T H E S I S

HISTOCHEMICAL AND MORPHOLOGICAL STUDIES OF CARNATION STEM ROT

Submitted by

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196

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY DOUGLAS J. PHILLIPS

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BE ACCEPTED AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter I

INTRODUCTION

Parasitism is one of the most intriguing of all biological phenomena. In years of study, the host-parasite relationships have presented many unanswered questions. Parasitism, which extends from a delicate balance of the nearly symbiotic pathogen to the extremely destructive organism, can be examined by many methods and from many points of view. Specific knowledge of physiologic interactions can provide us with valuable knowledge not only in the directly applied methods of control but also can yield information concerning the nature and variability of the relationship between hosts and parasites (25).

It has been recognized structural differences do not play too important a part in disease resistance, but, especially in the case of wound parasites, structural changes may be found that profoundly influence the disease development (33).

Fusarium roseum f. cerealis (cke.) Snyd. & Hans. is of primary importance as a wound pathogen attacking carnations. In carnation culture this pathogen is found even in greenhouses which practice a vigorous disease control program. Studies on the nature of parasitism may serve to insure an intelligent approach to this problem of control. In addition chemical and morphological information is important in understanding the basic relationship between the host and parasite.

The problem

What are the morphological and chemical changes associated with infection of carnation by Fusarium roseum f. cerealis?

Problem analysis.--The problem may be sub-divided into the following phases:

1. Detecting and tracing the presence of F. roseum f. cerealis in carnation tissue.
2. The use of histochemical stains to demonstrate a change in the chemical composition of carnation cuttings as a result of infection by the pathogen.
3. The demonstration of morphological differences between carnation varieties which differ in their resistance.

Delimitations.--This study will be limited to one virulent strain of the pathogen. The study will be limited further to stem cuttings of the commercial varieties of carnation, Dianthus caryophyllus L. varieties Red Sim and Miller's Yellow. Red Sim is a variety relatively resistant to F. roseum f. cerealis as compared to Miller's Yellow; however, both can be infected. Histochemical tests for pectin, cellulose, starch, suberin, lignin, and callose will be used in this study. The classical paraffin technique, as well as sections of fresh material will be utilized, employing Conant's quadruple stain for the killed and fixed material. All investigations will be performed under controlled conditions in the greenhouse and laboratory.

Definition of terms.--1. Histochemical test - A test which is used to indicate the presence of a specific chemical within plant tissue.

2. Infection - The conditions of being infected: The establishment of a parasitic relationship by the pathogen within the host.

3. Conant's quadruple stain - The combination of four specific coal-tar dyes in a staining schedule: safranin, crystal violet, fast green and orange II.

4. Virulent strain of the pathogen - F. roseum f. cerealis (Strain 56-2) isolated from diseased carnations growing in greenhouses located in Denver.

Chapter II

REVIEW OF LITERATURE

Casual observation of stem cuttings infected with F. roseum f. cerealis indicates that there is a need for examination of the development of this disease during the propagation period. The resistance to the disease increases as the cutting becomes established as a mature plant (11). This suggests that certain morphological factors are involved in the relationship between host and parasite. The literature dealing with the problem can be divided into several parts: (1) The causal agent, (2) Histology of defense, (3) The development of adventitious roots in carnation.

The causal agent

The disease commonly known as Carnation Stem Rot has also been called "die-back" (29), "Root Rot" (27,28), "Root and Crown Rot" (13), "Stem Rot or Wilt" (12). Confusion from these many names was compounded by the fact that the various species making up the genus Fusarium were not well defined. At the present time there seems to be general agreement that Fusarium roseum f. cerealis (30) is the causal agent of the disease called "Carnation Stem Rot" (3,4,11,30).

Symptoms.--The symptoms assigned to this disease by various workers vary only slightly (2,13,29,32). They are summarized by the following description: the tips of the plant wilt and die

due to a stem rot at the soil level. Both roots and the base of the stem are usually involved and infected cuttings root poorly or not at all. A discoloration is evident in the dermal tissues surrounding the region of infection. From such infection loci, which are usually wounds, tissue discoloration extends into the underlying tissues. Commonly these areas remain localized. The fungus often forms a pink spore crust on the surface of the lesion.

The development of the disease within the carnation.--The development of this disease within the carnation stem was noted in 1929 by Dowson (12) who found intra and intercellular hyphae invading the plant tissues. The xylem was commonly involved and an accumulation of gum-like substances in tracheids and vessels served as an indication of the presence of hyphae. Wickens comprehensively reviewed the early literature on the disease (32) and gave evidence supporting the view that the causal agent is a wound pathogen. He further noted that infection usually results during the propagative period. The contention that fungal penetration is accomplished through wounds was further supported by Guba (13) who also indicated that favorable conditions for root growth were not conducive to invection.

The development of an infection incited by F. roseum f. cerealis was described in detail by Moreau (14). The infection was shown to spread rapidly through the parenchymatous tissue of the carnation and to stimulate a proliferation of host cells in advance of the hyphae. The existence of a lignified ring of "pericyclic-like" tissue and the presence of lignified xylem elements were shown

to be a barrier to the advance of the fungus through the stem. Upward progression of the fungus was generally through the vessel elements, since in the nodal areas growth of the fungus in the cortex was restricted due to the presence of a sclerified "pericyclic-like" layer of tissue. The hyphal filaments came in contact with the cell membrane, swelled and pushed against the cell wall. The wall was distended so as to produce a swelling in the wall of the adjoining cell. A rupture then occured and the wall was penetrated by the fungus. In sclerenchyma the mycelium appeared to penetrate at the pits in the cell wall.

Resistance.--Since the stem rot organism has been shown to be a wound pathogen, the cutting with its exposed base is particularly vulnerable to infection (3,11,13,32). The cuttings, while rooting, become progressively more resistant to infection (11). However, varieties of carnations differ in acquiring this resistance (4,5,9). Baker has shown that the carnation variety, Miller's Yellow, remains susceptible for 24 days as opposed to a Sim variety which is moderately resistant in 4 days. Moreau (15,16) has shown a difference in lignification and in enzymatic activity between resistant and non-resistant varieties. This suggests that a lack of lignified tissue may indicate a susceptible variety.

