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Source: Southeastern Naturalist, 15(sp8):40-50.
Published By: Eagle Hill Institute
https://doi.org/10.1656/058.015.sp804
URL: http://www.bioone.org/doi/full/10.1656/058.015.sp804

2016 *Everglades Invasive Species Southeastern Naturalist* 15(Special Issue 8):40–50

The Effects of Heat on Spore Viability of *Lygodium microphyllum* and Implications for Fire Management

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Abstract - The vining fern *Lygodium microphyllum* (Old World Climbing Fern), which is native to the Old World tropics, has invaded central and southern Florida, disrupting native habitats, reducing biodiversity, and altering fire-line intensity and behavior. Prescribed fire, one of several methods used to manage Old World Climbing Fern infestations, reduces the fern's above-ground biomass over large areas, but its effects on spore viability are unknown. To determine the heat tolerance of spores, we exposed spores to temperatures ranging from 50 °C to 300 °C for durations of 5 sec to 1 h, then assessed their germination on agar in Petri plates. Temperatures of 50 °C had little effect; 300 °C killed spores for all durations. Results indicate that spore viability decreases with increasing temperature and duration of heat exposure, and that spores are killed at relatively low temperatures (≥100 °C).

Introduction

Lygodium microphyllum (Cav.) R. Br. (Old World Climbing Fern, hereafter OWCF), native to the subtropics of Africa, Asia, and Australia, has become a major invasive exotic species in central and southern Florida. Since its introduction to Palm Beach, FL, in the late 1950s (Pemberton and Ferriter 1998), OWCF has infested more than 49,000 ha of central and southern Florida, forming dense rachis mats that smother and shade native vegetation, damage natural habitats, and alter fire-line intensity and behavior (Ferriter and Pernas 2006, Lott et al. 2003, Stocker et al. 2008). In the invaded range, it is found in wetlands and uplands, including sawgrass marshes, pinelands, hardwood hammocks, cypress stands, bayheads, and mangrove communities (Pemberton and Ferriter 1998). The fern has been designated one of Florida's most serious invasive species by the Florida Exotic Pest Plant Council (FLEPPC) because of the severe ecological damage it has caused; the species even threatens the success of Everglades restoration (Hutchinson et al. 2006). Prescribed fire is one of several methods currently used to manage OWCF infestations (Hutchinson et al. 2006, Stocker et al. 2008). Despite the use of this management tool, the species is still spreading and infestations in southwestern Everglades National Park (ENP) are expanding (Rodgers et al. 2014); thus, efforts to manage and control OWCF need refinement. Three components to consider in OWCF management are fern biology, invasive species characteristics, and fire relations.

Although occasionally regarded as the only genus within the family Lygodiaceae, *Lygodium* is increasingly treated as a genus within the family of Schizaeaceae (Gandolfo et al. 2000; Lott et el. 2003; Madeira et al. 2008; Mueller 1982a, 1982b,

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Manuscript Editor: Richard Baird

1983; Pemberton 1998; Wikström et al. 2002). Recently, Christenhusz and Chase (2014) have reaffirmed its placement in Schizaeaceae based on molecular phylogenetic analyses. OWCF is a homosporous fern composed of a dichotomously branching rhizome with adventitious roots and climbing leaves (fronds; Mueller 1982a). The primary leaves are determinate and usually less than 10 cm long, while secondary leaves are indeterminate and twining, sometimes reaching over 30 m in length (Mueller 1982a). The indeterminate twining fronds make this plant very unusual morphologically, and produce the characteristic and easily recognizable climbing habit. Climbing leaves have alternate pinnae, each possessing opposite pinnules that are further subdivided into alternate pinnulules and a resting leaf bud, which can resume indeterminate growth if the leaf apex becomes damaged (Mueller 1982a, 1983). Pinnae are sexually dimorphic with fertile and sterile pinnae often occurring on the same climbing leaf. Fertile pinnulules form sorophores of revolute leaf tissue (Gandolfo et al. 2000), which contain sporangia on the abaxial surface. A typical pinnulule may produce more than 28,000 spores, which are wind-dispersed and can be produced throughout the year (Volin et al. 2004). A single spore can give rise to a new sporophyte through intragametophytic selfing (Lott et al. 2003); this ability likely contributes greatly to OWCF's long-range dispersal and colonization ability (de Groot et al. 2012).

