesian inference analyses were run with the same substitution model for 1 million generations, with sampling

every 1,000 generations. Each analysis consisted of four independent runs, each using four coupled Markov chain Monte Carlo chains. The run convergence was monitored by finding the plateau in the likelihood scores (standard deviation of split frequencies <0.01). The first 25% of each run was discarded as burn-in for the estimation of a majority rule consensus topology and posterior probability for each node. Trees were visualized, edited, and rooted in FigTree version 1.4.2. (Rambaut and Drummond 2012). Trees included reference sequences from nine, three, and two *Neopestalotiopsis* spp. published by Maharachchikumbura et al. (2014), Ayoubi and Soleimani (2016), and Rebollar-Alviter et al. (2020), respectively, and one from Chamorro et al. (2016), A. L. Dann et al. (*unpublished*), and Essa et al. (2018) (Table 1). Two *Pseudopestalotiopsis* spp. and two *Pestalotiopsis* spp. published by Maharachchikumbura et al. (2014) were also used (Table 1).

Morphological characterization. *Colony and spore characterization.* Twenty isolates (Table 2) were revived from the UF GCREC culture collection on PDA (Difco™ and Oxoid), according to Maharachchikumbura et al. (2014), and incubated for 7 days at 24°C with a 12-h photoperiod. Cultures were observed for colony color and shape, spore color, shape, length and width, and number and length of apical and basal appendages. Spore suspensions of each isolate were prepared in sterile deionized water, and spore measurements were determined by arbitrarily selecting 30 spores observed by using an Olympus BX41 microscope (Olympus America, Center Valley, PA) coupled to a camera and a computer, with OMAX ToupView (Toup-Tek, Zhejiang, China) software.

Mycelial growth at different temperatures. A mycelial plug (5-mm diameter) was taken from the edge of a 4- to 7-day-old actively

growing colony of each isolate and placed upside down in the center of three replicates of 10-cm diameter Petri dishes containing PDA. The effect of temperatures on mycelial growth was evaluated after 7 days of incubation at 5, 10, 15, 20, 25, 30, and 35°C, with a 12-h photoperiod, by measuring colony diameter in two perpendicular directions and discounting 5 mm of the mycelial plug (average colony diameter). Two assays, with two experiments each, were conducted in two different periods. The first assay was performed in June 2018 with 10 isolates collected from 1997 to 2017 (Table 2). The second assay was conducted in March 2020 with two isolates from the first assay and 10 isolates collected during the 2018–2019 and 2019–2020 outbreaks (Table 2).

Spore production at different temperatures. Spore production was quantified for the aforementioned isolates 7 days after isolates were transferred to PDA and incubated at 5, 10, 15, 20, 25, 30, and 35°C with a 12-h photoperiod. Two assays were performed, with two experiments each. Sterile deionized water (3 ml) was added to the plates containing the pathogen cultures. Structures were scraped with a flame-sterilized glass rod, and a 1-ml suspension was recovered. Spores were counted with a hemocytometer, and data were expressed as the number of spores per milliliter. In the first assay, performed in June 2018, spores of five isolates were quantified, whereas in the second assay, conducted in March 2020, spores of the 12 isolates characterized in the second mycelial growth assay were counted (Table 2).

Pathogenicity tests. *Fruit inoculation.* Pathogenicity of strawberry isolates was evaluated in two fruit assays, with two experiments each. The first assay was performed in March 2017 with nine isolates collected from 1997 to 2017 (Table 2). The second assay was

Fig. 1. Disease outbreak in Florida strawberry fields caused by the new *Neopestalotiopsis* sp. **A**, Early stage symptoms on ripe fruit; **B**, late stage symptoms on ripe fruit containing black acervuli of the pathogen; **C**, light to dark brown spots of different sizes developing from the margins or from the tips of the leaves; **D**, detail of leaf spots containing signs of the fungus; and **E**, overall symptoms in the field with the presence of stunted plants and symptomatic leaves.

conducted in March 2020 with three isolates from the first assay and eight isolates collected during the 2018–2019 and 2019–2020 outbreaks (Table 2). Strawberry fruits (Sensation ‘Florida127’) were harvested before commercial maturity (white-pink stage) from experimental plots at UF GCREC; sepals were removed, and the fruits were washed in 0.05% sodium hypochlorite for 2 min and rinsed twice in deionized water. Six nonwounded strawberry fruits per isolate were placed in egg trays, inoculated with 40 µl of a spore suspension (10^4 spores/ml) and incubated at 24°C ($\pm 2^\circ\text{C}$) under light in sealed plastic boxes containing water, serving as humidity chambers. Spore suspensions were obtained from 7- to 10-day-old colonies grown on PDA. In control treatments, 40 µl of water was placed on strawberry fruit. Disease incidence (percentage of symptomatic

fruit based on total inoculated) was assessed 7 days after inoculation. Three replicate boxes were used per isolate, and the experiments were conducted twice for each assay.