Control.--The common measures recommended to control this disease are sanitation and careful handling (2,8,13,32). In addition control sprays have been used including both general fungicides and antibiotics (11,23,24). Dips for cuttings and soil

drenches have been found to be effective (6,7,18,19). Regardless of protective measures, the ease of reinfestation of soil by water born spores remains a serious problem (10). A very few contaminating spores are able to cause significant infection (11).

Histology of defense

Akai (1) has reviewed mechanisms that interfere with invasion of the host. He used a broad 2 part classification for these mechanisms: (1) the static resistance to spread, already present prior to infection, and (2) the dynamic defense reactions which become apparent after infection occurs.

Static resistance is sometimes associated with histological characteristics of walls and, as such, may present a barrier to subsequent infection. The dynamic defense reactions are grouped according to their origin. Autonomous defense reactions can appear as a histological barrier which acts to demarcate the infected lesion (histogenic defense reaction), or as a plasmatic activity in the cell itself. Histogenic defense reactions include demarcation of infected lesions by forming cicatricial layers, abscission cells, tyloses, or gums; as well as callus-like swellings or callositites formed on the wall.

The incidence of gums in tracheids and vessels (12), the proliferation of host cells (14), and barriers of lignified tissues (14), have been reported as mechanisms occuring in carnations infected with F. roseum f. cerealis. Other morphological and developmental characteristics, however, may contribute to

susceptibility. For instance a related pathogen, Gibberella saubinetii (Mont.) Sacc., has been shown to enter corn seedlings in ruptures in the cortex produced by the emergence of adventitious roots or by the pulling apart of cells in rapid growth (17).

The development of adventitious roots in carnation

Stangler (26) described the development of adventitious roots in stem cutting of carnation, variety Northland. He found that the stem of 6-inch cuttings was composed mainly of primary tissues: an epidermis; an endodermoid layer at the inner boundary of the cortex; a radially wide pericycle consisting of mature fibers, immature fibers and parenchymatous cells; and vascular tissues in a continuous band surrounding the pith. The cambium produced only small amounts of secondary tissues. The roots arose 0.1 to 2 millimeters above the base of the cutting, and grew to the sheath of mature pericyclic fibers. The roots did not penetrate this sheath, but grew downward and emerged from the cut surface. Only a small amount of callus was present near the cut surface.

Summary and implication

It has been established that F. roseum f. cerealis is a wound pathogen of carnation. Its infection may be localized by such mechanisms as barriers formed by gums, ligneous deposits, and cell proliferation. It has also been demonstrated that, in the case of corn seedlings, penetration by Gibberella saubinetii can occur through the damaged tissue caused by adventitious root emergence.

This may imply that carnations may be infected from wounds produced by adventitious roots emerging through the base of the cutting. Further, subsequent limitation of invasion by the pathogen and increasing resistance to infection may be due to morphological barriers.

Chapter III

MATERIALS AND METHODS

Six to 8-inch terminal cuttings were taken from plants grown in a propagative mother bench. The cuttings were from 2 commercial carnation varieties, Millers Yellow and Pink Sim. The cuttings were trimmed with a straight cut across the stem midway between two nodes, and were placed in a greenhouse under an intermittent mist propagative system in flats containing horticultural grade perlite. The base of each cutting was dusted with a commercial rooting hormone, Stim Root (98 per cent talc, .1 per cent indole butyric acid, .25 per cent alpha naphthaleneacetic acid, and 1.65 per cent tetrachlorobenzoquinone).

Inoculum was obtained from week-old cultures of F. roseum f. cerealis grown in shake culture on Czapek's liquid medium (31). The suspension was passed through cheese cloth to remove hyphal filaments, washed by centifugation and decantation, and the spores were counted with a standard hemacytometer (22). The method of inoculation used consisted of direct application of a suspension of spores to carnation cuttings placed in previously steamed propagative flats.

Microtechnique methods.--Plants were selected arbitrarily from each test group. The base of the cutting was removed and examined as fresh material or processed in Craf III killing and

fixing solution (21). In cases where root growth was excessive, the roots were trimmed close to the stem. The killed and fixed plant material was dehydrated with tertiary butyl alcohol and embedded in Tissuemat. Ten to 15 μ serial sections were cut from the embedded stem pieces and mounted with Haupt's adhesive and 4 per cent formalin. The sections were stained with Conant's quadruple stain (21), or were used for histochemical tests. The histochemical tests included standard methods (20) employing the Zinc-chloroiodine cellulose test; KI-I test for starch; KI-I and sulfuric acid test for lignin and cellulose; Sudan IV stain for suberin, fats, and oils; ruthenium red stain for pectin; phloroglucinol test for wound gums and lignin; and lacmoid staining for callose.

Confirmation of inoculation technique.--Since characteristic fruiting bodies of the fungus were not always present in the tissue, it was considered necessary to confirm that the mycelium in the tissue was the pathogen. Inoculated and non-inoculated cuttings were placed under propagation. Lesions were allowed to develop on the cuttings. The base of each cutting was cut off and split aseptically. From half of the stem base, a 1 cc. section was taken from the pith and placed on potato-dextrose-agar (PDA). Only F. roseum f. cerealis was recovered from the infected sections and no fungi were found to be present in the control cuttings. The second half of this base was processed for staining. The stained sections from this base were used as standards for comparison in subsequent tests in order to verify the presence of the pathogen.

Chapter IV

RESULTS

To obtain diseased and normal cuttings in all stages of development, groups of cuttings were inoculated at the time of striking and 6, 12, and 18 days after the start of propagation. Spore suspensions were applied to cuttings at these intervals at the rates of 10, 100, and 1000 macroconidia per cc. of perlite. A control group was not inoculated.

The flats were sampled over a 26-day period. The infested flats were sampled every 2nd day for 8 days following inoculation, then every 4th day until the termination of the sampling. The control flats were sampled each day. All samples contained 4 stem bases and these were killed and fixed immediately after they were collected.

The average temperatures of the greenhouse during the propagation were as follows: day temperature 79°F, night temperature 52°F.