Typical traits associated with invasive species include fast growth-rate, exceptional propagule pressure, tolerance of variable habitat (Jose et al. 2013), and in some cases, allelopathy. OWCF has all of these characteristics, which facilitate its displacement of native plants (Lott et al. 2003, Pemberton and Ferriter 1998, Wang et al. 2014). OWCF's ability to produce spores year-round combined with the potential for multi-year spore viability (Hutchinson et al. 2006) may also contribute to its ability to invade new sites. These traits should be addressed in management strategies, for example, when determining the length of time to monitor a site post-treatment to ensure that spores in the soil do not germinate and allow OWCF to re-infest the area.

Although prescribed fire is used to reduce the above-ground biomass of OWCF infestations (Hutchinson et al. 2006, Stocker et al. 2008), the fern, in turn, affects fire-line intensity and behavior in habitats that it invades. *Pinus elliottii* Engelm. (Slash Pine), which is adapted to ground fires, can be severely affected by OWCF infestations (Lodge 2010, Pemberton and Ferriter 1998). Fire generally burns through only the lower vegetation in these pineland habitats, while the pines' sensitive meristematic and reproductive tissues remain protected in the canopy above the height of the fire (Whelan 1995). OWCF's climbing leaves produce vertical rachis mats that function as a fuel ladder, linking the understory vegetation to the forest canopy and lifting the fire into the vulnerable crown, often resulting in tree death (Osborne et al. 2010).

Fire also affects OWCF's growth—in its native range, this species resprouts from the rhizome after fire (Goolsby et al. 2006). Although prescribed fire has been used successfully in fire-adapted ecosystems to reduce above-ground fern biomass, it is unknown what effects fire has on sporulation or spore viability. Fire-created

updrafts may aid spore dispersal (Stocker et al. 2008), although there is some evidence to the contrary (Osborne et al. 2010).

Fire behavior is complex and depends on many factors, including fuel, soil moisture, temperature, relative humidity, heat released, duration, and other variables (Bond and van Wilgen 1996, Whelan 1995). A heterogeneous horizontal and vertical distribution of fuels tends to produce a patchy fire, while more evenly distributed fuels produce more homogeneous fires (Whelan 1995). A patchy prescribed fire, which mimics natural fire systems, is often a desirable management outcome because it creates refugia for native plant propagules, facilitating their reestablishment in the burned areas. However, reproductive OWCFs may also survive in unburned patches, producing spores and furthering invasive spread. Fires create convective airflows, and mature spores might be caught in an updraft and dispersed to new sites. Alternatively, a passing fire may expose spores to common fire temperatures that damage the plant or kill the spores even without ignition, thus reducing dispersal concerns (Whelan 1995).

Although burning can kill OWCF (if the rhizome is sufficiently damaged), the effects of lower temperatures vary. Plant cells are damaged by heat via several mechanisms, including protein denaturation (Whelan 1995), which typically occurs between 40 °C and 65 °C (Hopkins and Hüner 2004). Temperatures resulting in cell death, however, vary depending on the duration of heat exposure and hydration of the cells (Whelan 1995). The cells of common mesophytes die at temperatures between 50 °C and 55 °C (Hare 1961), but dehydrated organs can tolerate higher temperatures (Bond and van Wilgen 1996). Non-green spores, like those of OWCF, are relatively dehydrated, with water comprising just over 20% of their total weight, as opposed to other plant cells, where water accounts for over 90% of plant weight (Tryon and Lugardon 1991); thus, the temperature at which OWCF spores become non-viable could exceed the 50–55 °C range. The purpose of our study was to experimentally determine the effects of temperature and duration of heat exposure on spore germination. We hypothesized that spore viability would decrease with increasing temperatures and exposure durations.

