Development of Resistance Against *Geotrichum candidum* in Lemon Peel Injuries

A. B. A. M. Baudoin and J. W. Eckert

Former graduate research assistant and professor of plant pathology, respectively, Department of Plant Pathology, University of California, Riverside 92521. Present address of senior author: Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg 24061.

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ABSTRACT

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Injuries in the peel of lemons became progressively more resistant to infection by *Geotrichum candidum* when inoculated 4–20 hr after wounding. No significant accumulation of antifungal compounds was detected in 2- or 5-day-old arrested infections. The deposition of ligninlike substances around the inoculated tissue paralleled the development of resistance. Deposition was more rapid in the flavedo than in the albedo and was stimulated more strongly around inoculated wounds than around sterile wounds. The development of ligninlike zones and of resistance were strongly inhibited by cycloheximide (1 μg/ml). The ligninlike zones were not resistant to maceration when infiltrated with enzymes of the pathogen, but infiltration with water also overcame the resistance and activated arrested infections. Although the ligninlike zones may represent a defense mechanism against infection, they did not appear to be responsible for differences in resistance. The more resistant light-green or subturgid lemons produced less ligninlike material after inoculation than the more susceptible yellow or turgid fruits. The greater amount of ligninlike substances in susceptible fruit may be a response to greater fungal development.

Additional key words: *Citrus limon*, sour rot.

The incidence of sour rot, which is caused by *Geotrichum candidum* Link ex Pers., in stored citrus fruits varies with season, harvest date, and growing area (9–11,13). We showed earlier that there is great variability in the susceptibility of California lemons to sour rot and that genetic differences account for only a minor part of this variability (2). Yellow color, prolonged storage, and high water potential of lemons were associated with high susceptibility to wound infection by *G. candidum*. Inoculation of wounds in the lemon peel produced either a rapidly advancing soft rot or an arrested, dry lesion of 2–3 mm diameter. Fruit lots differed markedly in the percentage of inoculation sites that developed soft rot, whereas there were only minor differences in the rate of lesion expansion. Inoculation sites that had not developed an active soft rot within 5 days at 25 C and high relative humidity rarely did so thereafter. These characteristics suggested that inoculation or injury induced a response in the lemon which, within a few days, prevented further invasion by the sour rot pathogen. Wound responses of citrus fruit that might be involved in disease resistance include an increase in free phenolics, the deposition of lignin, and the production of a phellem and phellogen (4,14,15,17).

The objectives of this study were to characterize the factors in the lemon peel that prevented lesion development and to determine whether differences in susceptibility to sour rot were related to the rate or intensity of an induced host response.

MATERIALS AND METHODS

The procedures used for disease development and evaluation have been described previously (2). Briefly, 5 μl of arthrospore suspension was injected 2.5 mm deep into the peel of lemon fruits (*Citrus limon* (L.) Burm.). The fruits were held at 25 C and 100% relative humidity, unless otherwise noted. Disease was evaluated after 5 days, and the susceptibility of a fruit lot was expressed as the percentage active lesions or the inoculum dose required for 50% active lesions (ED_{50}). A soil isolate of *G. candidum* that does not infect citrus (cf, 8), isolate 310, was kindly provided by E. E. Butler, University of California, Davis.

The development of resistance was studied by wounding lemons with a sterile inoculation needle (1 mm diameter, 2.5 mm deep) and incubating the fruit at 25 C and high humidity. At various times after wounding, the injuries were inoculated with 5 μl of spore suspension. Polyvinyl chloride rings (7 mm inside diameter, 7 mm high) were attached with melted paraffin to the peel around the aging wounds so that the wounds could be covered with various solutions. The rings were filled with 0.1–0.2 ml of liquid.