Developmental study

The control group was examined until the rooting process was evident. Also a careful examination of the healthy stem for abnormalities, which might have been confused with disease damage, was conducted. This examination showed many damaged areas in the wounded surface of the cutting which extended into the stem. These were usually the result of growth cracks or damage associated

with adhering perlite particles at the cut surface (Figs. 1 and 2). The growth of the adventitious roots also caused damage as these roots pushed their way through the stem tissue (Fig. 3).

The formation of callus cells and general cell proliferation of the wounded area was more extensive than described by Stangler (26). The outermost cells at the cut surface apparently died within the first 2 to 4 days of the propagation period. This was followed by a cork-like proliferation of the parenchyma cells of the cortex and pith. This cork-like proliferation will be referred to as the "area of proliferation." Areas of the cambium commonly gave rise to thin-walled callus cells which formed mounds of irregular tissue at the cut surface. The callus and proliferation caused a macroscopic swelling of the base of the cutting which is commonly referred to as the "callus pad."

The origin and development of the adventitious roots were as described by Stangler (26). Emergence of the roots from the stem occurred after 11 to 16 days of propagation, with the average being 14 days.

The incubation period from inoculation to the development of symptoms averaged 11 days. This time was shortened for cuttings receiving a higher inoculum level as follows: 16 days for 10 spores per cc. of perlite, 11 days for 100 spores per cc. of perlite, and 9 days for 1000 spores per cc. of perlite.

The development of the disease followed a recognizable pattern. (It should be noted; however, that in only 2 of the stems

Fig. 1.--A cross section of a non-infected carnation cutting showing an area of damage associated with an adhering perlite particle found at the base of the cutting (X35).

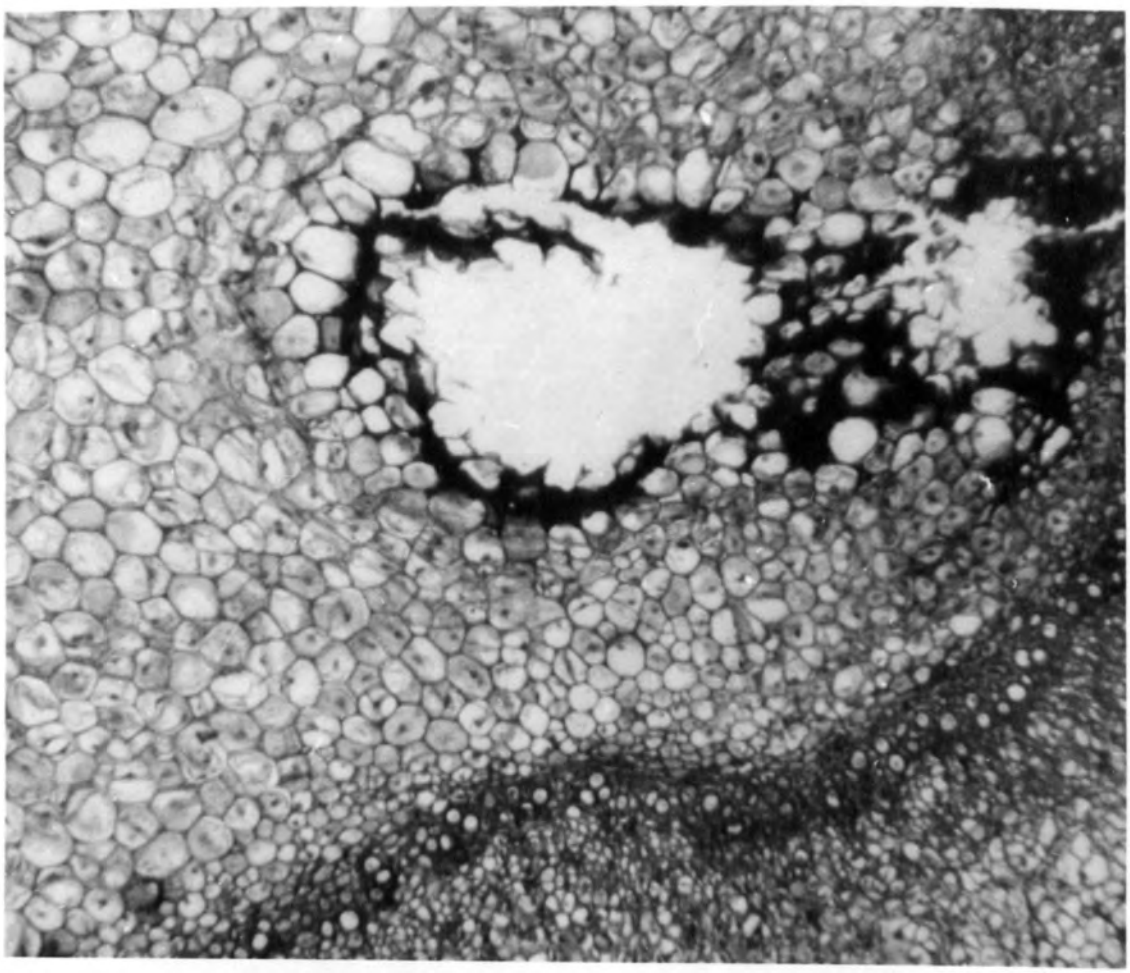
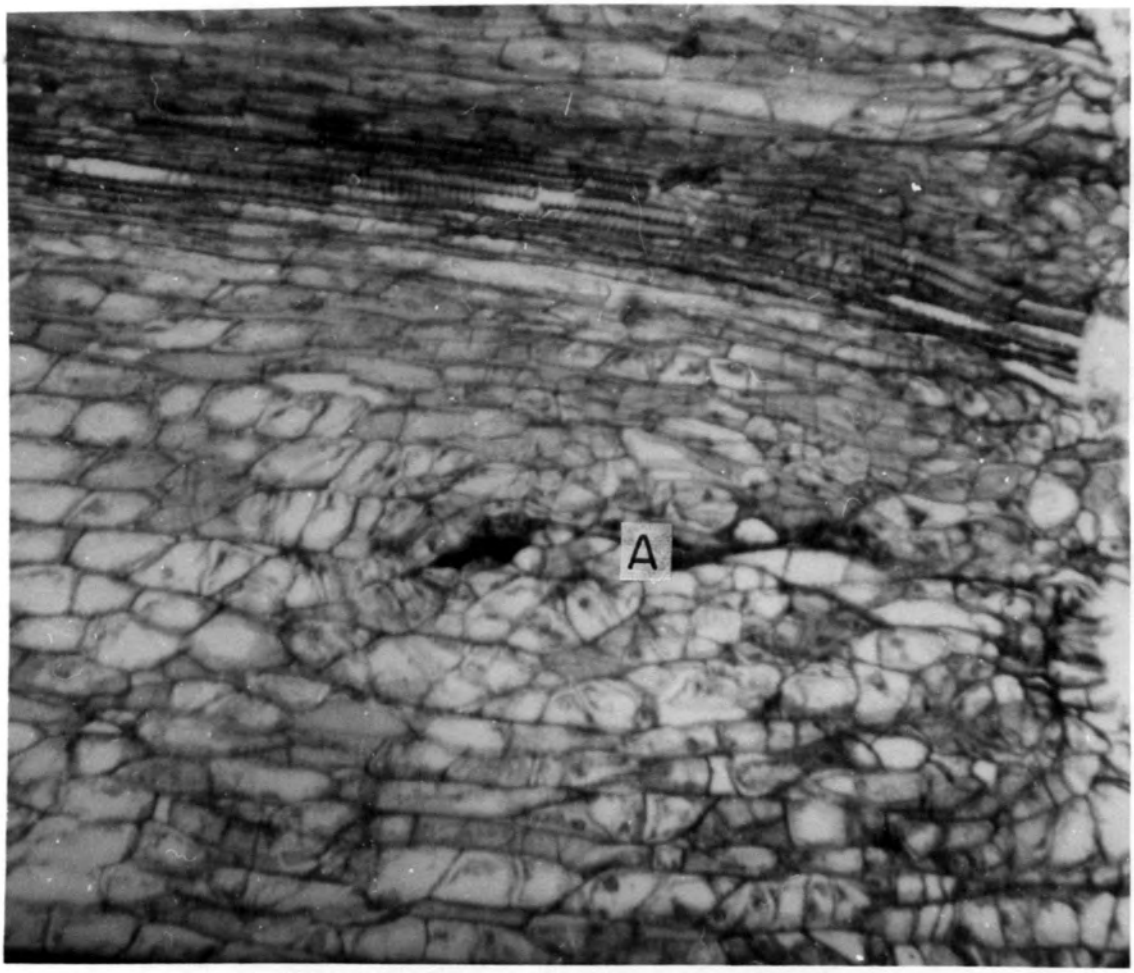


