Biological Control 56 (2011) 91–97

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

The Brazilian peppertree seed-borne pathogen, *Neofusicoccum batangarum*, a potential biocontrol agent

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ARTICLE INFO

Article history:
Received 16 June 2010
Accepted 27 September 2010
Available online 8 October 2010

Keywords:
Botryosphaeriaceae
Dieback
Fungi
Germination
Neofusicoccum batangarum
Schinus terebinthifolius
Seed-borne

ABSTRACT

The invasive exotic Brazilian peppertree, *Schinus terebinthifolius* Raddi (Sapindales: Anacardiaceae) has become a serious threat to the delicate ecosystem of Everglades National Park in Florida, USA. More than 4000 ha in the Hole-in-the-Donut (HID) area within the park have been infested with Brazilian peppertree. Brazilian peppertree is a prolific seed producer, which enhances its invasive potential. Native phytopathogens can be a viable tool in the management of exotic species; no prior studies have reported on the occurrence of native seed-borne pathogens of Brazilian peppertree in Florida. This study showed that drupes of Brazilian peppertree are affected by seed-borne fungal pathogens. These fungal pathogens either cause germination failure or attack seedlings after germination, which results in reduced vigor or seedling death. The seed-borne fungal isolate BPSPF-1 was found to be virulent, and when inoculated it was able to kill Brazilian peppertree seedlings in seedling assays, and 1 year old saplings in greenhouse trials. Field inoculation of Brazilian peppertree branches with BPSPF-1 resulted in dieback symptoms. Host range studies on one related native species (winged sumac, *Rhus copallinum*) and one non-native species (mango, *Mangifera indica*) showed that neither was affected by girdle inoculation of stems. The BPSPF-1 isolate produced dark melanized mycelium on agar media and did not produce conidia or other fruiting structures. Based on ITS DNA sequence analyses, the isolate was identified as *Neofusicoccum batangarum*.

1. Introduction

Florida along with Gulf Lowlands is second only to Hawaii in the USA in the magnitude of invasion by non-indigenous species (Cox, 1999). Brazilian peppertree, *Schinus terebinthifolius* Raddi (Sapindales: Anacardiaceae) is an invasive exotic evergreen, dioecious, insect-pollinated hardwood tree species (Loope and Dunevitz, 1981; Loope, 1992) native to Argentina, Brazil, and Paraguay (Mytinger and Williamson, 1987). Introduced to the United States in the mid-1800s as an ornamental, Brazilian peppertree is currently established in California, Florida, Hawaii, Louisiana, and Texas (Hight et al., 2003; Williams et al., 2005; Cuda et al., 2006).

Brazilian peppertree is a pioneer of disturbed sites, but is also successful in undisturbed natural environments (tropical hardwood forests, pine rocklands, sawgrass marshes, and mangrove swamps) in Florida (Jones and Doren, 1997). It can be an aggressive weed that displaces native vegetation. Brazilian peppertree now covers large areas in south and central Florida, as well as many of the islands on the east and west coasts of the state. Biannual surveys of exotic organisms conducted by the South Florida Water Management District indicate that Brazilian peppertree is the most widespread exotic plant in the state – occupying more than 283,279 ha (Ferriter, 1997). It has been placed in the Category-1 of Florida's Most Invasive Species list by the Florida Exotic Plant Pest Council because of its ability to alter the structure of terrestrial habitats and negatively impact biodiversity of native ecosystems.

A single Brazilian peppertree produces tens of thousands of single-seeded, small, fleshy, red fruits (drupes) annually between November and February, which are consumed by native birds and mammals (Ewel et al., 1982; Jones and Doren, 1997). The germination rates of seeds increase when consumed by frugivores (Panetta and McKee, 1997). Seed germination is also increased if the seed is released from the exocarps (Panetta and McKee, 1997; Tassin et al., 2007). Invasive success of Brazilian peppertree is attributed to its broad range of environmental eurytolerance (Snyder, 1999; Spector and Putz, 2006; Ewe and Sternberg, 2007) and allelopathic activity (Bennett and Habeck, 1991; Morgan and Overholt, 2005).