For histological examination, excised peel tissue cubes with inoculation sites were vacuum infiltrated with FAA (formalin, glacial acetic acid, ethanol, and water; 13:8:95:95, v/v) and fixed overnight or longer. They were dehydrated in an ethanol-*t*-butyl alcohol series or an ethanol-xylene series, embedded in paraffin, and sectioned with a rotary microtome (16). Fresh or fixed tissue was also sectioned freehand or with a Hooker model 1225 plant microtome (Labline Instruments Inc., Melrose Park, IL). Safranin-fast green (16) was used as a general stain. Host tissue degradation was followed by staining for cellulose with zinc-chlor-iodide (16) and for pectins with alkaline hydroxylamine hydrochloride (16) or by iron absorption (19). Structural barriers were evaluated using the following stains: phloroglucinol-HCl, chlorine sulfite (16), Maüle reagent (19), or crystal violet (18) for lignin; Sudan IV (19) for suberin; aniline blue (16) for callose; nitroso reagent, ferric chloride (16), or 1 M KOH (4) for phenolic compounds and tannins; and orcinol-HCl for aromatic aldehydes (19).

The intensity of the phloroglucinol reaction in a zone around the inoculation sites was evaluated semi-quantitatively at 2–5 days after inoculation. Arrested infections were excised, and two slices (about 5 × 5 × 1 mm), each containing approximately half of the infected tissue, were vacuum infiltrated with a 1% (w/v) phloroglucinol solution in 4.8 N aqueous HCl. The resulting red color was rated visually by two observers on a scale from 0 (no color) to 6 (intensely red zone) with the unaided eye or under low magnification. Zones in the flavedo (exocarp, the outer 1 mm of the

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peel) and albedo (mesocarp) were evaluated separately. Each arrested infection (replicate) thus yielded four readings which were averaged for analysis.

The presence of fungitoxic compounds was investigated as follows. Arrested infections were excised and trimmed to remove as much healthy tissue as possible. Equivalent amounts of healthy peel tissue served as controls. The tissue was comminuted in 2–3 ml of ethyl acetate per gram of fresh tissue and filtered. The residue was extracted twice more with similar volumes of ethyl acetate. In some tests, the tissue samples were not comminuted but were vacuum infiltrated with ethyl acetate and leached for 12 hr with three successive quantitites of ethyl acetate. The extracts were filtered, pooled, and dried under vacuum or in a stream of nitrogen. The dry residue was redissolved in ethyl acetate, and portions of these solutions were placed in either microbeakers (1 cm diameter, 7 mm high) containing 0.2 ml liquid asparagine-glucose medium (8) or potato-dextrose agar (PDA), or on disks (12 mm diameter, 4 mm thick) of PDA or water agar in petri dishes. In most experiments, three dilutions of each extract were tested. The amount of extract applied per square centimeter of bioassay surface (agar or glass) was prepared from a range of 16–826 mg of peel tissue or 0.21–6.36 arrested infections. After the solvent had evaporated, 200 or 30 μl of spore suspension (2×10^4 spores per milliliter) was placed in the microbeaker or on the agar, respectively, and the cultures were incubated for 16 hr at 25 C. Fungus growth was rated on a 0–3 scale: 0, little or no growth, less than 10% of growth in the control; 1, growth strongly inhibited, less than 50% of that in the control; 2, growth distinctly inhibited, but more than 50% of the control; 3, no detectable inhibition. Germ tube lengths were measured in some of the tests.

RESULTS

Development of resistance to infection in aging wounds. Sterile puncture wounds, aged for 4 hr before inoculation, were partially resistant to infection by *G. candidum*, and wounds aged for 20 hr were very resistant to infection (Fig. 1). Wound resistance appeared to increase faster in light-green lemons than in yellow lemons, but this is difficult to interpret since the level of infection in fresh wounds was higher in the yellow lemons than in the light-green fruit.

When wounded lemons were held at 18 C instead of 25 C or in an atmosphere containing 1–2% oxygen until inoculation, resistance development was retarded slightly. An atmosphere containing 5% carbon dioxide did not influence resistance development. Resistance development was not due to wound dehydration, since wounds that were covered with sterile water until inoculation became as resistant as wounds aged in air at high humidity. Wounds that were covered with cycloheximide solution (1–5 μg/ml) until inoculation were at least as susceptible to infection by *G. candidum* as were fresh wounds. Spore suspensions containing the same concentrations of cycloheximide were much more infective to lemons than inoculum in buffer only. For example, addition of 1 μg cycloheximide per milliliter to inoculum containing 10^6 spores per milliliter resulted in 93% active lesions compared to 18% without cycloheximide. Cycloheximide at these concentrations did not influence the growth of *G. candidum* in liquid asparagine-glucose medium.

