Selective Isolation Procedures for Differentiation of Two Strains of *Colletotrichum gloeosporioides* from Citrus

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ABSTRACT

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Citrus postbloom fruit drop is caused by a slow-growing orange strain of *Colletotrichum gloeosporioides* (SGO). A semiselective medium was developed to isolate the pathogen and differentiate it from the ubiquitous fast-growing gray saprophytic strain of *C. gloeosporioides* (FGG). Addition of streptomycin to potato-dextrose agar (PDA) at 300 mg/L suppressed contaminants without affecting growth of either strain of *C. gloeosporioides.* When plates were incubated for 4 days at the optimum temperature of 27 C for growth of the two strains, FGG colonies often overgrew SGO colonies. Incubation of plates for 4 days at 18 C restricted colony size of both isolates. When incubated for one more day at 27 C, SGO colonies produced abundant orange conidia, whereas FGG colonies developed characteristic dark pigmentation of hyphae. Addition of copper hydroxide (42 mg/L Cu) to PDA + streptomycin reduced contamination only slightly but enhanced orange color development of SGO colonies, facilitating counting of colonies. Plating of citrus tissue washings on PDA + streptomycin + copper hydroxide and incubation for 4 days at 18 C plus 1 day at 27 C optimized recovery of the SGO isolates.

Citrus postbloom fruit drop (CPFD) is caused by a strain of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (7). The fungus infects flower petals, inducing peach- to orange-colored necrotic spots and drop of fruitlets. The peduncle, calyx, floral disk, and nectaries remain to form the persistent "buttons" that are characteristic of the disease (7,13).

The disease was first described in Belize in 1979 (7) but has subsequently been reported from many citrus-growing areas of Central and South America, the Caribbean, and Florida (4,11,13). Little is known about the survival of the fungus between bloom periods or the population dynamics of the fungus in the canopy of citrus trees. Denham and Waller (5) suggested that the fungus persists as conidia on the leaf surface or as latent infections. Studies conducted in Florida (L. W. Timmer, *unpublished*) and in Belize (9) using spore traps to collect rainwater runoff from the canopy were hampered by the inability to distinguish the strain of *C. gloeosporioides* causing CPFD from the ubiquitous, saprophytic strain of the fungus that reproduces on senescent and necrotic citrus tissues (14).

In culture, the strains causing CPFD produce slow-growing orange colonies (SGO) whereas the saprophytic form produces fast-growing gray colonies (FGG) (7,8,12). These strains also differ in conidia size and shape, setae formation, and appressoria size and shape, as well as in pathogenicity (1). However, conidia of the two strains are not readily distinguishable microscopically and mycelial fragments and other possible propagules of the pathogenic strain cannot be identified microscopically.

To facilitate studies of survival and population dynamics of *C. gloeosporioides* on citrus, we developed a semiselective medium for the fungus and devised procedures to readily enumerate colonies of the two strains commonly found on citrus.

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MATERIALS AND METHODS

Growth of strains of *C. gloeosporioides* on different media plus additives. Three single-spored isolates of the SGO strain of *C. gloeosporioides* were obtained from infected flowers from sweet orange (*Citrus sinensis* (L.) Osbeck) orchards near La Belle, Indiantown, and Lake Placid, Florida, and two isolates of the FGG strains were obtained from necrotic twigs and leaves of sweet orange trees near Lake Alfred not affected by CPFD. Growth of the isolates was compared on potato-dextrose agar (PDA), oatmeal agar, cornmeal agar, and *Pseudomonas* F agar.

The effects of the following ingredients were evaluated on PDA at the indicated concentrations per liter: streptomycin sulfate, 300 mg; vancomycin hydrochloride, 250 mg; kanamycin monosulfate, 500 mg; a mixture of rifampicin, 25 mg, and ampicillin, 125 mg; iprodione (Rovral 50WP), 15 mg; and copper hydroxide, 42 mg Cu (Kocide 101). Appropriate amounts of these ingredients were dissolved or suspended in 10 ml of sterile distilled water and added to PDA that had been cooled to about 50 C. The pH of the media was about 5.3 with or without additives.

All isolates were transferred to three plates of each medium and incubated for 96 hr at 27 C; colony diameters were then measured. The experiment was repeated three times, and the means of the three experiments are reported.

Effect of temperature. Eight isolates of the SGO strain and four isolates of the FGG strain recovered from various citrus orchards around Florida were grown at different temperatures. Three PDA plates of each isolate were incubated at 10, 15, 18, 23, 27, 30.5, and 33.5 C for 7 days. Colony diameters were measured and the areas calculated.

