Influence of Temperature, Light Intensity, and Isolate on the Development of *Neofusicoccum parvum*-Induced Dieback of *Eugenia*, *Syzygium paniculatum*

Randy C. Ploetz, Jose M. Pérez-Martínez, Aaron J. Palmateer, and Tara L. Tarnowski, Department of Plant Pathology, University of Florida, Tropical Research & Education Center, 18905 S.W. 280th Street, Homestead, FL 33031-3314 USA

ABSTRACT

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Syzygium paniculatum (Myrtaceae) is an important plant in the South Florida ornamental industry. Known as eugenia in the trade, the plant was relatively free of diseases before Hurricane Wilma (2005). Since then, a serious dieback disease has become prevalent in local nurseries, especially during late summer. Symptoms included wilting and death of terminal and lateral branches, and vascular discoloration in dead and dying branches and the main stem. Several fungi were isolated from diseased plants, but *Neofusicoccum parvum* was usually the only fungus isolated from symptomatic tissue. Most isolates were sterile, but all that were tested produced significant ($P < 0.05$) dieback on, and reduced growth of, the cultivar Monterrey Bay. *Glomerella* spp. and a *Pestalotiopsis* sp. that were recovered from asymptomatic portions of diseased plants and *Mycroplectodiscus terrestris* recovered from healthy liners of Monterrey Bay did not cause dieback symptoms in pathogenicity studies or affect host growth. In incubator studies, *N. parvum* caused significant external symptoms, vascular discoloration, and mortality at 25 and 30°C; in general, only vascular symptoms developed at 20°C and no symptoms developed at 15°C. Thus, temperature may be associated with the seasonal development of this disease. Significant differences in disease development were not observed under a wide range of light intensities (2,000 to 300 $\mu\text{mol s}^{-1} \text{m}^{-2}$). *S. paniculatum* is a new host record for *N. parvum*.

Syzygium paniculatum (Myrtaceae; syns. *Eugenia myrtifolia*; *E. paniculata* var. *australis*) is a medium-sized rainforest tree, up to 15 m in height in its native eastern Australia (4). It has become important in ornamental trades worldwide, where it is known as eugenia (6). It is used as a potted plant, in espaliers and topiaries, and in hedges in USDA hardiness zones 10 and 11.

Eugenia is an economically important containerized ornamental in Florida. Although monetary values for the crop are not published, 50 wholesale nurseries offered the plant in a recent catalog (8), and Miami-Dade County nurseries produce approximately 600,000 plants per year (A. J. Palmateer, personal communication).

Eugenia's popularity stems from its dense, attractive foliage, the ease with which the canopy can be sculpted, and its relatively carefree management (6); in general, it requires little besides adequate water and fertilizer.

Shortly after Hurricane Wilma (November 2005), a serious dieback disease began to affect nursery production of eugenia in the Homestead area (southern Miami-Dade County) (Fig. 1A). Symptoms included wilting and death of terminal and lateral branches that developed in a unilateral fashion or involved the entire canopy (Fig. 1B). Disease developed in both sculpted (pruned) and nonsculpted plants. Leaves remained attached for a week or longer after they died, although branches and plants that were killed by the disease eventually defoliated. Brick red, vascular discoloration was extensive in dead and dying branches and at their insertion points along the main stem (Fig. 1C). In general, the disease developed during late summer and became infrequent as temperatures dropped in the late fall and winter.

In previous work, we demonstrated that *Neofusicoccum parvum* caused this disease (=“dieback” hereafter) (9,10). We examined different factors in the present work to better understand how and when dieback develops. To reliably reproduce dieback symptoms, different inoculation and incubation conditions, as well as pathogenic

variation in a collection of isolates from different commercial operations, were examined. Other fungi that were recovered from diseased plants and healthy liners were tested for pathogenicity to determine whether *N. parvum* was the sole cause of these symptoms. The influence of temperature was assessed to understand seasonal variations that are observed in dieback development, and light intensity was tested to determine whether dieback development would be affected under shade (commercial production occurs under full sun).

MATERIALS AND METHODS

Isolation and identification of fungi.