A significant infestation of Everglades National Park by Brazilian peppertree has occurred. Perhaps the largest and most infamous of the Everglades National Park Brazilian peppertree infestation

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doi:10.1016/j.biocontrol.2010.09.016

involves an area of over 4000 ha of abandoned agricultural lands in the midst of natural subtropical ecosystems, hence the name "Hole-in-the-Donut", (HID). Since the whole of Everglades National Park has over 40,000 ha that are affected by Brazilian peppertree, the infestation within the "Hole-in-the-Donut" is only part of a much larger issue. However, the "Hole-in-the-Donut" site has reached an almost monospecific stand stage of succession where change occurs very slowly (Loope and Dunevitz, 1981; NPS, 1998).

Eradication and management of Brazilian peppertree involves herbicides and mechanical methods (Dalrymple et al., 2003; Cuda et al., 2006). These are labor intensive and expensive (Manrique et al., 2009), and additional concerns include pollution and the effects on non-target species in the natural areas (Jones and Doren, 1997). Alternative possibilities mostly include classical insect biological control agents (Hight et al., 2003; Cuda et al., 2005; Manrique et al., 2009).

Propagule pressure (i.e. the number of propagules and the frequency of introduction events) is an important determinant of habitat invasibility of natural systems (Lockwood et al., 2005; Von Holle and Simberloff, 2005). The invasiveness of Brazilian peppertree is largely driven by its enormous reproductive potential. For management and eradication, identifying and understanding recruitment and dispersal pathways of non-native plants is critical (Davies and Sheley, 2007; Tassin et al., 2007). Evidence from various systems demonstrates that fungal pathogens can severely limit seed survival for species that recruit from persistent seed banks, and may play an important role in structuring plant populations and restricting host distributions within communities. During the course of our studies on Brazilian peppertree in Everglades National Park – "Hole-in-the-donut", we came across the persistent occurrence of seeds infected with fungi. This contamination was observed even after following strict surface sterilization procedures. It was presumed that the fungi originated from within the seed. These results were the impetus for an effort to study Brazilian peppertree seed-borne pathogens in the Everglades National Park – "Hole-in-the-Donut". If a large collection of seed-borne pathogens were made available, some of the selected pathogens may have the potential to be developed further as a bioherbicide system to control Brazilian peppertree.

The objectives of our study were to isolate and characterize indigenous seed-borne pathogens of Brazilian peppertree in Everglades National Park – "Hole-in-the-Donut", Florida, screen and evaluate the pathogenicity of seed-borne pathogens in greenhouses and field, and determine the host range of seed-borne pathogen on selected non-target plant species.

2. Materials and methods

2.1. Brazilian peppertree seed-borne fungi

Ripe Brazilian peppertree drupes on branches were collected randomly from 15 peppertrees along Research Road in Everglades National Park, Homestead, Florida, during the month of December each year from 2002 to 2008. Healthy drupes without damage or lesions were picked and air dried for 2 weeks. Different collections of healthy drupes sample from each tree were pooled into one sample. The dried outer coverings of the drupes were carefully removed manually by gently pressing and rolling. Seeds were separated from the debris; and were again examined for any damage or abnormality. Only clean and healthy seeds were selected, transferred to plastic bags, labeled and stored in the refrigerator at 4 °C.

In order to exclude fungal contaminants, the seeds were first thoroughly washed in mild liquid soap solution and then surface sterilized using 0.6% solution of sodium hypochlorite (10% Clorox commercial bleach) for 6 min and rinsed three times in sterile tap water before plating. Then, a single seed was placed inside a sterile Petri plate lined with two sterile wet filter paper discs (Whatman #2). The Petri dishes were wrapped with aluminum foil to prevent the loss of moisture and avoid contamination. The plates were then incubated at 28 °C in the dark. A seed was considered germinated when a 5 mm radicle had protruded from the seed coat (EPA, 1996). Seeds were observed for germination and fungal growth on the 7th and 14th days of incubation. During each year, a total of 120 seeds were tested with 40 seeds in each of the three replications.

2.2. Axenic Brazilian peppertree seedlings

Brazilian peppertree drupes were collected from Everglades National Park, Homestead, Florida during December, 2008. Healthy drupes without any blemish were separated from the collection and air dried for 4 weeks. The dry seed cover was removed gently; seeds were washed in mild soap solution, blotted dry using clean paper towels and stored in the refrigerator. To obtain axenic Brazilian peppertree seedlings, the seeds were first surface sterilized using 0.6% solution of sodium hypochlorite (10% Clorox commercial bleach) for 8 min and rinsed three times in sterile tap water before being placed into water agar (amended with half strength Hoagland's nutrients solution) in seedling tubes.

