Some Factors Affecting the Occurrence and Development of Foot Rot on Citrus Trees

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ABSTRACT

No infection of suberized stems occurred in greenhouse tests on sweet orange and rough lemon seedlings inoculated with zoospores of *Phytophthora parasitica* except when large numbers of zoospores were applied to wounds made through the outer bark. The fungus was able, however, to penetrate young intact stems in which the periderm had not yet developed. Following inoculation of Page and Pineapple orange trees in the field, some trees that were not visibly wounded developed foot rot, and in these cases infection probably occurred through naturally formed breaks.

Both naturally and mechanically induced breaks in the outer bark were detected by observing the penetration of triphenyltetrazolium chloride.

Zoospores settled in larger numbers on exposed parenchymatous tissue than on the suberized bark. No penetration of intact bark by germ tubes was ever observed. Wounds were invaded most easily while still fresh. In greenhouse tests, foot rot seldom appeared on stems wounded more than 10 days before inoculation.

Foot rot development was favored by covering the infected part of the trunk with soil. Phytopathology 61:1233-1238.

Additional key words: inoculation technique.

Foot rot denotes a diseased bark condition on the lower trunk or crown roots of citrus trees after invasion near ground level by *Phytophthora* spp. It is here considered distinct from another type of damage caused by attacks of fibrous roots by the same fungi. The causal organism isolated from foot rot lesions in Florida is *Phytophthora parasitica* Dastur (syn. *P. nicotianae* B. de Haan var. *parasitica* [Dast.] Waterh.) (3, 6, 19).

In contrast to the considerable information on fibrous root infection (4, 9, 14, 15), little is known about the process of trunk infection. What knowledge exists is mostly empirical, based on observations that foot rot is favored by high soil moisture, heaping of soil against the trunk, deep planting, low budding, and cultivation injury (3, 5, 7, 8, 11). The lack of experimental support results largely from difficulties in methodology. Techniques have been worked out for fibrous root infection using zoospores (2, 6, 9), but those for trunk infection have been limited to placing mycelium, together with the agar in which it was grown, in contact with the cambium after removing a disc of bark (5, 7, 10, 14). Doubt has remained as to whether unbroken bark provides a completely effective barrier against *Phytophthora* penetration.

This paper reports experiments to determine the mode of penetration of citrus stems by zoospores of *P. parasitica,* and to determine postinfection requirements for foot rot development.

MATERIALS AND METHODS.—For greenhouse studies, seedlings of Pineapple sweet orange (*Citrus sinensis* [L.] Osbeck) and rough lemon (*Citrus limon* [L.] Burm.) were grown in 46-oz cans containing a steam-sterilized 1:1 peat-soil mixture. At time of inoculation, all plants had active growth flushes. In the field, inoculations were made on 12-month-old trees of Page budded on rough lemon, and on 6-month-old Pineapple orange seedling trees. The Page cultivar originates from a tangelo (hybrid of grapefruit, *C. paradisi* Macf., and mandarin, *C. reticulata* Blanco) × mandarin cross.

Production of zoospore inoculum.—A modification of methods by Grimm & Whidden (6) and Menyonga & Tsao (12) was used to produce nutrient-free zoospore suspensions. Isolates of *P. parasitica* were grown on 100 ml lima bean broth (decoction prepared from 10 oz dry lima beans in 3,000 ml water) contained in 16-oz prescription bottles. The broth was inoculated with portions of agar culture. After 24 hr, the bottles were shaken to fragment the mycelium, then incubated in a horizontal position for 3-5 days. The mycelium was collected and washed on cheesecloth and resuspended in 100 ml autoclaved lake water in a 150-mm petri dish. After incubating for 2 days at 25 C for sporangium production, abundant zoospores were obtained by decanting the original water, replacing it with demineralized water previously cooled to 15 C, and pouring the suspension through cheesecloth to remove hyphal fragments. Zoospore concentrations were determined with a hemacytometer after heating the counting chamber to terminate motility. Concentrations were adjusted by dilution with water. Because zoospores congregated rapidly at the water surface, thorough stirring was essential before making spore counts and inoculations. The inoculum was used within 1 hr of preparation while the zoospores were still motile. Because variations in pathogenicity between cultures of *P. parasitica* have been reported (6), the inoculum consisted of a mixture of zoospore suspensions derived from at least six different isolates.