Activation of arrested infections with water. Six-day-old arrested infections were often activated after they were covered with water. The percentage activated in this fashion decreased with the age of the arrested infections. In one test, 83% of 6-day-old arrested infections were activated compared to only 4% after 18 days. In a second test, the percentage activated was 42 (6 days), 25 (11 days), 20 (16 days), and 14 (22 days). About half of 6-day-old arrested infections were also activated by injecting 1 ml of water into the albedo at a distance of 1 cm from the inoculation site.

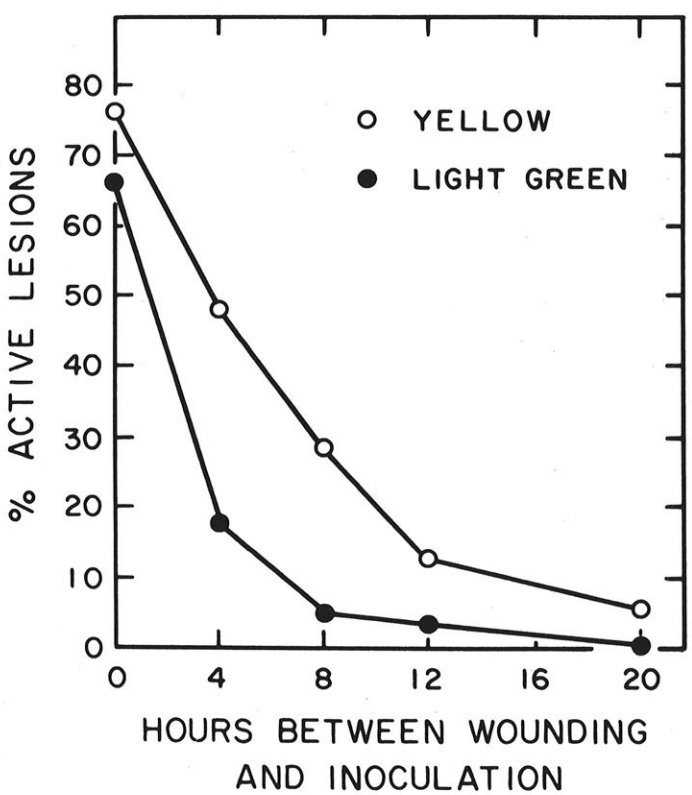

Fig. 1. Relationship between age of wounds in the peel of yellow and light-green lemons and their susceptibility to infection by *Geotrichum candidum*. Each point is based on 36–41 inoculations.