The tubes were incubated under 12/12-h light 30 °C day/dark 28 °C in a growth chamber. Seedling tubes showing failed germination, seed rot, or microbial growth were discarded during the first 2 weeks. Seedling tubes showing healthy Brazilian peppertree seedlings were kept under observation for up to 8 weeks in the incubator. Seedlings showing external fungal growth along with disease symptoms (leaf spot, chlorosis and tissue necrosis) as well as without disease symptoms were taken out of the seedling tube under sterile conditions. Isolations of fungi were made by transferring small pieces of fungal structures; mycelia, conidia, microsclerotia, pycnidia and ascomata onto half-strength Difco Potato Dextrose Agar (PDA) and water agar plates. Tissue pieces from symptom free healthy seedlings were also plated on PDA and water agar plates to test for the presence of fungi.

2.3. Pathogenicity screening of seed-borne fungal isolates

Individual fungal isolates from Brazilian peppertree seedlings were grown on PDA agar plates for 4 weeks. Out of the 20 seed-borne fungal isolates obtained, 12 isolates were selected for pathogenicity test on seedlings. A 5 mm × 5 mm agar block from each of the fungal plates was inoculated onto 3 week old axenically grown healthy Brazilian peppertree seedling in a seedling tube. Using a sterile forceps the agar block was carefully placed at the contact point between a petiole and the stem. There were 10 replications for each fungal isolate and an un-inoculated control. The control seedlings were inoculated with sterile agar blocks. The tubes with inoculated and the un-inoculated seedlings were incubated under 12/12-h light 30 °C/dark 28 °C in a growth chamber for 4 weeks and observed for symptom development. The experiment was repeated once.

2.4. Greenhouse inoculation experiments

In the greenhouse study 1-year-old pot grown Brazilian peppertree saplings were used. Plants were fertilized monthly with a granular (Osmocote) and liquid fertilizers (half strength Hoagland's nutrient solution). The two treatments consisted of BPSPF-1 inoculated and non-inoculated control saplings. The fungal inoculation was done by first making a circular surface incision (1–2 mm deep) at the base of sapling with a sterile sharp scalpel blade. The BPSPF-1 mycelial agar plug was placed on the incision and sealed with parafilm. Control plants were inoculated with ster-

ile agar block. There were 10 replicate pots in each of the two treatments. Average greenhouse temperature during the experiments was 30 ± 5 °C with a relative humidity of 90 ± 5%. The plants were kept under observation for symptom development for 6 weeks. The experiment was repeated once.

2.5. Statistical analyses

Greenhouse inoculation experimental pots were arranged in a completely randomized design, and the experiment was repeated once. Data were tested for homogeneity of variance, all percentage data were transformed by arcsine. All multiple comparisons were first subjected to ANOVA and significant differences among treatment means (at $P = 0.05$) were determined with Waller–Duncan multiple range test using SPSS version 13.0 statistical software package (SPSS Inc. Chicago, Illinois).

2.6. Field inoculation experiments

The field experiment was conducted inside the Nature Preserve site within the Florida International University, Modesto Maidique campus, Miami, Florida. In the field inoculation experiment, 15 Brazilian peppertrees, each with multiple branches, inside the Florida International University Nature Preserve were used. With four branches from each of the 15 Brazilian peppertrees a total of sixty healthy branches on the trees were selected and randomly assigned to four treatments. The four treatments included three native fungal isolates BPSPF-1, *Cylindrocladium* sp. (native foliar fungal pathogen of Brazilian peppertree, from Florida International University collection), BPSPF-8, and one non-inoculated control. Treatments were replicated 15 times. The inoculation procedure was similar to the one described for the greenhouse experiment, but the incision was made on a terminal branch approximately 2–3 ft below the terminal bud. In all the experiments, a circular incision (1–2 mm deep) was made on each branch with a sterile scalpel. A square of BPSPF-1 mycelial agar plug, 10 mm × 5 mm, was placed around on the top of the incision and sealed immediately with parafilm. There were three sets of controls. One set of control branches was inoculated with *Cylindrocladium* sp. The second set of branches was inoculated with a non-pathogenic seed-borne fungal isolate BPSPF-8. The third set of branches was inoculated with sterile agar block. The branches were observed for 8 weeks. If the branches showed dieback and wilting symptoms, the extent of vascular discoloration above and below the point of inoculation, and the development of the following symptoms, were recorded: necrosis = death of the terminal bud above the inoculation, dieback = progressive necrosis advancing in a basipetal fashion from the terminal bud, and gummosis = conspicuous discharge associated with inoculation. The experiment was repeated once.

