Histopathology of Carnation Infected with Fusarium oxysporum f. sp. dianthi

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ABSTRACT

Carnations, cultivar Improved White Sim, were inoculated with three isolates of *Fusarium oxysporum* f. sp. *dianthi* and examined histologically. *Fusarium oxysporum* f. sp. *dianthi* isolates from Pennsylvania (A-31), California (A-15), and Denmark (A-80) were used. Isolates A-31 and A-15 caused vascular plugging, hypertrophy and hyperplasia of xylem parenchyma cells, xylem parenchyma cell disintegration, and the formation of vascular cavities. Isolate A-80 incited more cell proliferation in the xylem parenchyma and less vascular cavity formation than did A-15 and A-30. No tyloses were seen in the xylem vessel elements regardless of the isolate used. No conidia were observed in advance of the mycelium in xylem vessel elements. Absence of conidia in advance of mycelium in the xylem vessel elements is probably the primary reason for the success of culture indexing as a control measure for Fusarium wilt of carnation.

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Fusarium wilt, one of the first recorded diseases of carnation, was originally reported in southern France during the late 1800's (23) and in the United States in 1897 (36). Much of the early work on this disease is questionable, due to confusion about the identity of the pathogen. Until 1935, the pathogen was identified only as *Fusarium* sp. (35, 36). In 1935, Wickens identified the causal organism of Fusarium wilt as *Fusarium dianthi* (38). Since then, the fungus has been reclassified as *F. oxysporum* Schlecht. f. sp. *dianthi* (Prill. & Del.) Snyd. & Hans. (33).

Symptoms exhibited by infected carnations were thoroughly described by Bickerton (7). Symptoms initially consist of chlorotic leaves and crook-neck shoots. Leaves and shoots wither and turn brown, and infected stems may also turn brown. At first the symptoms occur on one side of the plant, but they eventually involve the entire plant. The vascular system frequently exhibits a brown discoloration which may spread into the pith and cortex. Shredding of the internal tissue is the final step in pathogenesis. The soft, wet stem rot, reported by Wickens (38) and Hellmers (17), is probably due to the action of secondary organisms.

Symptom expression, however, may be delayed in infected carnations (24, 26). Since carnations are propagated vegetatively, such symptomless plants are a major factor in the spread of *F. oxysporum* f. sp. *dianthi*. As early as 1899, Mangin suggested a method of culture indexing as a control for Fusarium wilt (23). It was not until Dimock (11) introduced an economically sound method of culture indexing in 1941 that this method became popular in the USA. Although the method has since been modified, culture indexing and strict sanitation are currently the only successful means of control (11, 26).

Most investigations of Fusarium wilt have considered disease distribution, symptom expression, and disease control. Little work, beyond that of Bickerton (7), has been done on the histological response of the host to the pathogen.

This study was initiated to examine the effects of the fungus on the host, to determine whether there was any anatomical basis for the success of culture indexing as a control measure, and to determine whether several different isolates of the pathogen cause similar responses in carnation.

MATERIALS AND METHODS.—Culture-indexed, rooted cuttings of *Dianthus caryophyllus* L. 'Improved White Sim' (Yoder Brothers, Barberton, Ohio) were planted in 5-inch pots of a steam-treated 1:1:1 mixture of peat, perlite, and soil, and grown for 2 weeks prior to inoculation. Pots were placed on steam-treated wooden blocks, and watered with a Chapin watering system to eliminate splashing and to insure uniform watering.

Isolates of *F. oxysporum* f. sp. *dianthi* from Pennsylvania (A-31), California (A-15), and Denmark (A-80) were chosen from a group of six isolates on the basis of a pathogenicity test made on plants inoculated as described later. Cultures for inoculum were grown for 9 days on potato-dextrose agar slants under 40-w fluorescent lights suspended 43 cm above the cultures. The lights operated on a 12-hr day/night cycle. All culture tubes were sealed with cigarette paper and sealer (10% gelatin and 2% $CuSO_4$ in water) to reduce the possibility of contamination (34). The spores were suspended in sterile distilled water, and the suspension was standardized to 100,000 spores/ml with a hemacytometer.

We inoculated sixteen plants with each isolate by pouring 100 ml of the spore suspension onto the soil surface. Prior to inoculation, we disturbed the soil around each plant by inserting a small sterilized

spatula ca. 4 inches into the soil 5 times to wound the plant roots. Sterile water was poured on the soil of the check plants.

Plants were sampled at weekly intervals for 5 weeks, starting 1 week after inoculation. Samples consisted of the main stem of one plant, stripped of side shoots and leaves, inoculated with each isolate. The carnation stem was traced on paper and cut into 5-mm numbered pieces. The sections were cut with sterile razor blades, and the even-numbered pieces were fixed in formalin-aceto-alcohol (FAA) (19). The odd numbered pieces were divided in half transversely, surface-sterilized in 10% Clorox (5.25% sodium hypochlorite) for 5 min, placed on either Nash's medium plus neomycin (22) or in nutrient broth, and examined after 10 days. At that time, the location of the fungal and the bacterial isolations was noted and the fungi were identified (37).

