Susceptibility of *Melaleuca quinquenervia* to *Botryosphaeria ribis*, a Potential Biological Control Agent

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ABSTRACT

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Isolates of *Botryosphaeria ribis* from *Melaleuca quinquenervia* and *Rhizophora mangle* were evaluated for pathogenicity to *M. quinquenervia* clones under greenhouse conditions. Stem inoculations revealed (i) *B. ribis* induced cankers on stems that were similar to those observed in natural infections under field conditions, (ii) the mid-height segment of the main stem was more readily colonized than the root collar, (iii) a slightly positive correlation occurred between diameter of stems at the point of inoculations and canker lengths, (iv) hyphae and macroconidia were similarly effective as inocula for stem cankers, (v) more pronounced callusing occurred following inoculations during March than during October, (vi) all isolates initiated cankers on stems of all clones but some isolates exhibited greater aggressiveness, (vii) *M. quinquenervia* clones showed a differential susceptibility to infection by *B. ribis*, and (viii) establishment of this fungus in stem tissues requires a wound or some form of injury that stresses the tree.

Additional keywords: dieback, hyphal inoculum, *Pestalotia*, stem canker

Melaleuca quinquenervia (Cav.) S. T. Blake (Myrtaceae; common names: melaleuca, paperbark tree, cajeput tree), a tree species native to Australia, has recently been listed as a noxious weed and a prohibited aquatic weed in South Florida environments (4). Its ability to rapidly colonize the Everglades ecosystem and surrounding areas is well documented (15, 16,21) as are its environmental impacts through displacement of native flora, deterioration of wildlife habitat, increasing fire hazard, and negative effects on human health (3,8,15,20). Mechanical and chemical control methods are currently used despite their expense (3,4). Extensive use of chemicals stimulates concerns over possible long-term environmental impacts in the sensitive environment of the Everglades.

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Recently, increased attention has focused on biological control of this weed using suitable herbivorous insects and fungal pathogens (3,4,16,25). Because of its contiguous pattern of distribution in monoculture, *M. quinquenervia* is considered a suitable target weed for biological control in South Florida (3). In this respect, indigenous pathogens are desirable biological control agents from the standpoint of safety to nontarget plants (7). For example, *Chondrostereum purpureum* (Pers.:Fr.) Pouzar, a native saprophyte on wood of numerous deciduous trees and parasitic to many hardwood trees including domesticated *Prunus* spp., is under evaluation as a mycoherbicide to control some hardwood weeds including black cherry (*Prunus serotina* Ehrh.) in coniferous forests (10, 22). Discovery and development of an indigenous pathogenic fungus as a mycoherbicide may hold promise for biological control of *M. quinquenervia* in South Florida.

Some fungi, including *Botryosphaeria* spp. have been recorded on *M. quinquenervia* (1). During 1989 to 1990, only one fungus, the *Fusicoccum* anamorph of *Botryosphaeria ribis* Gross. & Duggar, was consistently isolated from the margins of cankers on the main stems of trees in the Acme-2 management area of the Loxahatchee National Wildlife Refuge in South Florida (25). In the affected areas of the Refuge, this fungus was associated with dieback and subsequent mortality of tissues distal to the cankered portion of the main stem (25).

Botryosphaeria ribis has been reported as pathogenic on numerous tree species (9, 13,24,27,30,31,33). The fungus is normally associated with stressed trees. Schoeneweiss (30,31) reported an increased aggressiveness of a close relative (*B. dothidea*) toward woody plants in the presence of host-stressing factors such as low temperature, drought, and defoliation. Variation in aggressiveness of *B. ribis* isolates (35) has been studied, as has variation in host susceptibility to some fungal species according to point of inoculation on the stems (2,17,26). *Botryosphaeria ribis* has been recognized as the cause of branch and twig dieback in *Prunus persica* (L.) Batsch (33), *P. amygdalus* Batsch 'Nonpareil' (11), *Malus pumila* (L.) Miller (6,17), *Eucalyptus marginata* Sm. (9), *E. camaldulensis* Dehnh. (34), *Rubus* spp. (18), *Juglans regia* L. (27), and *Myrica faya* Ait. (13). Recently, Ramos et al. (24) found *B. ribis* to be the cause of tip dieback of mango (*Mangifera indica* L.) in South Florida. In older stems, the cankers caused by *B. ribis* are not visible on stem surfaces but are evident after removal of bark tissues (9). Generally, the cankered xylem and phloem tissues are characterized by brown discoloration (27) and are sometimes associated with insect damage (9).

