Population Dynamics and Survival of Strains of *Colletotrichum gloeosporioides* on Citrus in Florida

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ABSTRACT

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The slow-growing orange (SGO) strain of *Colletotrichum gloeosporioides* causes postbloom fruit drop disease of citrus, whereas the fast-growing gray (FGG) strain is primarily saprophytic on senescent and dead tissue. Propagule densities of the SGO and FGG strains were compared on various tissues under field conditions and on inoculated leaves in the laboratory and greenhouse by assay on a selective medium to compare the survival of the two strains. In field studies in Florida, the SGO strain reached high populations densities on flower petals during bloom. This strain persisted on vegetative tissues, but population densities declined with time after bloom. Propagule densities of the FGG strain were relatively stable throughout the year. When conidial suspensions were sprayed onto foliage of greenhouse-grown seedlings, conidia of both strains germinated to form appressoria, but the percentage of conidia forming appressoria was much higher with the FGG strain. The number of propagules recoverable in leaf washes declined to near zero in 30 days with both strains, but these strains could still be isolated from leaf pieces. When plants that had been sprayed a month earlier with a conidial suspension were treated with water or a flower extract, water alone stimulated germination of some conidia and appressoria of both strains. The flower extract greatly increased germination of appressoria of the SGO but not the FGG strain. The SGO strain produced many new conidia on hyphae from germinating appressoria on the leaf surface in response to treatment with the flower extract, but the FGG strain produced few. The cycle of postbloom fruit drop caused by the SGO strain appears to be as follows: conidia produced in abundance in acervuli on petals are washed onto vegetative tissues, where many germinate to form appressoria and possibly some quiescent infections; appressoria survive on vegetative tissues until the next bloom, and substances from petals stimulate germination of appressoria to form conidia on the surface; these conidia are splash-dispersed to flowers and reinitiate the cycle.

Postbloom fruit drop (PFD) of citrus is caused by the slow-growing orange (SGO) strain of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (3,9,17). This strain infects petals of most citrus species producing orange- to peach-colored spots. Infection induces fruit drop and formation of persistent "buttons" consisting of the floral disc and calyx (5,9). The fast-growing gray (FGG) strain of *C. gloeosporioides* is an ubiquitous saprophyte in citrus orchards. This strain invades damaged or senescent tissue and is not considered a primary pathogen (21). However, fruit that has been subjected to ethylene degreening in the packinghouse may develop postharvest anthracnose caused by this strain (4). The strains can be distinguished by colony color and morphology, growth rate, conidium size and shape, and appressorium shape as well as pathogenicity (1,3).

The FGG strain of *C. gloeosporioides* forms appressoria on the surface of citrus tissues, with subsequent formation of infection pegs and development of quiescent infections (22). When tissues senesce, the fungus invades the tissues and produces abundant acervuli under favorable conditions (22). *C. gloeosporioides* survives and reproduces in a similar manner on many perennial tropical and subtropical crop plants (13,16). Other species of *Colletotrichum* may survive as conidia on living plant parts (11), as acervuli on plant debris on alternate hosts (12), or in soil (8). The spore matrix is important for the survival of *Colletotrichum* spp. on plant surfaces and in debris (7,14,15).

The means of survival of the SGO strain between bloom periods is unknown. Denham and Waller (6) only rarely isolated this strain from vegetative tissues. They speculated that this strain formed quiescent infections similar to those of the FGG strains on citrus and other forms of *C. gloeosporioides* on tropical crops.

Population dynamics of *C. gloeosporioides* on citrus have been difficult to study because strains could not be readily distinguished. Recently, strains have been well characterized (1,3,17), and selective isolation procedures have been developed to differentiate them (2). The objectives of this study were to follow the population dynamics of the SGO and FGG strains in citrus orchards affected by PFD and to determine the disease cycle and means of survival of the SGO strain between bloom periods.

