AN INVESTIGATION OF A SAGUARO SEEDLING DISEASE

by

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I hereby recommend that this dissertation prepared under my direction by John A. Booth

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Alice Boyle — Dissertation Director — Sept. 5, 1963 — Date

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INTRODUCTION

During the course of studies involving the saguaro cactus (Carnegiea gigantea (Engelm.) Britt. & Rose) large numbers of seedlings being propagated in the greenhouse were found to be dying. Subsequent investigation showed the cause of death to be due to either simultaneous or independent infection by two species of Fusarium (3).

The disease was primarily noted on crowded seedlings between 0.5 and 2.5 cm in height, but was not uncommon on isolated seedlings. Although excessive watering seemed to promote disease, seedlings growing under normal conditions of soil moisture also succumbed to infection on occasion. Initial symptom expression was characterized by a dark green to black, water-soaked spot without a contrasting margin of advance. The lesions were most frequently observed near the soil line or base of the stem and occasionally near the apex. Within 48 hours after the first water-soaked spots were noted, the entire stem of a naturally infected plant was usually reduced to a semi-liquid mass contained by a more or less intact epidermis. Rupture of the epidermis revealed a black tissue decomposition similar to a bacterial soft rot. As the progressive decomposition entered the central vascular system, light brown streaks developed acropetally and basipetally from the point of contact. In the final stages the non-lignified vascular system of the seedling stem was decomposed as well as the entire root system. Figure 1 shows the external and internal symptoms of naturally-infected

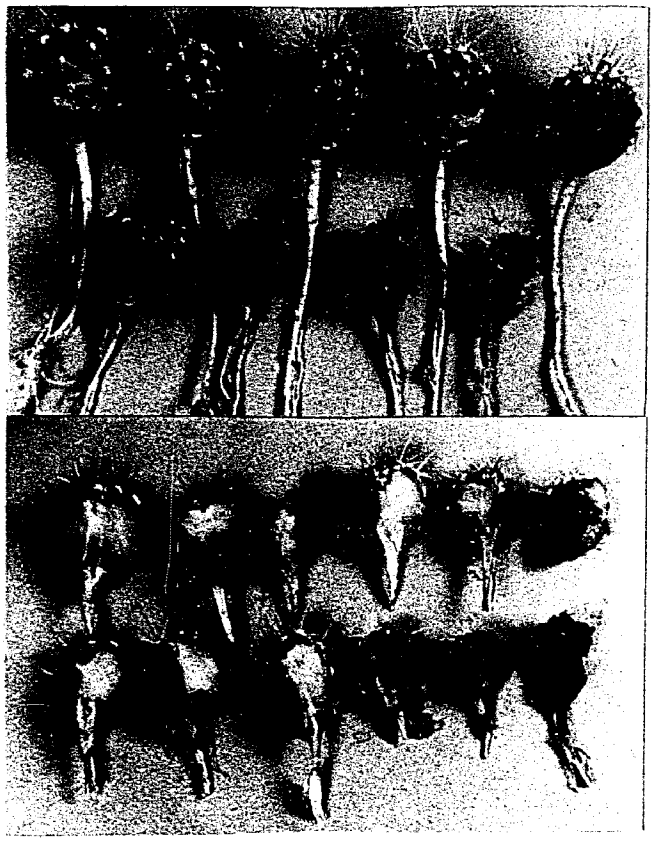

Figure 1. Saguaro seedlings showing varying degrees of natural infection by Fusarium spp. The upper two rows are entire plants, while those below have been sectioned longitudinally to show internal injury. The upper left seedling in each group is apparently healthy.

seedlings.

The decomposed black tissue was always found to contain living Fusarium mycelium, but the organisms were never observed in or cultured from the adjacent apparently healthy tissue. In addition it was noted that mechanically wounded saguaro tissue rapidly turned black, suggesting the presence of phenolic compounds and a high polyphenol oxidase activity.

The research herein described was divided and is presented in two parts. The first part has to do with the description of the host-pathogen relationship and the in vitro behavior of the causal organisms. The second part concerns the investigation of certain aspects of the physiology of the host-pathogen relationship.

THE DISEASE AND THE CAUSAL ORGANISMS

Literature Review

A survey of the literature revealed no previous published report of a fungus disease of saguaro seedlings prior to the report by Booth and Alcorn (3) in 1959. Other species of cacti however have been reported to be susceptible to attack by species of Fusarium. Petrak (17), in 1931, investigated a watery stem rot of various species of cacti representing five genera but not including the saguaro. The causal organism was a species of Fusarium, "possibly identical with F. blasticola Rostrup" (17). A "variety" of F. oxysporum Schlecht. causing a stem rot of mature Cereus schottii Engelm. was recorded in 1934 by McLaughlin (14). Pasinetti and Buzzati-Traverso (16) isolated and described two new species, F. cactacearum Pasin. & Buzz. and F. cacti maxonii Pasin. & Buzz. from Thelocactus nidulans (Quehl) Britt. & Rose and Cactus maxonii Rose, respectively. Both organisms caused a dry basal rot. It is assumed that the report concerned mature plants rather than seedlings. A lethal root disease of mature Opuntia ficus-indica (L.) Miller caused by F. oxysporum Schlecht. var. opuntiarum (Speg.) Pettinari was reported in 1951 by Pettinari (18). Preti (19), in 1935, described a collar rot and tap root rot of seedlings of Cephalocereus senilis (Haworth) Pfeiffer caused by a species of Fusarium closely resembling F. dianthi Prill. Carpenter (5) reported that progressive softening and eventual collapse of mature Opuntia megacantha

Salm-Dyck is caused by a variety of F. oxysporum.

The taxonomy of the Fusarium genus was indefinite and confused for many years. The work of Wollenweber and Reinking (28) in 1935 led the way to a broad form of organization of the genus. The system they devised provided for sixteen sections of the genus as an aid to classification. Natural variation of many isolates, however, often left the investigator on unsure ground in deciding with which species he was dealing. In some cases even the choice of section was in doubt. Snyder and Hansen (22, 23, 24), recognizing the importance of such natural variation, devised a more workable classification system. They reduced the number of sections to nine and the number of species to one per section. Where physiologic specialization, as expressed by selective pathogenicity, was treated on the species level by Wollenweber and Reinking, Snyder and Hansen reduced this to the category of forma. The new system has had wide acceptance by mycologists and has done much to bring order to the classification of new pathogenic isolates of Fusarium.

Isolation, Identification and Pathogenicity of the Causal Organisms

Materials and methods. Naturally infected saguaro seedlings growing in soil were removed at stages of symptom expression ranging from the first water-soaked spots to collapse of about half of the plant. Isolations were not attempted from completely collapsed seedlings. The plants were washed in distilled water, dipped in $HgCl_2$

solution (1:1,000) and, without rinsing, aseptically split apart from the side opposite the lesion. Portions of internal tissues were then removed and cultured from, 1) the black decomposed area, 2) the line of demarcation between healthy and decomposed tissue, and 3) from apparently healthy tissues immediately ahead of the advancing lesion. The tissues were cultured on Difco potato-dextrose-agar (PDA) in culture tubes.

Pure cultures were obtained by making mass hyphal tip transfers to petri dishes containing PDA. Single-spore colonies were then obtained by streaking dilute spore suspensions on water agar and transferring single germinated spores after 18 to 24 hours. Subsequent maintenance of cultures was by mass transfer to fresh PDA.

Fusarium isolates from saguaro and The American Type Culture Collection were inoculated into saguaro tissue of various ages by hypodermic inoculation techniques. Syringes and needles (22 gauge) were sterilized by boiling in distilled water for 30 minutes or by autoclaving for 15 minutes at 15 lb pressure. In all cases, in this and subsequent studies, the epidermis was washed with distilled water, surface sterilized with 5% Clorox solution and then inoculated within 30 minutes. The volume of inoculum and the depth of needle penetration varied according to the size of the plant material used. Seedlings from 1.0 to 12.0 cm as well as segments of branches cut from mature saguaros were inoculated. When the latter were used, multiple inoculations at least 15 cm apart were made into each segment. The inoculum was prepared in some cases by macerating mycelium from PDA

cultures or from nutrient broth cultures. Most inoculum consisted of sterile distilled water spore suspensions washed from the surface of PDA cultures in petri dishes. When standardization of spore concentration was necessary, counts were made using the Improved Neubauer Counting Chamber followed by appropriate dilution with sterile distilled water. Hypodermic inoculation was performed with care to prevent rupture of the succulent plant tissue, but a water-soaked appearance always developed immediately at the site of needle penetration. This condition remained visible for only a few hours after inoculation. Appropriate control inoculations with sterile distilled water were always made. Inoculated plants were placed on the laboratory bench at room temperature and observed daily.

In a study of natural penetration of saguaro seedlings, 500 ml of a dense suspension of Fusarium spores were poured on the surface of vermiculite medium in which 100 seedlings, each 1.0 cm in height, were growing. Eight replications were prepared with each Fusarium species; four contained intact seedlings and the remainder contained seedlings which were wounded immediately following application of the inoculum. Wounds were made by scraping the epidermis at the base of the stem next to the planting medium. Each of two control replications of intact and wounded seedlings were treated with sterile distilled water. The plants were observed daily and representative specimens which developed symptoms were cultured to confirm the presence of the inoculated Fusarium species.

Further studies of methods of inoculating healthy saguaro seed-

lings with one Fusarium sp. included: 1) transplanting into chloropicrin-sterilized soil, subsequently infested with applications of dense spore suspensions; 2) transplanting seedlings whose roots had been dipped into dense spore suspensions; and spraying; 3) mechanically wounded; and 4) intact epidermis with dense spore suspensions. Each inoculated pot contained three 18-month-old seedlings approximately 3.0 cm tall. The pots were placed on the greenhouse bench and observed daily. Half of the pots received water once weekly while the others were watered three times per week. Seedlings which developed symptoms were cultured to confirm the presence of the test organism.

Results and conclusions. Cultures of diseased tissues from 163 naturally-infected saguaro seedlings resulted in the isolation of Fusarium spp. from 154. Bacterial growth was observed in seven cultures and subsequent inoculation of pure cultures of those organisms showed them to be non-pathogenic. The Fusarium isolates were obtained from the black, decomposed tissue and from the slightly brown face of the advancing lesion. Fusarium growth was never observed in cultures from apparently healthy tissue taken from 0.5 mm in advance of lesion margins. The fungi, when observed on PDA in petri dishes, were of two types based on growth habit and pigmentation. Of the 154 isolates, 27 produced a prostrate, greasy-appearing growth and caused a dark purple to light red discoloration of the medium. A white aerial growth was produced by 118 isolates. Nine cultures were mixtures of the two growth types. Spore suspensions of all crude isolates were inoculated into sections of mature saguaro branches or into test seedlings of

various ages. Some degree of pathogenicity was displayed by all isolates, but lesions produced in mature saguaro tissues were often arrested before extensive damage occurred. All control inoculations were negative. Subsequent single-spore isolations from the pigmented types yielded 102 pigmented clones and 48 which were microscopically indistinguishable from the original white aerial type. All single-spore clones (100) from the original white isolates were identical to the parent cultures. Three "type" clones of the pigmented cultures were selected and designated "A" (dark purple), "B" (red) and "C" (light red). One "type" clone of the predominant white aerial growth was also selected.

The white clone was identified as F. solani (Mart.) emend. Snyd. & Hans. and clones A, B and C were F. oxysporum (Schlecht.) emend. Snyd. & Hans.[1]

The F. solani clone, growing on PDA (pH 5.6) at 30°C, produced abundant white aerial growth with spores borne in pseudopionnotes or sporodochia. Macrospores were 5.0-12.0 μ x 2.0-2.5 μ and thick-walled. Septations (one to three) were present but very thin and difficult to resolve. Foot cells were poorly developed but slightly tapered toward the base. The larger terminal cells were rounded or blunt at the apex. The sickle-shaped characteristic was not usually pronounced. Micronidia were abundantly produced and were usually non-septate. Terminal and intercalary chlamydospores were produced

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[1]The writer is indebted to Dr. W. C. Snyder, Professor of Plant Pathology, University of California, Berkeley, California for examination and identification of the "type" clones.