Evaluation of the semiselective medium. Composite samples of leaves, twigs, buttons, and leaf debris were collected from 15 trees each in two sweet orange orchards near Frostproof and Lake Placid. Disks 1 cm in diameter were cut at random from the leaf samples, twigs were cut into pieces 1–2 cm long, whole buttons were separated from the other tissues, and leaf debris was broken into small pieces. Eight 0.5-g subsamples of leaves, twigs, or buttons from each orchard were placed in a flask with 20 ml of sterile distilled water and agitated on a mechanical shaker for 15 min. Then, 0.3 ml of a 1:100 dilution was plated on PDA, PDA + streptomycin (S), PDA + copper hydroxide (Cu), and PDA + S + Cu. Four 20-g subsamples of leaf debris from each orchard were added to 60 ml of sterile distilled water and shaken for 15 min. The suspension was then filtered through cheesecloth and centrifuged at 5,640 *g* for 15 min. The supernatant was discarded and the pellet

Table 1. Effect of culture medium on growth of slow-growing orange (SGO) and fast-growing gray (FGG) strains of *Colletotrichum gloeosporioides*

	Colony area (cm^2)	
Medium	**SGO**	**FGG**
Potato-dextrose	5.7 a[z]	18.1 a
Cornmeal	3.8 b	14.1 b
Oatmeal	6.2 a	20.4 a
Pseudomonas F	5.1 ab	11.7 c

[z] Mean separation by Duncan's multiple range test, $P \leq 0.05$.

resuspended in 10 ml of sterile water, diluted to 10^{-3}, and plated as described. Plates were incubated for 4 days at 18 and 27 C. Since results from both orchards were similar, means from the two were calculated and expressed as propagules per gram fresh weight of tissue.

The percentage of the surface area of each plate that was contaminated was estimated by placing the plates on a backlit grid of 1-cm squares. Areas covered by fungal and bacterial colonies other than *C. gloeosporioides* were considered contaminated even if overgrown by colonies of *C. gloeosporioides*.

RESULTS AND DISCUSSION

Colletotrichum spp. grow rapidly on a wide range of media, and highly specialized selective media are not generally required for isolation of these fungi. Addition of common antibiotics is usually sufficient to suppress growth of bacteria and some aggressive fungi (2,3,6).

Isolates of *C. gloeosporioides* from citrus grew most rapidly on PDA and oatmeal agar (Table 1). SGO and FGG isolates grew more slowly on cornmeal agar than on other media, and FGG isolates grew more slowly on *Pseudomonas* F agar than on PDA or oatmeal agar. PDA was chosen for further studies.

Of the additives, only iprodione and the combination rifampicin + ampicillin suppressed growth of the SGO and FGG isolates at the concentrations tested (Table 2). The addition of streptomycin was adequate to suppress rapidly growing bacteria and some fungal contaminants without affecting the growth of either strain of *C. gloeosporioides*.

On PDA + S, the FGG isolates rapidly covered the plates and made SGO colonies difficult to identify and count when plates were incubated at room temperature (22–25 C). The optimum temperature for growth of several isolates of the two strains was determined in an attempt to use temperature to manipulate growth rates (Fig. 1). There were no significant differences in growth among isolates of the same strain, so the means for SGO and FGG strains are presented. Both strains had an optimum temperature for growth of 27 C. Growth curves at different temperatures were similar except that the FGG isolates grew faster than the SGO isolates at 30.5 and 33.5 C.

When leaf washes from CPFD-affected orchards were plated on PDA without additives and incubated for 4 days at 18 C plus 1 day at 27 C, neither SGO nor FGG colonies were readily discernible and plates were heavily contaminated