To date, the pathogenicity and relative aggressiveness of *B. ribis* toward *M. quinquenervia* have not been studied. Identification of aggressive isolates of this fungus could be an important component of the biological control program for *M. quinquenervia* in South Florida. The specific objectives of this research were to evaluate pathogenicity and compare aggressiveness of *B. ribis* isolates on *M. quinquenervia*.

MATERIALS AND METHODS

Tree clone production. Seven *M. quinquenervia* trees, each located at least 50 m apart at the Acme-2 management unit of the Loxahatchee National Wildlife Refuge, were selected and approximately 20 stem cuttings were taken from each tree. The cuttings were transported to Gainesville and rooted by placing them in water in plastic buckets. The rooted cuttings (ramets) were planted in a 1:1 mixture of peat and sand in 5-gallon plastic containers. The ramets produced from these seven trees were designated as tree clones MQ-1 through MQ-7. Cuttings from each clone

were rooted to produce additional ramets that were planted in half-gallon plastic containers containing the mixture of peat and sand and watered once a day. These ramets were grown for 18 months at ambient light and the daytime temperature below 30 ± 5°C, and fertilized every 6 months with NPK (12:6:7). Unless otherwise noted, these ramets with stem diameters of 4.5 to 12.0 mm (1.0 to 2.0 m tall) were used in all experiments.

Isolate acquisition. During 1989 to 1990, six isolates of *B. ribis* were obtained from canker margins of the stems of *M. quinquenervia* trees from the Loxahatchee National Wildlife Refuge (25). Each of these six isolates was derived from a single spore (macroconidium); the isolates were designated as BR-1 through BR-6. During 1993, two more isolates of *B. ribis* were obtained from necrotic tissues in perennial stem galls encircling branch stubs of red mangrove trees (*Rhizophora mangle* L., Rhizophoraceae) from coastal areas of South Florida. These isolates were single-spored as described above and designated as BR-7 and BR-8.

Inocula preparation. Unless otherwise noted, inocula of *B. ribis* isolates used in all experiments were prepared at room temperature according to the following descriptions. Hyphal inoculum was developed by growing each isolate on potato-dextrose agar (PDA) under 10 h fluorescent light for 3 days, removing 5-mm-diameter disks from the colony margin, placing the disks in sterile test tubes containing 3-mm glass beads and deionized water, and shaking the tubes vigorously until the disks were completely macerated. Three drops of the resulting hyphal suspension were added to potato-dextrose broth (PDB) (Difco, Detroit, MI) in 500-ml Erlenmeyer flasks that were maintained under continuous shaking (100 rpm) on a rotary shaker (Lab-Line Instruments, Inc., Melrose Park, IL) maintained under 10-h fluorescent light for 4 days. Four-day-old cultures were aseptically filtered through sterile cheesecloth, and the mycelial mass retained on the cheesecloth was weighed and then blended for 15 s under aseptic conditions. The hyphal suspension was then adjusted to 32% fresh weight/volume by adding sterile deionized water. This hyphal suspension was refrigerated and used as inoculum. Fresh inoculum was prepared for each experiment. Inocula of *Fusarium moniliforme* J. Sheld. var. *subglutinans* Wollenweb. & Reinking (FMS), a pathogenic deuteromycete affecting *Pinus* spp. (used as a control) were prepared using the same technique as described for *B. ribis* isolates.

Macroconidial inoculum of *B. ribis* isolates was obtained by incubating cultures on PDA under 12-h fluorescent light cycles at 30 ± 1°C for 7 days to induce the pycnidial stroma. Macroconidial suspensions were obtained by partially drying cultures under aseptic conditions to promote conidial extrusion from pycnidia, followed by washing the plates with sterile deionized water. The presence of a few hyphal fragments in the suspension was disregarded.

Inoculation techniques. Unless otherwise noted, the *M. quinquenervia* ramets in all experiments were wounded by making a drill-hole (1.5 mm diameter and 2.0 mm deep beneath the bark surface) in the stem. The drill holes were filled with hyphal or macroconidial inoculum of *B. ribis*. Following inoculation, the wounds were immediately wrapped with Parafilm (American National Can, Greenwich, CT).

Incubation period and stem canker evaluation. Unless otherwise noted, the *B. ribis*–inoculated ramets were incubated under greenhouse conditions (approximately 30°C and ambient light) for an 8-week experimental period and were watered once a day to field capacity. The ramets were evaluated weekly throughout the incubation period for evidence of die-back, wilting, and mortality. Wilted and dead plants were harvested during the day of observation; the remaining ramets were harvested at the end of the incubation period. The evaluation included (i) determination of the presence or absence of callus at the point of inoculation, (ii) measurement of distal and proximal spread of cankers (tissue necrosis beneath the bark) from the point of inoculation, (iii) assessment of sapwood and cambium necrosis at 5 mm distal to the point of inoculation, and (iv) reisolation of the fungus from discolored tissues. The dependent variable in the experiments was total canker length (proximal plus distal).

