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Potential for outcrossing in an apparently asexual population of *Fusarium circinatum*, the causal agent of pitch canker disease

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Abstract: *Fusarium circinatum* (teleomorph = *Gibberella circinata*) is responsible for the current epidemic affecting pine trees in native and urban forests along California's central coast. Only eight vegetative compatibility groups have been recovered from samples collected throughout the pathogen's range in California. This low level of diversity is suggestive of an asexually propagating population. However, crosses conducted in the laboratory on carrot agar, as well as on Monterey pine (*Pinus radiata*) twigs, reveal that California strains of the fungus are capable of undergoing sexual reproduction. Outcrossing was confirmed by demonstrating vegetative incompatibility between the progeny and their parents. These results indicate that sexual reproduction is possible within the California population of the pitch canker pathogen.

Key Words: forest pathology, fungal pathogen, *Gibberella circinata*, *Pinus radiata*

INTRODUCTION

Fusarium circinatum Nirenberg & O'Donnell [= *F. subglutinans* (Wollenw. & Reinking) Nelson et al. f. sp. *pini*], the causal agent of pitch canker disease, is spreading rapidly through both native and urban pine forests in coastal California. Pitch canker was described in the southeastern United States in 1946 (Hepting and Roth 1946) and was first observed on the west coast in 1986, when the fungus was isolated from a diseased Monterey pine (*Pinus radiata*) in Santa Cruz County, California (McCain et al 1987). The disease is now found as far south as San Diego Co., near the Mexican border, where it occurs in ornamental plantings, and as far north as Mendocino County, where native Bishop pine (*P. muricata*) is infected. The fungus can infect most pine species, but is particularly virulent on *P. patula* and *P. radiata* (Viljoen et al 1995, Gordon et al 1998a). *Fusarium circinatum* has also been reported to infect Douglas-fir (*Pseudotsuga menziesii*) (Storer et al 1995).

Based on collections from throughout the known range of the pathogen in California, a total of eight vegetative compatibility groups (VCG) have been identified (Correll et al 1992, Gordon et al 1996). Data obtained using polymorphic molecular markers show that isolates associated with the same VCG are identical at seven loci, as would be expected for a clonally propagating population (Wikler and Gordon 2000). The population in the southeastern USA is much more diverse; 45 VCG were found among 117 isolates collected in Florida (Correll et al 1992). This large number of VCG may indicate that the fungus has recently undergone outcrossing (Leslie 1993). However, the sexual state has not been observed in nature, and there are no reports of the fungus crossing on its natural substrate, i.e., pine trees.

Formation of perithecia in the laboratory has been reported (Britz et al 1998, Viljoen et al 1997), and the name *Gibberella circinata* was proposed for the teleomorph (Nirenberg and O'Donnell 1998). The sexual state has also been designated as mating population H in the *Gibberella fujikuroi* complex (Britz et al 1999). However, only two crosses between isolates of *F. circinatum* have been confirmed as outcrosses, and each of these crosses had a parent in common (Britz et al 1999). Because selfing is known among members of the *G. fujikuroi* complex (Britz et al 1999), further confirmation of the recombinant nature of the crosses is needed to establish the validity of this mating population. Furthermore, the potential for outcrossing within the California population is in doubt, as previous attempts to cross California isolates have been unsuccessful (Britz et al 1999, Correll et al 1992). Here we report that California isolates of the pitch canker pathogen are interfertile, based on laboratory crosses on carrot agar as well as on pine twigs.

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TABLE I. Identification of strains used to assess fertility in *F. circinatum* (teleomorph = *G. circinata*)