MATERIALS AND METHODS

Population dynamics of *C. gloeosporioides* in the field. Field studies were conducted in naturally infested orchards of 20-yr-old Valencia sweet orange (*Citrus sinensis* (L.) Osbeck) near Lake

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Placid, FL; of 15-yr-old navel oranges near Frostproof, FL; and in an inoculated, experimental planting of 3-yr-old Valencia sweet orange near Gainesville, FL. Ten source trees were selected arbitrarily in each orchard, and samples were collected from the same trees at each sampling date. The number of persistent buttons was counted on four branches selected at random on each sample tree approximately 4 mo after bloom as an indication of disease severity in each orchard during each year as in previous studies (19,20). Leaves, twigs, buttons, and flowers, when available, were collected separately from all designated trees in Lake Placid and Gainesville. In Frostproof, all vegetative tissue (leaves, twigs, and buttons) were composited. Samples were collected at approximately 3-wk intervals during the bloom period and at approximately 2-mo intervals at other times of the year.

Tissues were washed and suspensions plated in the laboratory to determine the number of surface propagules, then were surface-sterilized to determine the degree to which each strain had invaded tissues. For tissue washes, 1-cm-diameter disks were cut from the leaves, and the twigs were cut into 1- to 2-cm-long pieces. Buttons and flowers were separated from twigs. A 0.5-g fresh weight sample of each tissue was transferred to 20 ml of sterile distilled water and placed on a rotary shaker for 15 min. A 0.3-ml aliquot of dilutions of 10^{-3} and 10^{-4} for petals and 10^{-1} and 10^{-2} for vegetative tissues in sterile water was pipetted onto each of three plates of a selective medium (2). The medium consisted of 39 g of potato-dextrose agar (PDA), 300 mg of streptomycin, and 85 mg of copper hydroxide (Kocide 101, Griffin Corp., Valdosta, GA) (42 mg of metallic copper) per liter (2). Plates were incubated at 18 C for 4 days followed by 1 day at 27 C to differentiate the SGO and FGG colonies. After this procedure, the SGO strain produced compact colonies with white mycelium and dense orange centers due to abundant conidium production, whereas the FGG strain produced diffuse gray colonies with few or no conidia (2). Colonies of the two strains were counted and expressed as logarithms of the number of propagules per gram fresh weight of tissue. Suspensions were observed at ×400 to determine the types of propagules washed from the tissues.

For isolations, the different tissues from each sample were surface-disinfested with 0.525% sodium hypochlorite and rinsed in sterile distilled water. Tissue pieces from each sample were plated on the selective medium. The frequencies at which the SGO and FGG strains were isolated were expressed as the mean of the percentage of tissue pieces positive for each strain after incubation as above.

Leaf debris from beneath the 10 sample trees at Lake Placid and Frostproof was collected periodically from June 1990 to December 1991; no leaf debris was available from beneath the young trees at the Gainesville site. Twenty-grams fresh weight of debris was added to 60 ml of sterile water in flasks and agitated on a rotary shaker for 20 min. The suspension was filtered through cheesecloth and centrifuged at 8,700 *g* for 15 min. The supernatant was discarded, and the sediment was resuspended in 10 ml of sterile water. The suspension was examined at ×400 to determine the types of propagules present. A 0.3-ml aliquot of 10^{0}–10^{-3} dilutions was spread on three plates of the selective medium for each dilution and incubated as above. The number of propagules of the two strains was expressed as the logarithm of the number per gram fresh weight of debris.

Conidium germination and appressorium formation on greenhouse seedlings. Six-month-old Pineapple sweet orange and 4-mo-old Key lime (*Citrus aurantifolia* (Christm.) Swingle) seedlings growing in 2-L pots in a greenhouse were used to test the ability of SGO and FGG strains of *C. gloeosporioides* to survive on the surface of leaves. Conidia of isolates LP (SGO) and LA-1 (FGG) from 7- to 10-day-old cultures growing on PDA were suspended in sterile distilled water, and the concentration was adjusted to 5×10^{5} conidia per milliliter. Conidial suspensions were sprayed on seedlings to runoff with a hand-pump sprayer.