Reisolation of fungus. As a fulfillment of Koch's postulates, the presence of *B. ribis* in the symptomatic stem tissues in inoculated stems was evaluated by reisolation onto artificial media. A 5-mm-long segment of stem tissue was taken from a point 5 mm distal to the point of inoculation, surface sterilized by quickly dipping the sample in 95% ethyl alcohol and then flaming briefly. The surface-sterilized segments were placed on acidified PDA (APDA), incubated for 14 days at 25°C and approximately 10-h fluorescent light cycles, and evaluated for *B. ribis* colony development.

Influence of inoculum type (hyphal versus macroconidial). Most of the eight *B. ribis* isolates did not sporulate abundantly on artificial media (25). Isolate BR-1 was chosen to represent *B. ribis* isolates for this experiment because it produced abundant superficial pycnidial stroma and extraction of macroconidia was more easily accomplished. Macroconidia (11×10^4/ml) and hyphal fragments (two treatments) were inoculated at 10.0 to 15.0 cm above root collars of the ramets of seven *M. quinquenervia* tree clones. Each treatment was done three times. The inoculum type and tree clones were evaluated as fixed and random effects, respectively.

Role of *Pestalotia* in canker development. Isolation of putative causal agents from canker margins on naturally infected *M. quinquenervia* trees often yielded *Pestalotia* spp. along with *B. ribis*. Therefore, the ability of *Pestalotia* spp. to cause cankers on *M. quinquenervia* was evaluated.

Three treatments were used: (i) a composite inoculum of *B. ribis* isolates, i.e., mixture of equal proportions of mycelia of BR-1 through BR-6; (ii) mixture of the composite mycelial inocula of *B. ribis* and *Pestalotia* spp. (1:1); and (iii) mycelial inoculum of *Pestalotia* spp. For each treatment, four ramets of MQ-4 were inoculated at 10.0 to 15.0 cm above the root collars. The treatments were evaluated as fixed effects.

Point of inoculation on stem and canker development. Susceptibility of stem tissues to invasion by pathogenic agents may vary according to differences in characteristics of host tissues at different points along the stems. Accordingly, an experiment, using MQ-4 and a composite inoculum of *B. ribis* isolates, was designed to detect differences in host susceptibility with respect to inoculation points on the stem. The inoculation-points were root collars, mid-height segments, and apical segments (approximately 15 cm behind the apex) of the main stems. Each treatment was done five times. Fungal isolates and inoculation positions were analyzed as fixed effects.

Evaluation of pathogenicity and aggressiveness of isolates. A greenhouse-based experiment was used to evaluate the variation in aggressiveness among eight *B. ribis* isolates and susceptibility among seven clones of *M. quinquenervia*. Ramets were inoculated with isolates of *B. ribis*, and PDB and FMS (controls) at the mid-height of the main stem. There were a total of 10 treatments (eight for *B. ribis* isolates and two for controls) for each of the seven tree clones. The experiment was done three times; each was considered a block.

Botryosphaeria ribis–treated ramets were randomized within each block. Ramets in each of the two controls were grouped separately to minimize accidental contamination of the wounds. Fungal isolates and tree clones were evaluated as random effects.

Effect of time of year on canker development. Host susceptibility may be influenced by changes in environmental conditions that occur throughout the year. The influence of time (March versus October) of inoculation on canker development was evaluated on seven *M. quinquenervia* clones (MQ-1 through MQ-7) by inoculation with hyphal inoculum of the isolate BR-2.

Three ramets of each clone were inoculated at 10.0 to 15.0 cm above the root collars; three additional ramets were in-

oculated with sterile water at the same height to serve as controls. After treatment, all ramets were arranged in a randomized manner in a greenhouse; the control-treated ramets were maintained as a separate group to avoid possible contamination. During the incubation period, daytime temperature in the greenhouse was below 30 ± 5°C in both March and October, but the photoperiod and night time temperature were ambient. Time of inoculation and tree clones were evaluated as fixed effects.