The fixed sections were dehydrated in a tertiary butyl alcohol series (19), infiltrated, and embedded in Paraplast (Curtin Scientific). We softened embedded specimens for 48 hr in a solution of 90 ml of 1% sodium lauryl sulfate (Dreft) and 10 ml of glycerol (1) prior to sectioning at 10 μ on a rotary microtome. The longitudinal and transverse sections were mounted on chemically cleaned slides with Haupt's adhesive and stained with Johansen's Quadruple stain (19). The sections were examined under a Leitz Ortholux research microscope and photographed on Kodak Plus X Pan film with a Leitz Aristophot camera with a 4 X 5 inch Graflex back.

Selected sections from each inoculation were tested for pectin by the ruthenium red method (staining time 2 hr) and the Iron Absorption method, and for wound gum and lignin with phloroglucinol (18, 28). Sections were also examined under polarized light for the presence of cellulose.

RESULTS.—*Macroscopic symptoms.*— Improved White Sim carnation plants exhibited similar symptoms regardless of the isolate of *F. oxysporum* f. sp. *dianthi* used in inoculation. Plants developed chlorotic leaves, crook-neck shoots, and, eventually, a wilt on one side extending the entire length of the plant. The plants died soon after exhibiting the one-sided wilt symptom. Symptom expression was delayed in plants inoculated with isolate A-80.

Isolations.—Fusarium oxysporum was isolated from all inoculated plants sampled. The fungus extended the entire length of the stem in the wilted plants. Concurrently, the unidentified bacteria were generally restricted to the basal 13 cm of the stem, although they were occasionally scattered in the remainder of the stem. Isolations from noninoculated plants yielded bacteria but no *F. oxysporum*.

Histology of noninoculated plants.—Observations of stained, transverse, and longitudinal sections of carnation stems revealed an internal cellular arrangement which agreed with that previously reported (25). The stem is composed of an epidermis with a thick cuticle and sunken stomates. The cortex, several layers of large, isodiametric parenchyma cells, is adjacent and external to the fiber cylinder. The fiber cylinder varies in thickness from one to several cells depending on the age of the plant, and is occasionally broken by small groups of large parenchymatous cells. Several layers of parenchyma cells separate the fibers from the phloem cylinder. The vascular cambium divides the phloem from the xylem cylinder (Fig. 1). The xylem vessel elements have simple perforation plates. The pith is composed of large isodiametric parenchyma cells.

Histology of inoculated plants.—Isolates A-31 and A-15 incited similar anatomical responses. Isolate A-80 incited many of the same responses but there were also some notable differences.

The pathogen invades the carnation roots in an undetermined manner and enters the xylem vessel elements of the vascular system. Initially, the fungus is present in only a few vessel elements. It continues to grow and spread to adjacent vessel elements through the pit pairs (Fig. 2, 3). At this point, the fungus is restricted to the xylem vessel elements, and may eventually form masses of mycelium in them. After a time, the fungus becomes established in a large number of vessel elements (Fig. 4).

Once the fungus is established, cavities appear between the xylem cylinder and the pith, and also between the xylem and the phloem (Fig. 5). Cavities extend vertically and horizontally in the stem. Stems infected with A-31 had cavities extending a maximum of ca. 30 cm from the basal sections, whereas the maximum extent in A-15 infected stems was 12 cm.

The initial small cavities (Fig. 6) eventually enlarge to encompass the entire transverse area of the infected vascular tissue (Fig. 7). At this stage of cavity development, material that may be mycelium or host cell remnants is frequently found adjoining the pith tissue.

The vascular cambium and phloem to the exterior of the infected areas is frequently distorted and greatly reduced in size (Fig. 8). In many cases, a cavity occurs in the area usually occupied by vascular cambium and phloem tissue.

Conidia frequently begin to appear in the xylem vessel elements during cavity formation. They may be scattered (Fig. 9) or formed in masses in the vessel elements (Fig. 10). Masses of conidia often extend for some distance in the xylem vessel elements (Fig. 11). The conidia were always associated with the mycelium, and at no time were the conidia found in advance of the mycelium. They never appeared to be trapped by the perforation plates of the vessel elements. Concurrently, at no time were tyloses observed in vessel elements.

Hypertrophy and hyperplasia occur in the lower portion of infected stems. Hypertrophied cells occur in the xylem tissue adjacent to the pith, and eventually displace or separate xylem vessel elements from the rest of the vascular cylinder (Fig. 12, 13). The pith may also contain hypertrophied cells. Occasional cavities occur here, but they are more common in the upper portion of the stem.