Groups of eight seedlings of Pineapple sweet orange and Key lime were inoculated with either the SGO or FGG strain. After inoculation, each group of seedlings was placed in the dew chamber at 100% relative humidity (RH) and 23 C in the dark for 48 h. Because of limited space in the dew chamber and the desire to avoid cross-contamination, inoculations were not conducted simultaneously. These inoculations were conducted on three groups of sweet orange seedlings and three groups of Key lime seedlings inoculated with SGO and three groups of each citrus species inoculated with FGG. Data from two experiments with each citrus species inoculated with each strain are presented. After 48 h in the dew chamber, seedlings were placed in a screenhouse where temperatures ranged from 22 to 30 C.

Three leaves were selected arbitrarily from each group of inoculated plants at 6, 12, and 24 h and at 2, 4, 10, 15, and 30 days after inoculation. When wet, leaves were allowed to dry at room temperature for at least 2 h. Replicas of the surface of inoculated leaves were prepared by a stripping technique similar to that of Fitzell et al (10). A solution of clear nail varnish and acetone (1:1, v/v) was applied to half of each leaf and was allowed to dry at room temperature. A leaf disk with a diameter of 1 cm was cut, and the dried varnish was peeled from the leaf disk with forceps. Strips from the three leaves were mounted on slides and stained with 0.01% cotton blue in lactophenol. The number of conidia, percentage of germinated conidia, and percentage of appressoria formed were determined microscopically. A germinated conidium was defined as one with a germ tube as long or longer than the length of the conidium.

The survival of the isolates on the remaining half of the leaves was determined at each time by washing and plating the suspension on the selective medium as described above. After washing, the leaf pieces were surface-sterilized with 0.525% sodium hypochlorite for 30 s; three pieces of each were plated on the selective medium. The number of colonies from the suspension was expressed as the logarithm of the number of propagules per gram fresh weight, and the percentages of positive isolation for each strain were recorded after incubation as above.

Behavior of conidia and appressoria on inoculated greenhouse seedlings. Groups of eight Pineapple sweet orange or Key lime seedlings were spray-inoculated with conidial suspensions of either the SGO or the FGG strain as above, placed in the dew chamber at 23 C for 48 h, and transferred to the screenhouse for 28 days. At that time, four seedlings of each species inoculated with each strain were sprayed with a flower extract prepared by grinding one-part healthy Valencia sweet orange flowers with three-parts water (w/v). Another group of four plants was sprayed with water to determine the effect of addition of substances from flowers on the behavior of conidia and appressoria already present on the leaf surface. Prior to application of the treatments, nail-varnish replicas were prepared from three leaves per treatment, and the number of nongerminated conidia and appressoria per square centimeter was determined microscopically. After spraying with the flower extract or with water, all plants were placed in the dew chamber for 48 h in the dark at 23 C and transferred to the greenhouse.

At 2, 3, and 5 days after the application of the flower suspension, replicas were prepared and observed microscopically. The numbers of nongerminated and germinated conidia and appressoria as well as the number of conidia formed on newly formed hyphae were counted. Three replicate leaf samples were used for each treatment. The experiment was conducted on two groups of sweet orange seedlings and two groups of Key lime seedlings inoculated with the SGO strain and one group of each citrus species inoculated with the FGG strain.

RESULTS

Population dynamics of *C. gloeosporioides* in the field. The number of buttons counted per 12 branches per tree were as follows in the three locations during 1990 and 1991, respectively: Frostproof (16 and 139), Lake Placid (76 and 171), and Gainesville (13 and 1). Thus, disease was mild to moderate in the three locations during 1990 and severe in Frostproof and Lake Placid and very mild in Gainesville during 1991.