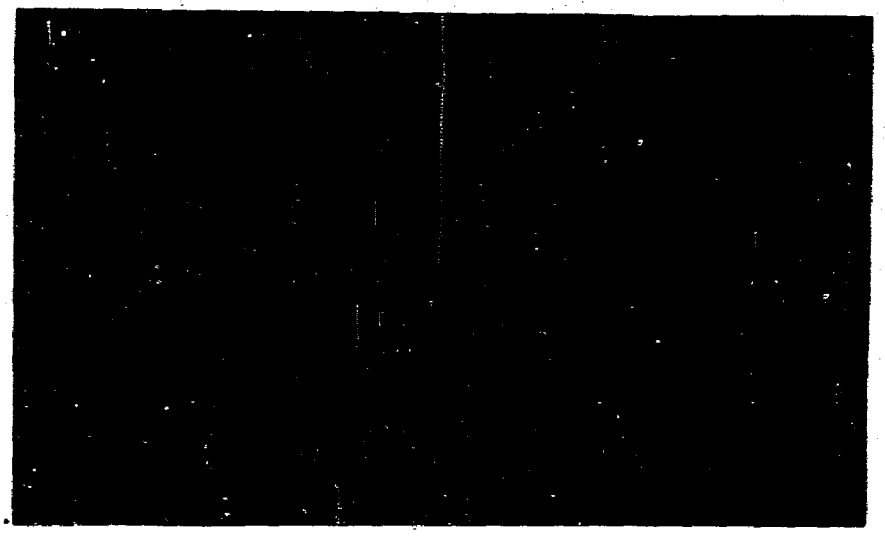
after six to eight days.

The clones of F. oxysporum, growing under the same conditions, were characterized by the production of macro- and microspores in sparse aerial mycelium at the margins of the cultures. The sickle-shaped macrospores were 6.0-14.5 μ x 2.5-3.0 μ with thin septations (two to four) and spore walls. Foot cells were well developed and terminal cells usually tapered to a point. Microconidia were abundantly produced and were usually non-septate. Both terminal and intercalary chlamydospores were sparingly produced. Spores of both Fusarium species are shown in Figure 2.

Pathogenicity was confirmed by hypodermic inoculation of spore suspensions from each "type" clone into separate groups of 22 two- to three-year-old (3.0-8.0 cm tall) saguaro seedlings. F. solani and clones A and B of F. oxysporum caused death and collapse of all seedlings within five days. Clone C of F. oxysporum caused symptom manifestation after 15 to 20 days on 14 seedlings and the remaining eight did not become infected. F. solani was observed to be somewhat more rapid in its pathogenic development than the clones of F. oxysporum. Eight inoculations with sterile distilled water were negative. The inoculated clones were recovered by culturing in all cases where symptoms developed. Two groups of six plants, inoculated with spore suspensions from cultures of F. solani and F. oxysporum obtained from The American Type Culture Collection, did not show a single case of symptom development.

The results of a study of natural infection (Figure 3) by the

A



B

Figure 2. Macro- and microspores of the two Fusarium isolates from saguaro seedlings. A) F. solani (approx. X1000). B) Clone A of F. oxysporum (approx. X1000).

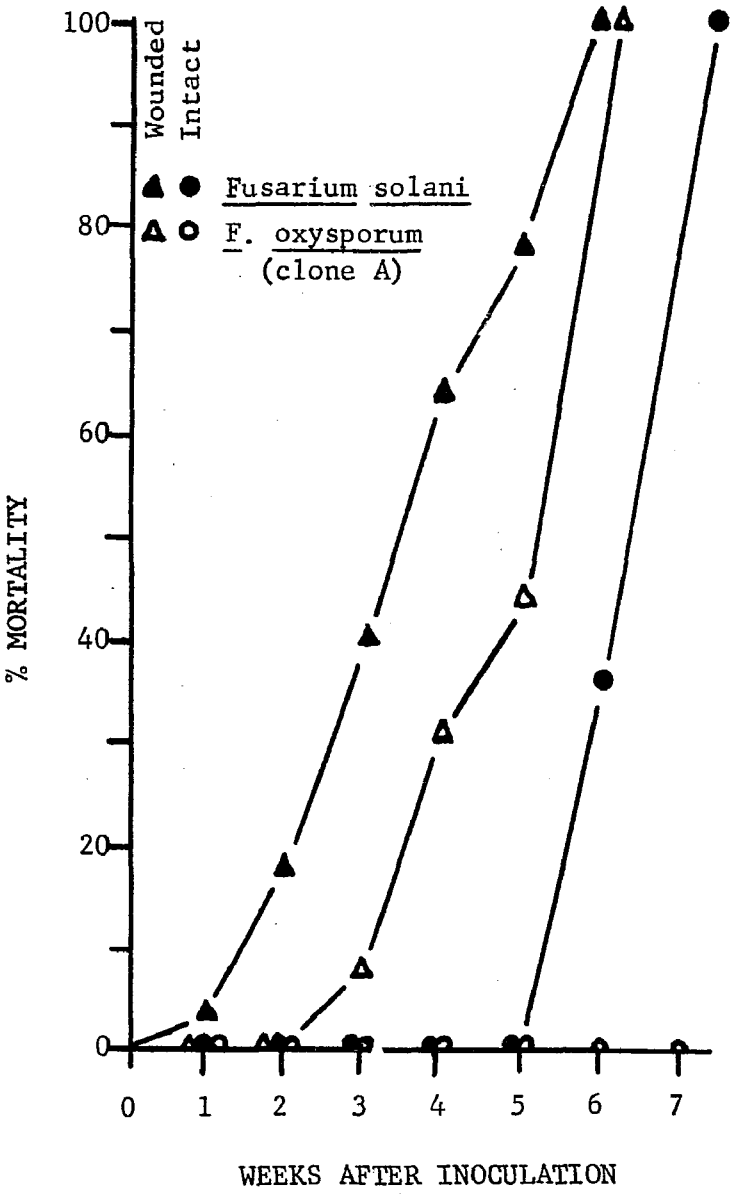


Figure 3. The rate of mortality of intact saguaro seedlings versus those with basal stem wounds. Inoculation was by application of spore suspensions of Fusarium solani or clone A of F. oxysporum to the planting medium. (Each line represents the average of four replications of 100 seedlings.)

two Fusarium species indicate that F. solani did not establish infection in intact seedlings before five weeks but killed all seedlings during the subsequent 14 days. It readily infected and killed all wounded seedlings within the first six weeks. Clone A of F. oxysporum did not infect non-wounded seedlings and was slow to infect those which had been mechanically wounded. Examination of seedlings in early stages of infection showed the initial penetration by both species to be where the stem base contacted the planting medium. Initial penetration of roots was not detected. All cultures of representative plants confirmed the presence of the inoculated Fusarium species. Control pots treated with sterile distilled water showed no loss of wounded or intact seedlings.

The experiment with F. solani having to do with inoculation methods and soil moisture was terminated seven days after the last infection was noted, or 21 days after inoculation. Table 1 summarizes the results of this test. It is noted that this fungus did not readily penetrate the older and larger seedlings used in this study. This suggests the development of a more efficient cuticular barrier to infection, but the short duration of the test may not have provided enough time for the pathogen to become established in the soil. There is some slight indication here, as was noted after observation of natural infections, that infection is enhanced by excessive watering. The results of the spray inoculations indicate the failure of the fungus spores to survive desiccation.

Table 1. Ratios of F. solani infected plants to total numbers of inoculated plants (3.0 cm tall) as influenced by inoculation method and frequency of irrigation.[a]

Inoculation method	Watered once weekly		Watered 3 times weekly	
	Inoculated	Check	Inoculated	Check
Spraying spore suspension on:				
Wounded epidermis	4/18[b]	0/9[c]	4/18	0/9
Intact epidermis	2/18	0/9	1/18	0/9
Dipping roots into spore suspension	1/18	0/9	0/18	0/9
Transplanting into infested soil	0/24[d]	0/9	3/24	0/9

[a]Observations made over a period of three weeks.

[b]Six replications; three plants per replication.

[c]Three replications; three plants per replication.

[d]Eight replications; three plants per replication.

Histological Investigations

Materials and methods. Root and stem tissues from saguaro seedlings inoculated with either F. solani or clone A of F. oxysporum were prepared for sectioning by the tertiary butyl alcohol method (10) and imbedded in Tissuemat[2] (mp 56.5°C). Serial sections 15 μ thick were cut, mounted on slides and stained by a safranin and fast green staining schedule (10).

Fresh stem tissues from similar inoculated seedlings were also examined. The tissues were impregnated under vacuum with 0.2% aqueous ascorbic acid (w/v) prior to sectioning in order to prevent browning. Sections 150 μ thick were cut with a freezing microtome and mounted, without staining, in 5% aqueous glycerine (v/v).

Polaroid photomicrographs were made of appropriate sections.

Results and conclusions. Morphologically, stems of saguaro seedlings 12 to 18 months old are characterized externally by longitudinal, parallel rows of slightly raised areoles bearing 13 spines. The rows begin near the base of the stem, just above the two barely discernible cotyledons, and converge at the apex of the stem where the areole primordia originate. The longitudinal surface depressions, which provide the ribbed effect seen in mature saguaros, are not present in seedlings of this age. Internally, a thick parenchymatous

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[2]Fisher Scientific Company

cortex surrounds a central vascular system of the dissectostele type. The number of vascular bundles corresponds to the number of rows of areoles. A parenchymatous pith is present in older seedlings. Vascular traces connect the areoles to the central vascular system. Cortical parenchyma cells are thin walled and often as large as 150 μ in diameter.

Examination of stained serial sections and fresh sections of inoculated saguaro seedlings revealed that cellular invasion by hyphae of both Fusarium species was inter- and intracellular. Penetration into non-discolored tissues was never observed, but two layers of slightly browned cells at the margins of the lesions usually contained hyphae. Cells in the mass of black, decomposed tissues were separated and full of mycelium. The mycelium tended to hold clumps of cells together (Figure 4). Hyphae were not observed in cortical vascular traces, although in some cases the discoloration extended through the vessels a few millimeters beyond the lesion margin. The pathogens were observed within vessel elements of the central vascular system but only when the over-all decomposition involved that area. In young seedlings, with only slightly lignified vascular elements, the pathogens reduced those tissues to an undifferentiated mass except that intact spiral vessel thickenings could be seen microscopically. In older, much lignified, vascular systems, the collapse of parenchymatous tissue left a somewhat intact xylem "skeleton." It was concluded that the two Fusarium species showed no differences, histologically, in their attack of saguaro seedlings.

Figure 4. A dissociated group of saguaro seedling cortical cells invaded by Fusarium solani. The cells are from an aqueous suspension of decomposed tissue removed from a rotting seedling (X 360).

Cardinal Temperature and pH Values for the Pathogens

Materials and methods. For these studies all cultures were grown in Richards' solution (KNO_3, 10.0 g; KH_2PO_4, 5.0 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; $FeCl_3$, 0.02 g; sucrose, 50.0 g; distilled H_2O, 1,000 ml).

Determination of the cardinal temperatures for the growth of F. solani and clone A of F. oxysporum was carried out in test tube cultures containing 10 ml of autoclaved medium (pH 5.6) seeded with one wire loopful of spore suspension. Prior to seeding, spore concentrations were equalized by the method previously described. Ten replicate cultures of each Fusarium species were grown in the dark at 6°, 10°, 15°, 20°, 25°, 30°, 34°, 38°, and 40°C. The controlled temperature facilities included refrigerators, dry heat incubators and water baths. Dry heat incubators were humidified to some extent by a water reservoir placed on the bottom shelf. Temperature effects were measured in terms of dry weight of mycelium produced after incubation for 14 days. The cultures were centrifuged three times at 15,000 x gravity for 15 minutes with resuspension of the mycelial pellets in distilled water between the second and third centrifugations. The final pellets were then transferred to tared aluminum foil squares, dried at 80°C for 24 hours, and weighed with an analytical balance. Weights were double-checked by drying for a second 24-hour period and reweighing.