As of August 2008, the Florida Extension Plant Diagnostic Clinic in Homestead, FL (=“the clinic” hereafter) had received a total of 29 eugenia samples with dieback symptoms from a total of 20 wholesale and retail nurseries in Miami-Dade County. Distal portions of discolored vascular tissue (5 mm²) were cut from specimens, surface-disinfested with 70% ethanol (15 s) followed by 10% bleach (2 min), and assayed for fungal colonists on ½-strength Difco potato dextrose agar (PDA; Becton, Dickson and Company, Sparks, MD). Hyphal tips of the most common fungi were transferred to PDA; isolates were then stored for future use both on filter paper and as mycelial plugs in 15% glycerol at –80°C.

A sterile gray fungus, identified previously as *N. parvum* (9,10), was isolated from every disease sample, virtually to the exclusion of other fungi. Seven isolates were cultured in the following ways to induce sporulation. Actively growing PDA cultures were used to seed plates of 1.5% Difco water agar (WA; Becton, Dickson and Company) and ½-strength PDA with and without sterile toothpicks, pine needles (*Pinus elliotii* var. *densa*), or eugenia stem pieces on the surface. Plates were incubated for 3 weeks at room temperature under cool-white fluorescent lights, or at 25°C under black light or in the dark.

Three consignments of liners of eugenia ‘Monterrey Bay’, which are used to propagate the plant in local nurseries, were obtained from a commercial provider (Agri-Starts, Inc., Apopka, FL). The liners did not exhibit external or internal symptoms of dieback, and were used to produce plants that were used in most of the disease studies described below. Two consign-

Corresponding author: R. C. Ploetz
E-mail: kelly12@ufl.edu

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*The e-Xtra logo stands for “electronic extra” and indicates that Figure 1 appears in color in the online edition.

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ments were assayed for endophytic fungi. Lateral branches and leaves were removed from stems, ca. 20 cm in length, and treated in two ways: (i) bark was removed and stems were submerged in 70% ethanol (2 min) followed by nondiluted household bleach (2 min); or (ii) bark was not removed and stems were washed in tap water for 30 s, dipped in 95% ethanol (30 s), 10% bleach (2 min), and 70% ethanol (2 min). Treated stems were placed in empty 15-cm-diameter petri dishes, and molten (45°C) ½-strength PDA amended with 100 µg of streptomycin sulfate/ml was poured over the stems. Plates were observed for 2 weeks for developing colonies of fungi. Ten and six plants from the respective consignments were assayed with each disinfection treatment (32 total plants).

Few fungi were isolated from asymptomatic tissues of diseased specimens and healthy liners. Representatives of the most common fungi were identified microscopically, and some of these were examined further with ITS DNA sequences. Isolates were grown for 3 to 5 days in potato dextrose broth at room temperature on a shaker. Mycelia were harvested and DNA was extracted using the simple DNA prep protocol from the University of Wisconsin Biotechnology Center (<http://www.biotech.wisc.edu/NewServicesAndResearch/Arabidopsis/>). DNA was amplified using standard PCR conditions with the ITS1/ITS4

primer pair (15) and sequenced (Interdisciplinary Center for Biotechnological Research, University of Florida, Gainesville). Sequences were edited visually and compared via BLAST searches to sequences deposited in GenBank.

Pathogenicity. In two initial experiments, two sterile isolates of *N. parvum* from diseased tissue, 280 and 284, and a representative isolate of *Pestalotiopsis* sp. from asymptomatic tissue, were used to inoculate healthy plants from local nurseries. Mycelium from PDA cultures of the fungi was inserted into flaps cut on the stem and sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI). Plants were grown outdoors under full sun in a randomized complete block design (RCBD), with four replications per treatment.

Three subsequent experiments were conducted in an air-conditioned greenhouse (ca. 28°C). A total of five sterile isolates of *N. parvum* and two that produced conidia were tested for pathogenicity on 2- to 6-month-old plants of Monterey Bay. In each experiment, treatments were replicated four or five times in a completely randomized design (CRD). Mycelium, as above, was compared to toothpicks as inoculum in two experiments. Sterile toothpicks were colonized by the respective fungi on the surface of PDA cultures and inserted into slits that

were produced in the lower stem with a sterile no. 11 scalpel (Feather Surgical Blades, Osaka, Japan). Control treatments included mock inoculations with noncolonized PDA or sterile toothpicks, as well as wounding with scalpels. Only colonized or noncolonized toothpicks were used in the third experiment. In the first two experiments, four endophytes were also tested with toothpick inoculum; two that produced asci on PDA were from asymptomatic tissue on diseased plants, and two isolates of *Mycocleptodiscus* sp. were from liners.