Washes of petals yielded high numbers of propagules of the SGO strain (10^{4}–10^{6} per gram fresh weight) during all years and

locations, except Frostproof during 1990 and Gainesville during 1991, when disease incidence was low (Fig. 1). Washes of vegetative tissues yielded much lower propagule numbers (10^2–10^4 per gram fresh weight). Propagule numbers of the SGO strain on vegetative tissues in Frostproof and on buttons in Lake Placid and Gainesville declined after the bloom periods during 1990 and 1991. Similar propagule densities were observed on leaf and twig tissues in Lake Placid and Gainesville (data not shown). However, in Gainesville, where disease incidence was low, the SGO strain was not detectable on leaf and twig tissue on some occasions during late summer, fall, and early winter. The SGO strain was recovered more consistently from washes of buttons than from leaves or twigs.

Isolations from surface-disinfested petals yielded the SGO strain in a high percentage of the cases in Lake Placid and Frostproof and less frequently in Gainesville (Fig. 1). The SGO strain was recovered from 15–30% of vegetative tissue pieces during the bloom period (Fig. 1). The SGO strain was recovered only rarely from surface-disinfested leaves and twigs outside the bloom period (data not shown). Even in Lake Placid during both years and Frostproof during 1991, where disease incidence was high, the SGO strain was isolated only from a low percentage of vegetative tissue pieces (Fig. 1). The SGO strain was isolated on more sampling dates from buttons than from leaves and twigs.

The FGG strain was isolated consistently from washes of all tissues at all times of the year at the three sites (Fig. 1). Isolations of the FGG strains from surface-disinfested tissue were more variable throughout the year. Recoveries of this strain were lowest when there was a high proportion of immature twigs and leaves during the spring.

When examined microscopically, washes of petals contained conidia almost exclusively. When vegetative tissue washes were examined, 56–61% of the samples contained conidia typical of *C. gloeosporioides*, 14–28% contained appressoria, and 16–33% contained septate mycelial fragments that could have been of *C. gloeosporioides*. Strain identity could not be determined from these structures. Ascospores typical of *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk, the teleomorph of *C. gloeosporioides*, were observed only in the January 1991 sample at the Lake Placid site.

Platings of washes of leaf debris from the Lake Placid and Frostproof sites yielded low levels ($\sim 10^1$ per gram fresh weight) of the SGO strain, whereas 10^4–10^5 propagules per gram fresh weight of the FGG strain were recovered (Fig. 2). The types of propagules observed microscopically were the same as in washes of living vegetative tissues. Ascospores typical of *G. cingulata* were observed in two samples from Frostproof. Perithecia of *G. cingulata* were never observed in any of the collections of debris or living tissues.

Conidium germination and appressorium formation on greenhouse-grown seedlings. In three experiments, the percent conidium germination, the percentage of conidia forming appressoria, the number of viable propagules on the leaf surface, and the percent isolation of the strains were followed with time after inoculation of sweet orange and Key lime seedlings. Analysis of variance (ANOVA) indicated some significant differences between experiments or interactions of some of the factors with experiment.