Host-infection through simulated storm-induced wounds. The experiment was designed to assess the ability of *B. ribis* to infect naturally wounded leaf and stem tissues. Eight-month-old potted ramets of *M. quinquenervia* (approximately 1.0 m tall), and macroconidial inoculum of BR-1 were used. The ramets were exposed to a sustained (5 min) wind (12.6 kg/cm^2 nozzle pressure) at a distance of approximately 15 cm from the sides and the top of the plants to simulate wounds that might naturally occur in field settings. This resulted in breakage of a few small twigs and detachment or tearing of some leaves. Additionally, the upper halves of the main stem of each member of a pair of adjacent ramets were deliberately rubbed against each other once to simulate injury that may occur during a storm. The injuries were limited to bark tissues of the rubbed portions of stems.

Using these injured ramets, four treatments (Trts.) were created: Trt. 1 (not inoculated); Trt. 2 (inoculated with 4.0×10^4 spores/ml); Trt. 3 (5.7×10^4 spores/ml); and Trt. 4 (11.6×10^4 spores/ml). The ramets in a fifth treatment (Trt. 5) were not deliberately injured by the simulated storm treatment but were inoculated with a macroconidial inoculum at 11.6×10^4 spores/ml. Each treatment contained five ramets. Macroconidia were suspended in sterile distilled water, diluted to the appropriate concentrations, and applied to the plants with an atomizer until the plants were completely wet. All the ramets in Trts. 2, 3, 4, and 5 were placed together in a dew chamber at 100% relative humidity and 27°C for 72 h. Ramets in Trt. 1 were placed separately in one corner of the same dew chamber to minimize contamination. After incubation for 72 h, surfaces of the leaves and small twigs were examined for spore germination using light microscopy. The ramets in Trts. 2 to 5 were then transferred to a greenhouse and placed together on a bench in a randomized manner. Ramets in Trt. 1 were also transferred to the greenhouse but were placed on a separate bench.

The plants were watered once a day and were evaluated weekly for leaf necrosis and development of stem cankers. Ramets were harvested 8 weeks after incubation and evaluated for canker development and the presence of *B. ribis* in the unwounded and wounded parts of the stem. A segment of bark and sapwood (5 mm long) taken from the margins of cankers (in Trts. 2 to 5) or calluses (Trt. 1) of each ramet was evaluated for *B. ribis* colony development on APDA.

Statistical analyses. Total canker length (proximal + distal canker length from the point of inoculation) was used as the response variable for all stem-inoculation experiments. Analysis of variance, linear contrasts among *B. ribis* isolates versus controls, and mean separations among variables were performed using GLM procedures in SAS (SAS Institute, Cary, NC). Pearson's correlation coefficient was used to test the relationship between stem diameter at the inoculation point and total canker length.

RESULTS

Influence of inoculum type. The influence of the inoculum type (hyphae versus macroconidial) on stem canker development in the seven *M. quinquenervia* clones and the interaction between the type of inocula and tree clone were not significant ($P = 0.05$). Callusing was observed among ramets from both macroconidial and hyphal inoculations.

Role of *Pestalotia* in canker development. Stem-inoculation experiments revealed that *B. ribis* induced much larger cankers than did *Pestalotia* (Table 1). Canker development resulting from the mixture of these two fungal species was somewhat smaller, though not statistically distinguishable from cankers caused by *B. ribis* alone (Table 1).

Point of inoculation and canker development. The point at which *M. quinquenervia* stems were inoculated with *B. ribis* influenced canker development ($P = 0.05$). The stem cankers were largest in the mid-height segments of the main stems of ramets (Table 1). Callusing was observed at all inoculation positions among ramets of all clones inoculated with *B. ribis*.

Evaluation of pathogenicity and aggressiveness of isolates. Under greenhouse conditions, mortality of *B. ribis*–inoculated *M. quinquenervia* ramets was not observed during the 8-week incubation period. Wilting and dieback of tissues distal to the point of inoculation were noted in about 20 and 10% of the ramets inoculated with BR-2 and BR-6, respectively. Except in the controls, cankers of various dimensions were observed among ramets inoculated with *B. ribis*.