Disease symptoms were assessed after 4 to 8 weeks, externally on a 1 to 5 subjective scale (1 = healthy, 5 = dead) and internally as the linear extent of vascular discoloration above and below the point of inoculation. Inoculated fungi were reisolated from plants as described under initial isolations, and fresh weights of the above-ground portions of plants were taken immediately after plants were harvested for disease assays. For each experiment, analyses of variance (ANOVAs) were conducted for external symptoms, vascular discoloration, and fresh weights. These and subsequent statistical evaluations were performed with SAS v. 9.0 (SAS Institute Inc., Cary, NC).

Temperature. To determine the effect of temperature on disease development, two experiments were conducted in tem-

Fig. 1. A, Commercial *Syzygium paniculatum* nursery in Homestead, FL throughout which dieback-affected plants are dispersed (X's identify symptomatic plants). B, External symptoms of dieback on a plant from a commercial nursery in Homestead. Internally, symptoms extend above and below the bracketed, externally affected portion of the plant. C, Internal vascular discoloration caused by dieback. Symptoms may have initiated at branch insertion points, indicated with black V's.

perature-controlled incubators (Percival Scientific) with 2- and 6-month-old plants of Monterrey Bay (experiments 1 and 2, respectively). Toothpick inoculum of a virulent isolate of *N. parvum* (276) was tested at 15, 20, 25, and 30°C, and treatments (mock and 276 inoculated) were replicated four times in a CRD. Cool-white fluorescent light on a 12:12 diurnal cycle was used, and light intensities were 150 $\mu\text{mol s}^{-1} \text{m}^{-2}$. Plants were evaluated as above for dieback development after 5 weeks. Regression analyses were used for each experiment to investigate the relationship between disease and temperature. To fit a linear regression model, data were $\log_n$ transformed.

In vitro growth was assessed for seven isolates of *N. parvum* (276, 280, 1-4, 130, 132, 284, and 395). Plugs, 5 mm in diameter, were taken from the margins of actively growing PDA cultures, placed mycelium side down in the centers of 10-cm-diameter plates of PDA, and incubated in the dark at the above temperatures. Three replicate plates were used per isolate at each temperature, and the experiment was conducted twice. Radial growth measurements were initiated 24 h after colony growth began at the different temperatures; colony extension was recorded at right angles in two directions over 24 h (25 and 30°C) or 48 h (15 and 20°C); growth rates for isolate $\times$ temperature combinations were calculated in centimeters per day. The experiment was repeated once, and the experimental design was a RCBD with experiments as blocks. ANOVAs for slopes of linear regressions were used to evaluate the relationships between growth rates and temperature among isolates.

Light intensity. The effect of light intensity on disease development was examined in two experiments. Treatments were full sun (ca. 2,000 $\mu\text{mol s}^{-1} \text{m}^{-2}$ at solar noon) and partial and full shade (respectively, ca. 750 and 300 $\mu\text{mol s}^{-1} \text{m}^{-2}$). At

solar noon, temperatures were slightly higher under full sun than in the shaded treatments in both experiments, respectively, 30.3 versus 29.8 and 29.6°C, and 35 versus 32.5 and 32.3°C. Toothpicks (isolate 276 and a mock control) were used on 5- and 6-month-old plants, and disease was evaluated after 4 weeks as above. Treatments were replicated five times in a split plot, RCBD, where main plots were light intensity, subplots were treatment (mock or inoculated), and experimental repetition was the blocking factor. To account for differences in plant size in the two experiments, the extent of internal discoloration in a given plant (mm) was divided by its fresh weight (g), and the relative values were used in ANOVAs.

RESULTS

Isolation and identification of fungi.

To date, *N. parvum* has been isolated from symptomatic tissue of every eugenia dieback sample that has been submitted to the clinic, usually to the exclusion of other fungi. Most isolates of *N. parvum* from eugenia have been sterile. Fruiting structures have not been observed on clinic specimens, and none developed for 8 of 10 isolates that were tested on different substrates and under different light regimes. Two isolates, 1-4 and 132, produced unicellular conidia in pycnidia on pine needles and toothpicks. Four isolates, 1-4, 132, 276 (sterile), and 284 (sterile), have been deposited with culture collections at the Forestry and Agricultural Biotechnology Institute (University of Pretoria, South Africa) and the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands) (accession numbers TBD).