Leaf inoculation. Pathogenicity of 13 strawberry isolates was evaluated in plant assays in growth room experiments (Table 2). Six cut-top, bare-root transplants of Sensation ‘Florida127’ per isolate were transplanted into 1-liter plastic pots filled with potting soil (Metro-Mix 820, Sun Gro Horticulture, Agawam, MA) in a greenhouse. Plants were overhead irrigated during daylight hours for 7 days, then irrigated twice daily through drip irrigation for 21 days to increase plant establishment. Afterward, plants were transferred to a growth room, and leaves were inoculated with an atomizer by spraying each plant with 5 to 10 ml of spore suspensions of each isolate (10^4 spores/ml),

Table 1. Details of isolates from Florida strawberry and the reference species used for sequence comparisons and phylogenetic analyses

Species	Culture accession	Host/tissue	Location	GenBank accession ^w			Reference
				ITS	<i>tefl</i>	β - <i>tub</i>	
<i>Pseudopezalotiopsis cocos</i>	CBS 272.29	<i>Cocos nucifera</i>	Indonesia	KM199378	KM199553	KM199467	Maharachchikumbura et al. (2014)
<i>Pseudopezalotiopsis indica</i>	CBS 459.78	<i>Hibiscus rosa-sinensis</i>	India	KM199381	KM199560	KM199470	Maharachchikumbura et al. (2014)
<i>Neopezalotiopsis rosae</i>	CBS 101057	<i>Rosa</i> sp.	New Zealand	KM199359	KM199523	KM199429	Maharachchikumbura et al. (2014)
<i>Neopezalotiopsis rosae</i>	CBS 124745	<i>Paeonia</i> sp.	United States	KM199360	KM199524	KM199430	Maharachchikumbura et al. (2014)
<i>Neopezalotiopsis rosae</i>	7927	<i>Fragaria</i> $\times$ <i>ananassa</i>	Australia	KY271740	KY271093	KY271094	N/A ^x
<i>Neopezalotiopsis rosae</i>	PEST3	<i>Fragaria</i> $\times$ <i>ananassa</i>	Egypt	KY688075	KY688074	KY688073	Essa et al. (2018)
<i>Neopezalotiopsis rosae</i>	CRMFRH	<i>Fragaria</i> $\times$ <i>ananassa</i>	Mexico	MN385719	MN268533	MN268530	Rebollar-Alviter et al. (2020)
<i>Neopezalotiopsis rosae</i>	CRMFRH	<i>Fragaria</i> $\times$ <i>ananassa</i>	Mexico	MN385718	MN268532	MN268529	Rebollar-Alviter et al. (2020)
<i>Neopezalotiopsis mesopotamica</i>	CBS 299.74	<i>Eucalyptus</i> sp.	Turkey	KM199361	KM199541	KM199435	Maharachchikumbura et al. (2014)
<i>Neopezalotiopsis mesopotamica</i>	CBS 137766	<i>Fragaria</i> $\times$ <i>ananassa</i>	Iran	KM074047	KM074054	KM074058	Ayoubi and Soleimani (2016)
<i>Neopezalotiopsis iraniensis</i>	CBS 137767	<i>Fragaria</i> $\times$ <i>ananassa</i>	Iran	KM074045	KM074053	KM074056	Ayoubi and Soleimani (2016)
<i>Neopezalotiopsis iraniensis</i>	CBS 137768	<i>Fragaria</i> $\times$ <i>ananassa</i>	Iran	KM074048	KM074051	KM074057	Ayoubi and Soleimani (2016)
<i>Neopezalotiopsis honoluluana</i>	CBS 111535	<i>Telopea</i> sp.	Hawaii (U.S.)	KM199363	KM199546	KM199461	Maharachchikumbura et al. (2014)
<i>Neopezalotiopsis foedans</i>	CGMCC 3.9123	Mangrove plant	China	JX398987	JX399053	JX399022	Maharachchikumbura et al. (2014)
<i>Neopezalotiopsis clavispora</i> ^y	TOR-802-803-804	<i>Fragaria</i> $\times$ <i>ananassa</i>	Spain	KU096879	KU096881	KU096880	Chamorro et al. (2016)
<i>Neopezalotiopsis clavispora</i>	NN043133	<i>Magnolia</i> sp.	China	JX398979	JX399045	JX399014	Maharachchikumbura et al. (2014)
<i>Neopezalotiopsis clavispora</i>	NN043011	<i>Magnolia</i> sp.	China	JX398978	JX399044	JX399013	Maharachchikumbura et al. (2014)
<i>Neopezalotiopsis asiatica</i>	NN0476380	Unidentified tree	China	JX398983	JX399049	JX399018	Maharachchikumbura et al. (2014)
<i>Neopezalotiopsis javaensis</i>	CBS 257.31	<i>Cocos nucifera</i>	Indonesia	KM199357	KM199543	KM199437	Maharachchikumbura et al. (2014)
<i>Pestalotiopsis rhododendri</i>	OP086	<i>Rhododendron sinogrande</i>	China	KC537804	KC537811	KC537818	Maharachchikumbura et al. (2014)
<i>Pestalotiopsis trachicarpicola</i>	Op068	<i>Trachycarpus fortunei</i>	China	JQ845947	JQ845946	JQ845945	Maharachchikumbura et al. (2014)
<i>Neopezalotiopsis rosae</i>	97-49F	<i>Fragaria</i> $\times$ <i>ananassa</i> (fruit)	United States	MK895141	MK903333	MK903337	This study ^z
<i>Neopezalotiopsis rosae</i>	14-691R	<i>Fragaria</i> $\times$ <i>ananassa</i> (root)	United States	MK895142	MK903334	MK903338	This study ^z
<i>Neopezalotiopsis rosae</i>	16-337C	<i>Fragaria</i> $\times$ <i>ananassa</i> (crown)	United States	MK895143	MK903335	MK903339	This study ^z
<i>Neopezalotiopsis</i> sp.	17-43L	<i>Fragaria</i> $\times$ <i>ananassa</i> (leaf)	United States	MK895144	MK903336	MK903340	This study ^z

^w β -*tub*, partial beta-tubulin gene; ITS, internal transcribed spacer; *tefl*, partial translation elongation factor 1-alpha gene.