Callus development was initiated within 2 weeks after inoculation in both the controls and the inoculated ramets. Further callus development ceased on those ramets that eventually developed dieback and wilting. Within 8 weeks, 86 to 100% of the inoculated wounds callused to various degrees (Table 2). All of the wounds in the controls were closed and no tissue discoloration was observed beyond 5 mm from the inoculation point. In contrast, *B. ribis*–inoculated wounds were still open and were laterally surrounded by callus tissues. These cankers were elliptical or evidenced long fissures. Discoloration of the bark, cambium, and sapwood occurred up to 100 mm beyond the callus margin on some stems. Morphology of these cankers was similar to that of those observed on the trees during 1989 to 1990 in the Loxahatchee National Wildlife Refuge (25). The cankers among noncallused stem

Table 1. Mean canker length (mm) at 8 weeks after inoculation of *Melaleuca quinquenervia* in greenhouse conditions with 1) *Botryosphaeria ribis* and *Pestalotia* spp., and 2) *B. ribis* at three different points along the stem

Experiments	Treatments	Mean canker length[z]
1. *B. ribis* vs. *Pestalotia*	*B. ribis* only	111.0 a
	B. ribis + *Pestalotia*	85.0 a
	Pestalotia only	5.5 b
2. Inoculation positions vs. canker development	Root collar	26.8 b
	Mid-height segment	129.8 a
	Apical segment	63.3 ab

[z] Means followed by the same letter(s) are not significantly different according to Scheffe's multiple range test at $P = 0.05$.

Table 2. Percentage of *Melaleuca quinquenervia* ramets that formed callus, and the percentage of cankers from which fungi were reisolated from 5 mm beyond the inoculation point on stems at 8 weeks after inoculation in March under greenhouse conditions.

	Isolates[w]								Controls	
	BR-1	BR-2	BR-3	BR-4	BR-5	BR-6	BR-7	BR-8	FMS[x]	Wound
Callusing	90	90	100	86	100	90	95	95	100	100
Reisolation	90	95	95	95	86	100	86	86	33[y]	14[z]

[w] BR = Botryosphaeria ribis.
[x] FMS = *Fusarium moniliforme* var. *subglutinans*.
[y] *F. moniliforme* var. *subglutinans* reisolated from inoculation point, but none from 5 mm above the inoculation point.
[z] *Botryosphaeria, Pestalotia,* and *Fusarium* were reisolated from the inoculation point, but none from 5 mm above the inoculation point.

wounds inoculated with *B. ribis* were marked on the stem surface by depressions located proximally and distally from the point of inoculation. Often, *B. ribis* produced pycnidial stroma on the bark on both the proximal and distal sides of inoculation point. *Botryosphaeria ribis* was reisolated from symptomatic tissues of 86 to 100% of the inoculated stems (Table 2).

Linear-contrasts analyses revealed the effects of *B. ribis* isolates to be significant (Table 3). Canker development in the controls was not significant compared with that induced by *B. ribis* isolates, and they were not included in further analyses of the main effects (Table 3) and the mean separations for isolates and clones (Fig. 1A,B). With or without controls, the variances of isolates and clones were highly significant, whereas their interaction terms were insignificant (Table 3).

Table 3. Effects of *Botryosphaeria ribis* isolates on canker length (mm) on stems of *Melaleuca quinquenervia* clones at 8 weeks after inoculation under greenhouse conditions

	Analysis of variance			
Sources[z]	df	MS	*F* value	$P_r > F$
Linear contrasts				
FMS and wound vs. BR isolates	—	—	—	0.0001
FMS vs. BR isolates	—	—		0.0001
Wound vs. BR isolates	—	—	—	0.0001
FMS vs wound	—	—	—	0.8087
Without controls				
BR Isolates	7	6,073.3	5.77	0.0001
MQ clones	6	4,986.0	4.74	0.0003
BR Isolates × MQ clones	42	1,051.9	1.00	0.4797
Error	112	1,048.7	—	—

[z] BR = *B. ribis*, FMS = *Fusarium moniliforme* var. *subglutinans*, and MQ = *M. quinquenervia*.

Stem-inoculation experiments showed BR-1 through BR-4, BR-6, and BR-8 to be more aggressive than BR-5 and BR-7 (Fig. 1A). Clones MQ-3 through MQ-7 were more susceptible than clones MQ-1 and -2 (Fig. 1B). Canker development from the inoculation point was greater distally than proximally (Fig. 2). Extent of discoloration of the cross-sectional area of sapwood, and circumference of cambium and phloem (an indication of the extent of fungal colonization of stem tissues) was greater in stems inoculated with BR-2 and least in the stems inoculated with BR-7 (data not presented). The correlation between stem diameter at the point of inoculation and total canker length was slightly positive ($P_r > R = 0.01$).

Effect of time of year on canker development. Regardless of the differences in inoculation time of year, *B. ribis* did not kill *M. quinquenervia* ramets in the 8-week experimental period. However, wilting and dieback of shoots distal to the inoculation points developed in 10 and 20% of the ramets inoculated in March and October, respectively. Accordingly, callusing did not occur in 10 and 33% of the total stems inoculated in March and October, respectively. Among the controls, the wounds were completely closed by callus tissues in both March and October inoculations. Characteristics of cankers and fungal sporulation on bark among callused and noncallused wounds on the stems were the same as described for March inoculations.