Inoculation technique.—The device for inoculating stems < 15 mm in diam is shown in Fig. 1. A collar, 30 mm high, was constructed around the base of the stem with a segment of Tygon tubing (25 mm inside diam). After making a longitudinal cut, the tubing was stretched around the stem, restored to a tubular shape, secured with plant tying wire, and then made

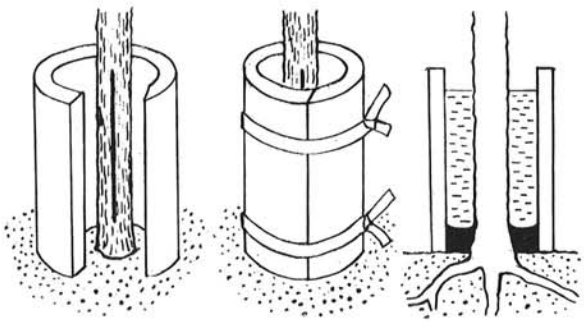

Fig. 1. Diagram showing construction of watertight collar around base of stem and location of vertical cut in bark made for most inoculations.

watertight by applying melted 1:1 mixture of paraffin wax and petrolatum to the open base and vertical seam.

Within each greenhouse experiment, all stems were inoculated with the same amount of inoculum. The concentration of inoculum varied between experiments from 0.5×10^4 to 8.0×10^4 zoospores/ml. In studies reported in Table 1, 10 ml of zoospore suspension were poured into each watertight collar; in all other greenhouse experiments, 5 ml were used.

Two other methods were used to inoculate larger stems. In the trial reported in Table 3, watertight waxed cardboard collars were fitted around stems to serve as chambers for zoospore suspensions. The collars were then filled to the brim with 200-400 ml suspension containing 1×10^4 zoospores/ml. In the trial reported in Table 4, similar chambers, though not watertight, were used to hold absorbent cotton in contact with stems: 200 ml of the same zoospore concentration were then poured onto the cotton.

Following inoculation, the watertight collars were kept filled with water for 2-3 days, and the absorbent cotton was kept moist for 3 days. The collars were subsequently filled with sterilized soil in greenhouse tests and unsterilized soil in field tests, except where otherwise stated.

Detection of injuries and natural breaks in bark.—Breaks that extended into living tissue were detected by constructing a watertight collar (as used for holding inoculum) and filling with a 0.5% solution of 2, 3,5-triphenyl-2H-tetrazolium chloride (TTC). Collars were immediately covered with aluminum foil to prevent sunlight-induced reddening of the solution. TTC is a colorless solution that turns red upon encountering metabolizing cells. It has been used for detecting surface injuries on citrus fruits (13), and has been shown to stain the cambial layer in dormant twigs (18). Removal of the suberized outer layer of the bark of citrus stems permitted some penetration into underlying living tissues, but it was only when deeper cuts were made through to the cambium that there was any movement of TTC into the xylem. Some indication of the depth of breaks in the bark could be obtained by observing whether red streaks, indicative of TTC translocation away from point of entry, were present in the wood. Observations were made 15-18 hr after the solution was applied.

Histological techniques.—Two procedures were used to observe the settling pattern of zoospores on injured bark. In one, the bark was first wounded by making a tangential cut down to the cambium before applying a zoospore suspension. Freehand tangential sections were then made parallel to the surface of the exposed tissue. In the other method, longitudinal cuts down to the cambium were made before applying the inoculum. Transverse sections were then obtained with a microtome after fixing pieces of stem in Formalin-acetic acid-alcohol, dehydrating in tertiary butyl alcohol, and embedding in paraffin. All sections were mounted in lactophenol-cotton blue.

Criteria used for determining Phytophthora penetration.—Invasion of stems by *Phytophthora* first causes a discoloration of tissues in the inner bark and cambium. This injury may remain localized, and is not visible externally. When the injury is more extensive, bark tissues disintegrate and produce the characteristic foot rot symptom. In experiments reported here, the presence of a yellowish-brown discoloration in the cambium was regarded as an indication of infection. Data given on lesion size were based on the width of cambial area stained, at the widest point.