Later a band of thick-walled, hypertrophied xylem parenchyma cells forms between the xylem cylinder and the hypertrophied tissue described above (Fig. 14). Xylem vessel elements along this band of

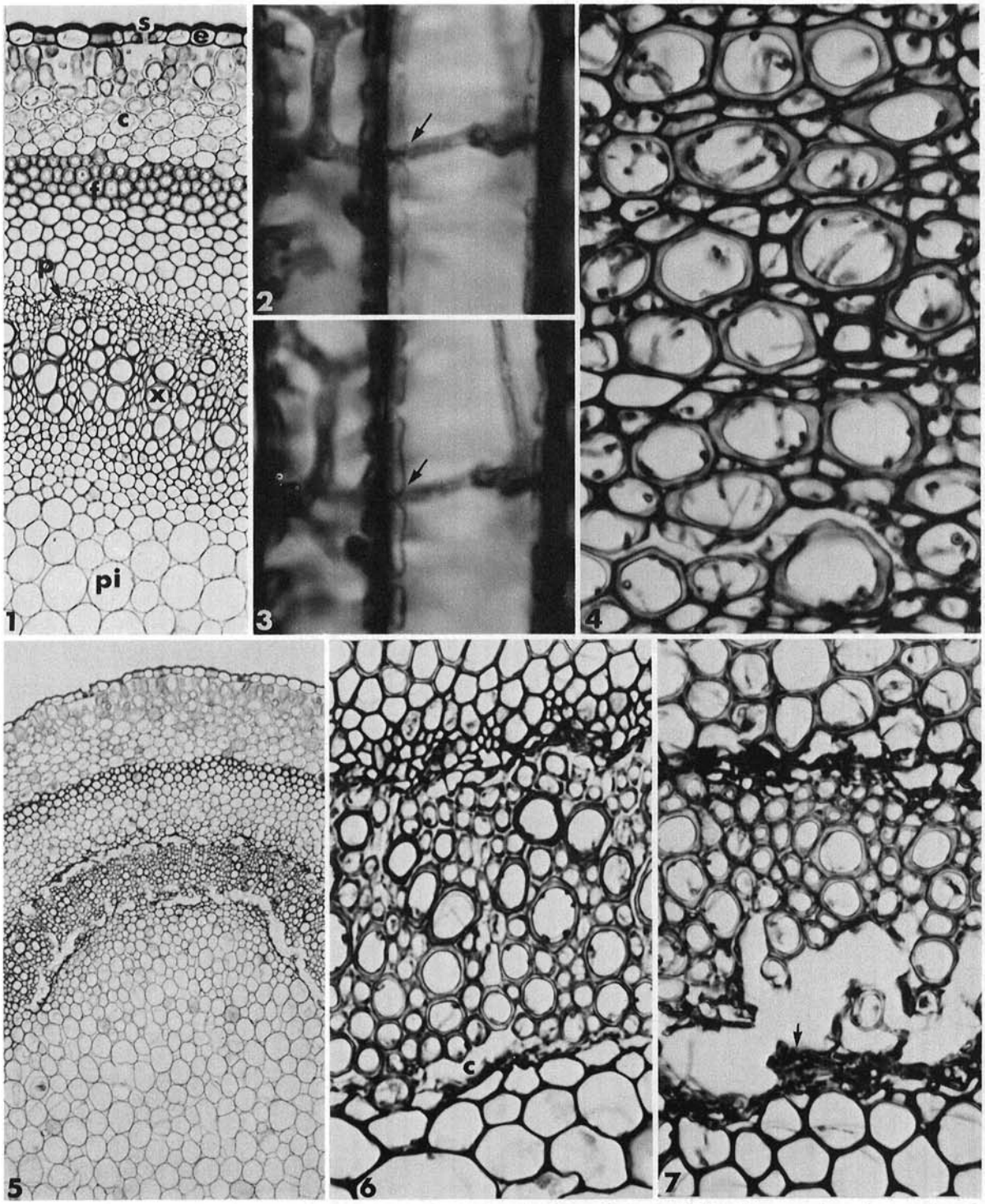

Fig. 1-7. Portions of transverse and longitudinal sections through stems of Improved White Sim carnation. Stem sections in Fig. 2-7 are infected with *Fusarium oxysporum* f. sp. *dianthi*. **1)** Transverse section through a portion of a healthy stem showing the epidermis (e); sunken stomate (s); cortex (c); fiber cylinder (f); phloem (p); xylem (x); and pith (pi) (× 120). **2, 3)** Longitudinal sections showing mycelium of the pathogen going from one xylem vessel element to the adjacent xylem vessel element through a pit pair. The sections are identical, but the pit pair is in focus in 3, and the mycelium in 2 (both ×1,680). **4)** Transverse section showing mycelium established in all xylem vessel elements (× 638). **5)** Transverse section in which cavities are visible on the exterior and interior of the vascular cylinder (× 80). **6, 7)** Transverse sections showing the development of a cavity between the xylem and the pith. **6)** Small cavity (c) is present at the xylem-pith margin (× 294). **7)** Cavity has enlarged and a mass of distorted host and/or pathogen tissue (arrow) is present at the interior edge of the cavity (× 470).