Mean canker length from March (81 mm) and October (93 mm) inoculations was not different ($P = 0.05$). The circumference of necrotic tissues on the sapwood surface was also larger (data not presented) in ramets inoculated in October than those inoculated in March. *Botryosphaeria ribis* was reisolated from 98% of the cankers on inoculated ramets.

Host infection through simulated storm-induced wounds. During the 8-week incubation period, mortality of *M. quinquenervia* ramets was not observed in any of the five treatments in this experiment. Germination of macroconidia and mycelial proliferation on the surface of leaves and stems were prevalent within the 72-h incubation period. During this period, some hyphal tips were observed to gain entry into the leaves through stomata of uninjured leaves. However, following transfer from the moist chamber to the greenhouse bench, further necrosis of uninjured as well as injured leaves was arrested. Other effects of the simulated storm on disease development on *M. quinque-*

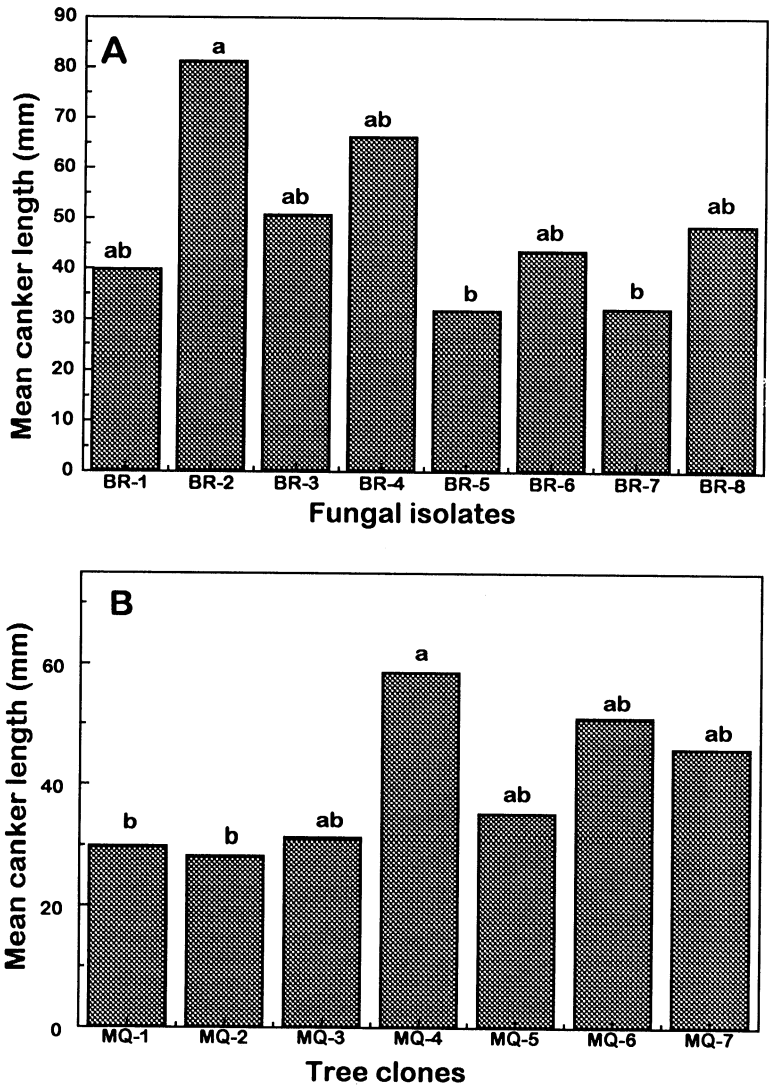

Fig. 1. (A) Pathogenic interaction between *Botryosphaeria ribis* (BR) isolates and *Melaleuca quinquenervia* clones; each bar represents the mean canker length from 21 stems. (B) Clonal susceptibility of *M. quinquenervia* (MQ) clones towards *B. ribis*; each bar represents mean canker length from 24 stems. Bars with the same letter(s) are not significantly different (Scheffe's Test ($P = 0.05$).

nervia stems are presented in Table 4. Regardless of the concentration of macroconidia used, all the wounds made by stem-rubbing were still open (Table 4) and were surrounded by thin strips of callus ridges. In contrast, the wounds on uninoculated stems were closed by callus ridges. Small bark-limited cankers (2 to 15 mm long) developed from leaf scars and unwounded upper parts of some wind-blasted stems (Table 4) where the bark was green at the time of treatment. Also, branch tip dieback was observed among some wind-blasted ramets inoculated with higher inoculum concentrations (Table 4). The percentage of cankered and uncankered part of the stems from which *B. ribis* was reisolated is presented in Table 4. In the controls (Trt. 1), 20 and 40% of the wounds yielded *B. ribis* and *Pestalotia* spp., respectively.