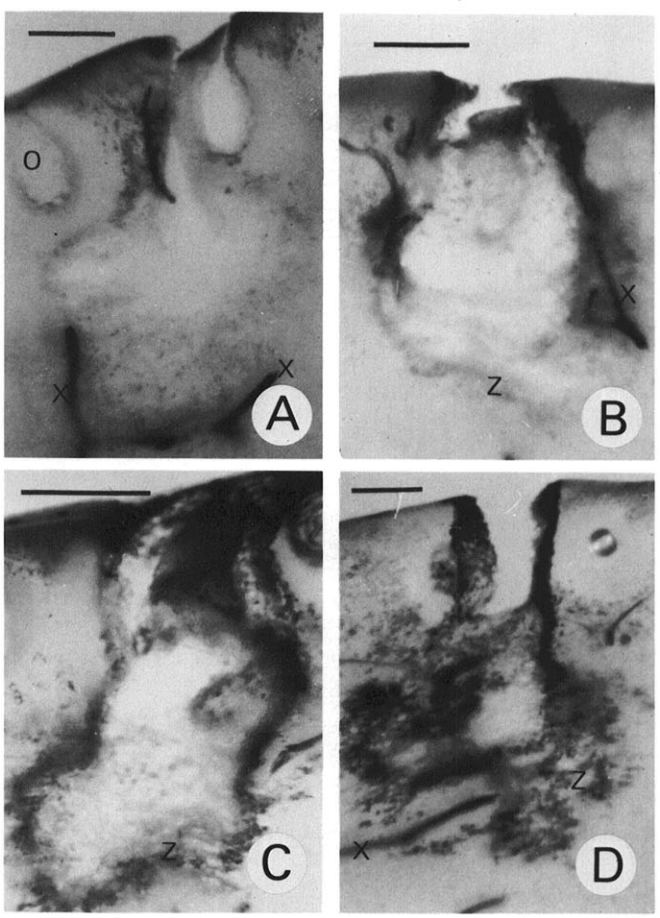

Fig. 2. Transverse sections (about 0.5 mm thick) through arrested infections of *Geotrichum candidum* in lemon peel stained with phloroglucinol-HCl. Development of lignified zones at 25 C after **A,** 1; **B,** 2; **C,** 3; and **D,** 5 days after inoculation. Bar represents 1 mm. X = xylem bundle, Z = ligninlike zone, and O = oil gland.

Histology of induced structural barriers. Spores germinated within 5 hr after inoculation, and albedo tissue around the germinating spores was visibly disintegrating within 8 hr. Cell wall disintegration was apparent ahead of the hyphae in some inoculation sites, while in others hyphae were found between cells without apparent wall disintegration. Degraded host tissue stained poorly with the pectin stains but stained blue with zinc-chlor-iodide, indicating degradation of pectins but not of cellulose.

One day after inoculation, a slightly browned zone was evident around the area invaded by the fungus. This zone was most apparent in fresh, thick tissue slices when viewed under incident light, and was better developed in the flavedo than in the albedo. After vacuum infiltration of the tissue with water or FAA, the zones could no longer be seen clearly in the albedo. Only a very faint reaction with phloroglucinol-HCl was observed around the invaded area at this time. Phloroglucinol-positive zones, consisting of lignin or ligninlike substances, became progressively better developed at 2–5 days after inoculation (Fig. 2). Staining was most intense in the flavedo and in the neighborhood of vascular bundles. After 5 days, the stained zone in the albedo parenchyma was only one or two cells thick and appeared discontinuous in thin sections (Fig. 3A and C). To test the resistance of the phloroglucinol-positive zones to maceration, slices of 5-day-old arrested infections were vacuum infiltrated with a sterile macerating extract from lemon peel decayed by *G. candidum*. When the tissue began to disintegrate, it was treated with phloroglucinol-HCl. The phloroglucinol-positive clumps of albedo cells were neither larger nor more coherent than non-staining albedo cell clumps, indicating that the ligninlike zones were macerated at the same rate as normal tissue. Ten days after inoculation, the phloroglucinol-positive zones were more strongly developed and, in some lemon lots, significant development of periderm was found at this time (Fig. 3B and D).

The phloroglucinol-positive tissue reacted with many other reagents and stains. Five days after inoculation, the reactions with chlorine sulfite (specific for syringyl groups [22]) and crystal violet were positive for lignin but less intense than the phloroglucinol reaction (which indicates the presence of cinnamaldehyde groups [22]). The Maüle reaction was inconclusive, since the zones stained brown, which might have masked the red color reaction of lignin. The phloroglucinol-positive zones stained more intensely red with safranin than did the surrounding tissue; they became yellow in zinc-chlor-iodide indicating encrustation with substances that mask cellulose. The intensity of the reactions with orcinol-HCl, 1 M KOH, and the nitroso-reagent was similar to that of the phloroglucinol reaction, whereas Sudan IV produced only a faint color. The reactions with ferric chloride and with aniline blue were negative. The reactions were similar both in fresh sections and in fixed, paraffin-embedded sections.

TABLE 1. Phloroglucinol reaction of lemon peel tissue surrounding arrested infections of *Geotrichum candidum*

Lemon peel	Susceptibility to infection[w]	Phloroglucinol reaction[x] Test 1	Test 2
Yellow, turgid	Medium	3.2 a	2.7 ab
Light green, turgid	Low	1.3 b	1.9 b
Yellow, subturgid	Low	0.9 c	···
Yellow, turgid within circular cut[y]	High	···	3.2 a
Aged[z] wound in yellow, turgid fruit	Very low	···	2.3 ab

[w] Fruit susceptibility to *G. candidum* as determined in numerous separate experiments.