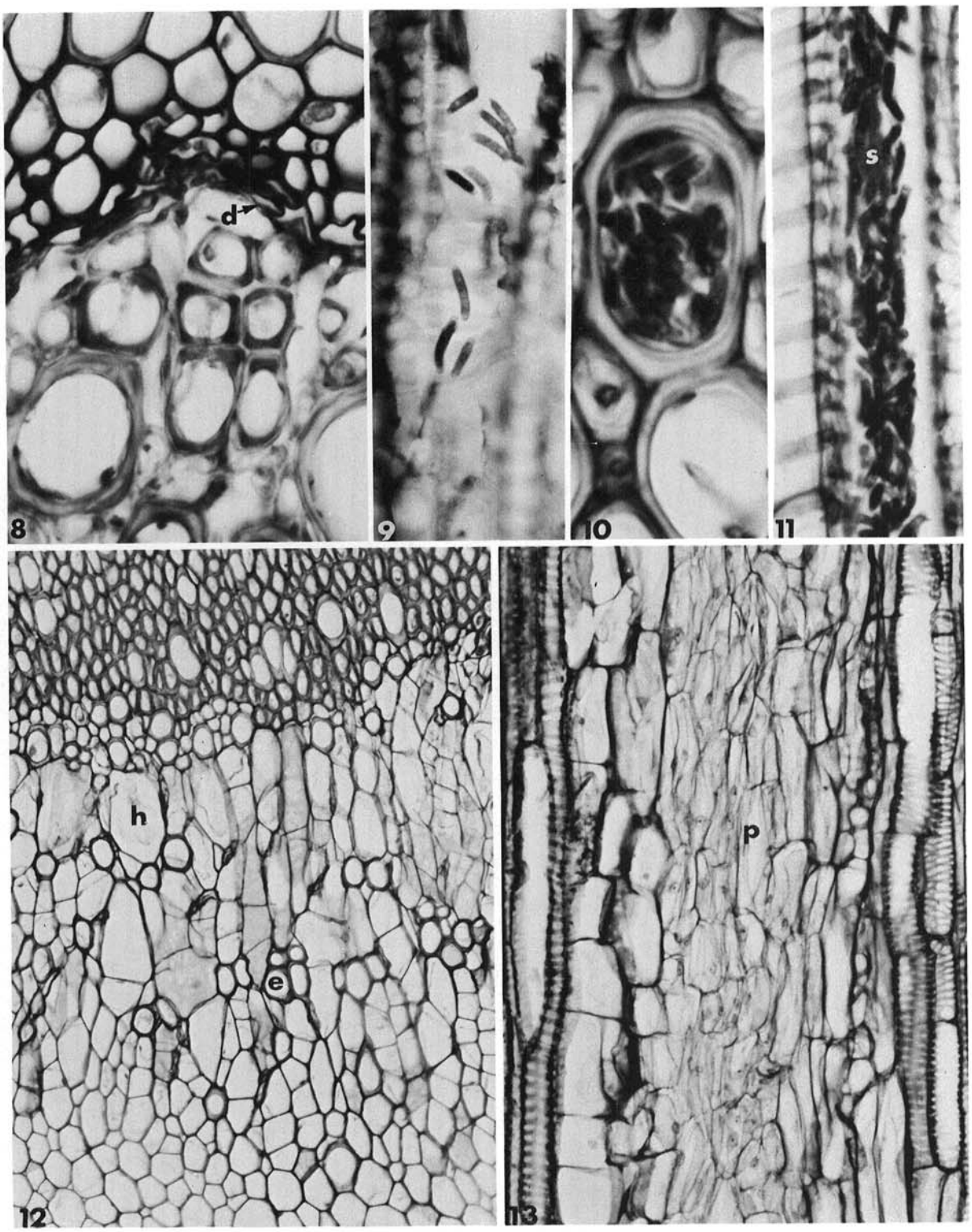

Fig. 8-13. Portions of transverse and longitudinal sections of stems of Improved White Sim carnation infected with *Fusarium oxysporum* f. sp. *dianthi*. **8)** Transverse section through the vascular region, showing distorted cells (d) in the area of the phloem and the vascular cambium (× 580). **9)** Longitudinal section of a portion of a xylem vessel element showing conidia of the pathogen in the lumen (× 816). **10)** Transverse section of a xylem vessel element showing the lumen plugged with a conidial mass (× 1,392). **11)** Longitudinal section of a portion of a xylem vessel element plugged with conidia (s) (× 860). **12)** Transverse section in which hypertrophied xylem parenchyma are visible (h) as well as displaced xylem vessel elements (e) (× 180). **13)** Longitudinal section through an area like that in Fig. 12. Note that the distorted hypertrophied xylem parenchyma cells (p) have separated the xylem vessel elements (× 196).