Fig. 2.--A tangential section through the base of a non-infected carnation cutting showing damaged tissue in association with a growth crack. A) Area of the growth crack (X35).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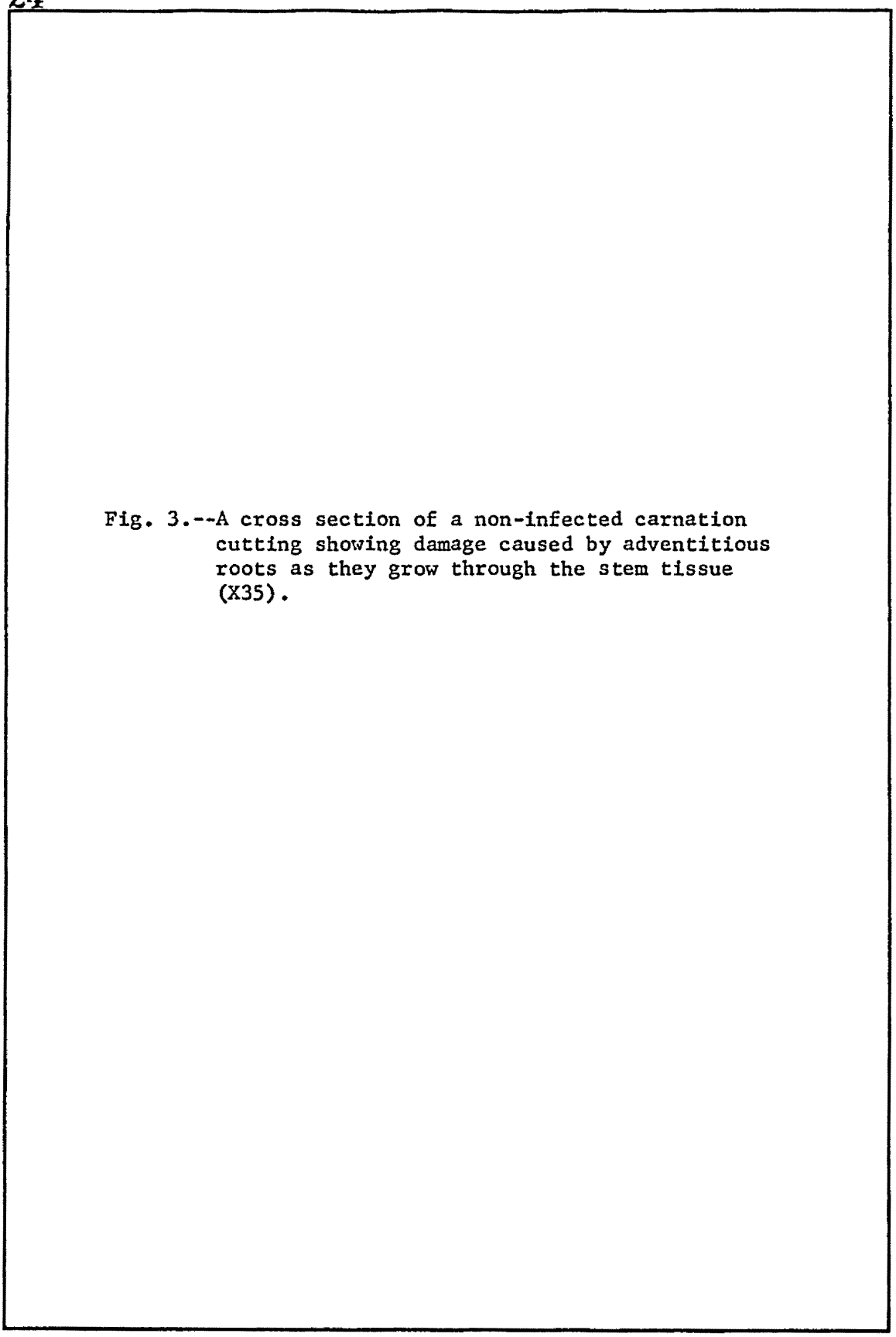
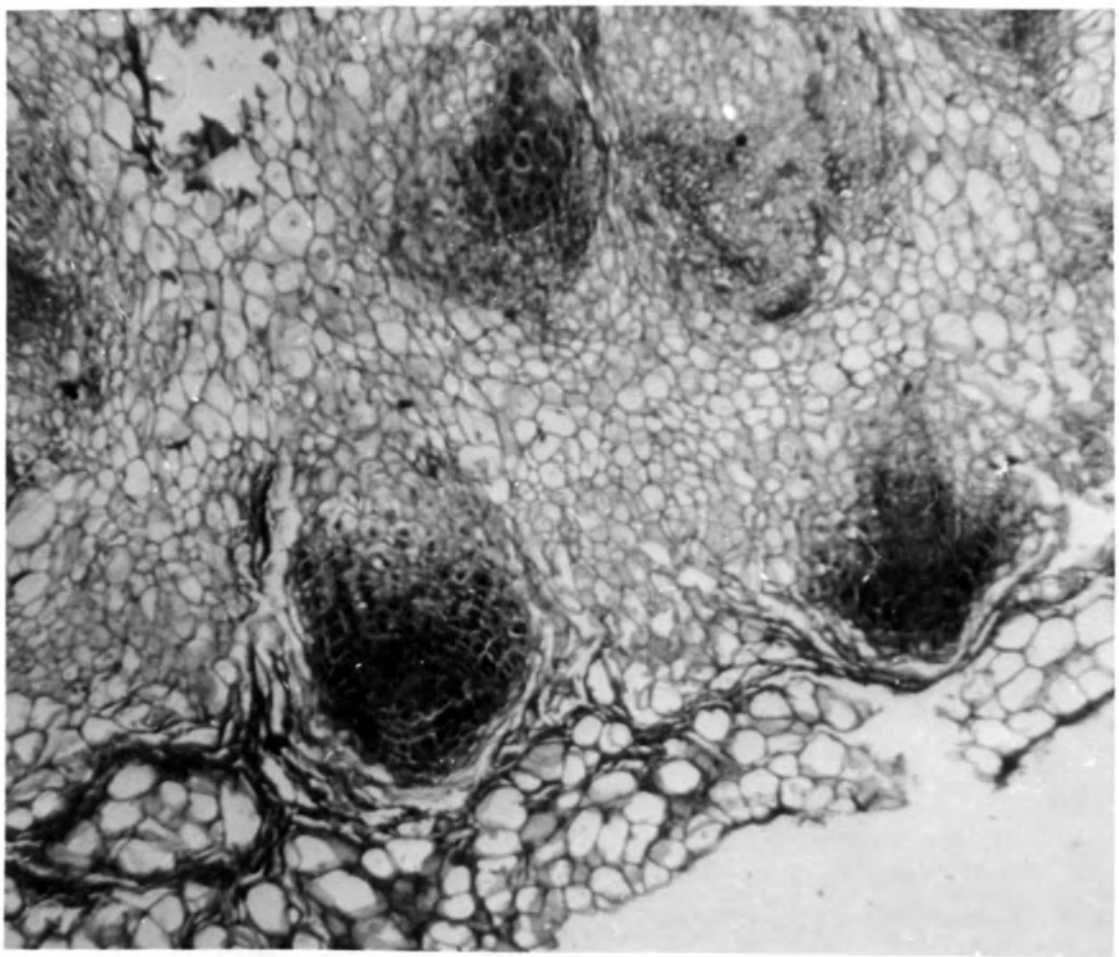