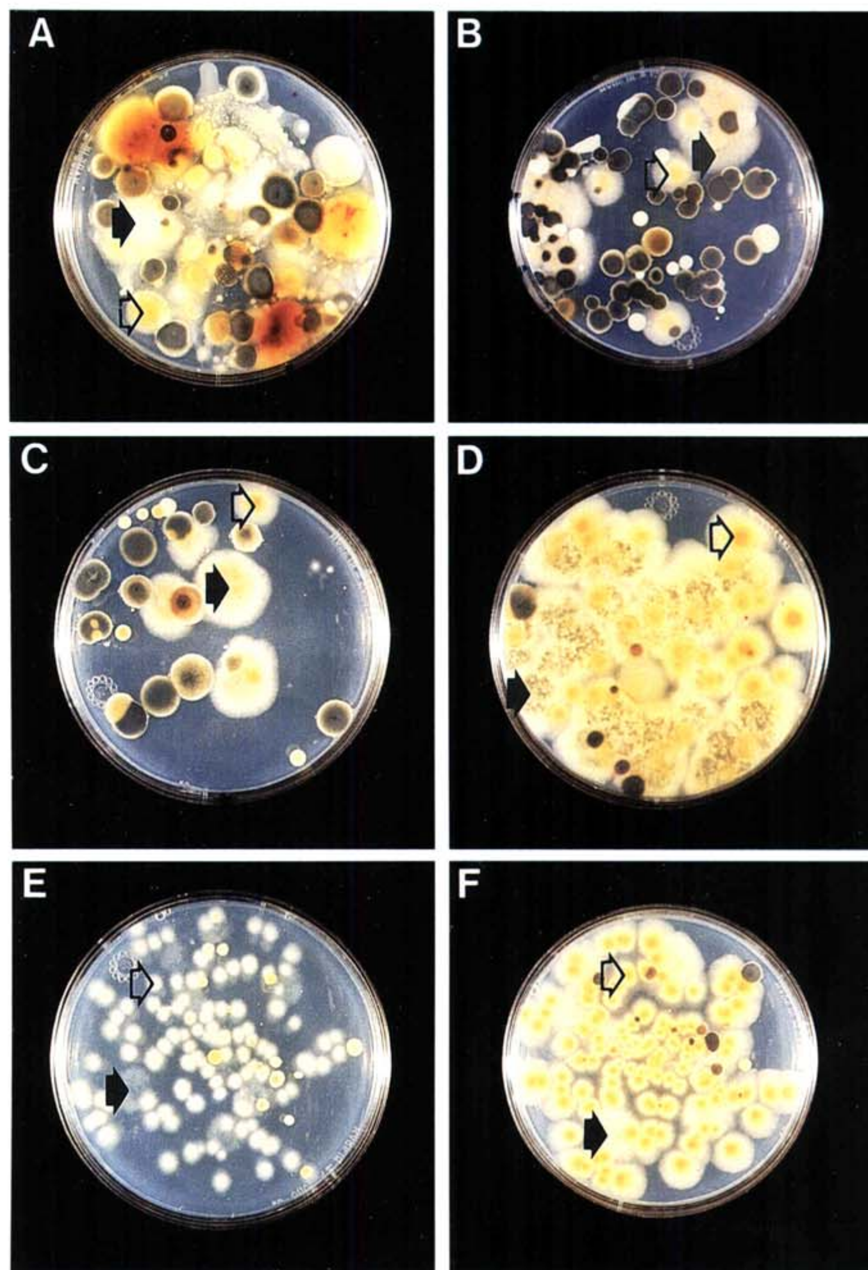

Fig. 2. Colony morphology of the slow-growing orange (SGO) and fast-growing gray (FGG) strains of *Colletotrichum gloeosporioides* from washings of citrus tissues plated on various media and incubated under different temperature regimes. Washings were from various sources, and the number of colonies are not indicative of the effect of the medium or temperature regime. Open arrows = SGO colonies, closed arrows = FGG colonies. **(A)** Potato-dextrose agar (PDA) without additives; SGO and FGG colonies barely discernible among the contaminants. **(B)** PDA + streptomycin; note the diffuse orange color of SGO colonies, lack of pigmentation of FGG colonies, and absence of bacteria. **(C)** PDA + copper; note the deep orange centers of SGO colonies. Plates shown in A–C were incubated 4 days at 18 C and 1 day at 27 C. **(D)** PDA + streptomycin + copper, plate incubated 4 days at 27 C; note gray pigmentation of FGG colonies and tendency to overgrow SGO colonies. **(E)** PDA + streptomycin + copper, plate incubated 4 days at 18 C; note small, barely identifiable colonies. **(F)** PDA + streptomycin + copper, same plate as shown in E but incubated an additional day at 27 C; note deep orange centers of SGO colonies, lack of pigmentation of FGG colonies, and small size of contaminating *Cladosporium* spp. (small dark colonies).

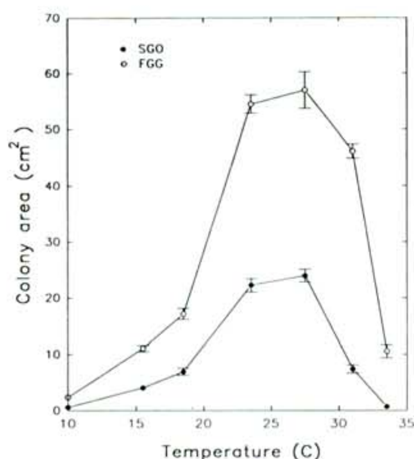

Fig. 1. Effect of temperature on growth of slow-growing orange (SGO) and fast-growing gray (FGG) strains of *Colletotrichum gloeosporioides*.