Methods

Material

ENP personnel collected sporulating OWCF fronds from sites in Everglades National Park, FL, in August 2014. Park staff double-bagged and transported to Florida International University (FIU) samples collected under Florida Department of Agriculture and Consumer Services permit no. 2013–022. In the lab, we placed the fronds in a plant press to dry and release spores. We used a Mettler AE240 analytical balance (accuracy = 0.01 mg) (Mettler Toledo, LLC, Columbus, OH) to weigh out 1.00-mg spore samples which we placed in small tin cups (5-mm diameter, 8-mm deep; Costech Analytical Technologies, Inc., Valencia, CA), pinched and folded closed, and stored individually in 16 mm x 50 mm glass vials at room temperature.

Experimental conditions

Heat alone can damage plant cells; thus, it may not be necessary to combust spores in order to reduce their viability. In our experiments, we examined a range of temperatures that we thought might damage the spores without actually igniting them.

We performed a series of preliminary experiments to establish growth conditions, appropriate experimental protocols, and temperature ranges and durations, followed by 2 final experiments, the results of which are reported here. The first of these final experiments utilized temperatures below 100 °C and heat durations from 5 min to 60 min. Results from the preliminary experiments indicated that these durations were relevant to viability at temperatures within the protein-denaturation range. The final experiment employed temperatures of at least 100 °C and short durations of <1 min, which are conditions typically found in a passing fire.

During our preliminary experiments, spore viability decreased with increasing spore age faster than expected from the literature, which reported that spores remain viable for 4 y (Hutchinson et al. 2006). Although OWCF spores have been reported to drop in viability (as indicated by germination rates) from 30% at 3.8 years of age to less than 3% after 5.8 years (Hutchinson 2010), viability of untreated control spores in our preliminary experiments was found to decline from 45% to 2% after only 2 years (Sebesta 2015). In our 2 final experiments, we used spores from a single collection; they were 4 months old at the time of the first experiment, and 7 months old at the time of the second experiment. There was some apparent loss of viability from 4 to 7 months of age, so we standardized germination rates for these experiments to their respective controls; thus we report data as percent of control germination. In both of these experiments, we assigned each 1.00-mg sample to a treatment or control for each temperature and duration combination, and replicated the control 3 times.

We sowed both heated and control spores on the same day that heat treatments were applied. We suspended spores in 1.0 ml of distilled water for 10 min, then pipetted 0.5 ml of the suspension onto 20-cm Petri dishes with 0.8% agar medium containing Parker-Thompson basal nutrients (PhytoTechnology Laboratories, Shawnee Mission, KS) and sealed them with Parafilm (American Can Company, Greenwich, CT). During preliminary counts, we determined that each 0.5 ml of suspension contained 800–900 spores. We cultured the plated spores in a growth chamber (Environmental Growth Chambers model GC8-2H, Chagrin Falls, OH) under a 13/11-h light/dark cycle with temperatures set to 26/24 °C (light/dark) (Philippi and Richards 2007). We placed temperature loggers in the chamber to monitor temperature fluctuations, which were minimal during the course of the experiments. After 2 weeks, we determined percent spore germination in each Petri dish by dividing each dish into 4 equal quadrants and using a dissecting microscope to scan each quadrant for spores. We scored the first 50 spores encountered in each quadrant scan as germinated or ungerminated. Germinated spores had a green, filamentous prothallus protruding from a cracked spore (level 4 in Philippi and Richards 2007). We estimated percent germination from the sum of the quadrants (number germinated/200).

