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Infection of the Native California Grass, *Bromus carinatus*, by *Fusarium circinatum*, the Cause of Pitch Canker in Pines

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Abstract

At Point Reyes National Seashore in California, *Fusarium circinatum*, the causal agent of pitch canker in pines, was isolated from *Pinus muricata*, the California native grass, *Bromus carinatus*, and the introduced grass, *Holcus lanatus*. All grass plants from which *F. circinatum* was isolated were symptomless. Pathogenicity of grass isolates was confirmed by inoculation of *P. radiata* trees, which developed symptoms similar to trees inoculated with a pine isolate of *F. circinatum*. Isolates from grasses were somatically compatible with isolates recovered from symptomatic pines. *B. carinatus* grown in a growth chamber was inoculated with a green fluorescent protein-expressing strain of *F. circinatum*. Segments of inoculated leaves were incubated in moist chambers; after 1 to 2 days, sporulating hyphae were observed growing from leaf tissue. Spores of *F. circinatum* removed from *B. carinatus* leaves were confirmed to be fluorescent when illuminated with ultraviolet light. These results raise the possibility that *B. carinatus* cryptically infected by *F. circinatum* may be a source of propagules capable of infecting pines.

Keywords: *Fusarium circinatum*, pitch canker, *Bromus carinatus*, *Holcus lanatus*, *Pinus muricata*, *Pinus radiata*

Pathogens are unlikely to survive over evolutionary time if they are restricted to a single host that they damage extensively (Gordon et al. 2003). Reduced host populations mean fewer opportunities for the pathogen to infect new individuals. A pattern observed in some plant pathogens is the capacity to infect multiple host species, manifesting a symptomless endophytic interaction with certain hosts and a pathogenic interaction with others (Delaye et al. 2013). Examples include *Fusarium oxysporum* f. sp. *fragariae*, which causes disease on cultivated strawberry but does not induce symptoms on infected asparagus, garlic, or tomato (Pastrana et al. 2017). In California, the oomycete *Phytophthora ramorum* causes sudden oak death in *Quercus* species but induces milder symptoms when infecting many other plant species (Garbelotto and Hayden 2012).

A similar pattern is seen with *F. circinatum*, which causes pitch canker in *Pinus* spp. and also colonizes both grasses and dicotyledonous plants without inducing symptoms (Swett and Gordon 2009). In California, this includes grasses at Point Reyes National Seashore on the California coast, north of San Francisco (Swett and Gordon 2012), where pitch canker is causing significant damage to native stands of bishop pine (*Pinus muricata*). It is currently not known whether grasses play a role in the epidemiology of pitch canker at Point Reyes or anywhere else the disease occurs.

It is known that conidia produced by *F. circinatum* on infected pines can be dispersed by insects (Gordon et al. 2001) and can also become airborne (Correll et al. 1991). If spores are produced on infected grasses, it would add another dimension to the epidemiology of pitch canker. Grasses could facilitate spread of the pathogen between widely separated pine stands and could serve as reservoirs of inoculum in pine seedling nurseries, compromising efforts to manage the disease through sanitation (Swett et al. 2014). This study was undertaken to determine whether grasses other than the invasive *Holcus lanatus* were cryptic hosts of *F. circinatum* at Point Reyes and whether infected grasses could support sporulation of the pitch canker pathogen.

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Materials and Methods

Isolations from plant material. Grass samples collected at Point Reyes on 17 July, 31 July, and 14 August 2017 were transported to the laboratory on the same day and kept at 4°C until examined. One day after harvesting, samples were identified to species using the *Jepson Manual* (Baldwin et al. 2012). As a physical record, representative samples of *Bromus carinatus* and *H. lanatus* were deposited in the University of California, Davis Center for Plant Diversity collection (Carter 2017a, b). Two days after harvesting, tissues were cut into 1-cm segments and submerged briefly in 0.1% Tween 20 with agitation on a magnetic stir plate, followed by similar treatment in 70% ethanol for 30 s and 1.0% NaClO for 1 min. Treated segments were placed on a *Fusarium*-selective agar medium (FSM) (Aegerter and Gordon 2006). Plates were incubated under fluorescent lights at room temperature and regularly observed for fungal growth. Colonies identified as *F. circinatum* based on colony morphology were transferred to carnation leaf agar (CLA) containing 0.6% KCl and incubated under fluorescent lights at room temperature.