[x] The intensity of the phloroglucinol reaction was rated visually on a scale from 0 (no reaction) to 6 (intensely red zone). Separate ratings for flavedo and albedo and for 3 and 5 days after inoculation were averaged. Within columns, values followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

[y] The inoculation site was isolated from surrounding peel by a cut made with a corkborer (20 mm diameter) at the time of inoculation.

[z] Wound aged 24 hr before inoculation.

Arrested infections in relatively resistant lemons sometimes produced a gummy exudate after about 5 days. This exudate was soluble in water but insoluble in 95% ethanol. It stained red in phloroglucinol-HCl, intensely red with safranin, and reacted to some extent with the pectin stains. The intensity of the color produced with phloroglucinol or safranin in sections of arrested infections was not visibly affected by leaching in water or by fixing in absolute ethanol, suggesting that only a small part of the phloroglucinol reaction was due to such water-soluble exudate.

The intensity of the phloroglucinol reaction around arrested infections was less in light-green and subturgid lemons than in the more sour-rot-susceptible yellow and turgid lemons, respectively (Table 1). Peel tissue isolated from surrounding peel by encirclement with a corkborer cut (20 mm diameter) before inoculation was much more susceptible to infection by *G. candidum* (1); however, the phloroglucinol reaction at the isolated inoculation sites was similar to that at sites that were not isolated (Table 1). The production of phloroglucinol-positive substances was stimulated by injection of a suspension of spores of *G. candidum*, both a citrus strain and a non-citrus strain, into wounds. An increase in the concentration of spores of the non-citrus strain increased the intensity of the phloroglucinol reaction (Table 2, Fig. 4). Adding spores of the non-citrus strain (10^7–10^8 spores per milliliter) to inoculum of the citrus strain (10^5–10^6 spores per milliliter) reduced the resulting percentage of active lesions: from 34, 32, 46, 50, and 34% (citrus strain only) to 18, 25, 16, 41, and 24% (with the non-citrus strain added), respectively, in five tests (the reduction was significant at $P = 0.01$). In these tests, however, the increase in phloroglucinol-positive substances due to addition of the non-citrus strain was small and not significant, presumably because the number of citrus strain spores required to produce 30–50% active lesions had already induced a relatively high level of phloroglucinol-positive substances. The intensity of the phloroglucinol reaction around wounds injected with sterile extracts of peel macerated by *G. candidum* was slightly, but not significantly, higher than in wounds injected with sterile water. Injection with cycloheximide (1 and 5 μg/ml) prevented the development of phloroglucinol-positive zones (Table 2).

Antifungal compounds. The effect of ethyl acetate extracts from healthy and infected lemon peel on germ tube growth of *G. candidum* was not consistent. In two tests, extracts from arrested infections were significantly more inhibitory than those from healthy peel, but in six subsequent tests there was no difference. Also, the inhibitory activity of healthy peel extracts from similar amounts of tissue varied widely between tests. The presence of variable amounts of peel oil or fungicide residues may have been

TABLE 2. Intensity of the phloroglucinol reaction around wounds in lemon peel injected with various solutions and spore suspensions

Injection (per 5 μl)		Phloroglucinol reaction[z] Test 1	Test 2
Sterile water		2.6 d	1.9 c
Cycloheximide, 5 ng		0.3 e	0.4 d
Macerating enzyme		2.9 d	2.7 bc
G. candidum, citrus isolate	100 spores	3.2 cd	2.7 bc
	500 spores	4.1 abc	···
	5,000 spores	4.0 bc	···
G. candidum, non-citrus isolates	100 spores	···	2.5 bc
	5,000 spores	4.2 abc	···
	50,000 spores	5.3 a	4.1 a
	500,000 spores	5.0 ab	···