Fig. 3.--A cross section of a non-infected carnation cutting showing damage caused by adventitious roots as they grow through the stem tissue (X35).



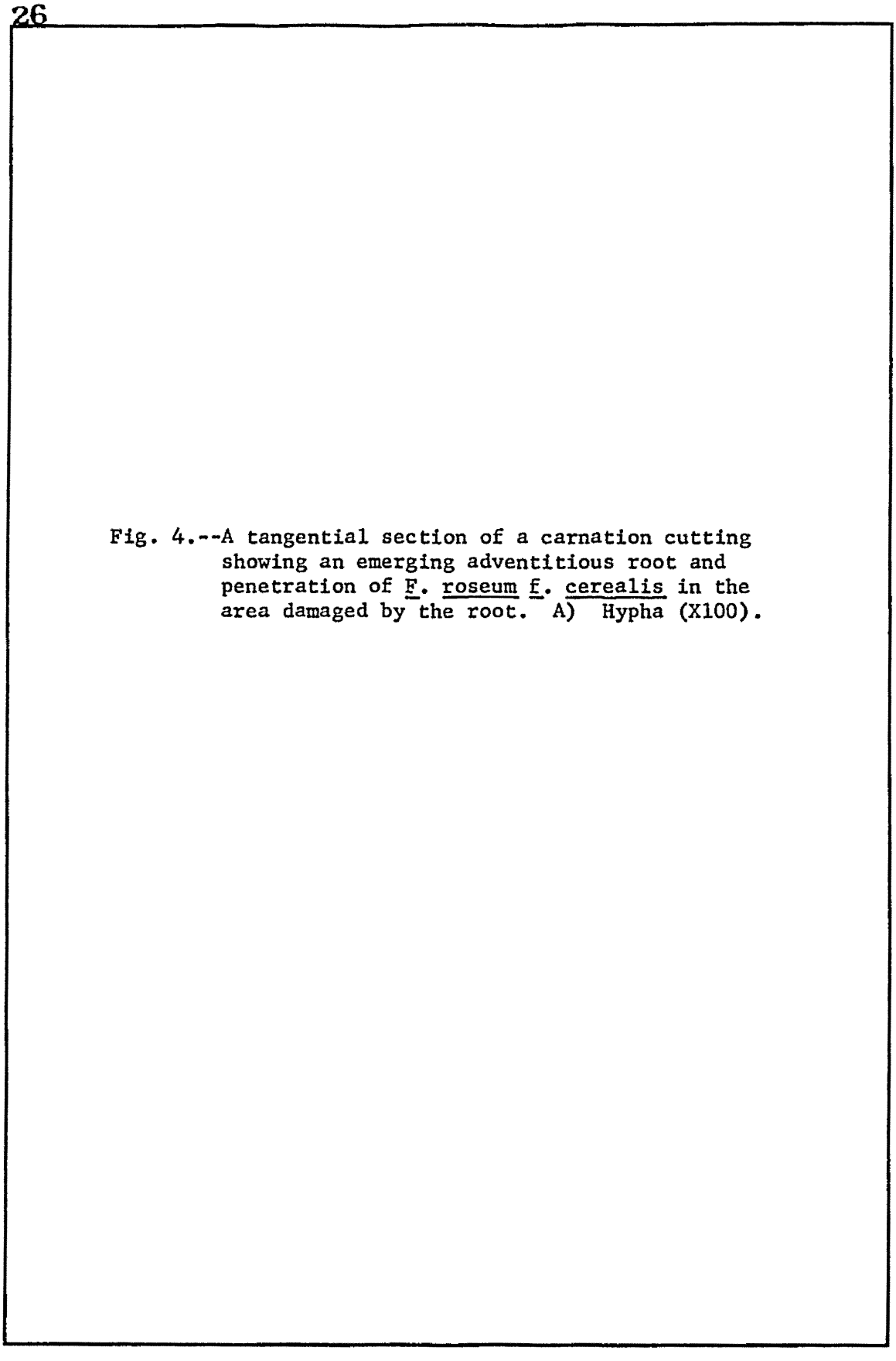
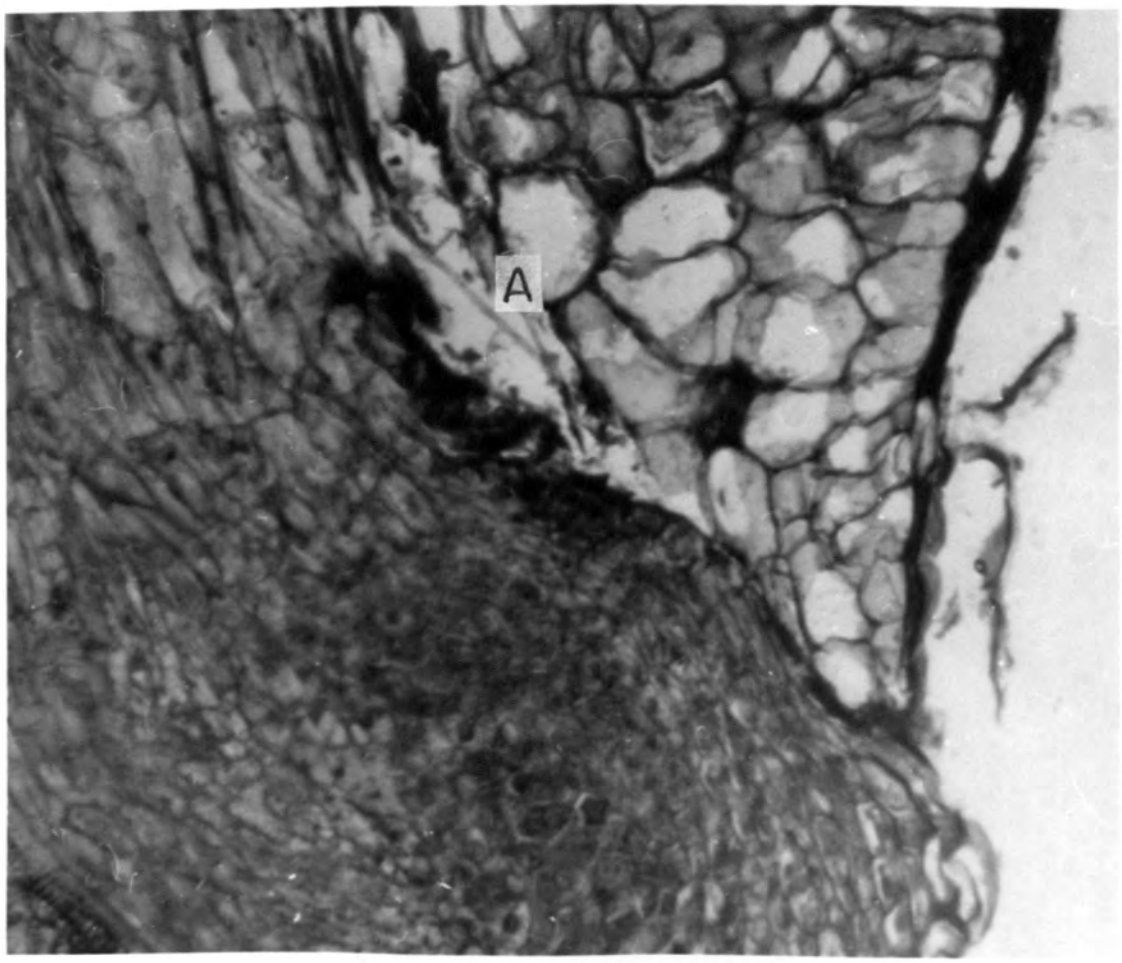


Fig. 4.--A tangential section of a carnation cutting showing an emerging adventitious root and penetration of F. roseum f. cerealis in the area damaged by the root. A) Hypha (X100).