2.7. Host-range study

For host-range study, mango (*Mangifera indica* L.), a non-native of commercial importance in Florida, and winged sumac (*Rhus copallina* L.), a Florida native plant species were selected. Both are classified in the family Anacardiaceae. Mango saplings cultivar Turpentine about 60 cm high were obtained from a local nursery and Winged sumac plants (30 cm high) from Fairchild Tropical Botanical Gardens. The inoculation procedure was similar to the one described for Brazilian peppertree saplings in the greenhouse, except in case of winged sumac the incision was made only at the surface level as its stem was soft and slender. The control plants were treated with sterile agar blocks. Each treatment included four replications. Additional Brazilian peppertree controls included three pots each of inoculated and un-inoculated treatments, with three saplings in each pot. The inoculation procedure was similar to the one described for the greenhouse experiment.

Another set of mango and winged sumac plants were inoculated on leaf using 1 cm × 1 cm agar blocks with BPSPF-1 mycelium and gently covered with parafilm. Leaves on control plants were treated with sterile agar blocks covered with parafilm. On each plant four leaves were inoculated, and both species had four replicate plants with inoculated and un-inoculated treatments. Average greenhouse temperature during the experiments was 30 ± 5 °C with a relative humidity of 90 ± 5%. The plants were kept under observation for symptom development.

2.8. BPSPF-1 fungus DNA analyses

Genomic DNA was extracted from approximately 50 mg of mycelium scraped from the surface of a 3–5 day old culture growing on PDA. The internal transcribed spacer regions 1 and 2 including the 5.8S rDNA were amplified using primers ITS 5 and ITS 4 (White et al., 1990). Gene fragments were amplified in 50-μL reactions on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, California, USA) under standard reaction conditions with 10–15 ng of genomic DNA, 200 μM dNTP, 2.5 units Amplitaq Gold (Applied Biosystems, Foster City, California, USA), 25 pmol of each primer, and 10 μl of the supplied 10× PCR buffer with 15 mM $MgCl_2$ in a 50-μl reaction. The thermal cycler program was as follows: 10 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, with a final extension period of 10 min at 72 °C. Amplified products were sequenced with the BigDye version 3.1 ready reaction kit (Applied Biosystems, Foster City, California) on an ABI 3100 automated DNA sequencer. The raw sequence was edited using Sequencher version 4.5 for Windows (Gene Codes Corporation, Ann Arbor, Michigan) and deposited in GenBank.

2.9. DNA data analyses

A BLAST search of the ITS region sequence data of sequences in GenBank provided a preliminary identification of *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers, & A.J.L. Phillips or a species closely related to it. The ITS sequence data from the isolate were subsequently aligned with sequences of the isolates of the *N. parvum* species complex presented in Fig. 2a by Begoude et al. (2010) using the default settings of Clustal X version 2.0.10 (Larkin et al., 2007), visually inspected, and the unaligned ends were excluded from later analyses. An unrooted, equally weighted maximum parsimony analysis of the resulting dataset was performed using the default settings of PAUP* 4.0b10 (Swofford, 2002) with MAXTREES set to auto-increase. Support for branches was also similarly obtained via a bootstrap analysis using the default settings of PAUP* 4.0b10 (Swofford, 2002) with 1000 replicates and MAXTREES set to 1000.

3. Results

Significant differences were found to exist in percent seed infection ($F_{6,14} = 70.84$; $P < 0.001$; $\eta^2 = 0.97$) and germination failure ($F_{6,14} = 49.49$; $P < 0.001$; $\eta^2 = 0.96$) between seed batches from different years (Table 1). On average around 10% of the seeds were found to be infected with seed-borne fungi, although the seed infection percentage varied from year to year. Among the seeds found to be infected with fungi, more than 50% of the seeds did not germinate.