[z] The intensity of the phloroglucinol reaction was rated visually on a scale from 0 (no reaction) to 6 (intensely red zone). Separate ratings for flavedo and albedo and for 3 and 5 days after injection were averaged. Within columns, values followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

responsible for the variation in inhibitory activity of extracts of healthy peel; however, extracts of flavedo and albedo tissue from the same peel sample gave the same results. In direct comparisons, no significant differences in inhibitory activity were found between ethyl acetate and ethanol extracts; between assays on water agar, PDA, or in liquid medium; between extracts from 2-day-old and 5-day-old arrested infections; between extracts dried under a stream of oxygen and a stream of nitrogen; between extracts from comminuted or merely leached tissue; and between assays in which liquid medium was preincubated with the dried extract for 24 hr

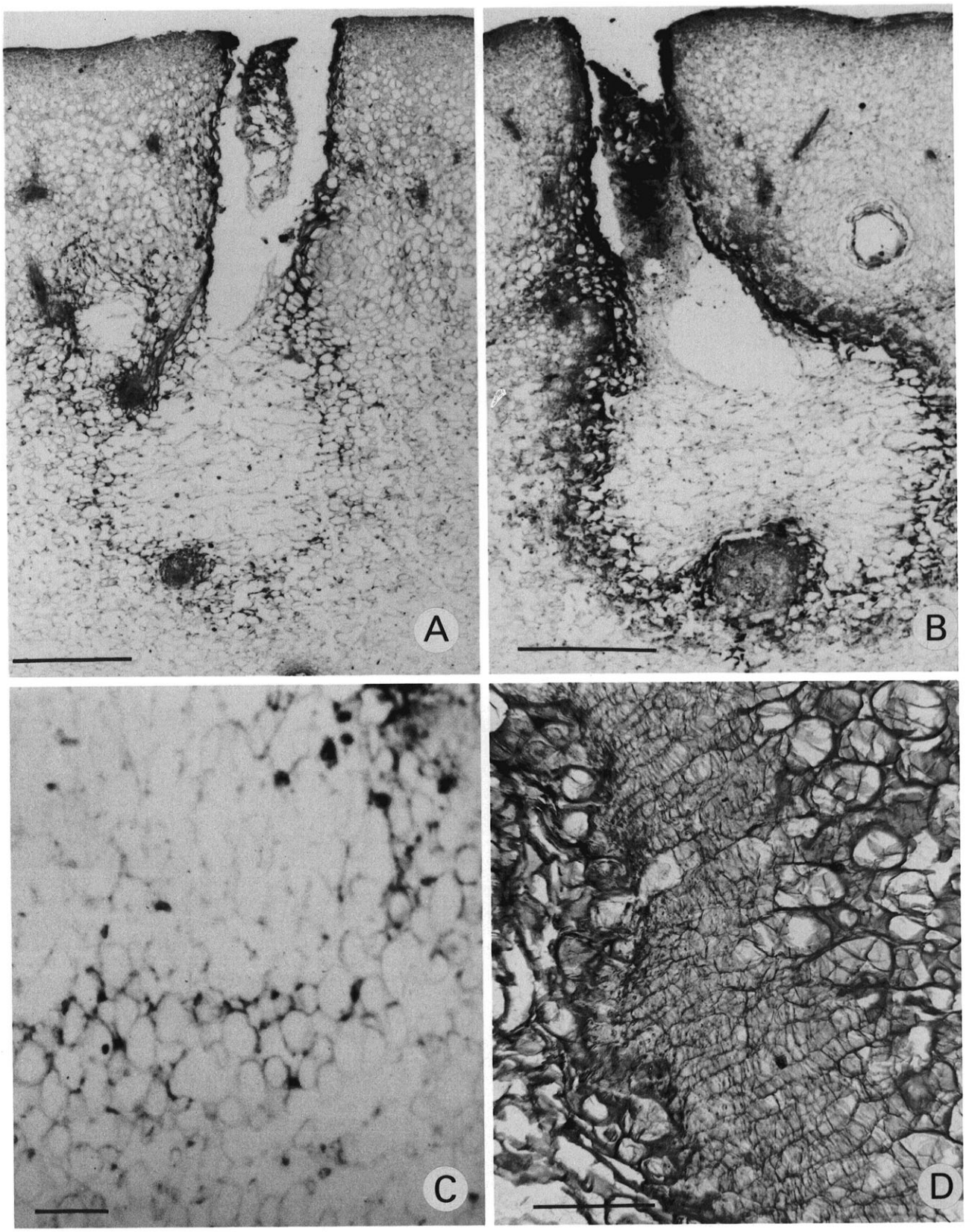

Fig. 3. Transverse sections (30 μm thick) through arrested infections of *Geotrichum candidum* in lemon peel. Sample at **A,** 5 and **B,** 10 days after inoculation (both stained with safranin). Bars represent 1 mm. **C,** Close-up of ligninlike zones in the albedo, 5 days after inoculation (safranin). **D,** Periderm and ligninlike zones, 10 days after inoculation (safranin-fast green). Bars in **C** and **D** represent 0.2 mm.