The combined influence of pH and temperature on mycelial growth was also determined for both Fusarium species. In preparing samples

of Richards' solution of various pH values it was noted that some salts precipitated at values above pH 8.0. To avoid differences in salt concentration, the entire volume of solution was adjusted to pH 12.0 with 1 N KOH solution. The precipitated salts were then filtered off and individual portions of the medium were adjusted to the desired pH values with 0.1 N HCl. All samples were then brought to standard volume with distilled water. The final pH values after tubing (10 ml per tube) and autoclaving were: 3.1, 5.4, 7.5, 8.8 and 10.8. Five replications of each pH value were prepared for each of the two fungi and for dark incubation at 20°, 25°, 30° or 35°C. Seeding of the tubes was carried out as in the temperature study except that spore concentrations were not equalized. The cultures were harvested as previously described after incubation for 10 days.

Results and conclusions. At pH 5.6 the cardinal temperatures for both F. solani and clone A of F. oxysporum were found to be 10°, 25° and 35°C (Figure 5). There was considerable difference between the growth of the two species at temperatures above the optimum; F. oxysporum was much more tolerant of higher temperatures and produced more aerial growth on the surface of the medium. Cultures which showed no growth at 6°C produced abundant growth when transferred to 25°C at the conclusion of the test. Those incubated at 40°C produced no growth when similarly transferred to 25°C. The final pH of the culture filtrates was 6.7 regardless of the incubation temperature.

The two Fusarium species also showed differences in their growth response to a range of pH values (Figure 6). F. oxysporum at

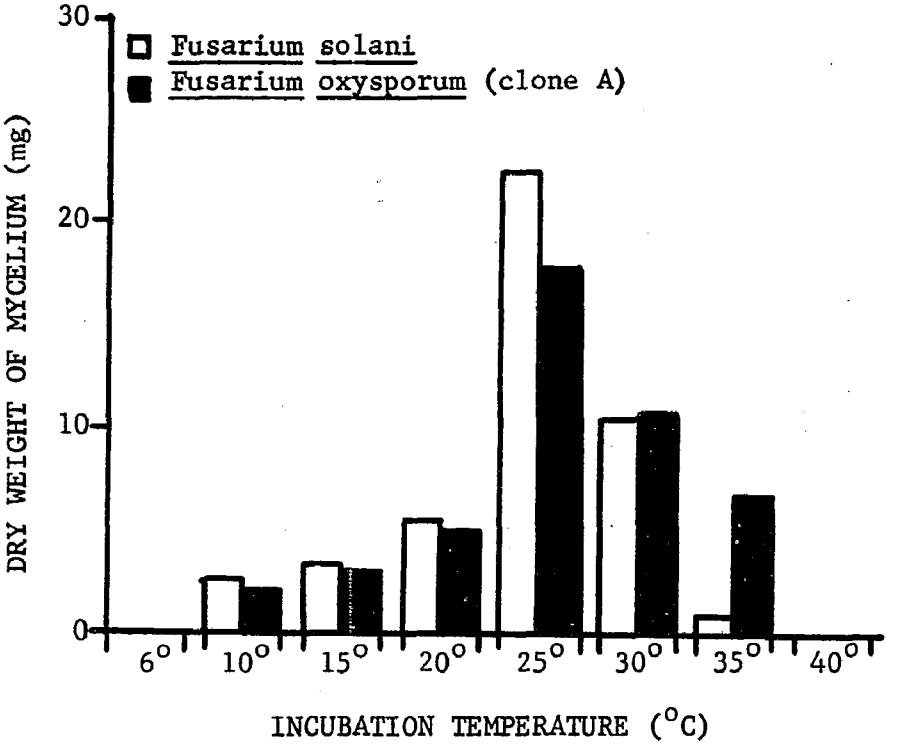

Figure 5. The effect of incubation temperature on mycelium production by Fusarium solani and clone A of F. oxysporum after 14 days of growth in Richards' solution at pH 5.6. (Each bar represents the average of ten replicate cultures.)

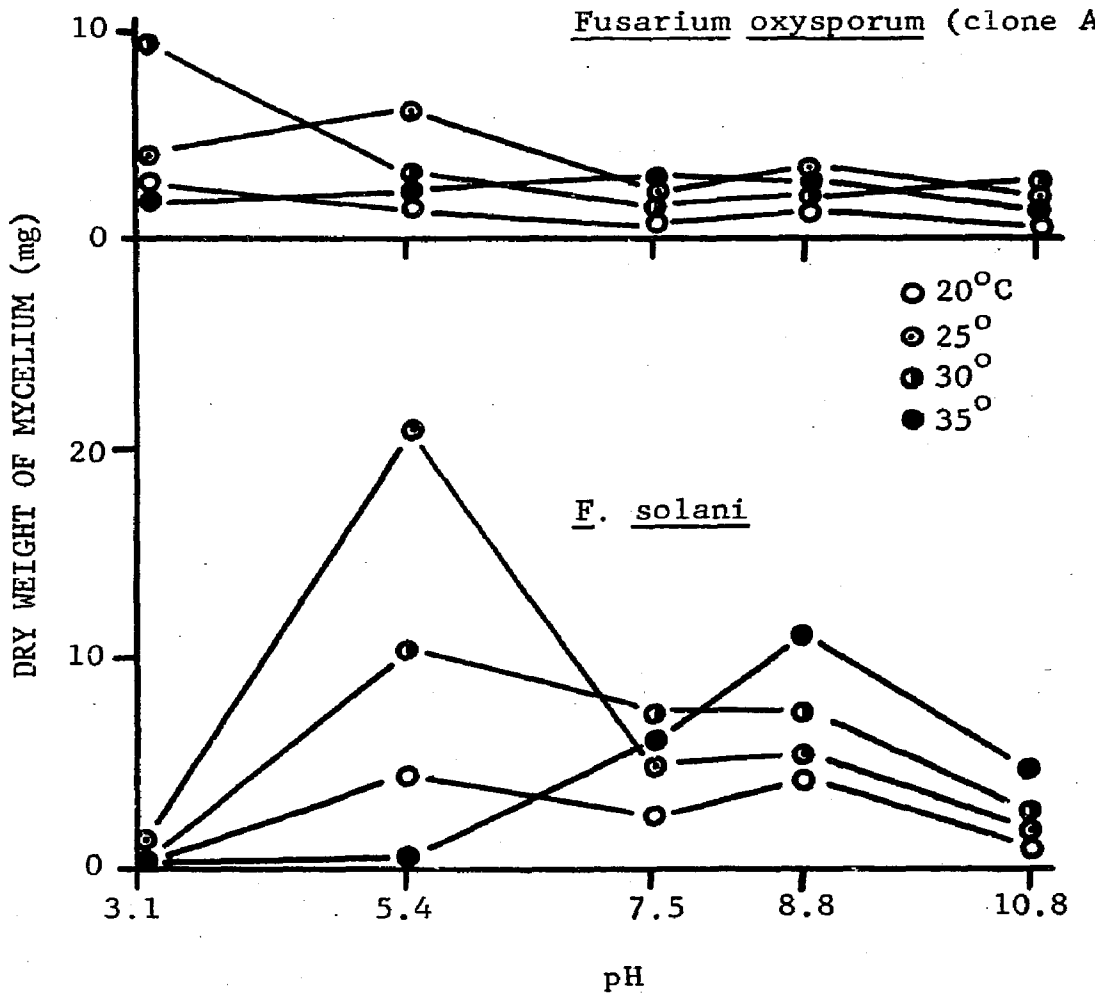

Figure 6. Production of mycelium by Fusarium solani and clone A of F. oxysporum as influenced by a range of pH and temperature conditions. Cultures were harvested and weighed after incubation for ten days. (Each point represents the average of five replicate cultures.)

30°C produced abundant growth at pH 3.1. This was subsequently confirmed by observation of five cultures in 50 ml of pH 3.1 medium at 30°C. F. solani, on the other hand, was intolerant of pH 3.1 at all temperatures. It is interesting that at 35°C the maximum growth of that species occurred at pH 8.8 and that at 20°, 25° and 30°C a slight secondary growth stimulation occurred at that same pH level.

Since the spore suspensions used were not of the same concentration, it is not possible to compare the two graphs with respect to the amount of growth shown by the two species. Qualitatively, however, the growth noted at 25°C and pH 5.4 for both organisms is comparable to the optimum noted at the same temperature in the previous experiment.

Neither species was observed to produce growth at 30°C in a subsequent test (two replicate cultures of each species) at pH 2.7 and and 11.1.

Effect of Temperature on Infection and Disease Advance

Materials and methods. The natural infection by F. solani of saguaro seedlings (0.5 cm tall) growing in petri dishes containing sterile vermiculite was observed under four controlled temperature conditions. The dishes contained 100 seedlings so arranged that no two seedlings were in contact. Eight dishes at each temperature were inoculated with 10 ml of dense spore suspension. The plants in four dishes were then wounded by scraping the stem epidermis near the surface of the vermiculite. Similarly treated controls were also prepared. The

dishes were kept in the dark at 20°, 25°, 30° or 35°C in humidified incubators. The number of diseased plants was recorded at two-day intervals. During the test, representative diseased plants were cultured to confirm the presence of the inoculated F. solani isolate.

The effect of incubation temperature on disease advance was also observed in three-year-old saguaro seedlings (8.0 to 10.0 cm tall) inoculated with F. solani. The plants, growing in 2.5" plastic pots of vermiculite were enclosed separately in polyethylene bags to prevent possible contamination of the incubators. Five plants each were placed in unlighted incubators at 20°, 25°, 30° and 35°C for 24 hours prior to inoculation. Four plants at each temperature were then inoculated hypodermically with 0.5 ml of a suspension of F. solani spores. The fifth plant in each set was similarly inoculated with sterile distilled water. The progressive manifestation of symptoms at the four temperatures was observed daily until complete collapse of seedlings at optimum temperature was noted. All seedlings were then split open and checked for internal decomposition.