Table 2. Effect of addition of antibiotics and fungicides to potato-dextrose agar on growth of slow-growing orange (SGO) and fast-growing gray (FGG) strains of *Colletotrichum gloeosporioides*

Additive	Concentration (mg/L)	Colony area (cm^2)	
		SGO	FGG
Streptomycin	300	5.1 a[z]	17.3 a
Vancomycin	250	4.4 ab	15.4 a
Kanamycin	50	5.1 a	15.6 a
Rifampicin + ampicillin	25 + 125	4.2 b	12.9 b
Iprodione	15	0.6 c	4.4 c
Copper hydroxide	42.5	5.0 a	17.8 a
None	...	5.1 a	17.8 a

[z] Mean separation by Duncan's multiple range test, $P \leq 0.05$.

Table 3. Recovery of slow-growing orange (SGO) and fast-growing gray (FGG) isolates of *Colletotrichum gloeosporioides* from washings of various citrus tissue sources on different media at 18 and 27 C

Tissue source	Medium[w]	SGO[x]		FGG[x]		Contamination[y]	
		18 C	27 C	18 C	27 C	18 C	27 C
Leaves	PDA	10.5	6.3	12.5	75.0	93	92
	PDA + Cu	25.0	18.8	37.5	37.5	88	93
	PDA + S	0.0	12.5	50.0	87.5	38	65
	PDA + Cu + S	33.8	75.0	75.0	162.5	25	45
Twigs	PDA	0.0	0.0	50.0	0.0	94	99
	PDA + Cu	25.0	12.5	75.0	100.0	95	95
	PDA + S	0.0	25.0	12.5	50.0	31	55
	PDA + Cu + S	66.6	62.5	50.0	112.5	20	60
Buttons	PDA	125.0	125.0	75.0	250.0	96	94
	PDA + Cu	137.5	75.0	300.0	287.5	93	97
	PDA + S	250.0	300.0	137.5	200.0	33	35
	PDA + Cu + S	350.0	337.5	116.6	412.5	21	42
Debris	PDA	2.8	0.0	5.0	8.3	75	99
	PDA + Cu	0.0	0.0	4.2	27.7	48	52
	PDA + S	5.5	0.6	38.8	22.2	34	45
	PDA + Cu + S	13.8	4.2	77.8	75.8	32	38
Source		***[z]		**		***	
Medium		***		**		***	
Temperature		NS		*		***	

[w] PDA = potato-dextrose agar, Cu = copper hydroxide at 42 mg/L, S = streptomycin sulfate at 300 mg/L.
[x] Number of propagules per gram of fresh weight of tissue.
[y] Percentage of plate surface covered by contaminants.
[z] Significant at * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$; NS = not significant.

(Fig. 2A). Addition of streptomycin alone reduced bacterial and some fungal contamination, but SGO colonies had a diffuse orange color (Fig. 2B) and at times could be confused with colonies of *Fusarium* spp. Addition of copper hydroxide alone decreased contamination only slightly but produced colonies with deep orange centers that were more readily identifiable (Fig. 2C). Previous work in Belize indicated that copper fungicides enhanced sporulation of SGO strains (10). The deep orange centers of colonies on media containing Cu were probably caused by high spore concentrations. When cultures were grown for 4 days at 27 C on PDA + S + Cu, FGG colonies produced gray pigmented mycelium and tended to overgrow SGO colonies (Fig. 2D). Plates of PDA + S + Cu incubated for 4 days at 18 C had small unidentifiable colonies (Fig. 2E), but when these plates were incubated for an additional day at 27 C, SGO colonies were readily identifiable and easily counted (Fig. 2F).

Washings from leaves, twigs, buttons, and debris were plated on PDA, PDA + Cu, PDA + S, and PDA + Cu + S and incubated at 18 or 27 C to determine the effect of the medium and temperature on the recovery of the two strains and the level of contamination. The source of tissue affected the recovery of both strains, with the highest recovery from buttons (Table 3). Source of tissue also affected contamination levels but only because the samples from debris were more highly diluted. The medium also had highly significant effects on recovery of the SGO and FGG isolates and on contamination levels (Table 3). PDA and PDA + Cu were highly contaminated, and colonies of SGO were reduced in numbers and difficult to count. Addition of streptomycin reduced contamination to acceptable levels and maximized recovery of both strains. Temperature had no significant effect on the number of colonies of the SGO isolates, but the 18 C temperature significantly reduced the number of FGG colonies detected. Thus, recovery of the SGO strain was maximized by plating on PDA + S + Cu and incubating at 18 C. However, maximum recovery of both strains can be achieved by incubating two sets of plates, one at 18 C for SGO and one at 27 C for FGG.

The medium and procedures described have been used routinely to assay populations of SGO isolates from washings of various tissues from citrus trees and for direct plating of tissues. They also could be used to assay rainwater in spore trap bottles and to isolate from possible insect vectors or surfaces of equipment and tools.

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