CLA plates were observed after 5 to 8 days for conidial false heads borne on polyphialides as described by Gordon et al. (1996). Those with such morphology (Table 1) were reisolated into pure cultures by excising the hyphal tip of a conidium germinating on water agar. Pure cultures were stored on dried filter paper at 4°C (Gordon and Okamoto 1991).

Somatic compatibility testing. To assign isolates to somatic compatibility groups (SCGs), nitrate nonutilizing (*nit*) mutants were generated on potato dextrose agar (PDA) amended with potassium chlorate and were tested for compatibility on Czapek medium (CzaPEK Solution Agar; BD) as described by Gordon et al. (1996). Mutants were tested for compatibility with tester *nit* mutants representative of previously characterized SCGs of *F. circinatum* in California (Gordon et al. 1996). Pairings between complementary *nit* tester strains served as positive controls, with pairings between incompatible *nit* mutants serving as negative controls. All *nit* pairings were repeated.

Pathogenicity testing. All isolates were tested for pathogenicity on *P. radiata* (Table 1). Trees were grown from seed (obtained from Forest Seeds of California, Placerville, CA) for 20 months under greenhouse conditions, wherein temperatures ranged from 6 to 44°C. Inoculum was prepared by growing isolates on PDA under

fluorescent light at room temperature for 10 days. A spore suspension was prepared by flooding plates with sterile 0.5% KCl and scraping the surface with a sterile glass slide to obtain a slurry. The slurry was passed through two layers of sterile cheesecloth to remove hyphae. The density of the resulting spore suspension was quantified using a hemocytometer. For inoculations, spore density was adjusted to 1.25×10^5 spores/ml by addition of sterile 0.5% KCl.

P. radiata trees were inoculated by making a shallow 1.6-mm-diameter wound on the stem and filling the wound with 2 µl of spore suspension (approximately 250 spores). Two trees were inoculated per isolate, for a total of 34 isolates. Two trees were also inoculated with a known virulent isolate of *F. circinatum* (GL1959). Inoculated pines were maintained in a greenhouse, wherein temperatures ranged from 14 to 34°C, for 6 weeks, after which lesion lengths at the site of inoculation were measured to the nearest millimeter.

A confirmatory pathogenicity test was conducted with a subset of grass isolates found to be virulent in the initial test. This group included at least one isolate from each location where *F. circinatum* was recovered from a grass (Table 2). GL1959 was again included as a positive control and a 0.5% KCl solution without spores served as a negative control, for a total of 12 treatments. Spore density was additionally confirmed by inoculating eight PDA plates with a dilution of the suspension to achieve a target dose of 50 colonies per plate. The number of colonies on each plate was enumerated 48 h later (Table 2). Using the method described above, we inoculated five trees per isolate. All trees used in the inoculations were grown from seed for 27 months under greenhouse conditions wherein temperatures ranged from 6 to 44°C. After inoculation, trees were randomly arranged in a grid and maintained in a greenhouse for 6 weeks. Lesion lengths were recorded as described above. The experiment was conducted twice.