ITS sequences for all (seven) eugenia isolates of *N. parvum* that were examined were identical, suggesting that a clonal population of this pathogen affects this host in South Florida. This sequence was

99% homologous with those for accessions of *N. parvum* in GenBank. However, the infrequent conidia that were produced, $7 \pm 0.9 \times 20.6 \pm 1.8 \mu\text{m}$, were ca. 2 μm wider than the reported dimensions for *N. parvum* (12). Sequences for five sterile strains (130, 276, 280, 284, and 395) and two that produced conidia (132 and 1-4) are deposited in GenBank as FJ478408, EU872493, EU882161, FJ478410, FJ478411, FJ478409, and EU882162, respectively.

N. parvum has not been recovered from asymptomatic tissue of dieback samples submitted to the clinic. Although it has been recovered up to 10 cm in advance of vascular symptoms in artificially inoculated plants, it appears that the fungus quickly induces vascular symptoms in the tissues it colonizes.

Representative isolates of the fungi that were isolated from asymptomatic tissue of disease specimens were identified as follows. *Pestalotiopsis* sp. was identified based on its conidial attributes (14) and was not recovered from symptomatic tissue. The asci-producing isolates, 598 and 599, were also not isolated from symptomatic tissue. The ITS sequence for isolate 599, FJ434136, was 99 to 100% homologous with those for accessions of *Glomerella cingulata/Colletotrichum gloeosporioides* in GenBank. But the identity of isolate 598 was equivocal in that several different taxa in GenBank were highly homologous. When the ITS sequence of 598, FJ434135, was manually realigned, it was most closely identified with *Colletotrichum* sp., an endophyte that has been recovered from several different tropical hosts; it may be a new species.

Few endophytic fungi were recovered from eugenia liners obtained from a commercial provider. Twice as many fungal colonies were isolated from intact than from bark-less stems, but *Mycocleptodiscus* sp., identified based on its characteristic conidia (2), was the most common fungus recovered for both treatments. *Aspergillus* sp. was also recovered from stems in both treatments, whereas *Alternaria* sp., *Fusarium* sp., *Pestalotiopsis* sp., and *Pithomyces* sp. were recovered from only intact stems. *N. parvum* was not recovered from these plants. An ITS sequence for the NE-4 isolate of *Mycocleptodiscus* sp., FJ478407, was most similar to two nonidentified fungal endophytes in GenBank (EU687173 and DQ508136, 98% homology), although two strains of *M. terrestris* were 94 and 92% homologous (respectively, EU364807 and U97332). NE-4 and other isolates from eugenia resemble closely Gerdemann's (5) original description of *M. terrestris*.

Pathogenicity. All of the fungi that were used in pathogenicity tests colonized eugenia and were easily recovered at the end of an experiment. However, only *N. parvum* caused dieback of eugenia and reduced host growth, measured as plant fresh weight accumulations (Table 1 and

Table 1. Impact of different fungi on dieback development and biomass accumulations in eugenia cv. Monterrey Bay

Treatment (taxon/isolate ²)	Source	Mean response ¹		
		External	Internal	Biomass (g)
Toothpick control	...	1.00 d	0.98 d	15.68 ab
Agar-only control	...	1.00 d	0.45 d	18.30 ab
<i>Glomerella</i> sp. 598	Diseased/clinic	1.00 d	1.18 d	18.68 ab
<i>Glomerella cingulata</i> 599	Diseased/clinic	1.00 d	1.25 d	16.00 ab
<i>Mycocleptodiscus terrestris</i> NE-4	Healthy/liner	1.00 d	1.90 d	17.85 ab
<i>Mycocleptodiscus terrestris</i> NNE-5	Healthy/liner	1.00 d	1.63 d	21.00 a
<i>Neofusicoccum parvum</i> 1-4	Diseased/clinic	4.25 a	22.25 a	4.45 d
<i>Neofusicoccum parvum</i> 276 (sterile)	Diseased/clinic	4.00 ab	20.88 ab	7.70 cd
<i>Neofusicoccum parvum</i> 132	Diseased/clinic	3.75 abc	19.75 ab	5.10 d
<i>Neofusicoccum parvum</i> 273 (sterile)	Diseased/clinic	4.75 a	22.50 a	3.15 d
<i>Neofusicoccum parvum</i> 284 (sterile)	Diseased/clinic	2.25 cd	17.13 bc	14.23 bc
<i>Neofusicoccum parvum</i> 395 (sterile)	Diseased/clinic	2.75 bc	15.00 c	17.10 ab