Results and conclusions. Penetration and infection of wounded and intact saguaro seedlings (0.5 cm tall) by F. solani were greatly influenced by incubation temperature (Figure 7). Penetration was apparently enhanced at 25° and 30°C and completely inhibited at 35°C. At 20°C the disease was considerably arrested. It is interesting that the minimum incubation period prior to the first infection was 14 days regardless of temperature. The graphs illustrate also the importance to the pathogen of wounds as portals of entry into the plant. All cul-

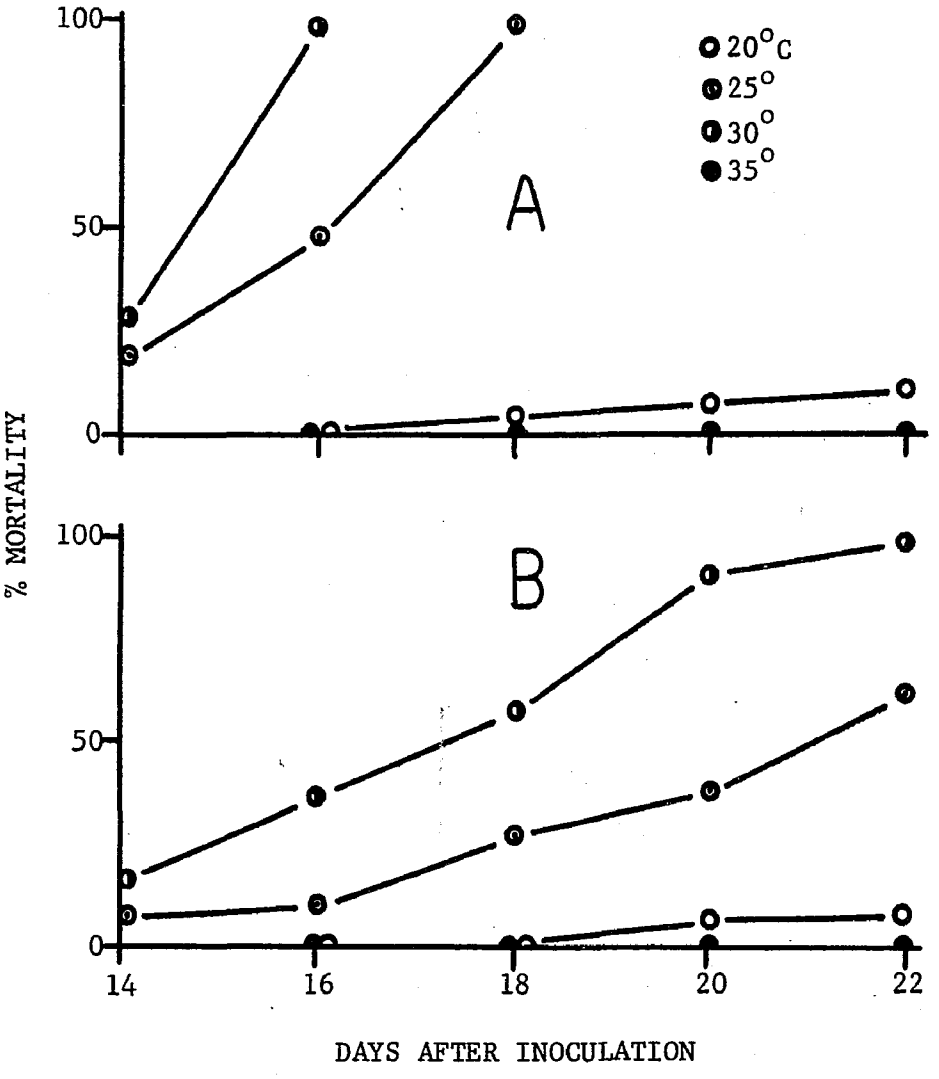

Figure 7. The effect of temperature on infection, by Fusarium solani, of four-week-old basally wounded (A) and intact (B) saguaro seedlings. (Each point represents the average of four replications of 100 plants.)

tures of representative diseased plants from each petri dish confirmed the presence of the causal organism.

Disease advance in three-year-old saguaro seedlings held at 20°, 25°, 30° and 35°C prior to and following inoculation presented a somewhat different picture (Figure 8). The most rapid symptom expression was observed in plants held at 25°C although the activity at 30° was only slightly less. Here again, the activity of the pathogen was completely arrested at 35°C; examination of the internal tissues of one of the plants held at that temperature revealed slight callus formation in the cortical tissue surrounding the needle puncture. Although external symptom expression by plants incubated at 20°C was only 15 percent, it was noted that nearly 60 percent of the internal tissues were decomposed. The decomposition was quite firm in contrast to the watery breakdown observed in plants held at higher temperatures. When seedlings incubated at 35°C were moved to 25°C they began to show symptoms after six days, indicating that the pathogen was still viable and capable of penetrating the thin barrier of callus tissue. Culture of representative infected plants from each temperature confirmed the presence of the pathogen.

Growth of *Fusarium* spp. on Special Media

Materials and methods. The growth of *F. solani* and clone A of *F. oxysporum* on PDA was compared with growth on saguaro seedling-dextrose-agar (SDA). The two media were identical except that a filtered broth prepared by boiling (30 minutes) 1.0 kg of fresh, coarsely chop-

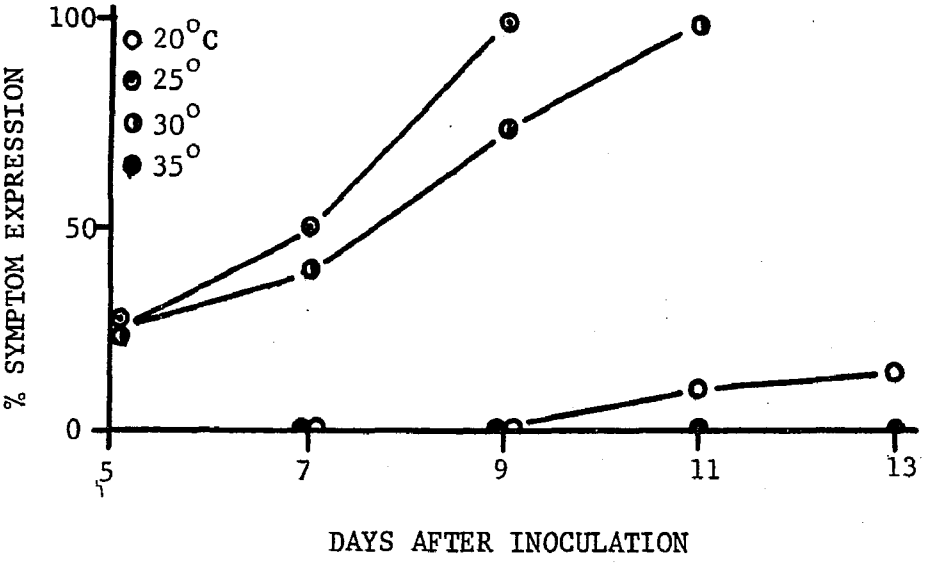

Figure 8. The rate of symptom development in three-year-old saguaro seedlings, inoculated with Fusarium solani, as influenced by inoculation temperature. (Each point represents the average of three inoculated seedlings.)

ped, saguaro seedlings (18-month-old) was substituted for the potato broth in one liter of standard PDA medium. Fifteen ml of each medium were poured into each of ten petri dishes. Each Fusarium species was seeded on five replicate plates of each medium by placing one wire loopful of standardized spore suspension in the center. The dishes were incubated at 30°C. Increase in colony diameter was measured along a fixed axis at 24-hour intervals for a period of five days. At the termination of the test the growth character of each isolate was recorded. In addition, spore production was estimated by counting the number of spores per milliliter of suspension obtained by flooding each plate with 20 ml of distilled water.

Growth of the two species was also compared on natural media prepared by the method of Hansen and Snyder (9). Dried, coarsely ground soybean, tomato and saguaro stems were sterilized by exposure to propylene oxide fumes for 24 hours. The materials were used by aseptically placing a pinch of the sterile material into flasks of 2.0 percent water agar prior to pouring into petri dishes. One ml of heavy spore suspension of F. solani or F. oxysporum was poured over the surface of plates of each medium. The plates were incubated in the dark at 30°C and observed periodically for unusual growth effects and particularly for the presence of the perithecia of the Hypomyces form of F. solani.

Results and conclusions. Comparison of the rates of increase in colony diameter (Figure 9) revealed very little difference due to the two test media. F. solani showed a more rapid growth on both

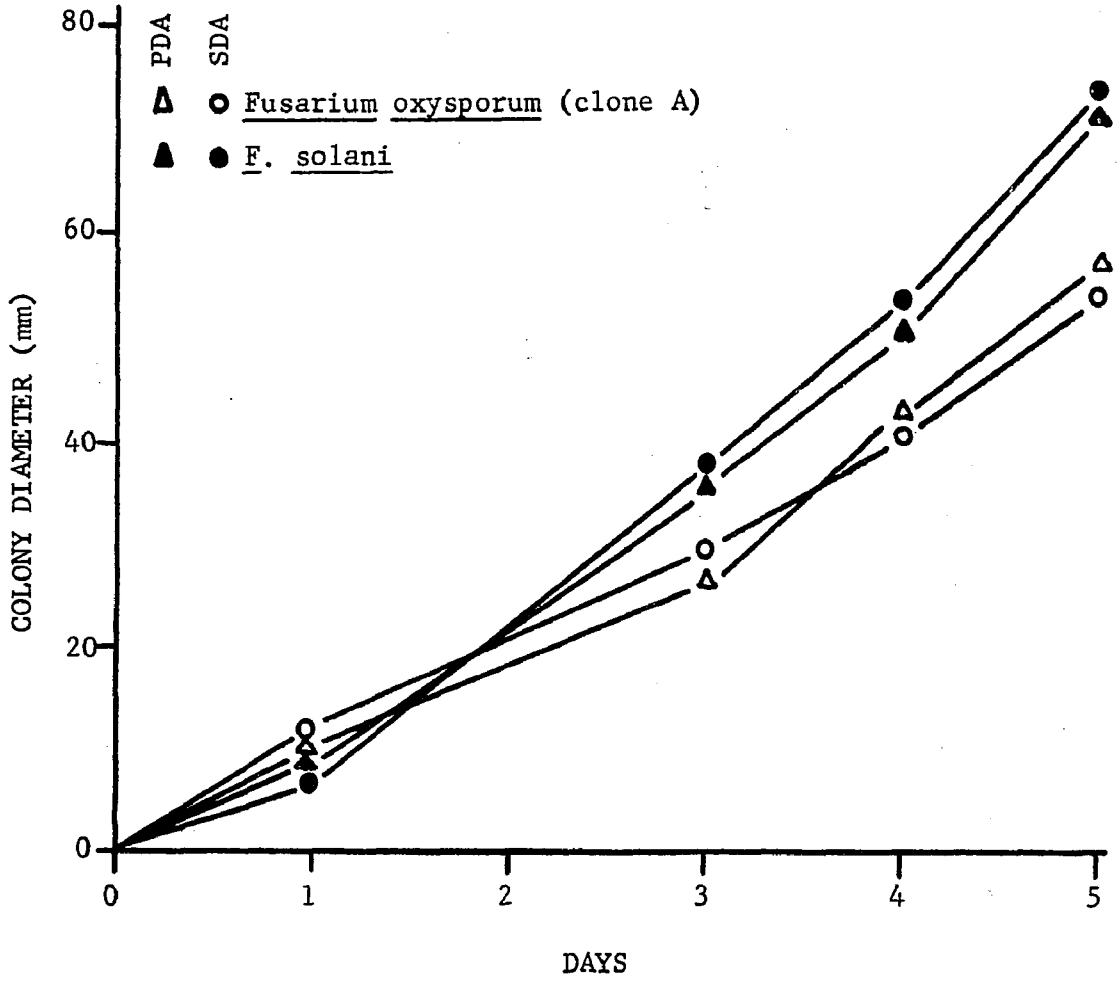

Figure 9. Comparison of the growth rates of Fusarium solani and clone A of F. oxysporum on potato-dextrose-agar (PDA) and saguaro-dextrose-agar (SDA). (Each point represents the average diameter of five replicate cultures.)

media than did clone A of F. oxysporum. Both organisms produced profuse mycelial growth on PDA but only thin growth on SDA. The SDA medium turned dark olive to brown following sterilization suggesting autoxidation of phenolic compounds. When spore production was compared, it was noted that F. solani produced 133,000 spores per square centimeter of mycelial growth on SDA and 300,000 on PDA. F. oxysporum (clone A) showed a similar trend with 610,000 spores per square centimeter on SDA and 1,500,000 on PDA.

Cultures of both organisms on preparations of dried plant materials suspended in water agar did not produce any unusual growth effects. Growth on soybean and saguaro materials was quite sparse, but ground tomato stem yielded profuse growth. No sign of the Hypomyces form of F. solani was noted after incubation for six weeks at 30°C.