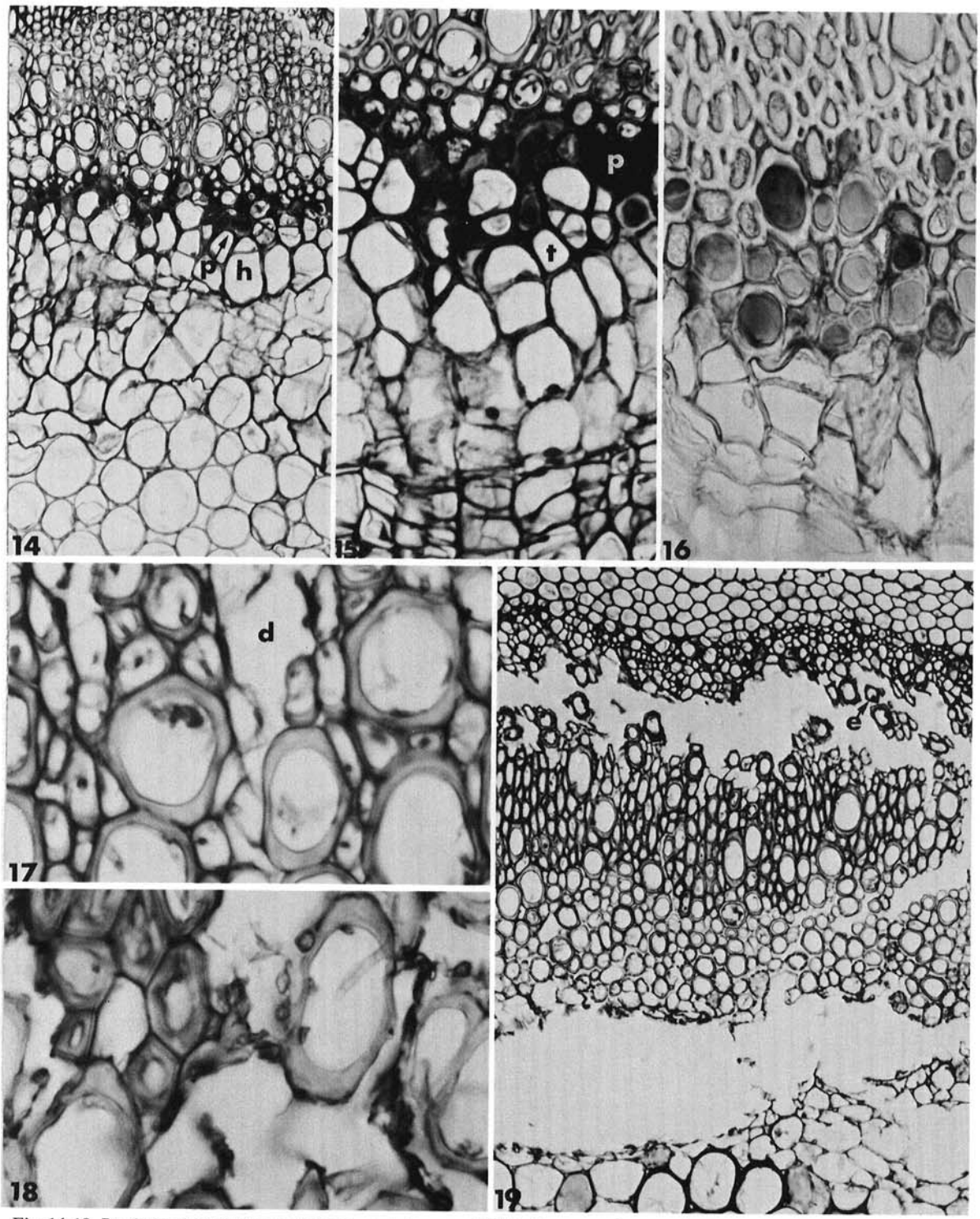

Fig. 14-19. Portions of transverse sections through Improved White Sim carnation stems infected with *Fusarium oxysporum* f. sp. *dianthi*. **14)** Preliminary stages of xylem parenchyma cell hypertrophy (h). Note the line of plugged cells (p) to the exterior of the hypertrophied cells (× 148). **15)** Plugged cells (p) and thick-walled cells (t) adjacent to the plugged cells are visible in this section (× 188). **16)** Plugs showing varying degrees of positive reaction to the Iron Absorption test for pectic substances (× 450). **17)** Preliminary stage in xylem parenchyma cell disintegration (d) (× 900). **18)** Advanced stage in xylem parenchyma cell disintegration in which no entire xylem parenchyma cells are visible (× 840). **19)** Final stages of xylem parenchyma cell disintegration in which cavities have formed between the xylem and pith and between the xylem and phloem. Note the isolated xylem vessel elements (e) in the exterior cavity (× 156).

distorted cells frequently become plugged, as do many of the vessel elements in the infected area (Fig. 15). Vascular plugs tested positive for wound gum and pectic substances (Fig. 16), and were not birefringent under polarized light, indicating the absence of crystalline cellulose.