A

examined did it appear that the infection resulted from the initial wounding caused when the cuttings were taken from the mother plant.) Typically, the hyphae first penetrated and infected the host at the base of the cutting in the phloem in the area of the phloem fibers and in conjunction with damage caused by a developing adventitious root (Fig. 4). Once established, the infection spread rapidly through the developing roots (Fig. 5) and tissues at the base of the cutting. The infection at the base was followed by the invasion of all tissues of the stem. The infection which followed, however, was limited by the phloem fibers and xylem elements found in the upper areas of the stem. The advancing margin of the infection was usually found in the pith parenchyma. A line of demarcation was apparent when the hyphae reached the nodal areas (Fig. 7). Here the cells of the pith are smaller. In sections in which infection had been limited by the nodal area, the base of the cutting contained a thick network of hyphae within the still intact cell walls. In the final stages of infection, the nodal area in many instances was invaded and the infection was evident throughout the section. Sporodochia were commonly formed when the hyphae were concentrated at the epidermis (Fig. 8).

The hyphae were primarily intracellular (Fig. 6). Width of individual hypha varied greatly and swelling of hyphal tips were not uncommon. There were pin-point constrictions of the hyphae at the point of cell wall penetration, but swelling of the wall due to penetration was not observed. Occasionally a mass of hyphae formed

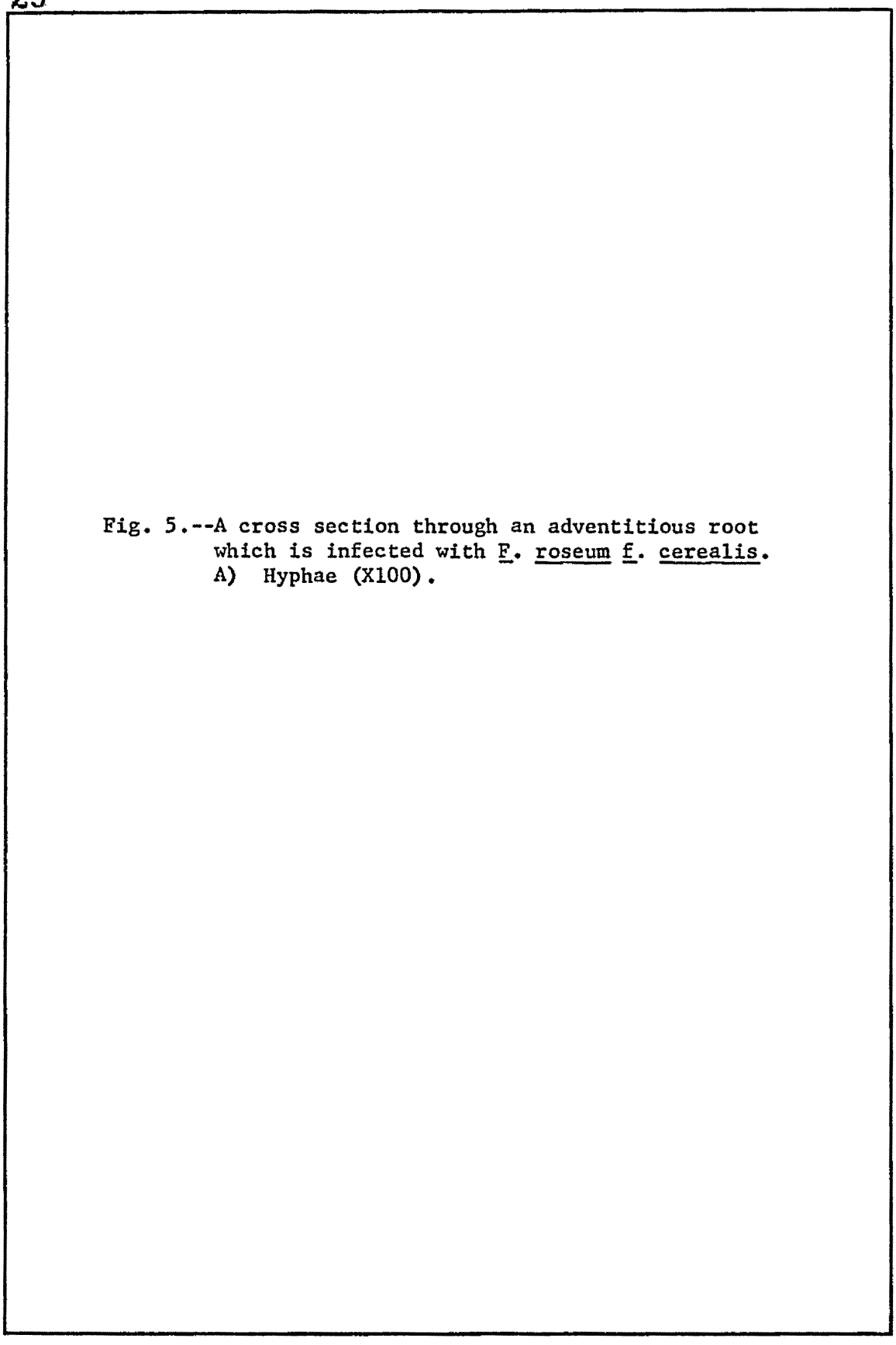


Fig. 5.--A cross section through an adventitious root which is infected with F. roseum f. cerealis. A) Hyphae (X100).