VCG	Strain designation	Phenotype	Use in study[a]
C1	A439/NitM	Nit M	female and VCG tester
C1	A439/*nit* 1	*nit* 1	male and VCG tester
C2	A362/NitM	Nit M	female and VCG tester
C2	A362/*nit* 1	*nit* 1	male and VCG tester
C3	SK2/NitM	Nit M	female and VCG tester
C3	SK2/*nit* 1	*nit* 1	male and VCG tester
C3	52/NitM	Nit M	female
C3	52/*nit* 1	*nit* 1	male
C3	fsp52	wild-type	female and male
C4	39/NitM	Nit M	female and VCG tester
C4	90/*nit* 1	*nit* 1	male and VCG tester
C4	fsp90	wild-type	female and male
C5	LA-4/NitM	Nit M	female and VCG tester
C5	LA-4/*nit* 1	*nit* 1	male and VCG tester
C5	LA-4	wild-type	female and male
C6	115/NitM	Nit M	female and VCG tester
C6	115/*nit* 1	*nit* 1	male and VCG tester
C6	118/NitM	Nit M	female
C6	118/*nit* 1	*nit* 1	male
C7	44/NitM	Nit M	female and VCG tester
C7	44/*nit* 1	*nit* 1	male and VCG tester
C8	63/NitM	Nit M	female
C8	63/*nit* 1	*nit* 1	male
C8	fsp63	wild-type	female and male
C8	fsp188	wild-type	female and male

[a] Strains were used either as males and/or females in crossing experiments, and in some cases also as VCG tester strains to assess vegetative compatibility between parents and progeny.

MATERIALS AND METHODS

Terminology.—To distinguish between assessments of sexual mating compatibility and vegetative compatibility, the former are referred to as crosses and the latter as pairings. Furthermore, two isolate designations separated by an x indicate the combination of isolates used in a given cross.

Isolates.—The complete list of isolates used to test for mating and vegetative compatibility is shown in TABLE I. For the initial set of crosses, one isolate was selected to represent each of the eight VCG known to occur in California (designated C1–C8), and these isolates were crossed in all possible combinations (see TABLE II). If the original representatives were infertile in all combinations, one or more additional strains were tested for fertility (see TABLE II). Additional strains were included in crossing experiments but not tested in all combinations with representatives of other VCG (see TABLE III).

Nitrate nonutilizing (*nit*) mutants were used for the crosses, except where otherwise noted, to facilitate collecting *nit* progeny that could be tested for vegetative compatibility with the parental VCG. The *nit* mutants were generated by culturing wild type isolates on a minimal medium amended with chlorate (3%) and asparagine (0.35 g/L) and retrieving the fast growing sectors (Puhalla 1985). For each combination of VCG, both of the isolates used were tested as the male and the female parent. The same isolate was used as the VCG representative for each gender of a cross. When *nit* mutants were used as the parental strains, females were all NitM (e.g., 63/NitM, where 63 refers to the wild type fsp63) and the males were all *nit*1 (e.g. 63/*nit*1, where fsp63 was the wild type). The only exception to this was in VCG C4, where 39/NitM was used as the female parent and 90/*nit*1 was used as the male. *Nit* mutant phenotypes were identified using the criteria described by Correll et al (1987).

Crosses on carrot agar.—Strains that served as female parents were grown on carrot agar and those that served as males were grown on complete medium slants; the crosses were performed as described by Klittich and Leslie (1988). The cultures were incubated in a growth chamber with a temperature and light regime that alternated every 12 h from 20 C with cool white and black lights to 15 C without light. After one wk, a spore suspension of the male isolate was prepared using 1.5 mL of 2.5% Tween 60. One mL of this suspension was poured onto the (female) isolate growing on carrot agar, and spread with a glass rod. After this "fertilization," plates were returned to the growth chamber.

Each combination of isolates was crossed at least twice. A VCG C3 (fsp52 or 52/NitM, female) × VCG C4 (fsp90 or 90/*nit*1, male) combination was included as a positive control for every set of crosses. Because the control combination never failed to produce fertile perithecia under the conditions described, this successful cross was repeated more than twenty times. Isolates representing VCG C5 (LA-4/NitM and LA-4/*nit*1) and VCG C8 (63/NitM and 63/*nit*1), were infertile in all combinations when crossed as *nit* mutants, so crosses were also performed using the corresponding wild type isolates. Because the crosses with the wild type C8 isolate were consistently infertile, all C8 crosses were repeated using a second wild type isolate (fsp188) associated with the same VCG.

Crosses on a natural substrate.—Five Monterey pine branch segments, 3–5 cm long and 8–12 mm diam, were surface sterilized in 1% NaOCL for 2 min and coinoculated with the two isolates that produced the most fertile crosses on carrot agar (judged by number and size of perithecia). Mycelial plugs of these isolates, fsp52 (VCG C3) and fsp90 (VCG C4), were inserted into a single, small wound created with a drill bit (1.6 mm diam). The twigs were placed in petri dishes with water-saturated filter paper, to maintain high relative humidity.