At this stage of host response, the mycelium is frequently found in cells other than xylem vessel elements. Soon the xylem parenchyma cells begin to disappear or disintegrate (Fig. 17, 18), isolating xylem vessel elements. Disappearance of xylem parenchyma cells does not necessarily follow the formation of hypertrophied tissue, but may occasionally precede it.

Simultaneously or following the beginning of xylem parenchyma cell disintegration, enlarged cavities frequently form through the region of hypertrophied, thick-walled cells. Isolated xylem vessel elements are scattered throughout the enlarged cavity (Fig. 19).

Reactions resulting from histochemical tests for pectin were negative to weakly positive in the area of xylem parenchyma cell disintegration and cavity formation (Fig. 20), indicating the absence or weakening of the middle lamellae in this area. Cell walls in unaffected areas of the same section were positive for pectin, indicating the presence of a middle lamellae between adjacent cells (Fig. 21). The final stage of disruption observed in plants inoculated with A-31 and A-15 was characterized by the occurrence of a large amount of hypertrophied tissue and dissolution of the vascular cylinder in the areas invaded by the fungus (Fig. 22).

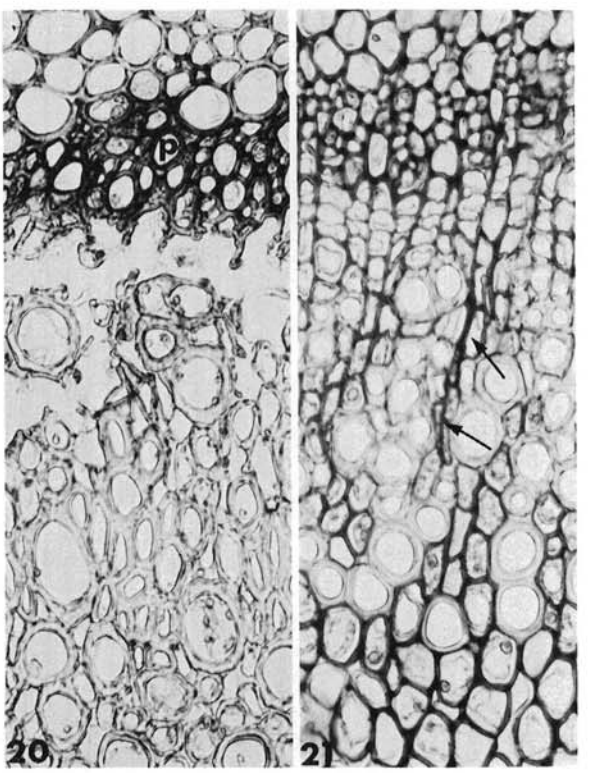

Fig. 20, 21. Portions of transverse sections of Improved White Sim carnation stems infected with *Fusarium oxysporum* f. sp. *dianthi* showing the results of the Iron Absorption test for pectic substances. **20)** Section in which the middle lamellae of the xylem parenchyma cells is negative, indicating a lack of pectic material. The phloem (p) tissue shows a strong positive reaction (× 390). **21)** Unaffected portion of the same stem section as in Fig. 20, showing a positive reaction (arrows) in the xylem parenchyma cells for pectic substances (× 390).

Plants inoculated with isolate A-80 had histological changes as described for the other isolates, except that infected areas in the stem were bordered on the pith side by hypertrophied xylem parenchyma cells which were always arranged in orderly columns (Fig. 23). A-80 also incited the formation of hypertrophied, thick-walled cells at the interior of the recognizable xylem border and plugging in the xylem vessel elements (Fig. 23). Unlike the other isolates, the first visible histological change was frequently the disappearance of xylem parenchyma cells (Fig. 24) rather than the formation of cavities. Another striking difference was the presence of hypertrophied xylem parenchyma cells extending into the vascular cylinder (Fig. 25). These wedges may be the sites of the future radial cavities which are frequently observed in plants infected with isolate A-80 (Fig. 26).

DISCUSSION.—The presence of *F. oxysporum* f. sp. *dianthi* in the carnation plant is characterized by mycelium and conidia in the xylem vessel elements, plugging of some xylem vessel elements, disintegration of the xylem parenchyma cells in infected areas, hyperplasia and hypertrophy xylem parenchyma cells, and the formation of cavities in the vascular cylinder. Although similar observations have been reported for other hosts infected with Fusarium wilt fungi, the situation in carnation is somewhat unique.

The fungus probably enters carnation roots through the zone of elongation as reported for the cabbage wilt Fusarium (32), but the exact mode of entry into carnation has not been determined. Once in the plant, the mycelium grows through the xylem vessel elements and spreads between vessel elements through pit pairs, as also occurs in tomato infected with *F. oxysporum* f. sp. *lycopersici* (8).