In about 5% of the seedling tubes which showed no visible signs of fungal growth during or immediately after germination, signs of fungal growth appeared after 3 weeks. In some tubes the presence

Table 1
Brazilian peppertree seed-borne fungi infection (%) and germination failure (%) within infected seed batches during different years.

Seed Batch	Fungi (%)	Germination failure (%)
December-2002	20 a[a]	75 ab[a]
December-2003	0 e	0 c
December-2004	11 cd	59 b
December-2005	16 b	80 a
December-2006	9 d	67 ab
December-2007	10 d	70 ab
December-2008	14 bc	69 ab

[a] Values within a column followed by the same letter(s) are not significantly different according to Waller–Duncan multiple range test ($P = 0.05$).

Table 2
Pathogenicity screening of seed-borne fungal isolates on axenic Brazilian peppertree seedlings.

Isolates	Symptom development		
	Necrosis[a]	Fungal growth on seedling[a]	Seedling death (%)[a]
BPSPF-1	++++	++++	100
Phomopsis sp.	+	+	0
Colletotrichum sp.	+	+	0
Verticillium sp.	+	+	0
Exserohilum sp.	+	+	0
Bipolaris sp.	+	+	0
BPSPF-5	+	+	0
BPSPF-6	NS	+	0
BPSPF-8	NS	NG	0
BPSPF-9	NS	+	0
BPSPF-13	NS	+	0
BPSPF-17	NS	NG	0

NS, no symptoms, +, low, ++, moderate, +++, high, ++++, extensive, NG, no growth.
[a] Based on 10 replications.

of fungal growth was also accompanied by leaf spots, chlorosis, and tissue necrosis. In some cases seedling death followed by development of external fungal structures on plant tissue or on agar adjacent to the plant was observed. Twenty fungal isolates and four bacterial isolates were obtained from seedlings with symptoms. Five of the isolates based on the fungal structures and spores, were identified as *Bipolaris* sp., *Colletotrichum* sp., *Exserohilum* sp., *Phomopsis* sp., and *Verticillium* sp. Following inoculation of seedling tubes, the fungal isolate BPSPF-1 aggressively colonized the seedling and killed them within 4 days (Table 2).

The BPSPF-1 was successfully re-isolated from infected seedling leaf and stem tissues and was found to cause blight symptoms and seedling death when inoculated onto new sets of axenic Brazilian peppertree seedlings. Weak mycelial growths on seedling with limited necrotic spots were observed in seedlings tubes inoculated with isolates of *Bipolaris* sp., *Colletotrichum* sp., *Exserohilum* sp., *Phomopsis* sp., and *Verticillium* sp. and BPSPF-5. These seedlings survived during the period of observation without showing any severe symptoms. Although fungal isolates BSPF-6, 9 and 13 showed weak growth on seedlings, no visible necrotic spots or other disease symptoms were observed.

In greenhouse inoculation studies on Brazilian peppertree saplings (Table 3), the saplings inoculated with BPSPF-1 started showing wilting symptoms within 7 days, and leaves on all of the saplings became blighted, followed by defoliation and death of the saplings within 3 weeks (Fig. 1). Progression of tissue necrosis following infection at the site of inoculation was observed to be in both acropetal and basipetal directions. Cross sections of stems of inoculated saplings taken 5–10 cm away from the site of inoculation showed dark brown discoloration of vascular tissue (Fig. 2). No gummosis was observed on inoculated saplings. The parafilm that was wrapped around the stem turned black due to extensive growth of BPSPF-1 on the parafilm. All of the Brazilian peppertree saplings in the control treatment remained free of disease symptoms.

The results of field inoculation experiments showed that the fungal isolate BPSPF-1 was pathogenic and that other isolates were not under these conditions (Table 4). Wilting or dieback symptoms were observed on all of the BPSPF-1 inoculated Brazilian peppertree branches. Initial wilting of leaves was observed 10 days after inoculation. The branches were all defoliated by the end of 4 weeks. In contrast branches inoculated with *Cylindrocladium* sp., BPSPF-8 and sterile agar did not show dieback or wilting symptoms.