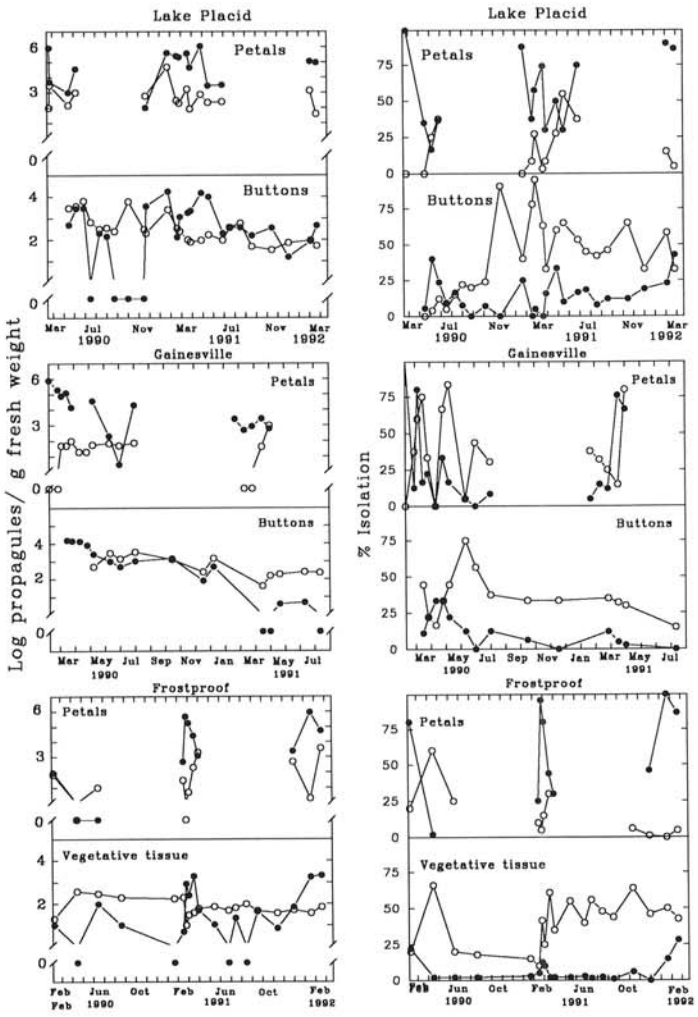

Fig. 1. Propagule numbers of the slow-growing orange (●) and fast-growing gray (○) strains of *Colletotrichum gloeosporioides*, determined by plating washes of tissues on a selective medium (left column), or percent isolation of the two strains, determined by plating tissue pieces on the selective medium (right column). Studies were conducted in a 20-yr-old Valencia sweet orange orchard near Lake Placid, FL, naturally affected by postbloom fruit drop (PFD) (top), in an artificially inoculated planting of 3-yr-old Valencia sweet orange near Gainesville, FL, (center), and in a 15-yr-old planting of navel oranges naturally affected by PFD near Frostproof, FL, (bottom).

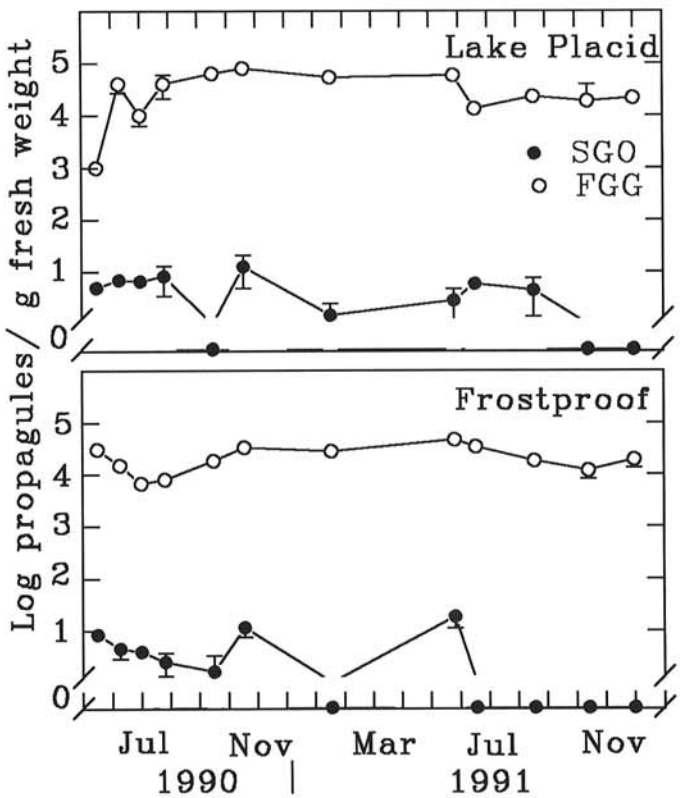

Fig. 2. Recovery on a selective medium of the slow-growing orange and fast-growing gray strains of *Colletotrichum gloeosporioides* from washes of leaf debris from the orchard floor plated on a selective medium at field sites near Lake Placid and Frostproof, FL. Bars on each point represent the standard error (SE) of the mean; absence of bars indicates that the SE was smaller than the point.

Thus, data from two of the experiments are presented (Fig. 3).