Contrary to the situation in banana, where conidia are reported to be abundant in the xylem vessel elements from the beginning of pathogenesis (2), conidia were not noted in the vessel elements of carnation until the mycelium was well established. Conidia were then formed abundantly. Sometimes the conidia and, occasionally, mycelium formed masses in the vessel elements. Such masses or fungal plugs occur in tomato infected with the Fusarium wilt fungus (8). They have been credited with causing a reduction in water flow through the vessel elements, and thus may contribute to the wilt symptom (29).

Conidia are apparently transported in the vascular stream and trapped by perforation plates and vascular plugs in banana, causing secondary infections in advance of the primary hyphae (2, 5, 12). A similar situation also may occur in tomato (30). Conidia of *F. oxysporum* f. sp. *dianthi* were not found isolated from the mycelium, and at no time were they trapped

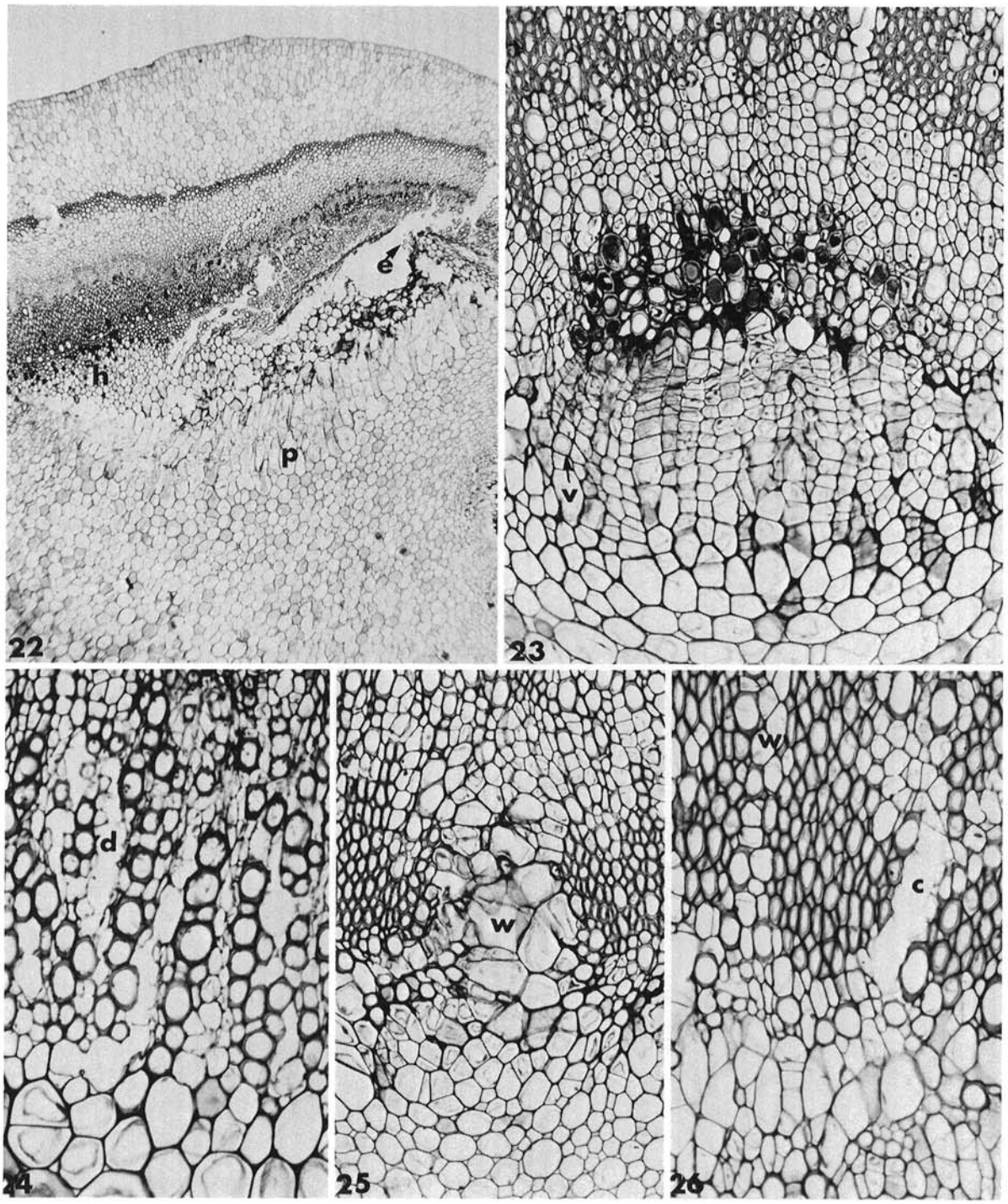

Fig. 22-26. Portions of transverse sections of Improved White Sim carnation stems infected with *Fusarium oxysporum* f. sp. *dianthi*. Stem sections in Fig. 23-26 were inoculated with isolate A-80 from Denmark. **22)** Advanced stage of vascular disruption in which hypertrophied xylem parenchyma cells (h) and pith cells (p) are visible, as well as a large cavity in which isolated vessel elements (e) are located (× 38). **23)** Advanced stage of host response to isolate A-80. Note the plugged xylem vessel elements and orderly hypertrophied xylem parenchyma cells in which occasional xylem vessel elements (v) are visible and the absence of cavities (× 124). **24)** Xylem parenchyma cell disintegration (d) was the first visible host response to isolate A-80 in some cases (× 184). **25)** Wedge of hypertrophied xylem parenchyma (w) extending into the vascular cylinder (× 128). **26)** Wedge of hypertrophied xylem parenchyma (w) and a cavity (c) which may have been the site of a wedge of hypertrophied cells are visible in this section (× 172).

by the perforation plates. If conidia were transported in the vascular stream as reported in other hosts, it should be possible to find conidia separated from hyphae and in advance of the hyphae. Our isolation studies indicated that no conidia occur in advance of the mycelium. *Fusarium oxysporum* was isolated in consecutive sections from the base of the plant upward in inoculated plants. Histological examination of the uncultured alternate sections revealed the presence of mycelium in all sections from which the fungus was isolated, but neither mycelium nor conidia were found in sections above the highest penetration of the fungus as determined by isolation techniques. The fact that conidia are not transported in advance of hyphal invasions probably explains the success of culture indexing as a control measure (26).

Studies of banana (4), tomato (8, 12), and sweet potato (21) revealed varying degrees of tylose formation in the xylem vessel elements. Tyloses in banana were numerous, and were considered to be manifestations of resistance. Beckman et al. (5) reported that the tyloses in banana xylem vessels successfully limit pathogen movement through those vessels. Tyloses were present but less frequent in both tomato (8) and sweet potato (21). Tyloses were not observed in stems of carnation, and do not appear to play a part in the response of Improved White Sim carnation to infection by *F. oxysporum* f. sp. *dianthi*.