before spores were added and assays in which spores were added immediately.

In an attempt to test inhibitory activity and its location more directly, peel tissue slices (0.5–1.0 mm thick) with 5-day-old arrested infections were placed in microbeakers. Some samples were sterilized by adding a few drops of 95% ethanol and allowing it to evaporate, or by keeping the microbeakers in propylene oxide vapors for 2 hr. Subsequently, a drop of melted (45 C) water agar or PDA, which—for the sterilized samples—contained spores of *G. candidum*, was placed on the tissue slices. After 24 hr, vigorous mycelium was growing from all areas of the invaded tissue of the unsterilized slices. On sterilized tissue slices, spores in PDA had germinated evenly and no areas of growth inhibition were visible. In water agar, growth was stimulated close to the peel tissue, whether infected or not, presumably because of diffusion of nutrients from the tissue.

Lemons with 5- to 7-day-old arrested infections in sterilized Mason jars were held in a freezer for several hours to injure or kill the host cells, and were subsequently thawed and kept at 25 C. Within 48 hr the fungus grew out from the infected area and formed a macerated lesion, although relatively little mycelium was found outside the originally invaded area.

DISCUSSION

When inoculated several hours after wounding, puncture wounds (1 × 2.5 mm) that penetrated into the albedo of lemon peel became resistant to infection by *G. candidum*. A similar phenomenon has been observed in the infection of oranges by *Penicillium digitatum* (4,6,12), but in that case resistance developed in the flavedo tissue only and 2–3 days were required for the development of a high degree of resistance.

When fresh wounds were inoculated, the incubation period for sour rot was generally less than 5 days at 25 C (2); development of resistance was sufficient at this time to prevent subsequent additional production of active lesions. Our experiments provided little support for a role for fungitoxic compounds in this induced resistance. However, the development of barrierlike zones did coincide with resistance development. After 3–5 days, these zones were well-developed in the flavedo and also clearly present in the albedo. Brown (4) reported that lignin was deposited in injured flavedo of orange peel but not in injured albedo. We confirmed that deposition of ligninlike substances in the albedo around sterile wounds was slight, but we found that around inoculated wounds it was significantly stimulated.

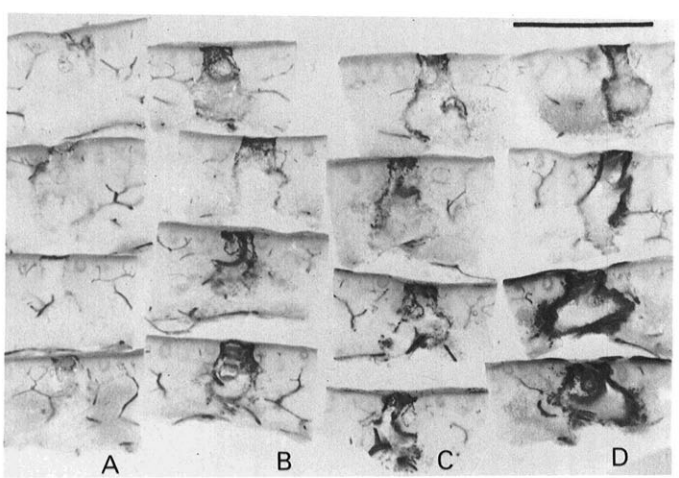

Fig. 4. Development of phloroglucinol-positive zones around 4-day-old wounds in lemon peel injected with 5 μl of **A,** water, **B,** a spore suspension containing 500 spores of a citrus isolate, **C,** 500 spores of a non-citrus isolate, or **D,** 300,000 spores of a non-citrus isolate of *Geotrichum candidum*. Four replicate sections are shown for each treatment. Bar represents 5 mm.