The first experiment examined the effect of temperatures below 100 °C and heat durations from 5 to 60 min. We heated spore samples in their tin cups in a muffle furnace (Fisher Scientific Isotemp model 550–12, Hampton, NH) at 50 °C, 65 °C, 80 °C, or 95 °C for durations of 5, 15, 30, or 60 min; each temperature/duration combination had 3 replicates. For each run, we used 3 temperature-logging iButton sensors (Maxim Integrated, San Jose, CA) to track internal oven temperatures for all but the 95 °C treatment, which was outside the range of the iButton. The iButtons time-stamped and logged the temperature every minute so that the measurements could be accurately matched to the treatments. We averaged readings from the iButtons to obtain the mean, standard error, and range of true temperatures in the furnace. For the controls (n = 4 Petri dishes with 0.50 ml spore suspension in each), we used spores that were identically stored but not heated.

Although the muffle furnace provided a wide range of suitable temperatures, its internal sensor was sensitive to dips in temperature from brief opening of the furnace door, which triggered the heating mechanism to produce a temperature spike and slow cooling back to the set point, slightly skewing the exposure temperatures, especially during the 80 °C treatments. We could not control humidity in the furnace and this variable may have affected spore viability.

In the second experiment, we exposed spores to 100 °C for 5 or 30 sec or 300 °C for 5 sec; each temperature/duration combination, as well as the controls, had 5 replicates. We heated spore samples (1.00 mg) in their tin cups by placing them into a hotplate-preheated 19 mm x 65 mm glass vial equipped with a TJ36 Series type K thermocouple (Omega, Westlake Village, CA). The thermocouple was connected to a CR23X Micrologger (Campbell Scientific, Inc., North Logan, UT) to verify exposure temperature every second. We removed samples from the vials quickly to halt residual continued heating. Control samples were from the same stored collection of spores but were unheated. Following treatments, we suspended the spores in 1.0 ml as before, pipetted 0.5 ml of the suspension onto prepared agar plates, and determined germination as in the first experiment.

Although the hotplate used in this experiment maintained a relatively constant temperature, it was difficult to remove the glass vials quickly from the stand. We considered holding the tin cups by tweezers over the hot plate, but verifying exposure temperature would have been very difficult in this scenario. Although the tin (melting point: 231.9 °C) cups were damaged during the 300 °C treatments, we heated another group of spores from the same collection in aluminum (melting point: 660.3 °C) cups, and then sowed the spores and assessed germination; these had the same charred appearance and germination rates as spores heated in tin cups.

We resealed and allowed to continue development for an additional week in the growth chamber all treatments that had zero germination after 2 weeks in the growth chamber (spores exposed to 95 °C for all durations, and all the treatments at ≥100 °C); germination was then reassessed. These secondary counts were more exhaustive—we counted and scored at least 100 spores in each quadrant. Of these, the spore samples exposed to 95 °C for 60 minutes were assayed a third time at 5 weeks with counts exceeding 800 spores per plate.

Data analysis

To analyze the overall trends for combined durations within each temperature group, we used a generalized linear model (GLM) assuming a binomial distribution of the dependent variable and logit link. We compared each temperature/duration combination to the control using the GLM, then compared durations within each temperature using Tukey tests from the multcomp package in R (Hothorn et al. 2008, R Core Team 2014).

Results

In experiment 1, temperature inside the muffle furnace varied from 1 °C to 8 °C from the set points (Table 1). Mean temperatures in the furnace were 0.6 °C to 4.8 °C greater than the target temperatures (Table 1). Similarly, in experiment 2, temperature varied across the bottom of the glass vial above the hotplate from 0.6 °C to 3.5 °C (with 1 exception of 99 °C below the 300 °C target) from the target temperatures. The mean logged temperatures during the 100 °C 5-sec and 30-sec targets were 103.1 °C and 102.5 °C, respectively. The 300 °C target averaged 286 °C due the single replicate with a 99 °C variation in treatment; excluding this sample, the mean logged temperature was 295.3 °C.