^x N/A, reference not available.

^y Isolate from Spain was misidentified as *Neopezalotiopsis clavispora*.

^z Isolates deposited in the GenBank were selected based on morphological, molecular, and pathogenicity studies to represent each strawberry tissue source and *Neopezalotiopsis* species. Letters R, C, L, and F represent the strawberry tissues from where they were isolated: root, crown, leaf, and fruit, respectively.

prepared as previously described. Control treatments were sprayed with water. After inoculation, plants were placed inside moist plastic boxes for 3 days and incubated at 24°C (±2°C), with a 12-h photoperiod. Disease incidence (number of symptomatic plants per total number of plants inoculated) and severity (number of lesions per total number of symptomatic leaves per plant) were evaluated 14 days after inoculation. Experiments were conducted twice.

Root and crown inoculation. Pathogenicity of 10 isolates to strawberry transplants was assessed in greenhouse experiments (Table 2). Spore suspensions were produced as previously described. Ten cut-top, bare-root transplants of ‘Strawberry Festival’ per isolate were inoculated by trimming roots to a uniform length and immersing roots in a suspension of 5×10^4 spores/ml or water (control) for 10 min. Plants were potted into 1-liter plastic pots filled with autoclaved soil (Myakka fine sand, Siliceous Hyperthermic Oxyaquic Alorthod) in a greenhouse at 28°C (±2°C) and overhead irrigated during daylight hours for 7 days to increase plant establishment, then irrigated twice daily through drip irrigation for the rest of the experiment. Above-ground symptoms such as stunting, wilting of foliage (drying and death of older leaves while center leaves remained green), or plant death were evaluated weekly. Final disease incidence, expressed as the number of symptomatic plants (stunted, wilted, and dead) per total number of plants inoculated, was evaluated 8 weeks after inoculation. Diseased plants were collected, and isolation from infected tissues onto general isolation medium was performed to confirm *Pestalotiopsis*-like infections. The experiment was conducted twice.

Data analysis. Mycelial growth and spore production trials were conducted and analyzed in a complete randomized factorial design with two fixed factors (species group and temperature). Mycelial growth data were fitted into a normal distribution, and spore production data were fitted into a Poisson distribution, and an analysis of variance was conducted to determine the significance ($P \leq 0.05$) of the fixed factors and their interaction. When models were significant, Fisher’s least significant difference ($\alpha = 0.05$) test was used for mean separation of the factor species group within the temperature with the highest mycelial growth or spore

production. Spore measurement data and disease incidence (proportion) data obtained in the fruit, leaf, and root and crown inoculation trials were fitted into a normal distribution, whereas leaf disease severity was fitted into a Poisson distribution. When models were significant, Fisher’s least significant difference ($\alpha = 0.05$) test was used for mean separation of the factor species group for spore measurement data and the factor isolates for disease incidence data. The experiment was considered a random effect, and analyses were performed with the procedure PROC GLIMMIX in SAS version 9.4.

Results

Molecular characterization. We first conducted phylogenetic analyses by using individual gene regions (data not shown). The divergence between ITS sequences was too low to separate species, and the *β-tub* sequences were the most variable to ensure more reliable delimitation of most species. To assess the phylogenetic congruence of the three gene regions, the partition homogeneity test was performed and revealed that these genes were heterogeneous ($P = 0.001$), suggesting different evolutionary histories of the genes and thus a need to use gene-specific substitution models for phylogenetic analysis.

Both maximum likelihood and Bayesian inference analyses revealed similar tree topologies and indicated that all the Florida strawberry isolates should be classified as *Neopestalotiopsis* sp. (Fig. 2). The phylogenetic tree also revealed a very low genetic diversity among the strawberry isolates from Florida examined in this study based on the ITS, *β-tub*, and *tef1* regions (Fig. 2). The alignments and phylogenetic trees were deposited in TreeBASE (<http://purl.org/phylo/treebase/phyloids/study/TB2:S26315>).

Twenty-nine strawberry isolates were clustered in the same sub-clade as *Neopestalotiopsis rosae*, *Neopestalotiopsis javaensis*, and *Neopestalotiopsis mesopotamica* in addition to the Israeli isolate (CBS638.68) (Fig. 2). Our isolates showed 99.45 to 99.78% identity with the strawberry isolate from Israel originally classified as *Pestalotia longisetula* (data not shown). The strawberry isolate from

Table 2. Origin and identification of the *Neopestalotiopsis* spp. isolates used in the morphological characterization and pathogenicity studies^a