Inoculation of *B. carinatus*. Three *B. carinatus* seeds (Larner Seeds, Bolinas, CA) were sown at a depth of approximately 2 cm in 15-cm-diameter pots containing Sunshine Professional Growing Mix (SunGro Horticulture Canada Ltd.). Pots were maintained in a growth chamber with a 14-h photoperiod and day/night temperatures

Table 2. Isolates of *Fusarium circinatum* tested for pathogenicity to *Pinus radiata*

	Lesion length at site of inoculation (mm)				
Isolate	**Range**	**Mean ± SEM**	**SCG[a]**	**Inoculum[b]**	**Host origin**
GL1959	4–63	25.2 ± 5.2	C1	120–210	*P. muricata*
GL1902	7–50	19.5 ± 4.4	C1	80–110	*Bromus carinatus*
GL1903	6–37	18.0 ± 3.0	C1	95–230	*B. carinatus*
GL1908	9–68	28.0 ± 6.5	C1	140–260	*B. carinatus*
GL1909	15–33	24.6 ± 1.9	C1	190–250	*B. carinatus*
GL1910	10–65	29.0 ± 6.0	C1	65–90	*B. carinatus*
GL1911	9–48	28.8 ± 4.0	C1	40–280	*B. carinatus*
GL1916	7–50	20.9 ± 4.8	C1	140–265	*B. carinatus*
GL1917	3–28	14.0 ± 2.6	C1	105–235	*Holcus lanatus*
GL1918	4–60	28.2 ± 5.3	C1	165–230	*H. lanatus*
GL1919	4–53	24.0 ± 5.5	C1	145–235	*H. lanatus*

[a] Somatic compatibility group (SCG) to which the isolate was assigned.
[b] Range of spore numbers delivered in inoculations.

Table 1. Source and location of *Fusarium circinatum* isolates obtained at Point Reyes National Seashore

Isolate	**Host origin**	**SCG[a]**	**Date collected[b]**	**GPS coordinates[c]**	**Elevation (ft)**
GL1902	*Bromus carinatus*	C1	31 July 2017	38.04636, –122.87521	240
GL1903	*B. carinatus*	C1	31 July 2017	38.04636, –122.87521	240
GL1904	*B. carinatus*	C1	31 July 2017	38.04636, –122.87521	240
GL1905	*B. carinatus*	C1	31 July 2017	38.04636, –122.87521	240
GL1906	*B. carinatus*	C1	31 July 2017	38.04636, –122.87521	240
GL1907	*B. carinatus*	C1	31 July 2017	38.04636, –122.87521	240
GL1908	*B. carinatus*	C1	31 July 2017	38.05688, –122.85641	560
GL1909	*B. carinatus*	C1	14 August 2017	38.04796, –122.87646	270
GL1910	*B. carinatus*	C1	14 August 2017	38.05753, –122.85618	590
GL1911	*B. carinatus*	C1	14 August 2017	38.05753, –122.85618	590
GL1912	*B. carinatus*	C1	14 August 2017	38.05753, –122.85618	590
GL1913	*B. carinatus*	C1	14 August 2017	38.05753, –122.85618	590
GL1914	*B. carinatus*	C1	14 August 2017	38.05753, –122.85618	590
GL1915	*B. carinatus*	C1	14 August 2017	38.05753, –122.85618	590
GL1916	*B. carinatus*	C1	14 August 2017	38.04547, –122.87763	190
GL1917	*Holcus lanatus*	C1	17 July 2017	38.04675, –122.87535	216
GL1918	*H. lanatus*	C1	17 July 2017	38.04612, –122.87531	180
GL1919	*H. lanatus*	C1	17 July 2017	38.04683, –122.86205	380
GL1920	*Pinus muricata*	C1	17 July 2017	38.04768, –122.87635	258
GL1921	*P. muricata*	C1	17 July 2017	38.04768, –122.87635	258
GL1922	*P. muricata*	C1	17 July 2017	38.04669, –122.87548	238
GL1923	*P. muricata*	C1	17 July 2017	38.04669, –122.87548	238
GL1924	*P. muricata*	C1	17 July 2017	38.04612, –122.87531	180
GL1925	*P. muricata*	C1	17 July 2017	38.04612, –122.87531	180
GL1926	*P. muricata*	C1	17 July 2017	38.04683, –122.86205	380
GL1927	*P. muricata*	C1	17 July 2017	38.04683, –122.86205	380
GL1928	*P. muricata*	C1	31 July 2017	38.04631, –122.87518	230
GL1929	*P. muricata*	C1	31 July 2017	38.04631, –122.87518	230
GL1930	*P. muricata*	C1	31 July 2017	38.05717, –122.85631	580
GL1931	*P. muricata*	C1	31 July 2017	38.05717, –122.85631	580
GL1932	*P. muricata*	C1	14 August 2017	38.04720, –122.87611	260
GL1933	*P. muricata*	C1	14 August 2017	38.04720, –122.87611	260
GL1934	*P. muricata*	–[d]	14 August 2017	38.05809, –122.85638	610
GL1935	*P. muricata*	C1	14 August 2017	38.05809, –122.85638	610