Confirmation of outcrossing and recombination.—Evidence for recombination was obtained by determining if the progeny were vegetatively compatible with their parents (see TABLES II, III and IV for the crosses from which progeny were collected). Progeny were collected by immersing the cirri oozing out of a perithecium in 1 mL of sterile water and spreading the ascospore suspension onto water agar plates. The following day, hyphal tips from germinating spores were transferred to PDA plates. The progeny were then

TABLE II. Fertility among *F. circinatum* strains representing all known California VCGs

		Females										
VCG		C1	C2	C3	C4	C5	C5	C6	C7	C8	C8	C8
Males	Strains crossed	A439/ NitM	A362/ NitM	SK2/ NitM	39/ NitM	LA-4/ NitM	LA-4	118/ NitM	44/ NitM	63/ NitM	fsp63	fsp188
C1	A439/*nit* 1	–[a]	–	+	–	–	–	–	–	–	–	–
C2	A362/*nit* 1	–	–	+	–	–	–	–	–	–	–	–
C3	SK2/*nit* 1	+* (4)	+* (2)	–	+	–	–	+	–	–	–	–
C4	90/*nit* 1	–	–	+	–	–	–	–	–	–	–	–
C5	LA-4/*nit* 1	–	–	–	–	–	NT	–	–	–	NT	NT
C5	LA-4	–	–	+* (4)	–	NT	–	–	–	–	–	–
C6	118/*nit* 1	–	–	+	–	–	–	–	–	–	–	–
C7	44/*nit* 1	–	–	+* (1)	–	–	–	–	–	–	–	–
C8	63/*nit* 1	–	–	–	–	–	–	–	–	–	NT	NT
C8	fsp63	–	–	–	–	NT	–	–	–	NT	–	NT
C8	fsp188	–	–	–	–	NT	–	–	–	NT	NT	–

[a] "–" indicates a strain combination where no perithecia developed, or if they did, no ascospores oozed out from the ostiole; "+*" indicates that perithecia formed and exuded ascospores, and recombination was confirmed by demonstrating vegetative incompatibility between progeny and parental VCG (the number of perithecia from which ascospores were collected follows in parentheses); "+" denotes crosses where perithecia exuded ascospores, but the progeny were not tested to confirm outcrossing; and "NT" indicates a combination that was not tested.

transferred to Czapek agar (Difco Laboratories) to determine if they inherited either of the nitrate nonutilizing phenotypes. The *nit* progeny were then paired with both a *nit*1 and a NitM tester strain from both parental VCG, on Czapek agar, to assess vegetative compatibility. To verify recombination in the wild type × wild type cross (fsp52 × fsp90), *nit* mutants of 19 wild type progeny were generated, as previously described, and paired with a *nit*1 and NitM mutant tester from each parental VCG. To provide further confirmation of outcrossing, evidence for recombination of the alleles at loci conferring the *nit*1 and NitM phenotypes was obtained by enumerating the recovery of wild type progeny in four additional crosses (see TABLE IV).

TABLE III. Additional fertile crosses between strains of *F. circinatum*

Female strain[a]	Male strain[a]	Outcome[b]
fsp52 (C3)	fsp90 (C4)	+* (7)
52/NitM (C3)	90/*nit* 1 (C4)	+* (5)
52/NitM (C3)	118/*nit* 1 (C6)	+* (3)
fsp90 (C4)	fsp52 (C3)	+
SK2/NitM (C3)	fsp90 (C4)	+
fsp52 (C3)	90/*nit* 1 (C4)	+
39/NitM (C4)	52/*nit* 1 (C3)	+
115/NitM (C6)	SK2/*nit* 1 (C3)	+
118/NitM (C6)	fsp52 (C3)	+
SK2/NitM (C3)	115/*nit* 1 (C6)	+
52/NitM (C3)	115/*nit* 1 (C6)	+

[a] VCG given in parentheses following strain.