The host range test on mango and winged sumac saplings in the greenhouse showed no lethal effect due to BPSPF-1 inoculation, the data are not presented. BPSPF-1 inoculation on leaves did not cause any disease symptoms on winged sumac, but on mango caused small leaf spots, which did not enlarge or spread further during the observation. Sapling stem inoculation with BPSPF-1 on both mango and winged sumac did not cause dieback or wilting symptoms. The BPSPF-1 inoculated parafilms wraps on stems of both mango and winged sumac turned black due to fungal growth, confirming that the mycelial inoculum was not inactive. The saplings were kept under observation well beyond the observation period of 4 weeks to 6 months and the saplings remained disease free. All of the inoculated Brazilian peppertree control plants showed wilting symptoms within 7 days, followed by defoliation and death of the saplings within 3 weeks.

A 531 bp DNA sequence of the ITS region (GenBank Accession No. HM357636) was obtained and a culture was deposited in the Centraalbureau voor Schimmelcultures as CBS 127348. The equally weighted parsimony analysis produced the same clades shown by Begoude et al. (2010) (Fig. 2a) in the one resulting unrooted tree (Fig. 3). BPSPF-1 was included in the clade that represents *Neofusicoccum batangarum*. Bootstrap values were similar to those obtained by Begoude et al. (2010). Additionally, the characteristic guanine of *N. batangarum* found at position 389 of the Begoude et al. (2010) ITS region dataset was present in the sequence of BPSPF-1.

4. Discussion

Invasiveness can potentially be limited by seed mortality and, for many plants, most mortality occurs during the seed stage (Fenner, 1992). The Brazilian peppertree seed germination failure due to seed rot fungi that was observed in this study clearly demonstrates the effect of native pathogens on this exotic invasive

Table 3
Symptom development on BPSPF-1 inoculated Brazilian peppertree saplings in greenhouse.

Treatment	Symptom development				
	Necrosis (%)[a]	Wilting and defoliation (%)[a]	Gummosis (%)[a]	Vascular discoloration (%)[a]	Basipetal spread of necrosis (numbers)[a,b]
BPSPF-1	100	100	0	100	10/10
Control	0	0	0	0	0

[a] Based on 10 replications.
[b] Number of saplings showing symptom out of 10 replicate saplings.

Fig. 1. Sapling death following BPSPF-1 inoculation.

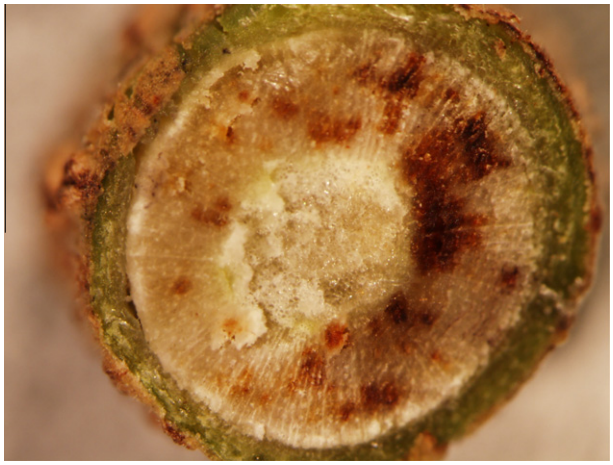

Fig. 2. Vascular discoloration following BPSPF-1 infection. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

species. Although the seed rot fungi were not characterized further in this study, fungi are a potential source of biocontrol agents.

Results from axenic seedlings indicate that Brazilian peppertree seed carry endophytic fungi. Not all of the seed-borne fungi were found to be pathogenic, some were weakly pathogenic, and one, isolate BPSPF-1, was found to be virulent. Repeated cycles of passage through the host over a period of time may present an opportunity for selection of virulent pathogenic strains within the endophytic population. Under natural conditions it is probable that some of these seed-borne pathogens may attack and kill or reduce seedling vigor of the Brazilian peppertree seedlings and contribute to reduced vigor of the exotic species. Although there is no direct evidence for this possibility, field observations of poor establishment of Brazilian peppertree seeds on certain sites (McMullen, 2003) support the need for additional studies.