Isolate ^v	Species	Strawberry cultivar	Farm, location ^w	Originating nursery ^x	Studies ^y
97-49F	<i>Neopestalotiopsis rosae</i>	‘Earlibrite’	GCRC, Dover	n3	MG, FA, LA, and RC
11-359R	<i>N. rosae</i>	‘Strawberry Festival’	AF, Plant City	n4	MG, FA, and RC
11-360C	<i>N. rosae</i>	‘Winter Dawn’	EM, Dover	n1	MG, SP, FA, LA, and RC
13-460P	<i>N. rosae</i>	—	JM, Wimauma	n1	MG, SP, FA, and RC
13-481R	<i>N. rosae</i>	‘Strawberry Festival’	MF, Plant City	n2	MG, SP, FA, LA, and RC
14-691R	<i>N. rosae</i>	‘Florida Radiance’	TJ, Wimauma	n2	MG, FA, and RC
16-337C	<i>N. rosae</i>	‘Florida Radiance’	SF, Plant City	n1	MG, FA, and RC
19-381C	<i>N. rosae</i>	‘Florida Brilliance’	AM, Plant City	n1	MG, SP, FA, and LA
17-10F	<i>Neopestalotiopsis</i> sp.	‘Florida127’ ^z	RG, Dover	n5	MG, SP, FA, LA, and RC
17-40F	<i>Neopestalotiopsis</i> sp.	‘Florida127’	RG, Dover	n5	MG, FA, and RC
17-43L	<i>Neopestalotiopsis</i> sp.	‘Florida127’	RG, Dover	n5	MG, SP, FA, LA, and RC
18-749P	<i>Neopestalotiopsis</i> sp.	—	GF, Wimauma	n1	MG, SP, FA, and LA
19-02L	<i>Neopestalotiopsis</i> sp.	‘Florida Radiance’	JM, Wimauma	n1	MG, SP, FA, and LA
19-44F	<i>Neopestalotiopsis</i> sp.	‘Florida127’	RB, Plant City	n5	MG, SP, FA, and LA
19-48F	<i>Neopestalotiopsis</i> sp.	‘Florida Radiance’	SZ, Plant City	n1	MG, SP, FA, and LA
19-54L	<i>Neopestalotiopsis</i> sp.	‘Florida Radiance’	KD, Plant City	n1	MG and SP
19-389L	<i>Neopestalotiopsis</i> sp.	‘Florida Beauty’	CG, Plant City	n1	MG and SP
19-459L	<i>Neopestalotiopsis</i> sp.	‘Florida127’	FS, Plant City	n5	MG, SP, FA, and LA
19-461L	<i>Neopestalotiopsis</i> sp.	‘Florida Brilliance’	GD, Duette	n6	MG, SP, FA, and LA
20-05L	<i>Neopestalotiopsis</i> sp.	‘Florida Brilliance’	FF, Plant City	n5	MG, SP, FA, and LA

^u —: Information not available.

^v First two digits of isolate codes correspond to the years when they were collected. Letters R, C, P, L, and F represent the strawberry tissues from where they were isolated: root, crown, petiole, leaf, and fruit, respectively.

^w Farm: abbreviation codes correspond to strawberry farms in Florida from where isolates were collected. Location: cities in Florida where isolates were collected.

^x Originating nursery: the codes represent the nurseries from where the strawberry transplants were obtained. Nurseries n1, n2, and n4 were located in North Carolina, and n3 and n5 were located in Massachusetts and California, respectively.

^y Morphological characterization and pathogenicity studies performed for strawberry isolates: MG, mycelial growth; SP, spore production; FA, fruit assay; LA, leaf assay; and RC, root/crown inoculation.

^z Cultivar ‘Florida127’ is marketed as Sensation.

The percentage identities to *N. rosae*, *N. mesopotamica*, and *N. jav-aensis* ranged from 99.67 to 100%, 99.45 to 99.67%, and 99.56 to 99.89%, respectively. We verified a maximum of 3-, 5-, and 4-bp differences in the combined sequences between our isolates and the

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previously mentioned species, respectively. Therefore, these strawberry isolates, mostly collected before 2017, were identified as *N. rosae*.

The remaining 37 strawberry isolates from our culture collection were clustered together but were not conspecific with any other species in the NCBI database. Most of the isolates obtained during the recent outbreaks in this cluster showed 100% identity among them and 99.67% identity to the Israeli strawberry isolate (data not shown). All isolates came from production fields in Florida that acquired transplants from the same nursery sources, including one in North Carolina and one in Ontario, Canada, which received plant stock from the North Carolina nursery. Their sequences were also 100% similar to an isolate from *Rhododendron* sp. collected adjacent to the strawberry field at the North Carolina nursery.

Morphological characterization. *Colony and spore characterization.* Fungal colonies were white, circular with undulate margins, and cottony on the upper surface, regardless of the isolate (Table 3 and Fig. 3A to E). However, on the reverse side, the isolates from the recent outbreaks (*Neopestalotiopsis* sp.) were white to pale yellow (Table 3 and Fig. 3I to J), and *N. rosae* were pale luteous to orange (Table 3 and Fig. 3F to H). Spores of all isolates were ellipsoid to fusiform, five-celled, and versicolored, with three median colored cells. Basal and apical cells were hyaline, whereas the second cell from the base had a light brown color, the third cell was honey brown, and the fourth cell was brown. Spore length measurements were different between the two groups ($P < 0.0001$) and ranged from 23.0 to 25.8 μm (average = 24.5 μm) for the *N. rosae* group and from 24.6 to 28.7 μm (average = 26.9 μm) for the new *Neopestalotiopsis* sp. group (Table 3). There was no significant difference between the species groups ($P > 0.05$) for spore width and basal and apical length measurements. Spore width measurements ranged from 6.8 to 8.5 μm (average = 7.4 μm) for the *N. rosae* group and from 6.3 to 8.8 μm (average = 7.4 μm) for the new *Neopestalotiopsis* sp. group (Table 3). Spores had one basal and two to four apical appendages (mostly three), with average lengths ranging from 5.1 to 12.3 μm (average = 8.5 μm) and 20.3 to 36.3 μm (average = 29.2 μm), respectively, for the *N. rosae* group and from 7.3 to 9.5 μm (average

= 8.3 μm) and 19.3 to 33.1 μm (average = 29.0 μm), respectively, for the new *Neopestalotiopsis* sp. group (Table 3).