[b] Outcome either "+*," where perithecia exuded ascospores, and recombination was confirmed by demonstrating vegetative incompatibility between progeny and parental VCGs (the number of perithecia from which ascospores were collected follows in parentheses); or "+," where perithecia exuded ascospores, but the progeny were not tested to confirm outcrossing.

RESULTS

Crosses on carrot agar.—For each California VCG, one or more isolates were fertile in at least one cross, except for VCG C8. Those combinations that were verified as outcrosses by assessing the progeny for vegetative compatibility with the parental VCG are shown in TABLES II and III. Because fsp52 crossed with the H− mating type tester, and fsp90 crossed with the H+ mating type tester (Britz et al 1999), the pattern of fertility among strains in this study indicated that isolates associated with VCG C3 correspond to the H+ mating type, and the fertile strains associated with the other VCG correspond to the H− mating type.

In fertile crosses, two-celled ascospores exuded from the ostioles of mature perithecia about 6 wk after they were fertilized. Crosses were rated as infertile unless they produced perithecia that oozed ascospores from their ostioles, based on the criteria established by Leslie (1995). However, some infertile combinations occasionally made perithecia, but they were small and never produced cirri. We initially observed that one out of twelve crosses between 118/NitM (VCG C6) × 90/*nit*1 (VCG C4) was weakly fertile (perithecia were small and few in number). This

TABLE IV. Recovery of *nit* mutant and wild-type progeny of *F. circinatum* from crosses of parental strains with complementary *nit* mutant phenotypes

Cross[a]		Number of progeny examined[b]				Number of progeny vegetatively compatible with parents	
Female parent	Male parent		*nit* 1	NitM	Wild-type	Female parent	Male parent
A362/NitM (C2)	SK2/*nit* 1 (C3)	148	43	67	38	1	0
118/NitM (C6)	SK2/*nit* 1 (C3)	147	34	83	30	2	0
A439/NitM (C1)	SK2/*nit* 1 (C3)	145	32	76	37	0	0
52/NitM (C3)	90/*nit* 1 (C4)	148	51	64	33	2	0

[a] The vegetative compatibility group for each parent is shown in parentheses.

[b] For each cross, between 45 and 50 ascospores were collected from each of three different perithecia.

result was anomalous because the two strains should have been associated with the same mating type based on their interactions with other strains. Consequently, this cross was repeated eight additional times, but there were no further indications of fertility. Therefore, this combination of strains is reported as infertile (TABLE II).

Not all mating-compatible combinations were equally prolific. Crosses between VCG C3 (female) and VCG C4 (male), regardless of which isolates were used, and between VCG C3 (male or female) and VCG C6 (male or female), regardless of which isolates were used, always produced abundant oozing perithecia. In contrast, very few perithecia were produced in the SK2/NitM (VCG C3) × LA-4 (VCG C5) and SK2/NitM (VCG C3) × 44/*nit*1 (VCG C7) crosses, compared to the C3 (female) × C4 (male) and C3 × C6 crosses.

No fewer than 12 progeny, and as many as 36, were collected from each fertile combination listed in Tables II and III. Except as noted below, the *nit* progeny in all crosses were vegetatively incompatible with the *nit*1 and NitM testers from both parents, indicating that recombination of alleles at the loci affecting vegetative compatibility had occurred. Such recombination could only result from outcrossing. The SK2/NitM (VCG C3) × LA-4 (VCG C5) cross, in which the C5 parent was wild type and the C3 parent was a *nit* mutant, was verified as an outcross, because none of the 12 progeny with the *nit* phenotype was vegetatively compatible with either the *nit*1 or NitM testers of VCG C3.

Further verification of outcrossing was provided by the recovery of wild type progeny in crosses where the two parents had complementary *nit* phenotypes (TABLE IV). A minimum of 145 ascospore progeny was examined for each of four crosses. Between 20 and 26% of the progeny were wild type, 22–34% were *nit*1 and 43–56% were NitM (TABLE IV). Nearly all of the progeny were vegetatively incompatible with both parents (TABLE IV).

In the fsp52 (VCG C3) and fsp90 (VCG C4) cross, three of the 19 progeny were vegetatively compatible with a VCG C3 tester; however, the remainder was not compatible with either parental VCG. All three VCG C3 compatible progeny were obtained from perithecia that also included progeny determined to be incompatible with both parents.