The conidia of the FGG strain germinated and formed appressoria rapidly, with the percentage of germination and appressorium formation reaching high levels by 30 days after inoculation (Fig. 3 A–D). In contrast, the SGO strain germinated more slowly, and the percent germination usually ranged from 20 to 40% after 1 mo. After the plants were placed in the screenhouse, conidium germination and appressorium formation continued with the FGG strain but increased only slightly with the SGO strain. ANOVA of factors within experiment indicated that strain and time after inoculation were highly significant for both conidium germination and appressorium formation ($P \leq 0.001$). Host was not significant in either experiment for either of these two factors.

Propagule densities, determined by leaf washes, varied between host and between experiments (Fig. 3 E–F). However, densities of both strains on both hosts declined with time after inoculation. ANOVA indicated time was a significant factor in both experiments ($P \leq 0.001$). Strains were significantly different in experiment 2 but not in experiment 1, and host was a significant factor in the first but not the second experiment.

Recovery of both strains by isolation from leaf pieces also declined with time after inoculation. Time was a significant factor in the ANOVA, but host was not significant in either experiment. Strain was a significant factor in experiment 1, with the recovery of the SGO strain declining more rapidly than that of the FGG strain. However, in experiment 2, no differences in isolation of the strains were observed.

Behavior of conidia and appressoria on inoculated greenhouse seedlings. Thirty days after spray inoculation of sweet orange and Key lime seedlings, nongerminated conidia as well as appressoria were present on the leaf surface as determined by the leaf-replica technique (Table 1). ANOVA was conducted within each factor in Table 1 to determine the effect of treatment, strain, and host on that factor. Individual means were compared by Student's t tests to determine the effect of treatment within host and strain. Strain was significant for all factors, except appressorium germination. The effect of host was not significant on any factor.

Many conidia germinated with the SGO strain, but relatively few of the conidia of the FGG strain germinated (Table 1). Application of the flower extract increased germination of conidia of SGO but not of the FGG strain. Treatment with flower extract greatly increased production of new conidia with the SGO strain but had little effect with the FGG strain. The number of propagules recovered was increased by treatment with flower extract only with the SGO strain on sweet orange.

Assays conducted on the same plants 1 and 3 days after removal from the dew chamber (i.e., at days 3 and 5 after initiation of