DISCUSSION

Very little is known about the diseases affecting *M. quinquenervia* although some fungal pathogens have been recorded from this host in South Florida (1). We report the first testing of the pathogenicity of *B. ribis* on *M. quinquenervia*.

In naturally occurring infections in the field in South Florida, stem cankers caused by *B. ribis* often were not visible on stems of *M. quinquenervia* trees. The elliptically shaped cankers were found only after removal of several layers of the thick, papery bark. In advanced cankers, callus tissues were discolored, imparting a gradient of brown to black coloration from the interface of the diseased and healthy tissues to the advanced stages of tissue decomposition at the surface of the exposed sapwood. Under greenhouse conditions, similar cankers and tissue discoloration patterns were observed on the stems inoculated with the isolates of *B. ribis*.

Some species of *Pestalotia* have been described as weak plant pathogens. This fungus was isolated along with some isolates of *B. dothidea* that cause gummosis disease on peach trees (33). Ramos et al. (24) reported *Pestalotia* from the tissues killed by the dieback diseases caused by *B. ribis*. In our study, the canker-causing ability of *Pestalotia* on *M. quinquenervia* was insignificant compared with that of *B. ribis* (Table 1). *Pestalotia* spp. appeared to be a secondary colonizer of *M. quinquenervia* tissues infected and killed by *B. ribis*. However, the ability of *B. ribis* to cause stem cankers on *M. quinquenervia* was somewhat retarded by adding *Pestalotia* to the inoculum. *Pestalotia* may be antagonistic to *B. ribis* in stem cankers on *M. quinquenervia*.

The relationship between total canker length and the diameter of the stem at the inoculation point was not strong. These results confirm the findings of Shearer et al. (31) who reported *Eucalyptus radiata* Sieb. ex DC. stems of all ages and diameters to be equally susceptible to invasion by *B. ribis*. However, this does not mean that stems of different diameters inoculated with *B. ribis* will be killed in the same length of time. Mortality of infected trees would depend on the ability of fungus to girdle the stem and/or occlude the vascular tissues, which, in turn, depends on the ability of fungus to grow vertically, radially, and tangentially through xylem, cambium, and cortex.

Our results from *B. ribis* inoculations revealed rapid stem canker development at the mid-height segment of the main stem when compared with the root collar (Table 1). These results are not in agreement with the findings for the close relative *B. dothidea,* which produced larger canker lesions on apple (*Malus* spp.) trees when inoculated at the lower portion of the trunk (17).

In general, the differences in inoculation time did not affect the length of the stem-cankers. However, wound callusing of *B. ribis*–inoculated stems was more frequent and pronounced following March inoculations than following October inoculations. Additionally, the decline and wilt of ramets was greater in October inoculations. This agrees with the findings of Filer and Randall (12) who inoculated *B. ribis* on 21 sweetgum families (*Liquidambar styraciflua* L.) in open pollinated progeny tests and found maximum infection of trees when inoculations were made in September and least infection among trees inoculated in May. Rapid disease development in September inoculations has been reported for *B. dothidea* on southern California chaparral vegetation (5). Our results do not agree with Davison and Tay (9), who reported *B. ribis* as an extensive invader of the phloem of *E. marginata* seedlings when inoculations were made in winter, spring, and summer. English et al. (11) reported *B. ribis* to be highly aggressive on almond (*P. amygdalus*) in spring, summer, and fall. Therefore, it appears that susceptibility of tree species to *B. ribis* may be influenced by the differences in the time of year when they are inoculated.