¹ Responses are means of four replications from one of three experiments. Disease was rated externally on a 1 to 5 (1 = healthy, 5 = dead) scale, and internally as the length of vascular discoloration in centimeters. Inoculated fungi were always recovered. Biomass was plant fresh weight in grams. Within a column, response means with different letters are significantly different (DMRT, $P < 0.05$).

² Identities of all but the NNE-5 isolate of *Mycocleptodiscus terrestris* were confirmed with BLAST comparisons of ITS DNA sequences in GenBank. The 1-4 and 132 isolates of *Neofusicoccum parvum* produced an anamorph on pine needles, but the remaining isolates were sterile.

data not shown). *Glomerella* spp., *M. terrestris*, and *Pestalotiopsis* sp. caused neither significant external nor internal symptoms of this disease, nor did they reduce host growth.

Disease development was generally comparable in plants that were inoculated with mycelium of *N. parvum* versus those that were inoculated with toothpicks that were colonized by the pathogen. Due to its convenience, toothpick inoculation was used in most of these assays. All isolates of *N. parvum* caused significant vascular discoloration, which developed even when no external evidence of dieback was present (Table 1 and data not shown). Most often, isolates also caused significant external symptoms, mortality, and reduced plant growth when compared to mock-inoculated plants. *N. parvum* was always reisolated from inoculated plants.

Temperature. Temperature had a significant impact on the in vitro radial growth of *N. parvum* and on the development of dieback on eugenia. Radial growth rates for all seven *N. parvum* isolates were similar, and increased significantly from 15 to 30°C ($P < 0.0001$, data not shown). Mean in vitro growth rates $\pm$ s.e. for the

isolate that was used in the disease studies, 276, were used in the comparison in Figure 2. Internal and external symptoms of disease increased significantly as temperature increased (Table 2, Fig. 2, and data not shown). With one exception, only vascular symptoms developed at 20°C, and no dieback symptoms developed at 15°C (*N. parvum* was not isolated from the minor discoloration that developed on plants at the latter temperature).

Light intensity. Significant disease, when compared with the mock control, developed at each light intensity that was tested, but no differences in disease development occurred within the range of light intensities that was tested (slopes of linear regressions, $P = 0.8402$ [internal symptoms] and 0.5449 [external]) (Table 3 and data not shown).

DISCUSSION

N. parvum appears to be the sole cause of this disease (9,10). This is in contrast to results from South Africa, where eight species in the *Botryosphaeriaceae* were recovered from the native *Syzygium cordatum*, including *N. parvum*, and shown to be pathogenic (7). *Syzygium paniculatum* is a

new host for *N. parvum*, although previous reports may have referred to this disease. Alfieri et al. (1) isolated *Botryosphaeria* sp., *Diplodia* sp., and *Phomopsis* sp. from plants with dieback symptoms, but provided neither identifying features of the fungi nor evidence for their pathogenicity.

Neofusicoccum was erected by Crous et al. (3) to accommodate *Fusicoccum*-like taxa that had DNA sequences that differed from those of *Fusicoccum sensu stricto* (*F. aesculi*) and produced two different types of conidia. Most species in *Fusicoccum sensu lato* were placed in *Neofusicoccum*, including the former *F. parvum*. Slippers et al. (12) argued that *Dothiorella dominicana* may be synonymous with this fungus. They indicated that *N. parvum* had been misidentified as *Botryosphaeria ribis* (anamorph: *Fusicoccum* sp.) and *B. dothidea* (anamorph: *F. aesculi*) due to overlapping host ranges and spore dimensions, and that the mango tip dieback fungus reported in Florida by Ramos et al. (11) as *B. ribis* was probably *N. parvum*.