[a] Somatic compatibility group (SCG) to which the isolate was assigned.
[b] Date plant tissue was collected.
[c] Location where plant tissue was collected.
[d] Isolate did not pair strongly with tester strains.

of 23/18°C. If multiple seeds in a pot germinated, they were thinned so each pot contained a single plant.

When senescence became visible in the oldest leaves, plants were inoculated with a pathogenic, green fluorescent protein (GFP)-expressing strain of *F. circinatum* (GL17-GH7) (Swett et al. 2018). A spore suspension was prepared as previously described, except that Silwet-L77 (a surfactant) was included at 0.2%. The same suspension without spores was included as a negative control. Silwet-77 was confirmed to have no effect on spore germination (*data not shown*). Five plants were sprayed to run-off for each of the two treatments, for a total of 10 plants, and plants were individually bagged for 48 h to maintain high humidity. After inoculation, plants were maintained in a growth chamber under conditions described above. This inoculation experiment was conducted twice.

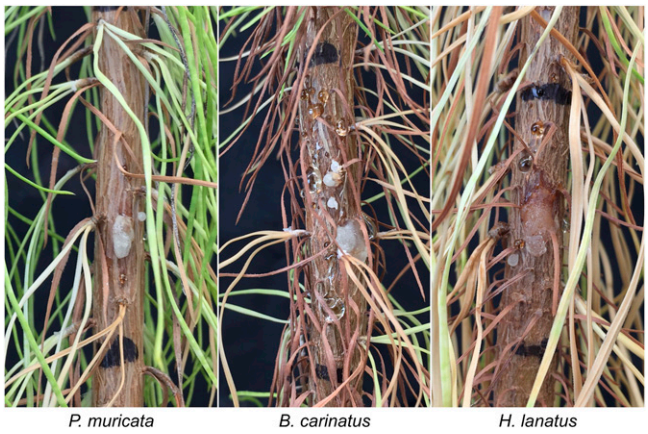

Fig. 1. External symptoms on *Pinus radiata* induced by isolates obtained from *P. muricata*, *Bromus carinatus*, and *Holcus lanatus*.