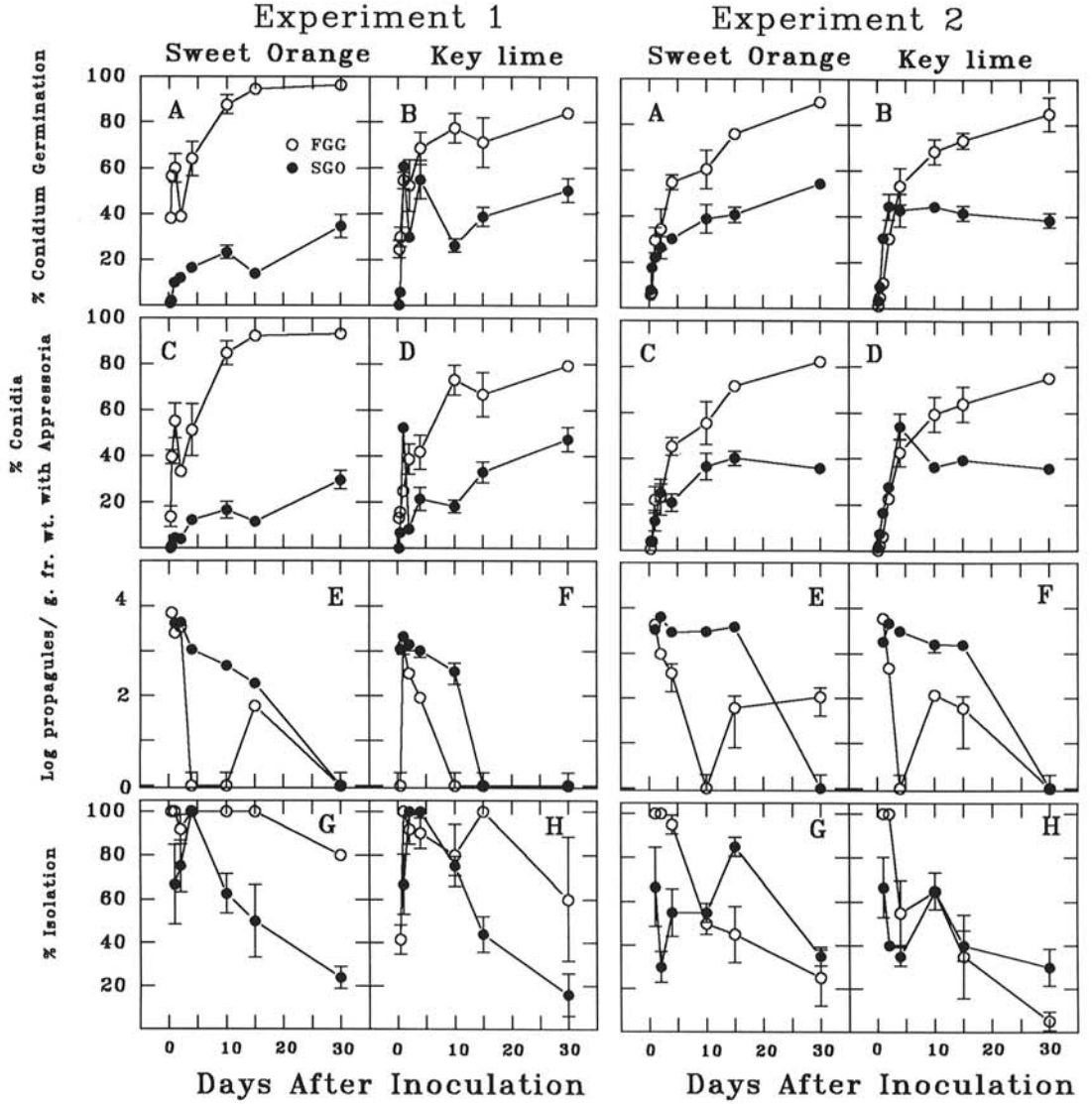

Fig. 3. Behavior of the slow-growing orange and fast-growing gray strains of *Colletotrichum gloeosporioides* on leaves of sweet orange and Key lime seedlings sprayed with conidial suspensions; **A–D,** percentage of the conidia germinated and germinated with appressoria, determined by stripping the leaf surface with nail varnish; **E and F,** propagule numbers, determined by plating leaf washes on a selective medium; **G and H,** percent isolation from surface-sterilized tissue pieces plated on a selective medium. Bars on each point represent the standard error (SE) of the mean; absence of bars indicates that the SE was smaller than the point.

TABLE 1. Conidium and appressorium germination of the slow-growing orange (SGO) and fast-growing gray (FGG) strains of *Colletotrichum gloeosporioides* on the surface of sweet orange and Key lime leaves[a]

Time (days)	Factor	Treatment	SGO strain		FGG strain	
			Sweet orange	Key lime	Sweet orange	Key lime
0	Conidia/cm^2	Pretreatment	77.7	50.0	79.6	28.2
	Appressoria/cm^2	Pretreatment	69.0	21.6	11.7	14.3
2	Conidium germination (%)[b]	Water	72.3	70.0	6.2	33.8
		Flower extract	100.0**[c]	100.0**	12.0	26.5
	Appressorium germination (%)[b]	Water	2.2	2.5	92.3	32.2
		Flower extract	89.9**	53.1**	59.7	33.9
	New conidia/cm^2[b]	Water	6.0	5.3	0.0	0.0
		Flower extract	465.0***	295.0***	0.0	20.7^{+}
	Propagules/cm^2[d]	Water	2.5	2.5	0.0	0.0
		Flower extract	21.3*	2.6	0.2	1.3

[a]Thirty days prior to the initiation of the experiment, plants were sprayed with a suspension of 5×10^5 conidia per milliliter, placed in a dew chamber for 2 days, and removed to the greenhouse. On day 0 (30 days after inoculation), plants were sprayed with an extract of crushed flowers or with water, placed in a dew chamber for 2 days, and removed to the greenhouse to dry.