Suscept Range Studies

Materials and methods. F. solani and clones A, B and C of F. oxysporum were tested for pathogenicity to various species of cacti native to southern Arizona. The cacti were used as seedlings or as vegetatively propagated, mature plant parts. Dense spore suspensions were used for hypodermic inoculations. Mature plants of prickly pear, Opuntia engelmanii Salm-Dyck, two species of cholla, O. fulgida Engelm. and O. versicolor Engelm. as well as seedlings of the barrel cactus, Ferocactus wislizeni (Engelm.) Britt. & Rose, and the organ pipe cactus, Lemaireocereus thurberi (Engelm.) Britt. & Rose, were used. Three

hypodermic inoculations with each Fusarium isolate were made in each plant species. Inoculations were made into separate stem pads or segments of the branched Opuntia species and into separate seedlings of the barrel and organ pipe cacti. Appropriate sterile distilled water control inoculations were made into each test species.

Two experiments conducted in the greenhouse were designed to ascertain the susceptibility of several economic crop plants to F. solani and clone A of F. oxysporum. Sterilized soil[3] in redwood flats was infested by adding one quart of a culture of either F. solani or clone A of F. oxysporum growing on steamed, unhulled oats. Four flats were prepared with each Fusarium species and four control flats contained sterile steamed oats. One week after infesting the soil, one row of each of the following plants were seeded in each flat: flax, Linum usitatissimum L., 'Punjab'; cabbage, Brassica oleracea var. capitata L., 'Copenhagen Market'; watermelon, Citrullus vulgaris Schrad., 'Klondike'; tomato, Lycopersicon esculentum Mill., 'Pearson'; cantaloup, Cucumis melo L. var. reticulatus Naud., 'Hale's Best'; bean, Phaseolus vulgaris L., 'Pinto'. Flax, cabbage and tomato were seeded at the rate of 30 seeds per row, while the rate for watermelon, cantaloup and bean was 10 seeds per row. The flats were observed daily for symptoms of Fusarium infection and plants suspected of being infected were removed, examined and cultured. After 60 days all plants were

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[3]Soil was sterilized with chloropicrin in sealed containers for one week and aired for three weeks prior to use.

removed from the soil, the roots were washed and a thorough examination was made for signs or symptoms of Fusarium infection.

The same plant species were tested in a second experiment in which healthy plants growing in 8" plastic pots of sterile vermiculite were inoculated with spore suspensions as previously described for saguaro seedlings growing under the same conditions. The numbers of replications and plants used for each Fusarium species and for controls were as follows: cabbage and tomato were planted in eight replicate pots with five plants per pot; watermelon, cantaloup and bean were planted in 10 replicate pots with two plants per pot; flax was planted in four replicate pots with 10 plants per pot. Immediately after inoculation, half of each replication was set aside and all plants in the remaining half were wounded by severing several rootlets and by scraping the epidermis below the surface of the vermiculite. The pots were examined periodically and those plants suspected of being diseased were removed, inspected and cultured. The experiment was terminated after 60 days.

Results and conclusions. The results of the inoculations of five cactus species are shown in Table 2. F. solani in one case caused disease of a stem segment of O. engelmanii; the other two inoculated segments did not develop symptoms beyond the site of inoculation, nor did any of those inoculated with the clones of F. oxysporum. The one diseased segment first showed a dark-green, water-soaked spot which became tan to brown with a slightly chlorotic margin. Abscission, before more than half the segment was rotted, prevented spread of the

Table 2. Ratios of disease occurrence to total numbers of inoculations of five cactus species with Fusarium solani and three clones of F. oxysporum.

Test plant	F. solani	Fusarium oxysporum A	B	C	Check
Opuntia engelmanii[a]	1/3	0/3	0/3	0/3	0/4
Opuntia fulgida[a]	1/3	2/3	3/3	2/3	0/4
Opuntia versicolor[a]	2/3	3/3	1/3	1/3	0/4
Lemaireocereus thurberi[b]	3/3	3/3	3/3	3/3	0/4
Ferocactus wislizeni[b]	3/3	3/3	3/3	3/3	0/4

[a]Three inoculations were made per plant in separate pads or segments.

[b]Single plants were used for each inoculation.

infection into the adjoining segment. Complete collapse of the abscissed pad was noted 18 days after inoculation; the internal tissues showed a moist, brown-black, pulpy consistency unlike the watery breakdown of saguaro seedlings.

The four Fusarium isolates caused similar symptoms in stem segments of O. fulgida and O. versicolor. F. solani caused disease of O. fulgida in only one case. Here again, abscission occurred prior to the advance of the organisms into adjoining segments. Symptoms included an initial browning with a slightly chlorotic advancing margin. Decomposed internal tissue was black and of a semi-dry, putty-like consistency. In all cases, segments became completely infected within seven days after inoculation.

Seedlings of L. thurberi were infected by all four Fusarium isolates and showed symptoms similar to those shown by saguaro seedlings except that breakdown of internal tissues was less watery. Collapse of the seedlings occurred within five days after inoculation.

Fero. wislizeni seedlings were also infected by all four Fusarium isolates. In all cases the plants became chlorotic and shriveled with internal tissues turning black. This type of symptom development could have been due to the reduced vigor and partial dehydration of the seedlings prior to inoculation.

Generally speaking, clone C of F. oxysporum did not attack the test plants as vigorously as did the other isolates. Each isolate was recovered from one representative diseased plant and reinoculated into three saguaro seedlings. All such inoculations were positive.

The tests for susceptibility of six economic crop varieties to F. solani and clone A of F. oxysporum were all negative. In the first experiment with plants growing in flats, a late soil infestation of fly larvae caused wilting of a few watermelon and flax plants. Examination of the roots showed extensive larval damage and in some cases the inoculated Fusarium isolate was cultured from the damaged tissue. Plant species growing in individual pots of sterile vermiculite in the second experiment were not attacked by either isolate. Mortality of similarly treated pots of saguaro seedlings in the tests described earlier was 100% except that no infection was noted on intact seedlings growing in vermiculite infested with clone A of F. oxysporum. The test with cabbage was inconclusive because of high greenhouse temperatures, but subsequent inoculations during cooler weather were also negative.

PHYSIOLOGICAL ASPECTS

Literature Review

Several aspects of the host-pathogen relationship observed during the course of the preceding investigation seemed worthy of further consideration. First the black discoloration of the decomposed tissue and the fact that the causal organism did not advance beyond the discoloration were of interest. The oxidation and condensation of phenolic compounds was suggested as a cause of discoloration which in turn suggested the possibility of polyphenol oxidase activity. Since mechanically wounded saguaro tissue turned brown and finally black, it was felt that the host itself was the source of the phenolic compounds and the enzyme if such were the cause of the reaction. Uritani (25) recently presented an excellent discourse on the role of phenolics in plant disease resistance. The saguaro seedling rot certainly does not present a resistance picture but it does suggest a mechanism bordering on resistance. As early as 1935 Walker and Link (26) demonstrated that protocatechuic acid and catechol were in some manner involved in the resistance of pigmented onions to attack by Colletotrichum circinans (Bark.) Vogl. In 1936 Dufrenoy (7) mentioned an accumulation of phenolic compounds in apparently healthy potato tissue next to areas infected by Synchytrium endobioticum (Schilb.) Perc. Reports of production by plants of specific phenolic compounds,

particularly chlorogenic and caffeic acids, in response to infection have been published in recent years (1,2,8,11,12,13,20). The toxicity of chlorogenic and caffeic acids to certain fungi, as shown by Schaal and Johnson (20), was largely dependent upon pH but they assumed that the oxidized or quinone form might be more efficacious. Clark et al. (6) reported a chlorogenic acid-amino acid addition product from potatoes which strongly inhibited the growth of Helminthosporium carbonum. Breakdown of the product into chlorogenic acid, caffeic acid and six amino acids resulted in marked decrease in inhibitory action. Uritani (25) speculated that the condensation of oxidized phenolic compounds into melanin-like substances might result in the formation of physical barriers to infection. The physiological role of polyphenol oxidase in plant diseases is not yet clear although Akazawa and Uritani (1) have demonstrated an increase in the activity of that enzyme in sweet potatoes infected with the black rot organism.

The nearly complete dissociation of the cortical cells involved in the saguaro disease lesions suggested the production of pectic enzymes by the pathogen. The role of such enzymes in plant pathogenesis has been discussed by Wood (29). In addition, the relationship of natural phenolic compounds to the function of pectic enzymes is also of interest. Byrde (4) concluded that the quinones produced in resistant apple fruit inoculated with Sclerotinia fructigena inactivated the pectic enzymes of the pathogen.

The following investigations were undertaken to ascertain the presence and mode of action of phenolic compounds, polyphenol oxidase

and pectic enzymes in the host-pathogen relationship of the saguaro seedling disease.

Saguaro Seedling Phenolic Constituents

Saguaro seedlings used in the following study were three to four years old and 5 to 8 cm in height. Hypodermic inoculations with F. solani spore suspensions were made as previously described.

Materials and methods. When saguaro cortical tissue was placed in boiling 95% ethanol, autoxidation of phenolic compounds with eventual formation of melanin-like substances readily occurred. Because of this, the extraction of phenolic compounds was carried out with pre-chilled (-10°C) 95% ethanol containing 0.25% ascorbic acid. Ten g of fresh cortical tissue were homogenized in a Waring blender. After filtration the filtrates were stored at -10°C until used for analysis.

Paper chromatography was used to characterize the phenolic constituents. The developing solvent was n-butanol: acetic acid: water[4] (4:1:1 v/v) used in the descending technique with Whatman #1 or 3 MM paper. After equilibration in solvent vapor for 12 to 14 hours the solvent was introduced and allowed to migrate downward until the front was within 2 to 5 cm of the edge, or a distance of 40 to 45 cm. Visualization of phenolic compounds was made as follows: equal volumes of solution A (0.9 g sulfanilic acid, 9.0 ml conc. HCl and 91.0 ml H_2O)

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[4]Deionized, distilled water was used in this and all subsequent procedures where the use of water is indicated.

and solution B (5% $NaNO_2$) were combined and immediately sprayed on the paper. After drying, the paper was sprayed with aqueous 20% sodium carbonate. This procedure caused a diazo coupling reaction in which phenolic compounds appeared red to brown.

Quantitative measurements of chlorogenic acid and related compounds were made, using the procedure devised by Zucker and Ahrens (30). A column of basic alumina 1 x 15 cm was prepared and washed with 200 ml of water. Solutions of chlorogenic acid or plant extracts were added to the column at the rate of 10.0 ml per sample, followed by washing with 10.0 ml of water. Next, a freshly prepared mixture of 2.0 ml of 5% acetic acid and 2.0 ml 0.5% $NaNO_2$ was introduced, followed by 30 to 40 ml of water. Chlorogenic acid turned orange to tan in a narrow band at the top of the column. Introduction of 5.0 ml of 5N NaOH caused the band to turn red and further washing with water moved the band downward. The collected eluate was then brought to 10.0 ml with water and its optical density was measured with a Bausch and Lomb "Spectronic 20" colorimeter set at 525 mμ. A chlorogenic acid standard curve was prepared for use in evaluating eluates from plant extracts (Figure 10).