RESULTS.—*Evaluation of the effectiveness of periderm as a barrier against infection by P. parasitica.*—Results of greenhouse inoculations showed that whereas some infection occurred on the younger seedlings, no infection occurred on stems completely covered by unbroken cork tissue (Table 1).

In other greenhouse experiments, different depths of tissues were excised from rough lemon stems immediately before inoculation. Some fungal penetration took place where only suberized tissue was removed, but infection occurred more frequently when deeper cuts were made (Table 2).

In field investigations on bark penetration by *P. parasitica,* the zoospores had access to both scion and

TABLE 1. Effect of seedling age, development of periderm, and wounding on susceptibility of sweet orange stems to *Phytophthora parasitica* infection

Age of seedlings, weeks	Stage of bark[a] development	Avg stem diam (mm)[b]	No. plants infected/5 inoculated	
			Unwounded	Wounded[c]
21	Epidermis still intact	1.5	2 (1)[d]	5 (3)
28	Periderm partially formed	2.0	1 (0)	5 (3)
36	Periderm encircling stem	4.5	0	5 (0)

[a] Refers only to 25-mm length of stem measured from soil level.
[b] Measurements made 25 mm above soil level.
[c] Two vertical 25-mm cuts on each stem.
[d] Values in parentheses represent numbers of plants dead 10 weeks after inoculation.

TABLE 2. Incidence of infection on rough lemon seedlings after wounding stem tissues to different depths and inoculating with *Phytophthora parasitica*

Wounding treatment[a]	Experiment 1[b], no. seedlings infected/5 inoculated	Experiment 2[c], no. seedlings infected/4 inoculated
No wounding	0	0
Suberized layer only removed	2	2
Tangential cut exposing inner bark	5	3
Bark slipped off to expose cambium	5	3
Vertical cuts penetrating cambium	5	4
Noninoculated water check, with vertical cuts penetrating cambium	0	0

[a] Wounds made at one location on each stem. The vertical cuts were 25 mm long. In all other wounding treatments, a square of tissue, 25 mm^2, was removed.
[b] Average stem diameter 25 mm above soil, 8 mm.
[c] Average stem diameter 25 mm above soil, 11 mm.

rootstock portions of the trunk of the Page trees, because the watertight collars were constructed around the bud union. The results again showed that intact bark provides considerable protection against *Phytophthora* infection (Table 3). In this test, foot rot did appear on some trees that had not been artificially wounded, but only above the bud union on the more susceptible scion variety.

Using the TTC indicator technique, I rarely observed reddening in the inner bark or cambium of rough lemon and sweet orange seedlings growing in the greenhouse. In contrast, Page trees growing in the field showed a much greater, though highly variable, incidence of TTC penetration. Whereas some of the penetrations coincided with obvious cultivation injuries, others appeared to be associated with breaks of natural origin. Deep breaks were often found at the bases of new suckers.

TABLE 3. Amount of foot rot on Page trees growing on rough lemon rootstock after inoculating with *Phytophthora parasitica* across the bud union in June and banking with soil

Treatment	Portion of trunk	Foot rot severity[a] on five trees[b]				
No wounding	Scion	5,	4,	0,	0,	0
	Rootstock	0	0	0	0	0
Suberized layer removed[c]	Scion	5,	4,	4,	3,	0
	Rootstock	0	0	0	0	0
Vertical cuts[d]	Scion	5,	5,	5,	5,	2
	Rootstock	1	1	1	1	1
Noninoculated water check with vertical cuts	Scion	0,	0,	0,	0,	0
	Rootstock	0	0	0	0	0

[a] 0 = No disease; 1 = bark healed over but brown stain present in cambium; 2 = girdling < 25%; 3 = 25-50% girdled; 4 = 50-75% girdled; 5 = >75% girdled. Data taken 16 weeks after inoculation.
[b] Average diameter 50 mm above bud union at time of inoculation, 38 mm.
[c] Suberized layer scraped away to reveal a square area of underlying tissue measuring 100 mm^2, at two places on each of scion and rootstock portions of trunk.
[d] Two vertical cuts down to cambium extending for ca. 25 mm above and below bud union.