Spore germination at ≤95 °C

OWCF spore-germination decreased with increasing temperatures (Fig. 1), and germination of controls was 31 ± 3.4 %. All temperature groups, which combined durations within each temperature treatment, differed from controls and from each other *(P* < 0.0001). No spores heated to 95 °C for any of the durations had germinated after 2 weeks, so we excluded this treatment from further statistical analysis. Although when counted at 2 weeks, all spores treated for 5 min or more at 95 °C remained ungerminated and were presumed dead, low levels of germination occurred over time in these plates. At 3 weeks, mean germination-counts per 800 spores (and percent germination) were: 5-min exposure, 4.33 (0.5%); 15-min, 10 (1.3%);

Table 1. Target temperatures and durations (Dur), measured temperatures of treatments (Mean Temp, SE, Range), and difference (Diff.) between target and mean measured temperatures for experiment 1 treatments. No data for 95 °C treatment.

Target (°C)	Duration (min)	Mean Temp (°C)	SE	Range (°C)	Diff. (°C)
50	5	54.81	0.11	55	4.81
50	15	54.11	0.22	52–55	4.11
50	30	52.39	0.04	52–53	2.39
50	60	51.54	0.23	42–53	1.54
65	5	67.64	0.03	68	2.64
65	15	67.50	0.21	66–68	2.50
65	30	66.13	0.04	66	1.13
65	60	67.29	0.16	66–71	2.29
80	5	81.78	0.46	81–83	1.78
80	15	81.69	0.14	81–82	1.69
80	30	81.46	0.09	80–82	1.46
80	60	80.64	0.03	80–81	0.64

30-min, 0.67 (0.08%); 60-min, 0 (0%). We assayed only the 60-min group again at 5 weeks; some germination occurred and reached 10 (1.3%). The gametophytes from these treatments were stunted, but green.

Each temperature/duration combination differed significantly from the control (all $P < 0.0001$) except for 50 °C at 5 min (Fig. 2). Spores exposed to 50 °C for 5 min showed no difference in viability compared to controls. All longer durations at 50 °C differed significantly from the 5-min treatment, but did not differ from each other, and averaged 70% germination of controls. All of the durations at 65 °C differed significantly from the controls, but did not differ from each other, and averaged 60% germination of controls. All of the durations at 80 °C differed significantly from the controls and from each other. The 15-min duration had significantly lower germination than the 5- or 30-min durations, but did not differ from the 60-min duration, and averaged 15% germination compared to controls. The 5- and 30-min durations did not differ from each other but had significantly greater germination than the 60-min duration and averaged 39% germination compared to controls.

Spore germination at ≥100 °C

When spores were exposed to temperatures ≥ 100 °C for durations ≤ 30 sec, no spores had germinated for any of the treatments after 2 weeks in the growth