Results and conclusions. Paper chromatographic analysis of crude saguaro tissue extracts showed a phenolic reaction at only one location (Rf 0.36) with the n-butanol: acetic acid: water solvent system. Rf values for pure chlorogenic acid and caffeic acid were 0.49 and 0.73 respectively. Subsequent rechromatograming of aqueous eluates of the phenolic material from streaked chromatograms did not

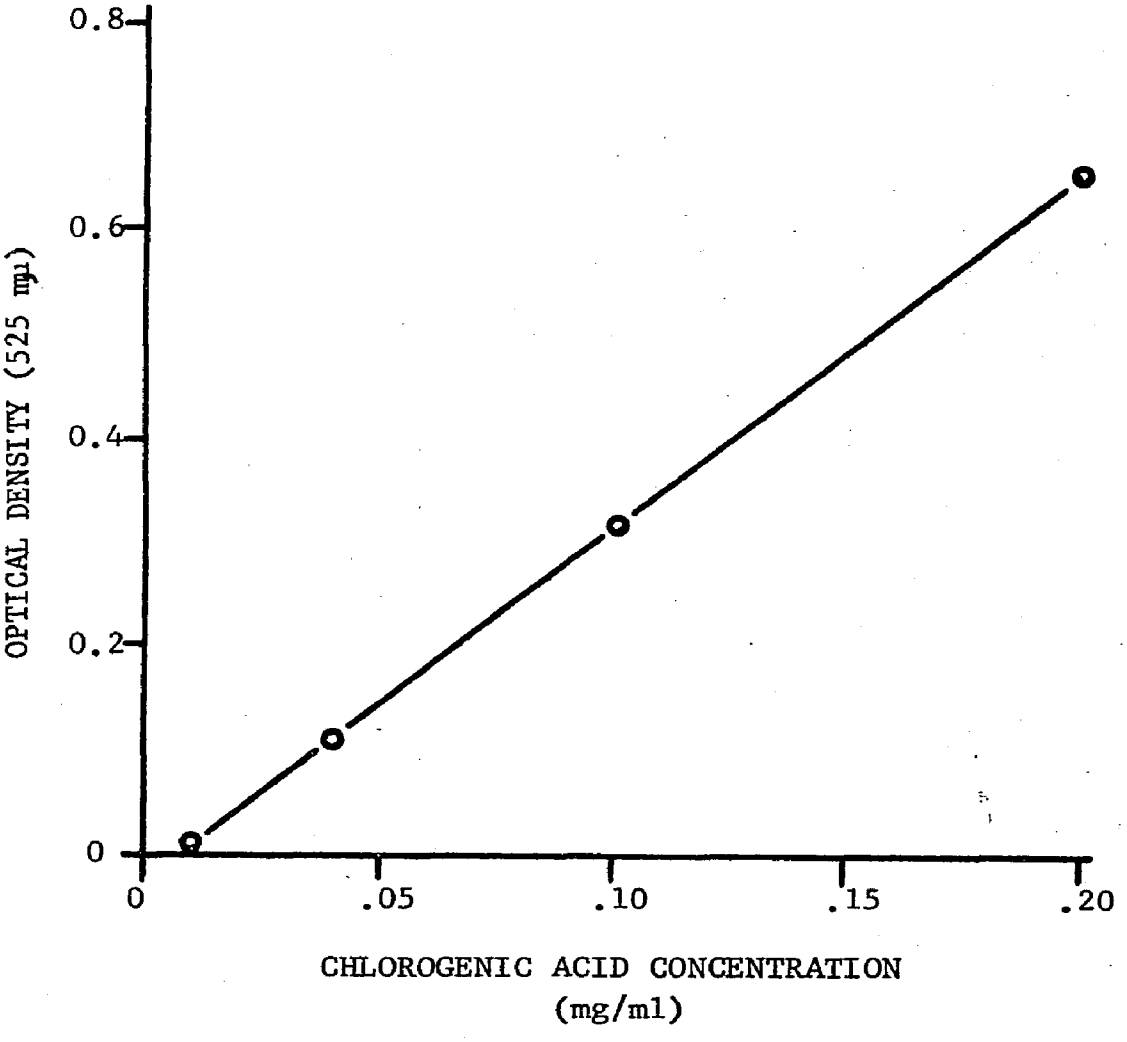

Figure 10. Standard curve for the spectrophotometric determination of chlorogenic acid concentration. The formula for the regression line is y = 3.38x -0.025. (Each point represents the average of three replicate determinations.)

show separation into more than one component. The material proved to be positive also to ninhydrin by the development of a red-violet color. The reaction to ninhydrin suggested the presence of a phenol-amino acid product such as that reported by Clark et al. (6). It was noted that when pure tryptophan and phenylalanine solutions were chromatographed with the same solvent they produced ninhydrin-positive spots of Rf 0.36 and 0.48 respectively. In addition, the two amino acids did not react to the diazo spray reagent with the same color reaction typical of the saguaro phenolic compound (SPC) as did chlorogenic and caffeic acids. SPC did not fluoresce under short-wave UV light but chlorogenic and caffeic acids showed a blue-white fluorescence. Table 3 shows a comparison of the reactions of the various compounds tested.

Visual comparison of paper chromatograms of extracts from healthy saguaro cortex with those from the apparently healthy tissues bordering disease lesions showed SPC to be present in slightly higher concentrations in the latter. Extracts from the black decomposed tissues, however, did not contain the compound in detectable quantities.

An eluate of SPC in 95% ethanol showed a UV-absorption maximum at 283 mμ. Rechromatograming did not change the absorption maximum of the second eluate.

SPC also produced a narrow red band on basic alumina columns which was indistinguishable from that produced by chlorogenic acid controls. Extracts prepared from equal amounts of tissue from groups of three healthy and three infected saguaro seedlings grown in the dark

Table 3. The chromatographic reactions of the saguaro phenolic compound compared to certain known compounds.

Test Compound	Rf	Reactions		
		Ninhydrin	Diazo reagent	UV Fluorescence
Saguaro phenolic	0.36	red-violet	rust-red	none
Tryptophan	0.36	violet	yellow	none
Phenylalanine	0.48	blue	---	---
Chlorogenic acid	0.49	none	rust-red	blue-white
Caffeic acid	0.73	none	rust-red	blue-white

[a]Descending solvent: n-butanol:acetic acid:water (4:1:1)

for seven days at 20°, 25° and 30°C were analyzed for concentration of SPC (Figure 11). The tissues from diseased plants were selected from the apparently healthy, 0.5 cm layer bordering the advancing lesion. The concentration of SPC was expressed as "milligrams of chlorogenic acid per gram of fresh tissue." The compound was found to be present in higher concentrations in tissues from diseased plants incubated at 25° and 30°C than in corresponding tissues from healthy plants. The concentrations found in healthy and diseased plants maintained at 20°C were identical but considerably lower than those found in plants held at the higher temperatures.

Effect of Phenolic Compounds on the Germination of *Fusarium solani* Spores

Materials and methods. Chlorogenic acid and aqueous chromatogram eluates of SPC from healthy plants (grown at room temperature) were tested for their effect on *F. solani* spore germination. Chlorogenic acid was tested in concentrations of 50 to 300 ug per ml. Eluates of SPC were concentrated *in vacuo* at 40°C and brought to a volume equivalent in milliliters to the fresh weight in grams of the original plant tissue. Regarding these solutions as 100%, dilutions downward to 1% were prepared and tested for their effect on spore germination. Hanging-drop spore suspensions were prepared by placing one drop of aqueous spore suspension on a cover glass which contained the dried residue from one drop of chlorogenic acid solution or chromatogram eluate. The cover glass was then inverted over a deep-

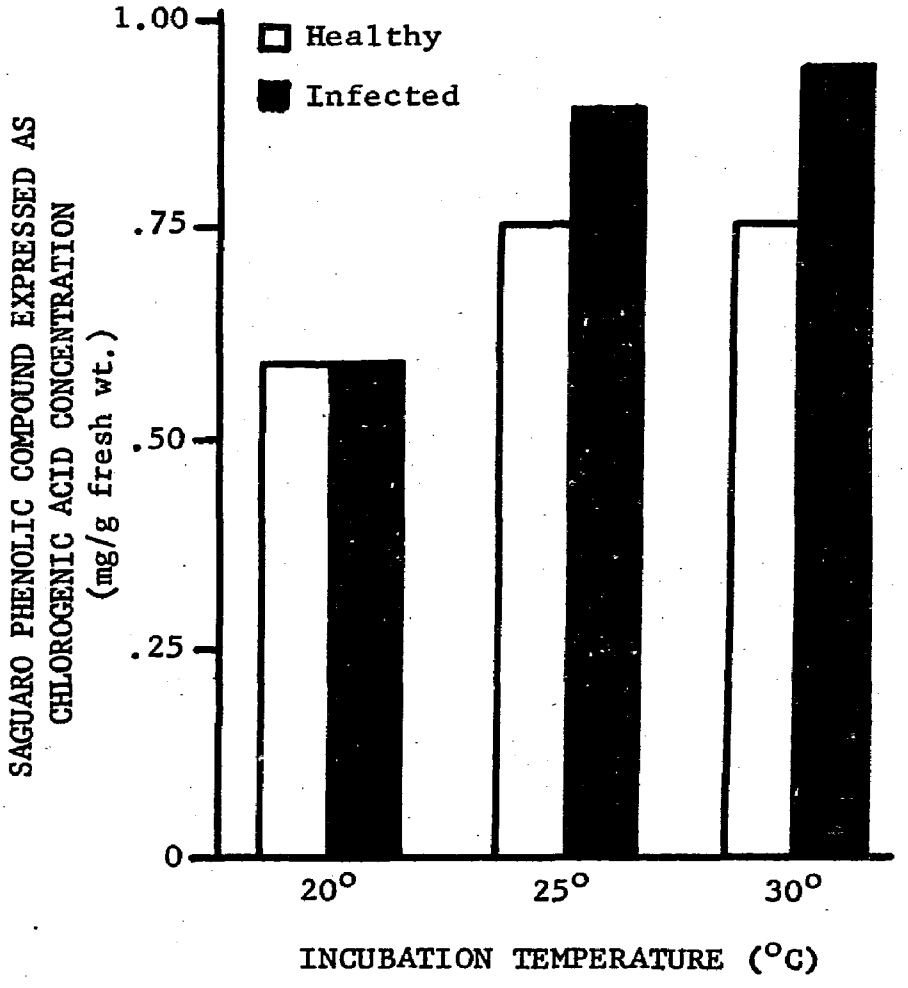

Figure 11. The effect, after seven days, of three incubation temperatures on the phenolic compound concentration of healthy and Fusarium solani infected saguaro seedlings. (Each bar represents the average of three determinations from separate plants.)

well slide. After incubation at 30°C for 16 hours, the percent germination was determined by microscopic observation of 100 spores.

Results and conclusions. Figures 12 and 13 show the results of F. solani spore germination tests with a range of concentrations of chlorogenic acid and SPC respectively. Percent germination is plotted on a "normal distribution" basis while chlorogenic acid and SPC concentrations are plotted logarithmically. It is interesting that the dosage response curves for the two compounds are quite similar in shape. It is probable that the natural SPC concentration was sufficiently high to prevent germination of F. solani spores. However, if the concentration of SPC never exceeded the level noted for healthy plants at 25°C (Figure 11) then it could be assumed that, on a molar basis, chlorogenic acid is approximately three times more effective than SPC in inhibiting spore germination.

Activity of Polyphenol Oxidase from Saguaro Seedlings

Materials and methods. Crude preparations from healthy and F. solani infected saguaro seedlings (8 to 10 cm tall) were tested for polyphenol oxidase activity by Warburg respirometer methods with chlorogenic acid as the substrate. The choice of substrate was prompted by the work of Sisler and Evans (21) with tobacco polyphenol oxidase. The preparations from diseased plants were made from sections (0.5 cm thick) of apparently healthy tissues bordering the advancing lesions.