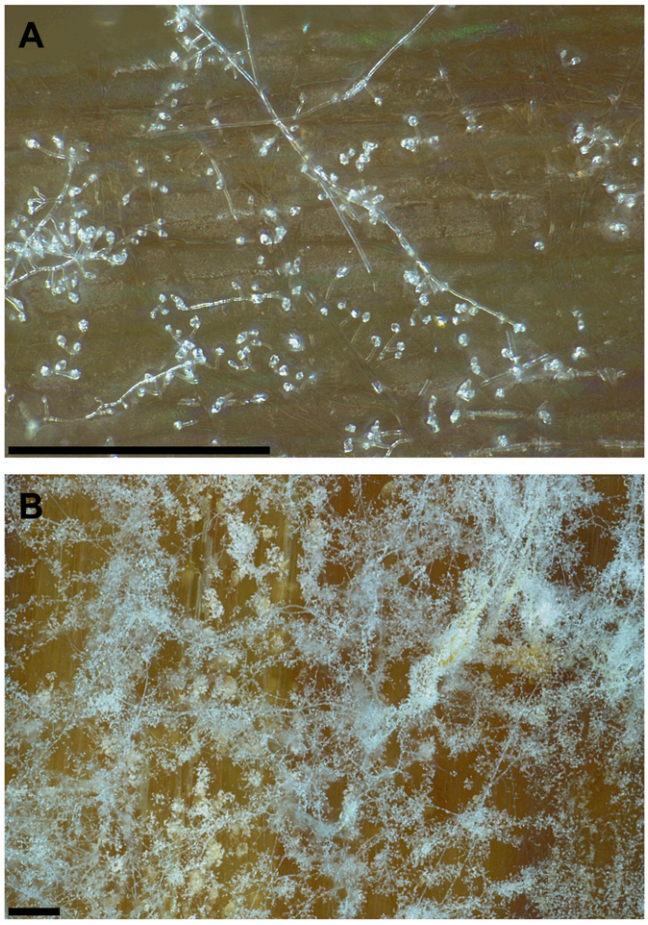

Fig. 2. A and B, Hyphae bearing abundant conidia growing out from *Bromus carinatus* leaf tissue inoculated with GL17-GH7. Tissue samples were incubated for 3 days in a moist chamber after removal from the inoculated plant. Scale bar = 250 μm.

After ≥10 days of incubation, leaf tissue was removed from inoculated plants, washed in 0.1% Tween 20 for 2 min, and placed in a moist chamber. At intervals thereafter, leaf tissues were observed microscopically, and fungal growth was photographed using a Leica DVM6 digital microscope. In addition, fungal biomass was scraped from leaf surfaces and placed on microscope slides in 50% glycerol. Slides were observed for fluorescence emanating from spores using a Leica DM5000B epifluorescent microscope with a GFP filter cube under 20× and 40× objectives.

Data analysis. All statistical analyses were conducted using the R computational language (R Core Team 2018) in the RStudio interface (RStudio Team 2016), along with the R packages agricolae (Xie 2018), knitr (de Mendiburu 2017), pander (Daróczi and Tsegelskyi 2018), and tidyverse (Wickham 2017).

Results

Isolations from plant material at Point Reyes. Branch segments were taken from nine symptomatic *P. muricata* trees. Twelve samples of *B. carinatus* and six of *H. lanatus* were each taken as flowering/fruiting shoots, pooling from plants growing within 3 m of a location marked by GPS coordinates (Table 1). From those samples, 117 *P. muricata* segments, 1,523 *B. carinatus* segments, and 504 *H. lanatus* segments were cultured on FSM. We retained all isolates recovered from grasses and a subset of those from *P. muricata*, which were representative of the range of GPS locations sampled. Based on morphological criteria observed on CLA, isolates from *P. muricata* (n = 16), *H. lanatus* (n = 3), and *B. carinatus* (n = 15) were identified as *F. circinatum* (Table 1).

Somatic compatibility testing. All isolates were somatically compatible with California tester strains associated with SCG C1 (Table 1), with the exception of isolate GL1934. Five GL1934 *nit* mutants failed to strongly pair with tester strains previously used by Wikler et al. (2000), with three mutants showing no compatibility with testers and two mutants showing only weak pairings with testers. We consequently did not assign GL1934 to an SCG. However, a second isolate recovered from the same *P. muricata* tissue segment (GL1935) strongly paired with SCG C1 testers. Positive control pairings consistently showed a compatibility reaction, whereas negative control pairings did not show a compatibility reaction.

Pathogenicity testing. In the initial test, all candidate *F. circinatum* isolates induced yellowing needles on at least one tree by 5 weeks post-inoculation. All but two isolates (GL1922 and GL1924) induced a lesion of at least 15 mm in length. All grass isolates produced a lesion ≥18 mm in length on at least one tree. All isolates included in a second round of testing induced the expression of resin at the site of inoculation (Fig. 1). A two-way analysis of variance (ANOVA) of lesion length data showed the experiment × isolate interaction not to be significant (P = 0.761) so data were pooled across experiments. Based on a one-way ANOVA, isolate was not a significant source of variation in lesion length (P = 0.346), indicating that the virulence of grass isolates was not significantly different than an isolate from pine.