Crosses on a natural substrate.—Dozens of fertile perithecia formed on all five twigs that were coinoculated with fsp52 and fsp90, reaching maturity 7–8 wk after inoculation. Although needles were removed from most of the inoculated twigs, where they were left on, perithecia formed on the needles as well as on the twig.

DISCUSSION

This study shows that California isolates of the pitch canker pathogen have the ability to outcross and give rise to VCG that differ from their parents'. If selfing had occurred, all the progeny should be vegetatively compatible with a single parent. On the other hand, outcrossing would be expected to result in recombination of the alleles at loci affecting vegetative compatibility (*het* or *vic* loci) (Leslie 1993). Where the parents differ at two or more of these loci, sexual recombination can generate progeny that are vegetatively incompatible with their parental VCG. Thus, where some or all of the progeny from a cross were vegetatively incompatible with both parents we conclude that outcrossing occurred and not selfing (TABLES II, III).

Assuming the genes responsible for the *nit* 1 and NitM phenotypes are unlinked, some progeny would

inherit both the *nit* 1 and NitM traits. Consequently, some of the progeny judged to be vegetatively incompatible with both parents might have failed to produce wild type growth at the interface of the pairing because they were double *nit* mutants, rather than because they were incompatible. Thus, the frequency with which progeny were incompatible with both parents may have been overestimated. However, for a double *nit* to be borne, recombination would have been required, again confirming that meiosis occurred during the cross. That such recombination did occur is further supported by the recovery of between 20 and 26% wild types from progeny of crosses between parents with complementary *nit* phenotypes (TABLE IV); this approximates the frequency expected for independent segregation of alleles at the loci affecting nitrate utilization. The high frequency of NitM phenotypes in the progeny (average of four crosses = 49.5%) is consistent with the inclusion of double *nit* mutants in this group.

Previous indications of infertility between isolate combinations that proved to be fertile in this study may reflect differences in the temperatures at which crossing plates were incubated. In the present work, a temperature of 20 C for 12 h alternating with 15 C for 12 h was used, in contrast to a constant temperature of 25 C in previous work, which found California isolate combinations to be infertile (Correll et al 1992). Similarly, Covert et al (1999) reported that crosses of *G. circinata* incubated at 20 C yielded viable ascospores, whereas the same combination of isolates incubated at 25 C did not.

On repetition, most of the crosses reported here gave consistent results. A notable exception was 118/NitM (VCG C6) and 90/*nit*1 (VCG C4), which appeared weakly fertile on only one occasion. Because the majority of the progeny collected from this cross were incompatible with both parental VCG, fertility could not be attributed to selfing. Given that the isolates in question are associated with the same mating type, chance contamination by a different strain is the simplest explanation for the original observation of fertility. However, attempts to document the presence of a contaminating strain were not successful, as 37 single conidial isolates from a fertile crossing plate were all associated with one of the parental VCG.

Previous findings support the view that the California population of *F. circinatum* is propagating clonally (Correll et al 1992, Gordon et al 1996, Wikler and Gordon 2000). If so, it may be due, in part, to the limited cooccurrence of opposite mating types, which can be inferred from the reported distribution of VCG in California (Gordon et al 1996). Two of the three sites where sexually compatible strains have been recovered were Christmas tree farms that are no longer planted to pine. At the third site, where C1 and C4 predominate, C3 (opposite in mating type to C1 and C4) has been recovered only infrequently. However, as the disease spreads, more extensive intermingling of sexually compatible strains may increase the likelihood of sexual reproduction. Of course, barriers to sexual reproduction not discernible from laboratory experiments may preclude outcrossing in nature, notwithstanding the cooccurrence of opposite mating types.

To the extent that recombination does occur in the California population of the pitch canker pathogen, it may have implications for disease control in the future. Resistance to the prevalent strains of *F. circinatum* has been identified (Gordon et al 1998b) and this is likely to be the principal tool for limiting damage caused by pitch canker to Monterey pine. If recombination increases the frequency with which new pathotypes are generated, it may adversely affect the durability of genetic resistance in Monterey pines, in both landscape and native settings.

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