[b]Determined microscopically after application of a nail varnish-acetone mix to the leaf surface, stripping, and staining.

[c]Mean significantly different from the water treated control according to Student's t test, $^{+} = P \leq 0.10$, $^{*} = P \leq 0.05$, $^{**} = P \leq 0.01$, $^{***} = P \leq 0.001$.

[d]Determined by dilution-plating leaf washes onto a selective medium.

the treatments) indicated similar effects of treatments on the number of new conidia and on the number of propagules recovered from leaf washes (data not shown). However, the number of new conidia and propagules was lower than on day 2.

DISCUSSION

The SGO strain of *C. gloeosporioides* produces most of the conidia in acervuli on the surface of infected petals (9,17). In contrast, the largely saprophytic FGG strain reproduces primarily on dead vegetative tissues (19,20). Denham and Waller (6) suggested that the pathogenic strain (SGO) survived on living tissues by forming appressoria and producing quiescent infections similar to the primarily saprophytic strain (FGG). Our field studies indicated that populations of the SGO strain decline after the bloom period. This suggests that this strain may not be reproducing on dead tissue within the canopy of the tree. Surface populations of the FGG strain, which reproduces on dead tissues, were relatively stable throughout the year. The low populations of the SGO strain in debris on the orchard floor also support the view that the SGO strain does not compete well for dead vegetative material. In in vitro studies, Agostini (1) found that, while the SGO strain readily colonized sterilized tissue, it was not detectable after 4 mo in infested detached buttons and petals exposed to ambient conditions.

On most tropical and subtropical crops, *C. gloeosporioides* produces appressoria and quiescent infections (16). All appressoria may not form infection pegs and quiescent infections. For example, Brown (4) found that removal of appressoria by washing citrus fruit reduced the incidence of postharvest anthracnose, implying that many appressoria were superficial. In our studies, the SGO strain was recovered from a very low percentage of surface-sterilized vegetative tissues even when populations recovered by surface washes were high. In contrast, the FGG strain was more commonly isolated from surface-sterilized tissue. Thus, the SGO strain, while forming appressoria on the surface, may produce relatively few quiescent infections.

Behavior of the SGO and FGG strains on the surface of inoculated leaves also differed greatly. The FGG strain showed little response to external factors such as moisture and the application of flower extracts, whereas these factors induced germination of conidia and appressoria with production of new conidia with the SGO strain. Most quiescent infections of *C. gloeosporioides* ultimately result in invasion and colonization of the same tissues (16). This does not seem to be the case with the SGO strain, which appears to be able to complete its life cycle without invasion of vegetative tissues. Light and electron microscopy have confirmed the production of new conidia by the SGO strain from germinating structures on the leaf surface (23). Also, the SGO strain is able to invade and colonize inoculated leaves once detached, so it is presumed that SGO also is capable of producing quiescent infections although they may not be essential to its survival (23).

Based on our studies, we suggest the following cycle for PFD of citrus. Conidia are produced abundantly on infected petals during the spring. These conidia are washed onto vegetative tissues where they may germinate to form appressoria or remain as nongerminated conidia; quiescent infections are probably formed to some degree. In the absence of bloom, these propagules gradually lose viability with time. When flowering resumes, petals that fall on the leaf surface provide substances stimulating germination of appressoria to form a few conidia. These conidia are rain-splash dispersed to new flowers, reinitiating the cycle.

These findings help explain some previous observations (18). PFD is much more severe on declining trees and on young trees. This is probably attributable to more off-season bloom that allows surviving inoculum to be replenished frequently. Likewise, in more tropical areas where trees bloom several times during the year, the disease is more of a problem than in more temperate areas with a single concentrated bloom period.

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