Mycelial growth and spore production at different temperatures. Overall, differences between species groups, temperatures, and the interaction species group $\times$ temperature were observed for mycelial growth and spore production. Mycelial growth was inhibited at 5 and 35°C for all the isolates tested, and spores were not produced at 10°C. The highest mycelial growth was observed at 25°C in both assays. The optimum temperatures for spore production were between 25 and 30°C. Mycelial growth (Fig. 4A) and spore production (Fig. 4B) were usually higher for the *Neopestalotiopsis* sp. isolates from the recent outbreaks than for *N. rosae* ($P < 0.05$).

Pathogenicity tests. *Fruit inoculation.* In pathogenicity tests, not all isolates were able to produce *Pestalotiopsis*-like symptoms on inoculated fruit (Table 4). In the first and second assays, disease incidence on inoculated fruit varied from 0 to 100% and from 13.9 to 88.9%, respectively (Table 4). Fruit inoculated with the *Neopestalotiopsis* sp. isolates from the recent outbreaks showed disease incidences ranging from 88.9 to 100% for the first assay and 41.7 to 88.9% for the second assay, which were usually higher than in fruit inoculated with *N. rosae* isolates (Table 4). Fruit inoculated with *N. rosae* isolates 97-49F, 11-359R, and 13-460P did not develop symptoms, whereas disease incidence were $\leq 25\%$ for the other isolates, regardless of the assay. Spores from symptomatic fruit were morphologically identical to the original colonies used for inoculum production. Noninoculated fruit did not show any disease symptoms.

Leaf inoculation. All tested isolates were able to produce *Pestalotiopsis*-like lesions on strawberry leaves (Table 4). However, *Neopestalotiopsis* sp. isolates from the recent outbreaks were more aggressive than *N. rosae* isolates (Table 4). Plant disease incidence varied from 25 to 41.7% in plants inoculated with *N. rosae* and from 58.3 to 100% in strawberries inoculated with *Neopestalotiopsis* sp. isolates from the recent outbreaks (Table 4). The number of lesions per symptomatic leaf per plant was greater in plants inoculated with the *Neopestalotiopsis* sp. isolates from the recent outbreaks ($P = 0.011$), ranging from 0.63 to 1.44 lesions/diseased leaves/plant,

Table 3. Morphological characteristics of the *Neopestalotiopsis* spp. isolates from Florida strawberry

Isolate ^a	Species	Conidium size (μm), mean (SD)		Basal appendage length (μm), mean (SD)	Apical appendage length (μm), mean (SD)	Colony color	
		Length	Width			Upper	Bottom
97-49F	<i>Neopestalotiopsis rosae</i>	23.0 (0.44)	7.0 (0.10)	6.7 (0.37)	29.8 (0.83)	Whitish	Pale luteous to orange
11-359R	<i>N. rosae</i>	24.1 (0.46)	8.5 (0.22)	9.0 (0.52)	29.7 (0.39)	Whitish	Pale luteous to orange
11-360C	<i>N. rosae</i>	23.1 (0.51)	6.8 (0.14)	7.7 (0.29)	20.3 (0.48)	Whitish	Pale luteous to orange
13-460P	<i>N. rosae</i>	24.5 (0.42)	8.0 (0.23)	8.7 (0.44)	30.0 (0.72)	Whitish	Pale luteous to orange
13-481R	<i>N. rosae</i>	25.5 (0.29)	6.8 (0.19)	5.1 (0.21)	25.1 (0.48)	Whitish	Pale luteous to orange
14-691R	<i>N. rosae</i>	25.0 (0.37)	7.0 (0.11)	9.7 (0.63)	32.1 (0.54)	Whitish	Pale luteous to orange
16-337C	<i>N. rosae</i>	24.9 (0.43)	8.2 (0.2)	8.4 (0.65)	30.6 (0.72)	Whitish	Pale luteous to orange
19-381C	<i>N. rosae</i>	25.8 (0.42)	7.0 (0.09)	12.3 (0.75)	36.3 (0.70)	Whitish	Pale luteous to orange
Average		24.5 (0.37)	7.4 (0.25)	8.5 (0.75)	29.2 (1.68)		
17-10F	<i>Neopestalotiopsis</i> sp.	24.6 (0.36)	7.1 (0.09)	7.7 (0.60)	26.1 (0.59)	Whitish	Whitish to pale yellow
17-40F	<i>Neopestalotiopsis</i> sp.	27.4 (0.31)	7.4 (0.10)	7.21 (0.31)	30.6 (0.63)	Whitish	Whitish to pale yellow
17-43L	<i>Neopestalotiopsis</i> sp.	26.0 (0.43)	7.2 (0.15)	8.4 (0.44)	30.8 (0.74)	Whitish	Whitish to pale yellow
18-749P	<i>Neopestalotiopsis</i> sp.	28.2 (0.43)	6.3 (0.13)	7.7 (0.33)	26.4 (0.47)	Whitish	Whitish to pale yellow
19-02L	<i>Neopestalotiopsis</i> sp.	27.3 (0.5)	7.1 (0.16)	7.6 (0.40)	30.7 (0.55)	Whitish	Whitish to pale yellow
19-44F	<i>Neopestalotiopsis</i> sp.	27.2 (0.62)	7.7 (0.16)	8.9 (0.54)	27.0 (0.54)	Whitish	Whitish to pale yellow
19-48F	<i>Neopestalotiopsis</i> sp.	26.2 (0.41)	6.8 (0.12)	7.3 (0.31)	28.5 (0.76)	Whitish	Whitish to pale yellow
19-54L	<i>Neopestalotiopsis</i> sp.	27.2 (0.37)	7.6 (0.18)	9.5 (0.41)	32.4 (0.51)	Whitish	Whitish to pale yellow
19-389L	<i>Neopestalotiopsis</i> sp.	28.7 (0.43)	7.9 (0.13)	8.9 (0.43)	33.1 (0.60)	Whitish	Whitish to pale yellow
19-459L	<i>Neopestalotiopsis</i> sp.	26.5 (0.45)	8.8 (0.25)	9.8 (0.78)	30.6 (0.77)	Whitish	Whitish to pale yellow
19-461L	<i>Neopestalotiopsis</i> sp.	28.7 (0.56)	8.0 (0.19)	9.0 (0.44)	32.0 (0.45)	Whitish	Whitish to pale yellow
20-05L	<i>Neopestalotiopsis</i> sp.	24.6 (0.63)	6.8 (0.14)	8.1 (0.42)	19.3 (0.52)	Whitish	Whitish to pale yellow
Average		26.9 (0.40)	7.4 (0.19)	8.34 (0.25)	29.0 (1.11)		
Pr > F ²		<0.0001	0.7649	0.6868	0.3629		