A test was made on stems of Pineapple orange seedlings, planted 6 months previously in the field, to determine whether there was any association between the frequency of *Phytophthora* penetration and numbers of breaks in the bark. Sixteen trees were inoculated with a zoospore suspension, and another 16 were treated with TTC. The TTC-treated stems were examined for internal red staining after 18 hr, and the inoculated trees were examined for *Phytophthora* penetration after 26 days. Fifteen trees showed one or more points of red staining by TTC, but these were mostly indicative of shallow injury. Six trees showed deeper breaks in the bark: one/tree on five trees, and two on the sixth tree. Six of the inoculated trees became infected, one zone of penetration being observed on each tree. One infection point was apparently associated with the base of a young side shoot. The number of stained areas in the cambium, indicative of successful *Phytophthora* penetrations, was less than the total number of breaks detected.

Histological examination of wounded and inoculated parts of sweet orange and rough lemon stems (Fig. 2-A, B) revealed that zoospores had settled mostly on the exposed parenchymatous tissue. Although some zooospores had encysted and germinated on the surface of the bark, no penetration of intact bark was observed. The zoospores sometimes failed to penetrate a narrow cut because of congestion at the entrance (Fig. 2-C), and in these cases, many of the germ tubes were directed towards the underlying exposed parenchymatous cells.

Time that wounds remain open to infection.—The results of greenhouse tests (Fig. 3, left) to determine how long time wounds could function as infection courts indicated that fungal penetration seldom occurred after 10 days even with severe wounds.

Effect of different amounts of inoculum on the incidence of infection and on lesion size.—In a greenhouse test on sweet orange seedlings, a reduction in disease incidence did not occur until the inoculum level dropped to below ca. 4,000 zoospores/stem. Above this level, increases in the number of zoospores applied to the stem continued to increase lesion width (Fig. 3, right).

Effect on foot rot development of postinfection soil-banking of stems.—In greenhouse studies, there was little increase in lesion size unless soil was banked around the inoculated part of the stem. In one test with sweet orange stems, the average width of lesions after 64 days measured 2.1 mm when stems were left exposed, and 3.7 mm when stems were banked with 3 cm soil. In a similar test with rough lemon stems, the average lesion width 41 days after inoculation was 0.6