Little is known about the epidemiology of this disease, including how and on what host *N. parvum* entered eugenia production areas in South Florida. *N. parvum* has a wide host range that includes fruit trees that are grown in South Florida, such as avocado and mango (12,16). Although isolates of *N. parvum* from the present study caused severe postharvest decay on 'Saigon' mango fruit (data not shown), we have not recovered *N. parvum* from avocado or mango in South Florida. More work is needed to determine the source of *N. parvum* on eugenia and what other hosts might be reservoirs for this pathogen in South Florida. Although species in the *Botryosphaeriaceae* are often endophytes (13), endophytic infection of eugenia by *N. parvum* was not observed in this work. Sources of the pathogen in commercial nurseries, how plants are infected, and whether cultural practices influence disease development, are important topics for future work. Based on results from this study, shade production of this crop would not reduce dieback development.

Temperature had a profound effect on the development of dieback. External and internal symptom development was far greater at 25 and 30°C than at 15 or 20°C, and host mortality and reduced host growth usually occurred only at the higher

Fig. 2. Influence of temperature on the in vitro growth rate of isolate 276 of *Neofusicoccum parvum* and internal symptoms of dieback that it caused on *Syzygium paniculatum* cv. Monterrey Bay. Bars are means $\pm$ s.e. for two experiments. In vitro growth rates and $\log_n$ internal symptoms increased significantly ($P < 0.0001$) as temperature increased.

Table 2. Influence of temperature on dieback development^z

Experiment	Regression analyses					
	External symptoms			Internal symptoms		
	Line equation	Slope P value	R^2	Line equation	Slope P value	R^2
Experiment 1	0.13 $\times$ -2.06	<0.0001	0.8272	0.09 $\times$ +1.62	0.0030	0.4794
Experiment 2	0.12 $\times$ -1.72	0.0008	0.6914	0.26 $\times$ -1.05	<0.0001	0.8926

^z Plants 2 and 6 months old were toothpick inoculated with isolate 276 in experiments 1 and 2, respectively, and replicated four times at 15, 20, 25, and 30°C. Six weeks after inoculation, disease was rated externally on a 1 to 5 (1 = healthy, 5 = dead) subjective scale, and internally as the extent of vascular discoloration, in centimeters, above and below the point of inoculation. Disease data were $\log_n$ transformed and regressed vs. temperature to obtain linear regressions. Data were not combined due to differences in plant size in the two experiments.

Table 3. Influence of light intensity on the development of dieback on eugenia cv. Monterrey Bay

Light intensity ^z	Treatment	Mean response ^y	
		External	Internal
2,000 (full sun)	Control	1.00 ± 0.00	1.69 ± 0.74
	276	3.56 ± 0.38	34.61 ± 4.89
750 (partial shade)	Control	1.00 ± 0.00	1.44 ± 0.47
	276	3.33 ± 0.29	37.94 ± 5.61
300 (shade)	Control	1.00 ± 0.00	10.00 ± 0.31
	276	3.89 ± 0.39	35.44 ± 5.39

^y Disease was rated externally on a 1 to 5 (1 = healthy, 5 = dead) scale, and internally as the extent of vascular discoloration, in centimeters, above and below the point of inoculation. Significant ($P < 0.001$) external and internal disease (greater than the mock controls) developed at each light intensity, but disease among the light intensities was not significantly different ($P = 0.8402$, internal, and 0.5449, external symptoms).

^z Approximate maximum photosynthetic photon flux in $\mu\text{mol s}^{-1} \text{m}^{-2}$ at solar noon in Homestead in July.

temperatures. Thus, temperature appears to be an important factor in the seasonal development of this disease. Since the relationships between temperature and disease severity and in vitro growth rate of the pathogen were similar, in that both increased over the range of temperatures that were tested (from 15 to 30°C), the observed disease relationships may be at least partially due to temperatures that favor the pathogen.

In conclusion, several fungi were recovered from diseased and healthy eugenia plants. However, only *N. parvum* was routinely isolated from diseased plants and caused significant damage to this host. Most isolates of *N. parvum* that were examined in this work were sterile (produced neither an anamorph nor teleomorph), but were identified as *N. parvum* with ITS DNA sequences. Although dieback development occurred over a wide range of light intensities and was favored by warm temperatures (25 and 30°C), much remains to be learned about the epidemiology of this

disease and tactics that could be used in its management.

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