^a First two digits of isolate codes correspond to the years when they were collected. Letters R, C, P, L, and F represent the strawberry tissues from where they were isolated: root, crown, petiole, leaf, and fruit, respectively.

² Probability of a greater *F* value associated with type III test of fixed effect for each species group.

compared with 0.25 to 0.92 lesions/diseased leaves/plant for *N. rosae* (Table 4). Noninoculated plants did not show any typical disease symptoms.

Root and crown inoculation. All isolates were able to infect and produce root and crown rot symptoms. Early aboveground symptoms such as wilting were observed as soon as 12 days after root inoculation, and plant stunting and death were observed starting 3 weeks after inoculation. Final disease incidence recorded 8 weeks after inoculation ranged from 35 to 90% and was not significantly different between the isolates (data not shown). Morphological characteristics of isolates obtained by culturing of necrotic crown and root tissue and spores produced were identical to those in the original inoculum. *Pestalotiopsis*-like fungi were not recovered from any of the noninoculated plants.

Discussion

In this study, we identified *Neopestalotiopsis* as the genus affecting strawberry production fields in Florida. Morphological characteristics and pathogenicity assays together with phylogenetic analyses and an appraisal of the literature demonstrated that although there was low genetic diversity among the strawberry isolates, there was

a clear separation of the isolates in two groups. The first group included isolates recovered over a period of several years, which were identified as *N. rosae*. Although it could be found on leaves and fruit, *N. rosae* was most commonly found causing symptoms on roots and crowns, contributing to plant establishment difficulties. Most isolates recovered during the recent outbreaks were genetically distinct and may belong to a new *Neopestalotiopsis* sp., and they were isolated mainly from leaves and fruit.

The taxonomy of these fungi is poorly understood and has gone through multiple reclassifications based on morphological characteristics in the past and phylogenetic analyses more recently. The first reports of the pathogen (*Pestalotia longisetula*) in strawberry (Howard and Albrechts 1973; Kenneth et al. 1968) followed the description of Guba (1961), who characterized the fungus morphology for isolates from canes of *Rosa* sp., which, like strawberry plants, belongs to the Rosaceae. Based on morphological characteristics, *P. longisetula* was subsequently reclassified as *Pestalotiopsis longisetula* (Steyaert 1949, 1955). More recently, strawberry isolates were identified as belonging to the genus *Neopestalotiopsis*, in reports describing a pathogen of strawberry roots and crowns (Chamorro et al. 2016; Essa et al. 2018; Mahapatra et al. 2018; Obregón et al. 2018).

Fig. 3. *Neopestalotiopsis* spp. **A to E**, Upper side and **F to J**, bottom side morphology of isolates from strawberry roots (**A, F**), crowns (**B, G**), petioles (**C, H**), leaves (**D, I**), and fruit (**E, J**) from *Neopestalotiopsis rosae* (**A to C, F to H**) and *Neopestalotiopsis* sp. isolates from the recent outbreaks (**D, E, I, J**) growing on potato dextrose agar for 7 days.

Fig. 4. A, Mycelial growth (cm) at 25°C and **B**, spore production ($\times 10^4/\text{ml}$) at 30 and 25°C for assays 1 and 2, respectively, of *Neopestalotiopsis rosae* and *Neopestalotiopsis* sp. strawberry isolates. Assays 1 and 2, with two experiments each, were performed in June 2018 and March 2020, respectively. Data are the means of two experiments for each assay. Ranges within bars represent mean standard error. Single and double asterisks indicate significant differences for assays 1 and 2, respectively.

Most of the recent taxonomy of *Pestalotiopsis*-like organisms is based on the reclassification suggested by Maharachchikumbura et al. (2014). However, some species were classified and named after only a couple of representative isolates. Moreover, overall branch-length support values in the *Neopestalotiopsis* genus were low, as also observed in our phylogenetic studies. Therefore, the current data might be insufficient to determine species boundaries within the *Neopestalotiopsis* genus, and additional gene loci might be needed for better species separations.