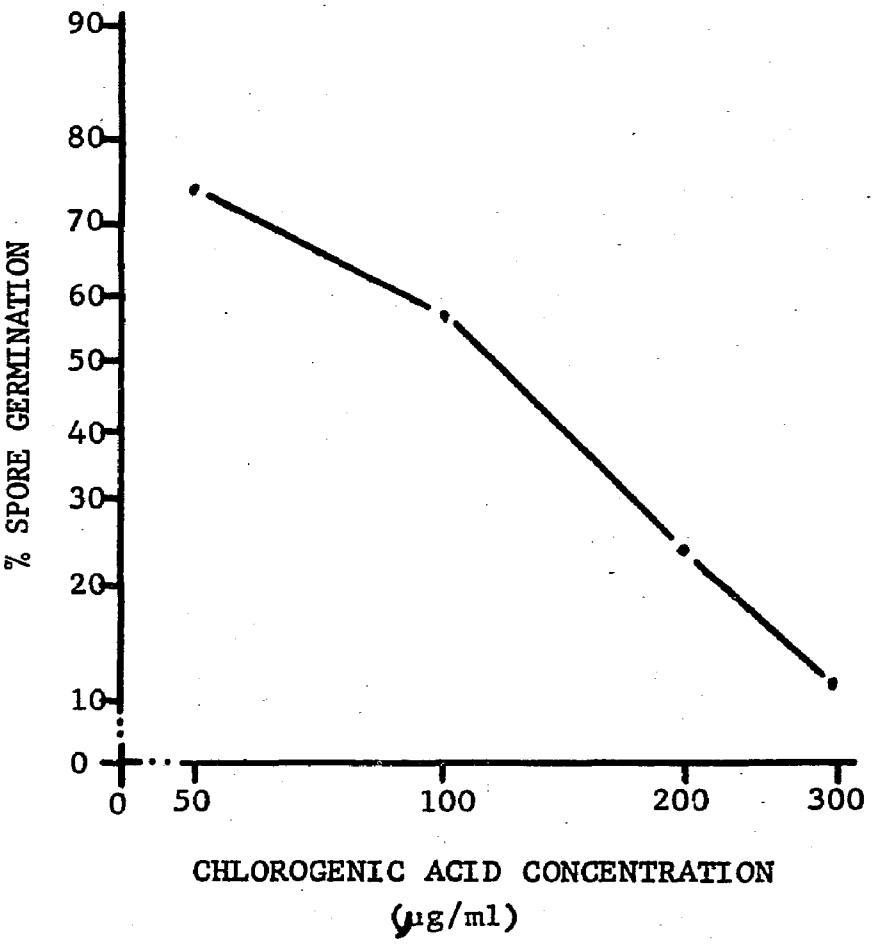

Figure 12. The effect, after 16 hours, of a range of chlorogenic acid concentrations on the germination of Fusarium solani spores. (Each point represents the average of four replications of 100 spores.)

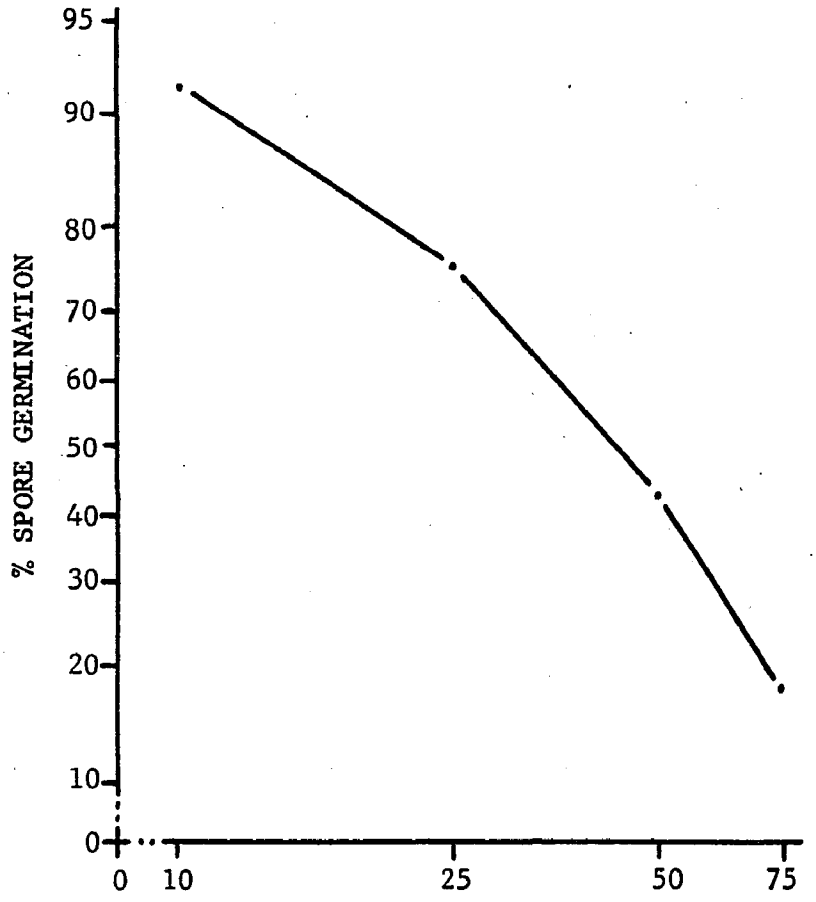

Figure 13. The effect, after 16 hours, of a range of concentrations of the saguaro phenolic compound on the germination of Fusarium solani spores. The compound was obtained from healthy plants maintained at room temperature. (Each point represents the average of four replications of 100 spores.)

According to Akazawa and Uritani (1), the homogenization of sweet potato root tissue causes oxidation of polyphenols and the resulting quinones inhibit polyphenol oxidase activity. The procedure they devised to overcome the difficulty was employed, with slight modification, in the present study of saguaro polyphenol oxidase. Ten g of plant tissue were added to 100 ml of pre-chilled (-10°C) acetone containing 0.2% ascorbic acid and were homogenized in a pre-chilled Waring blender receptacle. The suspension was filtered and the residue was washed twice with 100 ml of cold acetone followed by 100 ml of cold ethyl ether. The acetone powder was then dried over $CaCl_2$ *in vacuo*. Extraction of the powder was carried out with 20 ml of cold 0.1 M phosphate buffer (pH 7.0) in a pre-chilled mortar. The mixture was rapidly filtered with suction and the clear yellow solution was used for assay of polyphenol oxidase activity. The assay, based on oxygen uptake as measured with a Warburg respirometer, was carried out as follows: One ml of 0.1 M phosphate buffer (pH 7.0), 1.0 ml of 0.01 M chlorogenic acid and 1.0 ml of 0.04 M ascorbic acid were added to the main cell of the Warburg flask. The center-well contained 0.2 ml of 20% KOH. The enzyme preparation (0.1 ml) was added to the side arm. Appropriate controls containing boiled enzyme preparation were used. After equilibration for 10 minutes at 30°C, the enzyme preparation was washed from the side arm and the readings were begun.

In the first experiment, tissues from duplicate inoculated and control plants (maintained at room temperature) were used for extractions. Two Warburg determinations were made with each of the

four preparations. Two control flasks were also prepared for each preparation.

In a second experiment, the effect of seedling incubation temperature on polyphenol oxidase activity was investigated. Enzyme preparations were made from three pairs of healthy plants, each pair of which had been maintained in the dark at 20°, 25° or 30°C for six days. Duplicate tests with single controls were made with each of the six preparations.

Finally, the effect of reaction temperature was determined. One of the preparations from an infected plant (from the first experiment) was tested at water bath temperatures of 20°, 25° and 30°C. Triplicate determinations were made at each temperature. Duplicate controls were used for each test.

Results and conclusions. That polyphenol oxidase is present in saguaro seedlings was adequately demonstrated in the first experiment (Figure 14). In addition it was noted that the activities of the preparations from infected seedlings were approximately 21% greater than those from healthy plants.

As shown in Figure 15, polyphenol oxidase activity of the extracts from plants kept at 25° and 30°C was more than two and three times greater, respectively, than the activity of the extracts from plants incubated at 20°C.

The direct effect of reaction temperature on the *in vitro* activity of a polyphenol oxidase preparation is shown in Figure 16. At 20°C the activity was negligible, but at the two higher tempera-

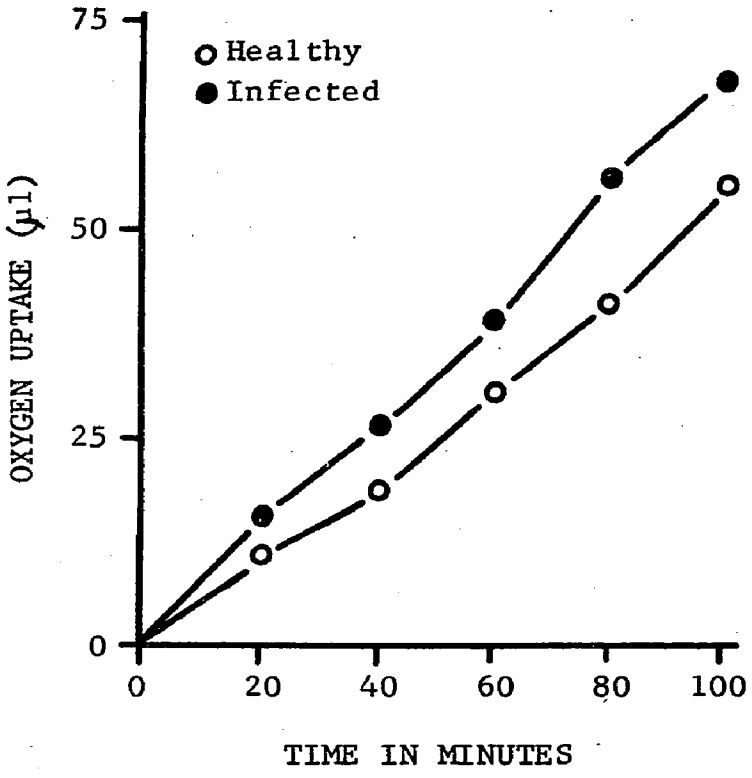

Figure 14. The activity of crude polyphenol oxidase preparations from equal weights of tissue from healthy and Fusarium solani infected saguaro seedlings. Plants were incubated at room temperature. (Each line represents the average of two preparations and two replicate determinations per preparation.)

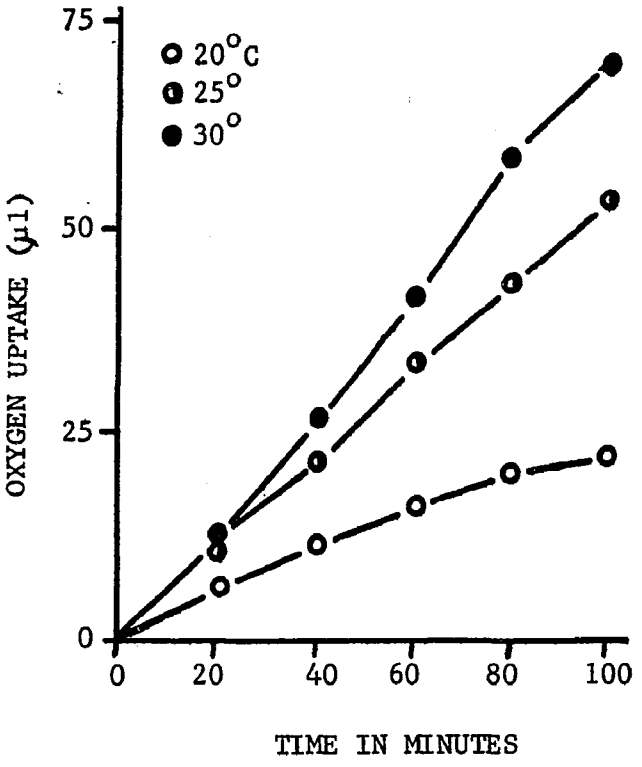

Figure 15. The effect of incubation temperature on the polyphenol oxidase activity of subsequent crude preparations from healthy saguaro seedlings. Preparations were made from equal weights of tissue from plants incubated for six days at 20°, 25° or 30°C. (Each line represents the average of two preparations and two replicate determinations per preparation.)