Substances that exhibit characteristic color reactions with phloroglucinol-HCl, crystal violet, etc, have been described in the literature as wound gum, lignin, suberin, or ligno-suberin (24). The ligninlike and phenolic nature of such deposits in healed orange flavedo was corroborated by spectrophotometry (6) and by additional histological tests (5). However, other phenolic substances in lemon peel (such as the water-soluble, gummy exudate that was produced by some older arrested infections by *G. candidum*) can give a positive phloroglucinol reaction. Therefore, our use of the term lignin is tentative.

Cycloheximide inhibited lignification in injured flavedo and albedo tissue and prevented the development of resistance to infection by *G. candidum*. Cycloheximide inhibited the synthesis of phenylalanine ammonia lyase and lignification in orange flavedo (15), lignification and resistance to leaf pathogens in grass leaves (3,23), and suberization and resistance to *Pseudomonas fluorescens* in potato tissue (25). We found that resistance of lemon peel injuries was increased by a treatment that appeared to stimulate lignification, viz, adding spores of a non-citrus strain to inoculum of the citrus strain of *G. candidum*. These data are consistent with the hypothesis that the ligninlike zones act as a defense mechanism against sour rot.

Differences in resistance to sour rot did not appear to be due to differences in the deposition rate of ligninlike substances. On the contrary, relatively susceptible lemons (turgid or mature) produced ligninlike material more rapidly than more resistant (subturgid or less mature) fruits. Also, inoculation sites within circular cuts developed more active lesions, although the rate of lignification was about the same as in the control inoculations. These results might be explained by the observation that lignification was enhanced by fungal attack (cf, 3,20). We found that inoculated wounds produced more lignin than sterile wounds and that an increase in spore concentration of the inoculum also increased lignin production. Lignification may either be a localized response that mainly occurs close to hyphae of the pathogen (3), or its intensity may depend on the number of host cells killed. The latter possibility is consistent with the observation that sterile macerating enzyme also appeared to increase the deposition of lignin. In the more resistant lemons, a reduction in the rate of fungal attack (fungal growth, macerating enzyme production, or macerating enzyme action) by other mechanisms could then result in reduced lignin deposition.

The mechanism by which structural barriers might prevent further invasion of peel tissue by *G. candidum* is not entirely clear. Ten-day-old arrested infections could not be activated by covering them with water, and periderm and deposits of ligninlike substances appeared sufficient to form a physical seal around the infected tissue. However, 5 days after inoculation the large intercellular spaces in the albedo, which comprise about 50% of the tissue volume, did not appear to be completely sealed. The lignified zones in the albedo were not resistant to maceration when the tissue was infiltrated with a macerating-enzyme suspension. However, the addition of water alone to 5-day-old arrested infections also overcame the resistance of the tissue in many cases. It is possible that the lignified zones are more resistant to cell wall degradation when free water is absent from the intercellular spaces. Brown (7) and Wood (24) have reviewed evidence that free liquid in the intercellular spaces strongly enhances the diffusion and activity of macerating enzymes. Water might also remove or dilute a macerating-enzyme inhibitor or fungitoxic chemical. However, our results do not support a role for induced fungitoxic compounds in resistance. We did not investigate the two remaining suggestions by Ride (21), namely that lignified barriers might restrict diffusion of toxins and enzymes or nutrients and water, or that fungal hyphae themselves might become lignified.

Several alternative explanations for the difference in resistance of lemons to *G. candidum* should be considered. Since the presence of intercellular water stimulated infection, the rate of liquid withdrawal from intercellular spaces by the surrounding host cells following wounding may determine the success of the defense reaction. This rate might differ in green or yellow or in turgid and subturgid lemons. Alternatively, the defense reaction may proceed

at the same rate in fruits that differ in resistance, but preformed characterisitics of the tissue may lead to differences in pathogen growth, production or activity of macerating enzymes, or in the susceptibility of the host tissue to maceration. A study of the role of preformed factors in resistance of lemons to *G. candidum* will be reported separately.

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