Most Florida strawberry isolates collected before 2017 and a strawberry isolate from Israel collected in the 1960s (CBS 638.38) were clustered in the same subclades as *N. rosae*, *N. javaensis*, and *N. mesopotamica*. However, branch-length support values for species separation were low, as was observed by Maharachchikumbura et al. (2014). For instance, the percentage identities of *N. rosae* to *N. mesopotamica* and *N. javaensis* were 99.67 and 99.89%, respectively. Moreover, Maharachchikumbura et al. (2014) reported a 2-bp difference in the ITS sequence between *N. javaensis* and *N. rosae* in addition to longer and thinner spores and shorter apical appendages in the former. Additionally, *N. rosae* is differentiated from *N. mesopotamica* by its wider spores and shorter apical appendages (Maharachchikumbura et al. 2014). Following morphological characteristics of the three species, our isolates fitted the description of *N. rosae*, with similar spore color, shape, and dimensions (Maharachchikumbura et al. 2014), as well as high genetic identity (99.67 to 100%). Moreover, four *N. rosae* isolates used in the phylogenetic analysis were isolated from strawberry tissues, mainly roots and crowns, in Australia, Mexico, and Egypt (Essa et al. 2018; Rebollar-Alviter et al. 2020) (Table 1). Furthermore, one *N. rosae* isolate originated from a stem lesion in *Rosa* sp. (Maharachchikumbura et al. 2014). Similarly, *P. longisetula* was isolated from *Rosa* sp. canes by Guba (1961), suggesting that *N. rosae* might be a synonym of *P. longisetula*.

Most Florida strawberry isolates collected from petioles, leaves, and fruit during the recent outbreaks were not clustered with any other species reported by Maharachchikumbura et al. (2014) or from the NCBI

database. Although spore morphology was similar to that of the other Florida strawberry isolates identified as *N. rosae*, spores were longer, and colony color was different (Table 3 and Fig. 3I and J). Moreover, mycelial growth and spore production were higher in *in vitro* assays (Fig. 4), and symptoms on fruit and leaves of strawberry were more severe (Table 4), suggesting they might be more aggressive and confirming observations from the recent outbreaks in commercial strawberry fields in Florida.

Interestingly, a similar outbreak was also observed in Mexico during the 2018–2019 strawberry season, where similar symptoms on leaves, petioles, and crowns were reported, and diseased plants showed reduced growth and died approximately 1 month after transplanting (Rebollar-Alviter et al. 2020). However, these isolates were identified as *N. rosae* and were genetically different than the isolates from the recent outbreaks in Florida. This was also true for one of our leaf isolates collected in 2017 (Fig. 2); however, the isolate was from transplants originating from a different nursery source than all other isolates associated with the recent outbreak. In the case of the report from Mexico, there is no reference about the nursery source for the transplants.

Although a recent report from Spain classified strawberry isolates as *N. clavispora* (Chamorro et al. 2016), and reports from India and Argentina followed this description (Mahapatra et al. 2018; Obregón et al. 2018), none of these studies performed a complete phylogenetic analysis by using the combination of multiple genes to fully characterize strawberry isolates, as done in our study and the ones by Ayoubi and Soleimanil (2016), Essa et al. (2018) and Rebollar-Alviter et al. (2020). Previous authors based their classification on similarities of individual genes, which might have led to misclassifications. For example, the strawberry isolate reported as *N. clavispora* by Chamorro et al. (2016) showed 100% nucleotide identity with *N. rosae* for the combined analyzed regions in our study. In addition, the isolate was similar to our isolates from before the disease outbreaks and did not phylogenetically group with other *N. clavispora*.

Before 2002, the pathogen was known for infecting strawberry fruit exclusively. Recently, reports worldwide have shown it also

Table 4. Disease incidence on strawberry fruit and leaves of Sensation ‘Florida127’ plants inoculated with *Neopestalotiopsis* spp. isolates^u

Isolate ^v	Species	Fruit ^w		Leaf ^x	
		Incidence (%) assay 1 ^y	Incidence (%) assay 2 ^y	Incidence (%) ^y	Severity (lesions/symptomatic leaves) ^y
97-49F	<i>Neopestalotiopsis rosae</i>	0.0 c	—	25.0 e	0.25 e
11-359R	<i>N. rosae</i>	0.0 c	—	—	—
11-360C	<i>N. rosae</i>	5.5 c	25.0 de	33.3 de	0.33 e
13-460P	<i>N. rosae</i>	0.0 c	—	—	—
13-481R	<i>N. rosae</i>	25.0 b	13.9 e	25.0 e	0.42 de
16-337C	<i>N. rosae</i>	25.0 b	—	—	—
19-381C	<i>N. rosae</i>	—	13.9 e	41.7 cde	0.92 abcde
17-10F	<i>Neopestalotiopsis</i> sp.	97.2 a	73.3 ab	75.0 abc	1.08 abcd
17-40F	<i>Neopestalotiopsis</i> sp.	100.0 a	—	—	—
17-43L	<i>Neopestalotiopsis</i> sp.	88.9 a	—	100.0 a	1.44 abc
18-749P	<i>Neopestalotiopsis</i> sp.	—	60.0 bc	83.3 ab	1.44 abc
19-02L	<i>Neopestalotiopsis</i> sp.	—	58.3 bc	100.0 a	1.76 a
19-44F	<i>Neopestalotiopsis</i> sp.	—	47.8 cd	58.3 bcde	0.78 bcde
19-48F	<i>Neopestalotiopsis</i> sp.	—	41.7 cd	75.0 abc	1.44 abc
19-459L	<i>Neopestalotiopsis</i> sp.	—	88.9 a	58.3 bcde	0.63 cde
19-461L	<i>Neopestalotiopsis</i> sp.	—	44.4 cd	75.0 abc	1.52 ab
20-05L	<i>Neopestalotiopsis</i> sp.	—	52.8 bc	66.7 abcd	0.78 bcde
Pr > F ^z		<0.0001	<0.0001	0.008	0.0041

^u —: Data not available.