Growth from inoculated *B. carinatus* leaf tissue. After 1 to 3 days of incubation in moist chambers, sporulation characteristic of *F. circinatum* was observed on leaves inoculated with GL17-GH7 (Fig. 2), but not on leaves of negative control plants. No fluorescence was observed from fungal biomass taken from negative control leaves, whereas GFP fluorescence was observed in spores and hyphae scraped from leaf tissue inoculated with GL17-GH7 (Fig. 3). Fluorescence was only observed when samples were illuminated with wavelengths required for excitation of GFP, no fluorescence was observed at wavelengths required for excitation of red or yellow fluorescent proteins, suggesting that observed fluorescence was from GFP and not a result of autofluorescence.

Discussion

Our observations are consistent with previous reports that *F. circinatum* can infect *H. lanatus* (Swett and Gordon 2012). In addition,

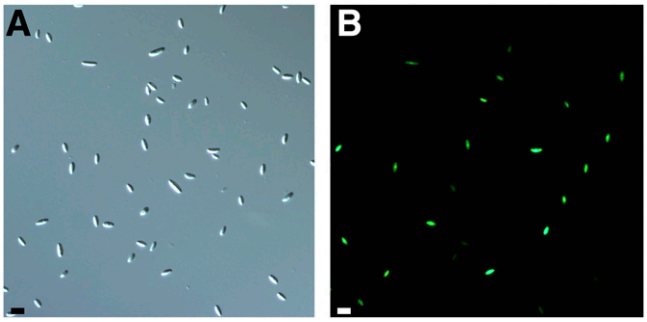

Fig. 3. Spores scraped from fungal growth on *Bromus carinatus* leaves previously inoculated with GL17-GH7. **A,** Illuminated for differential interference contrast optics. **B,** Illuminated for green fluorescent protein fluorescence. Scale bar = 10 μm.

we provide evidence that the native California grass, *B. carinatus*, is also a host of *F. circinatum* at Point Reyes and presumably elsewhere as well. Isolates of *F. circinatum* were obtained exclusively from symptomless grasses, but all isolates induced symptoms of pitch canker when inoculated into *P. radiata*.

Research in Spain has shown that nongrass species can also host cryptic *F. circinatum* infections (Hernandez-Escribano et al. 2018). These findings expand our view of the potential for the pitch canker pathogen to exist as an endophyte in plants that manifest no symptoms. This calls into question what constitutes the principal ecological activity of *F. circinatum*. Given the apparent breadth of its host range, it is possible that commensal relationships are the norm for this fungus and that virulence to pine is not central to its life history, and it may be a relatively recent evolutionary innovation. This possibility is supported by the observation that two rounds of selection for low virulence to *P. radiata* resulted in a significant reduction in mean lesion length, with 83% of F_3 generation progeny being avirulent (Slinski et al. 2016). This finding suggests that *F. circinatum* has not been subjected to strong selection for virulence to pine.

If grasses and other symptomless hosts are of primary importance in the life history of *F. circinatum*, the fungus must be able to perpetuate itself on at least a subset of plant species that can be infected. This could be accomplished by vertical transmission, a possibility suggested by recovery of *F. circinatum* from seed of *Hypochaeris radicata* (Hernandez-Escribano et al. 2018). In addition, horizontal transmission could occur if the fungus can sporulate on an infected host.

Our findings show that *B. carinatus* can support sporulation of *F. circinatum* when infected tissue is incubated in a moist chamber. Growth and sporulation were observed on both green and senescing leaf tissue, indicating that grasses may be reproductive hosts for *F. circinatum.* If so, spores produced on *B. carinatus* could serve as inoculum for other cryptic hosts and susceptible pine species. Sporulation of *F. circinatum* was not visible to the unaided eye on grass leaves at Point Reyes, perhaps because collections were made during the hot, dry summer. Based on our results, it may be possible for *F. circinatum* to emerge from colonized leaves and sporulate on leaf surfaces under the cool, moist conditions that commonly occur in winter and spring on the California coast.

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