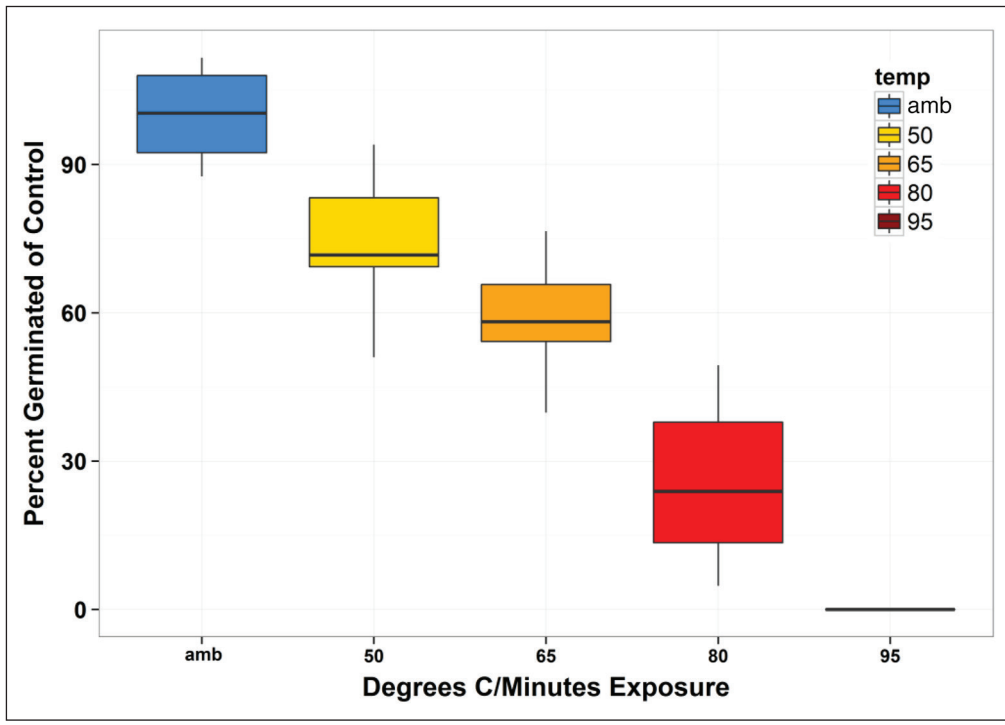

Figure 1. Final experiment 1: *Lygodium microphyllum* spore germination expressed as percent of germination of controls for each temperature treatment; heat duration of 5–60 min combined at each temperature. amb = ambient temperature for controls. All temperature treatments differed significantly from the control and from each other ($P < 0.0001$).

chambers, while germination of controls was 21 ± 4.5%. After 5 sec at 300 °C, spores were brown, appeared charred, and none germinated. After the initial assay, we resealed all of the 100 °C and 300 °C samples and placed them in the growth chamber for another 10 days. When reassayed, average germination reached only 0.23% for spores exposed to 100 °C for 5 sec; the other treatments remained at zero germination.

Discussion

In this study, we determined the lower limit of temperatures and durations required to significantly reduce OWCF spore viability. A temperature of 50 °C, when applied for durations ≥ 15 min, significantly reduced spore viability as compared to controls; exposure to temperatures ≥ 100 °C for only 5 sec resulted in nearly complete loss of viability. Thus, although desiccated and therefore tolerant of higher temperatures than hydrated (vegetative) tissue, OWCF spores are vulnerable to relatively low temperatures given that temperatures in a fire often reach 700 °C or more (Whelan 1995). Spore germination was reduced to less than 2% by a 5-sec exposure to temperatures of at least 100 °C, suggesting that spores dispersed following exposure to these temperatures would likely have greatly reduced viability. Osborne et al. (2010) reported similarly encouraging results when examining uninvaded *Cladium jamaicense* (Crantz) Kük. (Jamaica Swamp