Brazilian peppertree, like many other invasive plants, is a prolific seed producer, which contributes to their capacity for increase as well as their potential for dispersal (Tobe et al., 1998). A chalcid wasp *Megastigmus transvaalensis* Hussey, discovered in Florida was reported to damage the Brazilian peppertree drupe and make it incapable of germination (Habeck et al., 1989). Seed predators are frequently employed as biological control agents (Kremer, 2000), but there are no reports of microorganisms contributing to the damage incurred. Seed attacking pathogens have been tested as potential control agents of weeds (Massion and Lindow, 1986; Johnson and Baudoin, 1997; Medd and Campbell, 2005) and the efficacy of microbial seed damage can be augmented by combining it with selective seed-attacking insects (Kremer, 2000). A successful use of an approach combining both insects and microorganisms was the synergistic integration of the scentless plant bug (*Niesthrea louisianica* Sailer) and pathogenic fungus (*Fusarium* sp.) for attack of velvetleaf (*Abutilon theophrasti* Medik) seed (Kremer and Spencer, 1989a,b). These results demonstrate possibilities for integration of compatible biological agents for effective reduction of weed seed viability prior to entry into the seed bank.

On PDA media plate *N. batangarum* grew initially as a white colony, slowly turned to grey and later became dark and melanized. *Neofusicoccum batangarum* did not produce conidia either on synthetic/vegetable based media or on sterile pine needles, toothpicks, leaves and twigs of Brazilian peppertree under different light regimes. As a result it was not possible to conduct inoculation studies using conidial suspension. Results from greenhouse inoculation of Brazilian peppertree saplings using BPSPF-1 mycelial agar block clearly demonstrated the pathogenic capabilities of the fungal isolate. The symptoms on Brazilian peppertree saplings were similar to those that have been reported for fungal pathogens associated with mango decline (Ploetz et al., 1996), except that there was no gummosis. Field inoculation of Brazilian peppertree tree branches also resulted in similar dieback symptoms. The fungus was able to colonize and damage vascular tissue as indicated by extensive dark discoloration and necrosis in the affected areas. Inoculation of Brazilian peppertree leaves, inflorescences and drupes in the field with BPSPF-1 mycelial inoculum resulted in necrosis and blight symptoms (data not shown).

Table 4
Symptom development on inoculated Brazilian peppertree branches in field.

Treatment	Symptom development				
	Necrosis (%)[a]	Wilting and defoliation (%)[a]	Gummosis (%)[a]	Vascular discoloration (%)[a]	Basipetal Spread of necrosis (numbers)[a,b]
BPSPF-1	100	100	0	100	15/15
Cylindrocladium sp.	0	0	0	0	0
BPSPF-8	0	0	0	0	0
Control	0	0	0	0	0

[a] Based on 15 replications.
[b] Number of branches showing symptom out of 15 replicate branches.