Vascular plugging is frequently mentioned as a defense response to invasion of the host (3). Such plugs have been reported in banana (4, 5) and tomato (15, 27) infected with *F. oxysporum*. The plugs are generally considered primarily pectic in composition (10, 27), although wound gums (9, 21) and hemicellulose (3, 6) have also been reported as plug constituents. The plugs are thought to result from the action of pectolytic enzymes such as pectin methylesterase and pectin depolymerase on the primary cell walls and middle lamellae (10, 15). The enzymes may split constituents of the middle lamellae and primary cell walls into large molecular fragments which then move into the xylem vessel elements and form plugs (15, 27). Beckman & Zaroogian (6) suggest that the gels in banana result from swelling of the perforation plates, end walls, and side walls of the vessel elements. Vascular plugging was frequently found in carnation stems. The plugs gave a positive test for pectin and wound gum. It is quite possible that they also result from the action of pectic enzymes on the middle lamellae, although *F. oxysporum* f. sp. *dianthi* has not been previously reported to produce these enzymes. This possibility is strengthened by the weak reaction to the pectin test in the middle lamellae of cells in the affected vascular regions.

A proliferation of parenchyma cells adjacent to infected cells has been noted in tomato infected with the Fusarium wilt fungus (8, 10). Such proliferation is probably due to the action of auxins (12). Mace (20) isolated 3-indoleacetic acid (IAA) from *F. oxysporum* f. sp. *cubense*, and Sequeria (31) indicated that this hormone is the most common auxin synthesized by plants. Thus, it is possible that the hypertrophy and hyperplasia found in the xylem parenchyma cells of carnations with Fusarium wilt is due to either auxin produced by the fungus, auxin produced by the plant, or a combination of the two. Such hypertrophied cells might have weaker cell walls and be more susceptible to pressures that could result in tearing or disintegration of the cells.

The final response of carnation stems to *F. oxysporum* f. sp. *dianthi* was the formation of cavities within the stem. Bickerton (7) reported hollowness in carnation stems in advanced stages of pathogenesis. Our study of unfixed infected stems also revealed varying degrees of hollowness in advanced stages of the disease. It has been shown that several Fusarium wilt pathogens produce pectinases (9, 13, 16) which attack the cell walls and the middle lamellae. Such attacks in carnation would weaken and eventually destroy the bonds between cells in the vascular cylinder. Destruction of the middle lamellae would result in free-floating xylem vessel elements and cavities. The xylem parenchyma cells may lack secondary walls (14) and frequently are destroyed, perhaps by pectolytic enzymes. Such action might well result in cavities within the stem.

Different isolates of *F. oxysporum* f. sp. *dianthi* caused similar changes in carnation stems. The main difference was the increase in xylem parenchyma proliferation and a decrease in cavity formation in plants infected with isolate A-80. Such differences might be due to differences in the ability of the isolates to produce 3-indoleacetic acid and pectic enzymes, or differences in the rate of growth of the isolates in carnation tissue.

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