Callus formation around stem wounds is viewed as a host reaction to contain the

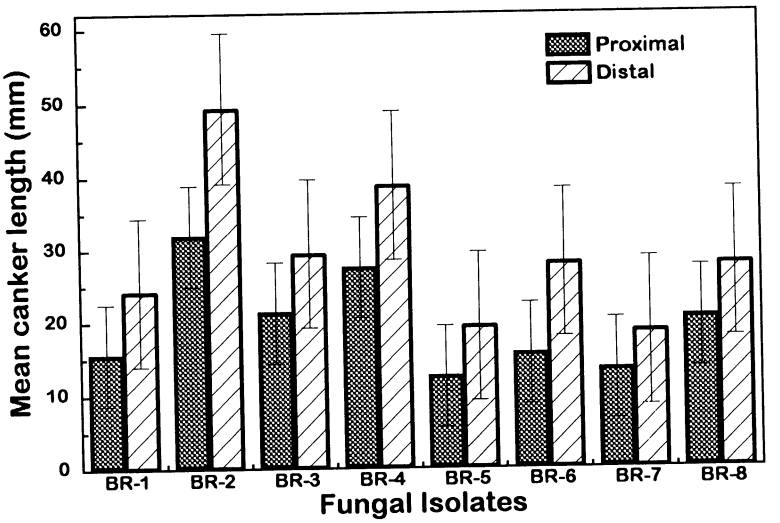

Fig. 2. Mean proximal and distal canker length from the point of inoculation of *Melaleuca quinquenervia* stems with *Botryosphaeria ribis* (BR) isolates. Each bar represents mean canker length from 21 stems.

Table 4. Effects of simulated storm damage on the establishment of BR-1 on stems of *Melaleuca quinquenervia* ramets inoculated with macroconidia and maintained in dew chamber for 72 h and then in greenhouse for 8 weeks

	Treatments[z]				
	1	2	3	4	5
A. Wounds	Closed	Open	Open	Open	None
B. Part of ramets exhibiting cankers					
Unwounded part of stems	0	40	60	60	0
Leaf scars	0	40	40	60	0
C. Dieback of stem tips	0	0	20	60	0
D. *Botryosphaeria ribis,* reisolation					
Wound cankers	20	100	100	100	0
Bark-limited cankers	0	80	100	80	0
Uncankered stems	0	60	80	20	29

[z] Treatments: 1 (injured, not inoculated with fungal spores), 2 (injured, inoculated with 4.0×10^4 spores/ml), 3 (injured, inoculated with 5.7×10^4 spores/ml), 4 (injured, inoculated with 11.6×10^4 spores/ml, and 5 (ramets not injured but inoculated with 11.6×10^4 spores/ml). The numbers in "B," "C," and "D" represent the percentage of ramets developing stem cankers, tip dieback of branches, and uncankered or cankered stems that yielded *B. ribis*, respectively.

tissue-invading fungus and to subsequently aid wound closure. Under greenhouse conditions, October inoculation of *M. quinquenervia* with *B. ribis* appeared more effective in slowing callus formation around wounds on stems than did March inoculations.

Under greenhouse conditions, some *B. ribis* isolates were more aggressive than others on *M. quinquenervia* (Fig. 1A). Similar variation in aggressiveness among isolates of *B. ribis* has been reported on currants (*Ribes* spp.) (14) and on blueberry (*Vaccinium* spp.) (35). Isolates of *B. dothidea* have shown similar variation in pathogenicity on apple cultivars in Chile (17). Based on the results from the inoculation on apple cultivars, the existence of host-specialized strains of *B. dothidea* has been suggested (17,32). Also, cultivar-dependent susceptibility toward isolates of *B. ribis* and *B. dothidea* occurs on currant (14), apple (17), and blueberry (19). These findings agree with our observations in which variable susceptibility among some *M. quinquenervia* clones was evident.

Uninjured ramets inoculated with macroconidial inoculum of *B. ribis* neither developed cankers nor showed any symptoms of decline (Table 4). On the other hand, some of the ramets exposed to the gust of wind and inoculated with *B. ribis* produced bark-limited cankers on tissues that did not appear to suffer obvious wounding (Table 4). These bark-limited cankers were similar to those described for *B. dothidea* infection of peach stems through lenticels that developed sunken necrotic lesions (23). It is assumed that establishment of *B. ribis* in *M. quinquenervia* also requires either a wound exposing the sapwood or injury stresses such as loss or damage of leaves or minute branch injuries. These results agree with Milholland (19) and Rumbos (27), who found that *B. ribis* requires wounds and/or some other form of injuries and stresses to establish and cause canker diseases leading to tree decline and dieback.

Schoeneweiss (28,29) found the close relative *B. dothidea* to be a more aggressive pathogen on stressed woody plants. After proper evaluation, this attribute of *B. ribis* may be utilized for biocontrol strategies of *M. quinquenervia*. The control strategy may be enhanced by applying this fungus to the trees in combination with phytophagous insects of *M. quinquenervia* or some environmentally safe chemicals.

The pathogenicity of *B. ribis* on *M. quinquenervia* has been established and our understanding of the infection biology and host response has been advanced. Further investigation of the biological control potential of this fungus on *M. quinquenervia* is suggested.

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