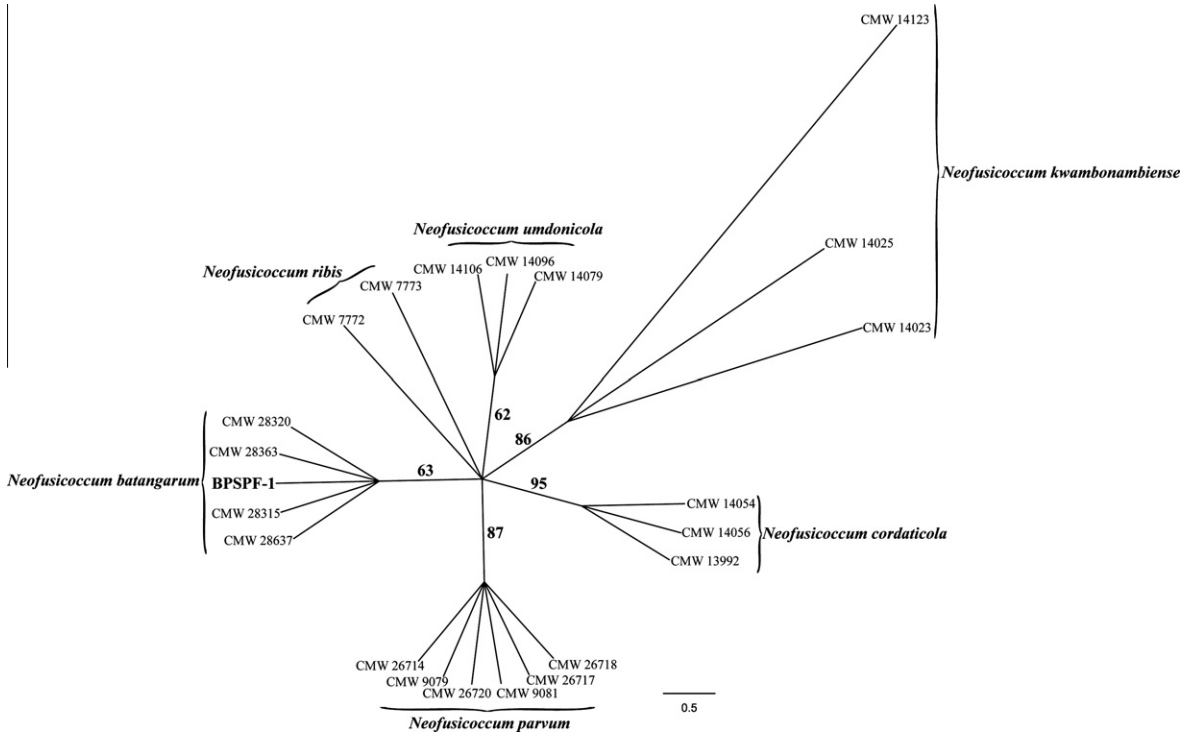

Fig. 3. The one unrooted tree resulting from the equally weighted parsimony analysis of the ITS region dataset. Bootstrap support values (%) from the 1000 replicates are given on the branches. CMW isolate numbers are from Begoude et al. (2010). *Neofusicoccum batangarum*: BPSPF-1 (culture number CBS 127348, GenBank Accession No: HM357636) from this study.

The DNA analyses of the fungal isolate BPSPF-1 identified it as *N. batangarum* Begoude, Jol. Roux & Slippers, a narrowly defined member of the *N. parvum* species complex in the family Botryosphaeriaceae (Begoude et al., 2010). Fungi of the Botryosphaeriaceae include both endophytes and pathogens of woody plants (Slippers and Wingfield, 2007) causing various canker and dieback diseases. They are also capable of infecting and colonizing inflorescences and fruit tissues (Johnson et al., 1992).

Neofusicoccum batangarum was first described and reported from isolates obtained from the branches of a tropical tree, *Terminalia catappa* L. (Combretaceae), in Cameroon, and the fungus was reported to be present as an endophyte inside the host tree tissue without causing any symptoms. However, it was found to be capable of causing pathogenic reactions when tested under greenhouse conditions (Begoude et al., 2010). A related species, *N. parvum* (Botryosphaeriaceae), has been reported to be the causal agent of lethal dieback of Bush Cherry (*Syzygium paniculatum* Gaertn.) in Florida nurseries (Ploetz et al., 2008). The origin of *N. batangarum* in south Florida is not clear; one possibility is that it may have been introduced with plant materials from Africa. However, GenBank Accession No. EU563590, which represents sequence data from seed originating in Panama, is identical in sequence to BPSPF-1 from seed in Florida. Thus, it is likely this fungus has a wider geographic range than is currently known. The pathogenic interaction between a South American host, Brazilian peppertree, with a fungus, *N. batangarum*, that is known previously only from Cameroon can be considered as a new association (Hokkanen and Pimentel, 1984).

We have been conducting field surveys in the Florida natural areas to record the occurrence of native phytopathogen-induced diseases on Brazilian peppertree and to collect disease plant samples for isolation of native pathogens. In the process incidences of severe foliar disease and inflorescence blight symptoms caused by native pathogens have been observed. In addition, the isolation and characterization of a virulent pathogen *N. batangarum* from seed further validates the need for concerted efforts to recover and develop phytopathogens against invasive exotic plant species. Control of invasive species can be made more effective by targeting different stages of invasive species' life cycle. Increasing seed and seedling mortality through seed-borne pathogens provides a promising mechanism to reduce the abundance of exotic invasive plants.

Acknowledgments

This paper is dedicated to the late Dr. Michael R. Norland, Everglades National Park, FL. We thank Dr. Craig Smith, Everglades National Park – "Hole-in-the-Donut" project manager for support and encouragement. We thank Dr. Jack Fisher of Fairchild Tropical Botanic Garden, Miami, Florida for donating winged sumac plants. We thank Dr. Paulette Johnson, FIU for help with statistical analysis. We also thank Jose Pacheco for technical help. This work was supported by Cooperating Agreement No. 5280-00-035 between the National Park Service (Everglades National Park, SFNRC) and FIU with funds allocated through the Miami-Dade County Mitigation Trust Fund/"Hole-in-the-Donut" Mitigation Bank.

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