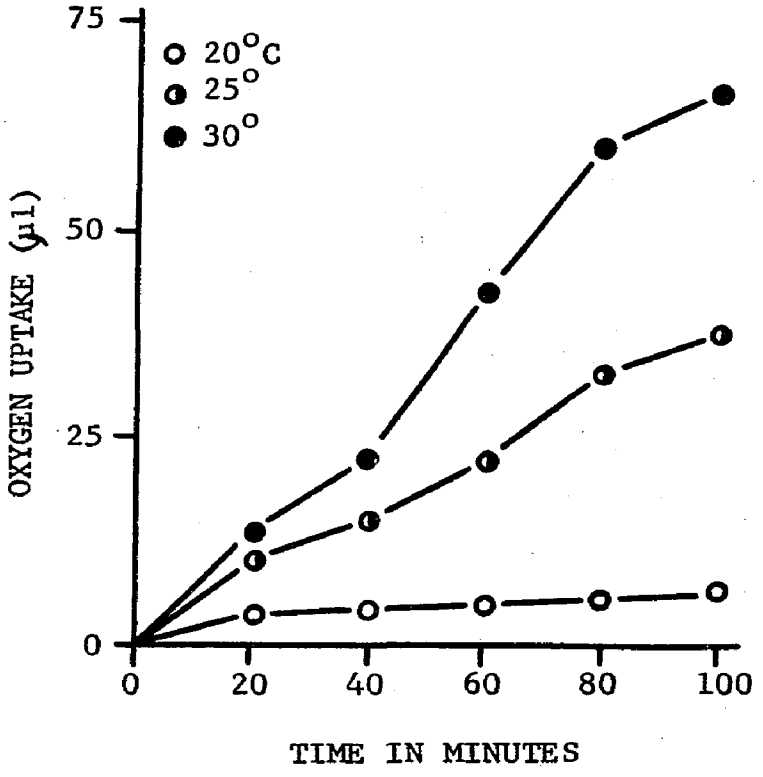

Figure 16. The effect of Warburg respirometer temperatures on the activity of a crude polyphenol oxidase preparation from an infected seedling. (Each line represents the average of three replicate determinations.)

tures the activity was greatly increased. Since the test was performed at each temperature on successive days, the experimental error due to loss of activity with increasing age of the preparation might have been considerable. This, however, was negated to some extent by making the 20°C test first.

Production of Pectin Methylesterase by *Fusarium solani*

Materials and methods. The *in vitro* production of pectin methylesterase (PME) was determined, with slight modification, by the method of Winstead and Walker (27). *F. solani* (one loopful of dense spore suspension per culture) was grown for eight days in shake culture in 250 ml flasks containing 50 ml of Czapek's solution (sucrose, 30.0 g; $NaNO_3$, 3.0 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; H_2O, 1000 ml). Three flasks contained medium with 1.5% pectin (Eastman, practical, #P2569); in three others the medium was unmodified. The culture filtrates were recovered after passage through autoclaved bacterial filters and half of each filtrate sample was then boiled for five minutes. Triplicate aqueous dilutions of 5, 10 and 15 ml of filtrate in 15 ml total volume were prepared for each sample. All dilutions were adjusted to pH 7.0 with 0.1 N NaOH. Each was then added to 10 ml of 0.5 M acetate buffer (pH 4.5) and the mixture was then added to 75 ml of 1.5% pectin solution. The reaction mixtures were stirred and incubated for three hours at 30°C. After incubation, each sample was titrated, with constant stirring, to

pH 7.0 using 0.1 N NaOH and a line-operated pH meter. PME activity was expressed as "milligrams methoxyl removed per milliliter of filtrate." The calculation was as follows:

$$3.1 \times \frac{\text{ml 0.1 N NaOH for unheated} - \text{ml 0.1 N NaOH for heated}}{\text{ml of filtrate}} = \text{mg methoxyl removed per ml of filtrate}$$

Results and conclusions. No activity was detected in filtrates from cultures grown on Czapek's medium without pectin. Slight PME activity was demonstrated, however, in filtrates from cultures on Czapek's medium plus 1.5% pectin. Those diluted 1:3 with water did not show measurable activity; the assays of the 2:3 dilutions and the undiluted filtrates showed, respectively, values of 0.047 and 0.060 mg of methoxyl removed per milliliter of filtrate. The values are comparable to those obtained by Winstead and Walker (27) for various Fusarium species.

Production of Polygalacturonase by Fusarium solani

Materials and methods. The technique of Winstead and Walker (27) was used for the preparation of polygalacturonase (PG)-substrate reaction mixtures. Flasks containing 50 ml of Czapek's solution with and without 0.5% sodium polypectate were seeded with one wire loopful of F. solani spore suspension. Flasks were incubated in shake culture on the laboratory bench for eight days. The culture filtrates were collected as in the previous test, adjusted to pH 7.0 with 0.1 N NaOH, and incorporated into sodium polypectate solutions so that the final

reaction mixtures contained 0.5% pectate and 10% filtrate. The mixtures were held at 30°C and 5.0 ml samples were removed after 0, 5, 30, 60, and 120 minutes of reaction time. Each sample was combined with 1.8 ml of 1 M Na_2CO_3 solution. The carbonate solution caused the mixture to gel. Vacuum filtration through Whatman #2 filter paper provided clear filtrates which were analyzed for free reducing groups by the spectrophotometric method of Miller (15). The aqueous solutions used were:

Solution A
2.0% 3,5-dinitrosalicylic acid
0.4% phenol
1.0% NaOH

Solution B
0.1% sodium sulfite
1.0% NaOH

Solution C
40.0% Na-K tartrate (Rochelle salts)

For analysis, tubes containing 1.0 ml each of solutions A and B and 2.0 ml of reaction mixture filtrate were placed in a boiling waterbath for five minutes. After removal from the bath, 0.8 ml of solution C was introduced into each tube. The optical density (OD) of the resultant blue solution was measured with a Bausch and Lomb "Spectronic 20" colorimeter at 575 mμ. The color development was based on the reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. The method proved accurate only for the determination of galacturonic acid concentrations up to 0.6 mg per ml. A standard curve obtained by analyzing quadruplicate samples of solutions of known galacturonic acid concentration provided the following regression equation:

$y = 2.28 x - 0.118$, where "y" is the OD and "x" is the galacturonic acid concentration in milligrams per milliliter.

Results and conclusions. The presence of PG was not detected in filtrates from cultures grown in Czapek's solution without sodium polypectate. The filtrates from five replicate cultures which contained pectate showed only slight PG activity. Winstead and Walker (27) expressed PG activity in terms of enzyme units per milliliter of culture filtrate; one unit was equal to the amount of enzyme required to liberate 0.05 meq of reducing groups per five minutes per five milliliters of reaction mixture. The average increase in galacturonic acid concentration observed in this experiment indicated an activity equivalent to 0.0058 PG units. That value compares favorably with the values reported by those workers for several Fusarium wilt pathogens.

DISCUSSION

The saguaro seedling disease, unknown in nature, does not present an impressive picture from the standpoint of economic loss. In fact its brief existence in greenhouse seedling propagations was of little consequence, especially since the use of sterile planting media and the reduction of soil moisture kept subsequent losses at a minimum.

The F. oxysporum clones are regarded as of secondary importance in the saguaro seedling disease. This conclusion is based on the low frequency of their presence in naturally infected saguaro seedlings as well as their failure to penetrate intact seedlings. The primary pathogen (F. solani), however, would by most standards be classed as a weak pathogen as evidenced by the extended lag period prior to natural penetration of intact seedlings. Although evidence presented here suggests that only members of the Cactaceae are susceptible, the conclusion cannot be made that the pathogen is a new forma. Controlled experiments with Fusarium formae of known specific pathogenicities would be essential before such a conclusion could be accepted or rejected.

The disease is, however, of considerable interest from the standpoint of the physiology of the host-parasite relationship. The present study has elucidated certain aspects of the physiology which help explain several of the observations made in connection with symp-

tom expression under various environmental conditions. The much reduced development of the disease at temperatures as low as 20°C is at least partly explained by experimental evidence as follows: The saguaro cactus produces a phenolic compound which, even in healthy plants at 20°C, is present at concentrations which are toxic to spores of F. solani. Polyphenol oxidase is also present but at that temperature its activity is almost negligible. Without inactivation of the toxic phenolic the establishing of the pathogenic relationship is hindered and symptom expression is only slight.

At higher temperatures (25° - 30°C) the host-pathogen relationship is better established, as evidenced by the increased rate of symptom development. At these temperatures there is an increased polyphenol oxidase activity which presumably converts the phenolic compound to the quinone form. The quinone, if stable, would probably be toxic to the pathogen according to Byrde (4), Kuć (11) and Kuć et al. (12). In the saguaro, however, the compound is converted to non-toxic melanin condensation products. As the phenolic is inactivated the cells at the lesion margin begin to turn brown and the pathogen advances inter- and intracellularly.

The present study shows that the pathogen is capable of in vitro production of pectin methylesterase and polygalacturonase. The demonstrable activity of these enzymes is very low. If their activity is as low in the disease situation, then the "weak pathogen" classification is further justified. Winstead and Walker (27) reported similar in vitro activities for Fusarium wilt organisms but up to 200-fold

greater activity for Botrytis cinerea Pers. ex Fr., a soft rot organism.

The presence of the phenolic compound is not associated with a specific response triggered by the infection. Increase in concentration of the compound in the apparently healthy tissue bordering lesions is, however, apparently related to infection. That the compound might be the same chlorogenic acid-amino acid addition product reported by Clark et al. (6) is not supported by the evidence given here. In addition to the failure of the compound to yield chlorogenic acid upon rechromatograming, it also failed to show a minor UV absorption peak at 320 mμ as reported by those workers.

This study did not include a search for the mechanism which triggers the increased production of the phenolic compound by the host. Perhaps a closer look at the pectic enzyme activity of the pathogen might reveal such a mechanism. In addition, the production of vivotoxins by the pathogen might also be important. It is likely that some of the reactions reported here for the saguaro seedling disease are closely related to certain specific defense mechanisms reported for other diseases (2, 11, 12, 13). The saguaro disease offers an excellent starting point for the study of the role of phenolic compounds in plant disease resistance. The fact that the pathogen is incapable of penetrating normal cells indicates enzymatic deficiencies which might simplify a resistance mechanism study.

SUMMARY

A new disease of saguaro cactus (Carnegiea gigantea (Engelm.) Britt. & Rose) seedlings caused by Fusarium solani (Mart.) emend. Snyd. & Hans. was described. F. oxysporum (Schlecht.) emend. Snyd. & Hans. was also reported as an infrequent and relatively unimportant causal organism. Both organisms caused a soft rot characterized by a black, watery decomposition of saguaro stem cortical tissue. The principal causal organism, F. solani, was also pathogenic to several other species of cacti but not to any of six economic crop varieties tested.

Cardinal temperature values for in vitro growth of the organism were 10°, 25° and 35°C. Optimum pH at 25°C was found to be 5.4 while maximum and minimum values were 10.8 and 3.1 respectively. Natural penetration of four-week-old, intact seedlings, growing in infested vermiculite, was observed after 14 days when plants were maintained at 25° and 30°C, but infection of plants at 20° and 35°C was nil. Similarly, symptom development in three-year-old, hypodermically inoculated seedlings was rapid at 25° and 30°C and nil at 20° and 35°C.

The organism did not invade cortical cells which appeared healthy but readily invaded tissue which had turned brown probably due to oxidation and condensation of a demonstrated but unidentified host phenolic compound. The concentration of the compound was greater in plants maintained at 25° and 30°C than at 20°C and greater in diseased

than in healthy plants at the two higher temperatures. The compound was highly toxic to spores of F. solani at concentrations equivalent to those found in diseased saguaro seedlings.

Polyphenol oxidase activities of extracts from diseased plants were greater than those of comparable extracts from healthy plants. In addition, extracts from healthy plants maintained at 20°, 25° and 30°C showed increasing activity with increase in incubation temperature.

Slight pectin methylesterase and polygalacturonase activities were demonstrated for F. solani culture filtrates.

The host reaction to the invasion of the pathogen was suggestive of phenolic defense mechanisms which have been reported for certain cases of plant disease resistance. The reaction was negated, however, as evidenced by the advance of the pathogen as the phenolic compound was oxidized and condensed into non-toxic melanin compounds.

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