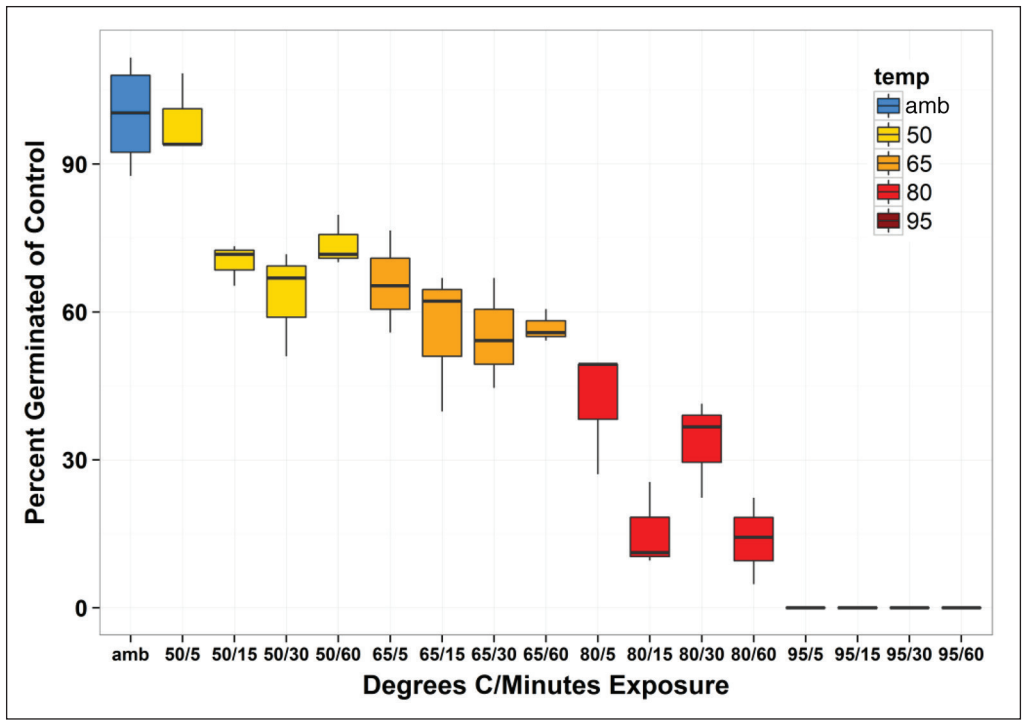

Figure 2. Final experiment 1: *Lygodium microphyllum* spore germination expressed as percent of germination of controls for each temperature and heat duration treatment. amb = ambient temperature for controls.

Sawgrass) plots adjacent to burned OWCF-infested plots. Following a prescribed burn, Osburne et al. (2010) found only 1 in 10 of the uninfested plots were colonized by OWCF. Although fire alone may not be sufficient to manage OWCF infestations (Hutchinson et al. 2006), its use in combination with herbicides or with biocontrols shows promise (Hutchinson and Langeland 2010, Stocker et al. 2008). Our findings show that relatively low temperatures reduce spore viability; this result suggests that fire-aided dispersal of viable spores is likely not as great a threat as has been hypothesized (Hutchinson et al. 2006). Thus, our data support the use of fire as part of a management strategy for OWCF infestations, particularly if temperatures near sporulating fronds can be raised to 100 °C.

Spore longevity in our experiments declined quickly, compared to other reports of viability over time (Hutchinson 2010, Hutchinson et al. 2006). Germination rates examined by Hutchinson (2010) remained between 30% and 45% for up to 3.8 y and then dropped to below 3% for spores older than 5.8 y. Germination rates for the younger spores used as controls in our experiments were similar to most rates reported by Hutchinson (2010). Differences in spore longevity may be influenced by storage conditions or attributed to differences among populations. All of the spores used in our experiments were from a single population in ENP, but some differences in germination have been reported among populations across the state (Hutchinson 2010). After germinating the spores from 12 different populations, Hutchinson (2010) found a north–south gradient of increasing rates of germination, ranging from 19% to 46%. Our collection was from a more southern location than all of those used in Hutchinson's germination evaluations; thus, we would expect those spores to have somewhat higher germination rates if the north–south gradient he observed can be extrapolated. Both the 31% and 21% germination rates obtained for our controls fall within the published range for OWCF germination rates. However, they are lower than would be expected according to population or age, suggesting that these unexplored factors also affect longevity. Additional evaluations of germination rates and long-term viability for more southerly populations may inform monitoring and retreatment protocols relevant to ENP, particularly if germination rates for these populations normally follow the trends shown by Hutchinson (2010). Additionally, experiments that explore whether viability declines for spores exposed to temperatures and humidity levels found in the field would be helpful for understanding spore longevity in vivo. This information would be especially useful in deciding whether a spore bank exists and how to adjust management practices accordingly.

Acknowledgments

Funding was provided by NSF-FL-GA Louis Stokes Alliance for Minority Participation Bridge to Doctorate Program FIU HRD #1301998, and Everglades National Park CESI #P12AC11125. We thank Hillary Cooley, Steve Oberbauer, Evelyn Gaiser, Lorenzo Menzel, Franco Tobias, Suzanne Koptur, and Michael Ross for their various contributions. We are also grateful to 3 reviewers who provided invaluable feedback on this manuscript.

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