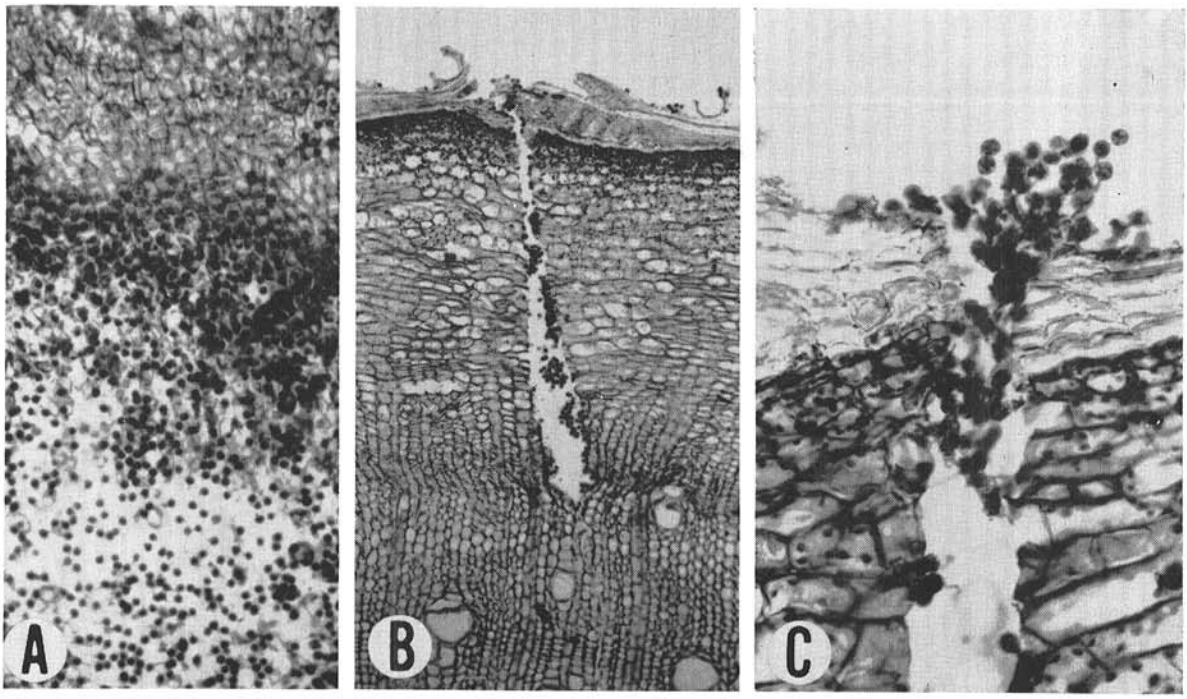

Fig. 2. Distribution of encysted and germinating zoospores of *Phytophthora parasitica* on exposed parenchymatous tissue and bark surface of sweet orange stems. **A)** Section made 30 min after inoculation, paralleling the surface of a tangential cut which was made through the bark at time of inoculation, showing encysted and germinating zoospores that had settled mostly on the cut surface beneath the suberized layer (×170). **B)** Transverse section, 15 min after inoculation, through vertical cut extending down to cambium made at time of inoculation, showing congregation of zoospores in wound (×90). **C)** Transverse section through a cut with narrow opening showing congestion of zoospores at entrance (×350).

mm for exposed stems and 2.3 mm for banked stems.

In a field test, postinfection soil-banking was studied on Page trees budded low on rough lemon rootstock. As in other inoculations of such trees (Table 3), foot rot development was confined almost entirely to the Page portion of the stem. Within each treatment, the trees showed considerable variation in lesion development (Table 4), but stems banked with soil generally developed more foot rot than stems not subsequently banked.

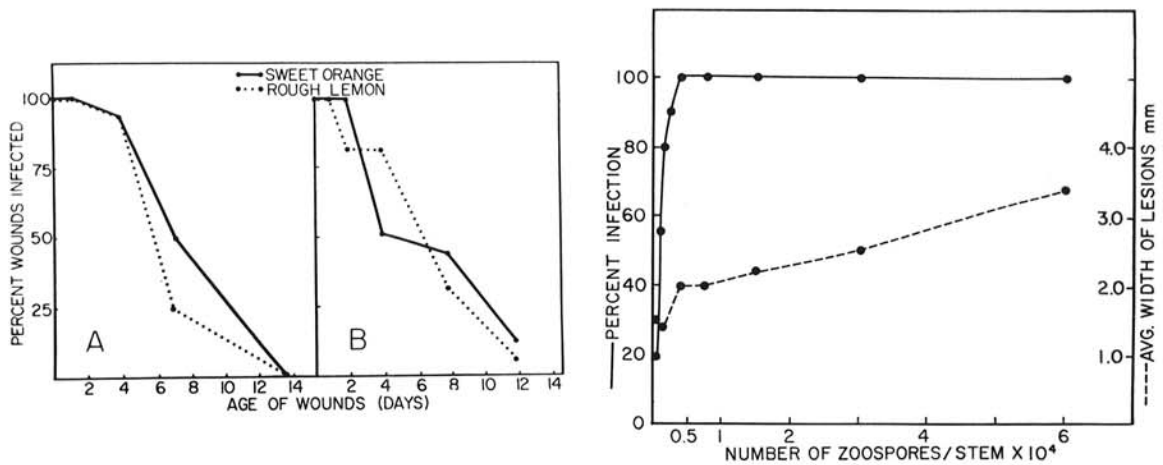

Fig. 3. (Left) Incidence of infection by *Phytophthora parasitica* through wounds in bark of sweet orange and rough lemon stems in relation to time after wounding. **A)** Wounds consisted of hairline cuts 25 mm long. **B)** Wounded by making two vertical cuts and stripping away from wood a portion of bark measuring 25 mm high and 2 mm wide. Avg stem width = 9 mm. Four plants of each variety were wounded at each time. (Right) Effect of *Phytophthora parasitica* zoospore numbers on wound penetration and lesion development on sweet orange stems. Wounds consisted of four vertical cuts, each 25 mm high, on each stem. Avg width of stem = 10 mm. Data recorded 26 days after inoculation. Five plants were inoculated at each concentration level.