^v The first two digits of isolate codes correspond to the years they were collected. R, C, P, L, and F represent the strawberry tissues from which they were isolated: root, crown, petiole, leaf, and fruit, respectively.

^w Nonwounded fruit harvested before commercial maturity (white-pink stage) were inoculated with a 40- μ l aliquot of conidial suspension (10^4 spores/ml) and incubated at 24°C ($\pm 2^\circ$ C) in sealed humid chambers in two fruit assays, with two experiments each. Assay 1 was performed in March 2017, and assay 2 was conducted in March 2020. Incidence was assessed 7 days after inoculation.

^x Strawberry plant leaves were inoculated with an atomizer by spraying 25 to 50 ml of spore suspensions of each isolate (10^4 spores/ml) and incubated at 24°C ($\pm 2^\circ$ C), with a 12-h photoperiod. Disease incidence (number of symptomatic plants per total number of plants inoculated) and severity (number of lesions per total number of symptomatic leaves per plant) were evaluated 14 days after inoculation.

^y Data are the means of two experiments for each assay. Values in columns followed by the same letters are not significantly different according to Fisher’s protected least significant difference test ($\alpha = 0.05$).

^z Probability of a greater F value associated with type III test of fixed effect for each isolate.

infects other parts of the strawberry plants (Ayoubi and Soleimani 2016; Camili et al. 2002; Chamorro et al. 2016; Van Hemelrijck et al. 2017). During the 2012–2013 Florida strawberry season, the pathogen was repeatedly isolated from roots and crowns of weak, stunted, and dying transplants (Supplementary Fig. S1; Mertely et al. 2015). However, it was not uncommon to observe concomitant infections by other crown and root rot strawberry pathogens. Thus, during the stressful establishment period after transplanting, weakened plants may be more susceptible to root and crown infections, and *N. rosae* might act as an opportunistic pathogen.

Conversely, the most recent *Neopestalotiopsis* sp. isolates, commonly isolated from symptomatic leaves and fruit, were responsible for the serious outbreaks in Florida (Fig. 1) and caused significant crop losses in commercial fields, suggesting higher aggressiveness. Moreover, the recent outbreaks occurred in commercial fields whose transplants originated from the same nursery sources, including one in North Carolina and one in Ontario, Canada, which received plant stock from the North Carolina nursery. Furthermore, all isolates originating from this nursery were 100% identical. These observations suggest that the pathogen might be transported with strawberry transplants from specific nurseries, as has been reported for other strawberry pathogens (Forcelini and Peres 2018; Howard et al. 1992; Oliveira et al. 2017; Pettitt and Pegg 1994).

Interestingly, the strawberry isolates from the recent outbreak were very similar to an isolate (19-62) obtained from native *Rhododendron* plants (Fig. 2) growing next to the strawberry transplant production field from where the plants originated (Supplementary Fig. S2). The sexual state of *Pestalotiopsis*, known as *Pestalotia*, is commonly found on leaves of *Rhododendron maximum* growing in North Carolina and was first reported in Macon County (Barr 1975), the same county where the strawberry nursery is located. Considering that some of the *N. rosae* isolates originated from transplants from this same nursery and that other isolates collected from native *Rhododendron* plants clustered with different *Neopestalotiopsis* and *Pestalotiopsis* species (Fig. 2), we could hypothesize that the pathogen was always present in the area and that genetic recombination might be happening. This question and the pathogen dissemination between hosts need to be explored further. Dissemination from the native *Rhododendron* to strawberry transplants may have been facilitated by Hurricane Florence in the fall of 2018, which was responsible for heavy rains and strong winds throughout North Carolina. However, host specificity and epidemiological studies are needed to confirm these observations and elucidate the disease cycle.

A trifecta of events may have contributed to the recent outbreaks. Two were the introduction of the pathogen with nursery transplants and reported failure of most fungicides registered for strawberry (Whitaker et al. 2019). The third was the occurrence of favorable environmental conditions at the end of December, characterized by unusually prolonged rains followed by days of warm weather (20 to 24°C). These conditions conform to the pathogen temperature requirements. In fact, our *in vitro* studies have demonstrated that temperatures between 25 and 30°C are optimal for pathogen growth and sporulation. Moreover, preliminary studies have shown that inoculation of different strawberry cultivars with isolates originated from leaves were able to show different levels of susceptibility between cultivars, but none were considered resistant (Mertely et al. 2019).

In this study, we add to the taxonomic knowledge of *Neopestalotiopsis* spp. affecting strawberry. Isolates identified as *N. rosae* are associated mostly with root and crown rots in weakened plants, being responsible for plant establishment difficulties. The new *Neopestalotiopsis* sp. isolates, commonly associated with leaf and fruit symptoms, seem to be more aggressive, confirming observations from the recent severe outbreaks and crop losses in commercial strawberry fields in Florida. Additional research is needed to elucidate the origin of the new pathogen and the disease cycle and to develop integrated strategies for disease management in strawberry nursery and production fields.

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