TABLE 4. Influence of postinfection soil-banking versus nonbanking on development of foot rot on Page trees growing on rough lemon rootstock

Treatment	Portion of trunk	Foot rot severity on six trees[a]					
Not wounded but banked[b]	Scion	5,	5,	4,	2,	0,	0
	Rootstock	0	0	0	0	0	0
Wounded[c] and banked	Scion	5,	5,	5,	5,	3,	2
	Rootstock	1	1	1	1	1	1
Wounded but not banked	Scion	4,	2,	2,	1,	1,	1
	Rootstock	1	2	1	1	1	1
Noninoculated wet cotton check, wounded and banked	Scion	0,	0,	0,	0,	0,	0
	Rootstock	0	0	0	0	0	0

[a] 0 = No disease; 1 = bark healed over but brown stain present in cambium; 2 = girdling < 25%; 3 = 25-50% girdled; 4 = 50-75% girdled; 5 = >75% girdled. Inoculated in July, data taken 8 months later.

[b] After removing the cotton collar, a bank of unsterilized soil rising to ca. 3 cm above the bud union was built around the stem.

[c] Wounded by making four vertical cuts down to the cambium, each extending ca. 25 mm above and below the bud union.

DISCUSSION.—Infection of stems by *P. parasitica* apparently occurs only when there are mechanically induced or naturally occurring breaks in the bark. Although some infection occurred where only the suberized layer of the bark was removed, infection appeared to be facilitated by wounding more deeply through to the inner bark or cambium, suggesting that some resistance may also be provided by tissues underlying the actual cork layer. Histological studies on wounded and inoculated stems showed accumulation of zoospores on the surface of freshly exposed parenchymatous tissue. This indicated a possible chemotactic response, a phenomenon that causes attraction of zoospores to citrus feeder roots in the region of elongation and to areas of older roots where a wound has occurred (1, 20).

Although naturally formed breaks in the bark were commonly detected on trees growing in the field, some of the trees tested showed no breaks at all. The variable occurrence of breaks in the bark might explain the usually scattered distribution of diseased trees, and the fact that trees adjacent to diseased trees often show no foot rot, even in very closely planted nurseries. Considering that wounds expose the stem to infection for only limited periods, the time at which a break develops in the bark before a favorable infection period occurs would also have an influence on disease occurrence.

The need for a sufficiently high inoculum level, as previously hypothesized as a requirement for fruit infection (16), was shown to be essential for stem infection. There were also indications that the number of zoospores entering an opening might affect fungus activity during the postpenetration phase, thereby influencing ultimate lesion size.

Sources of *P. parasitica* inoculum are present in the soil and originate from diseased citrus feeder roots. Growth of the fungus in the soil itself is, however, very limited, and the fungus persists in the soil, mostly in the form of oospores and chlamydospores (17). After germination, these structures give rise to sporangia that produce zoospores, thereby increasing the number of infective propagules. Although zoospores can be splashed onto the aerial parts of a tree from the soil, it seems unlikely that concentrations high enough to cause bark infection would reach the higher parts of the tree. This might explain in part the reason bark infections are seldom found more than 50 cm above ground under Florida conditions. The greater likelihood of bark infection occurring at the base of citrus trees may also be related to slower drying out of bark close to, or in contact with, soil (particularly if air movement is restricted by weed growth or low hanging branches).

It is generally supposed that low budding, deep planting, and heaping of soil against the stem increases foot rot incidence by bringing soil-borne inoculum closer to the susceptible scion portion of the stem and by keeping the bark surface moist, thereby favoring fungal penetration. The results presented here showed that heaping of soil against the stem of a highly susceptible variety also favored fungal activity after host penetration, due possibly to an increased moisture content of the inner bark.

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