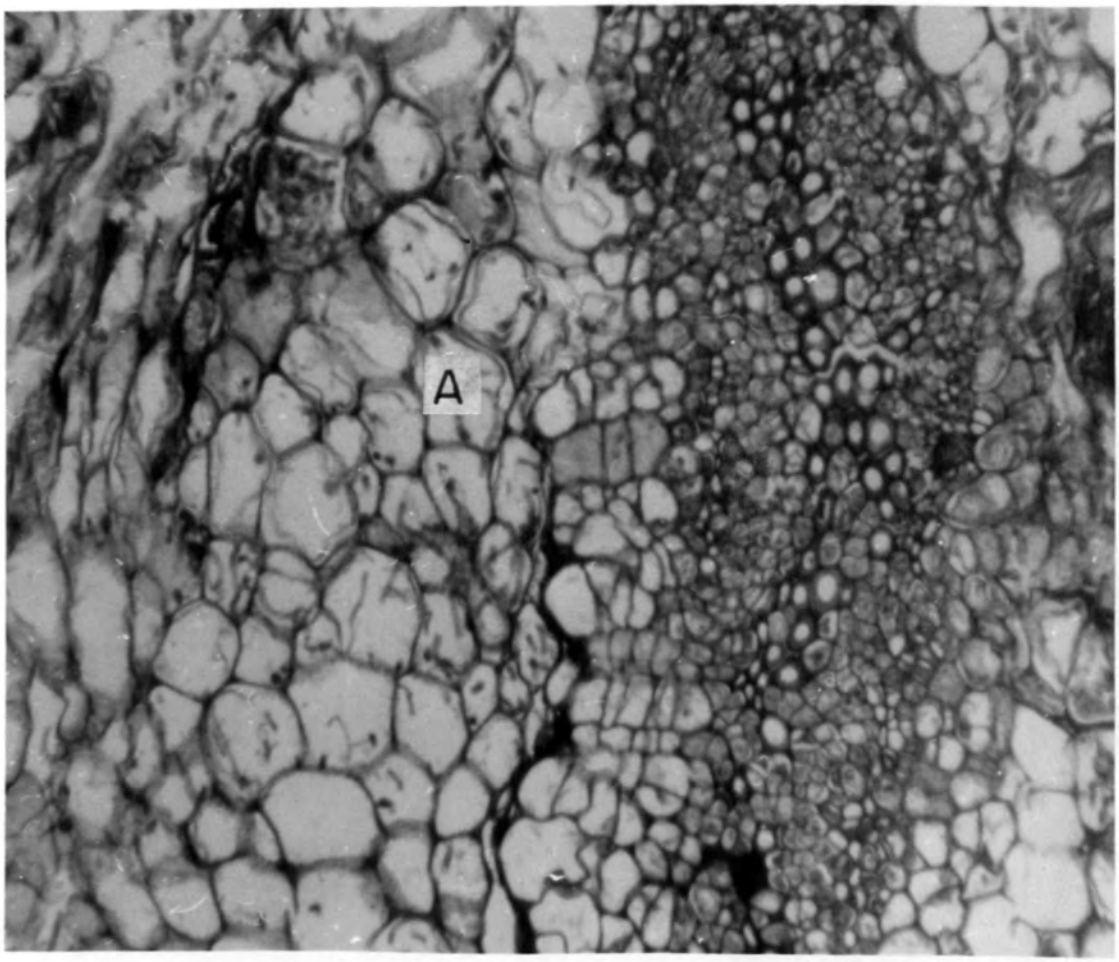

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Fig. 6.--A cross section of an infected carnation cutting showing the penetration of F. roseum f. cerealis through cell walls in the pith parenchyma. A) Hyphae (X450)

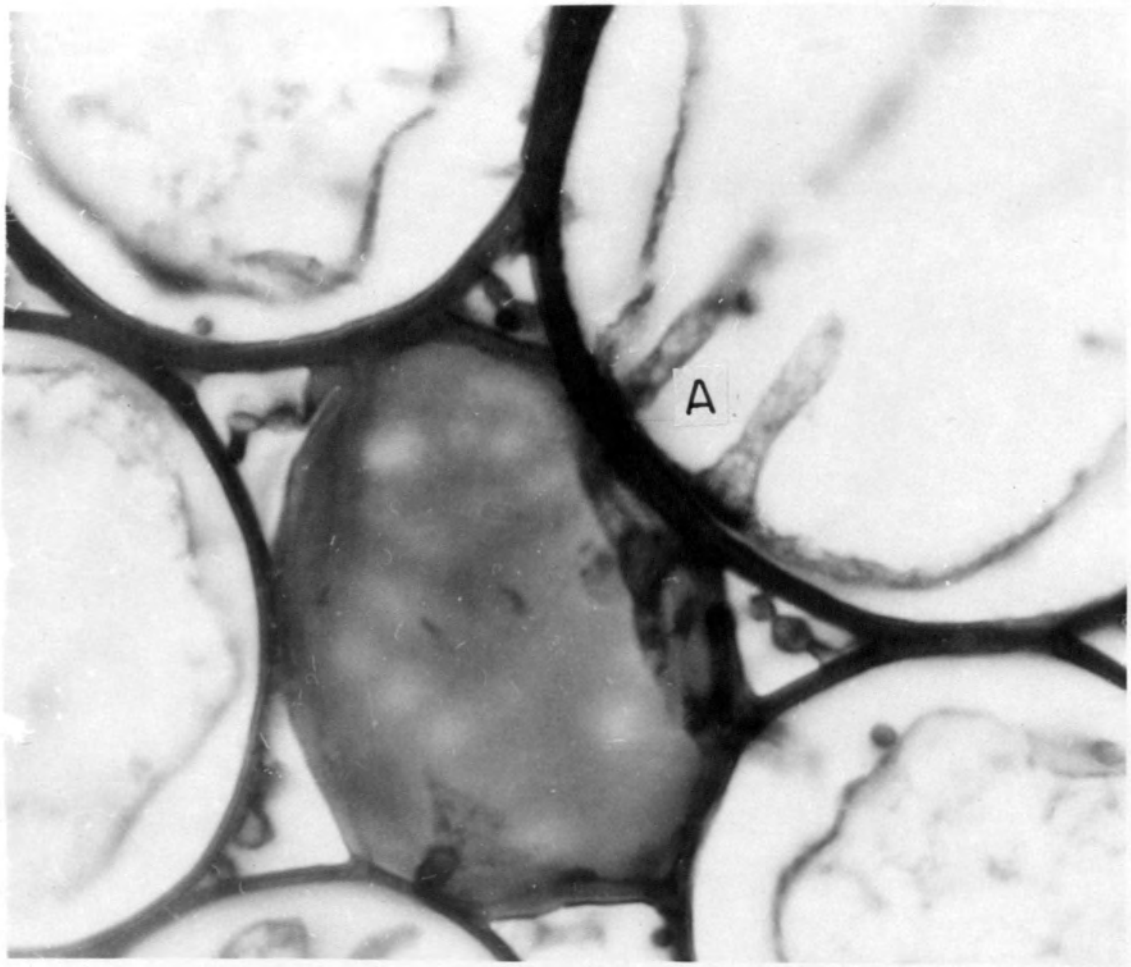
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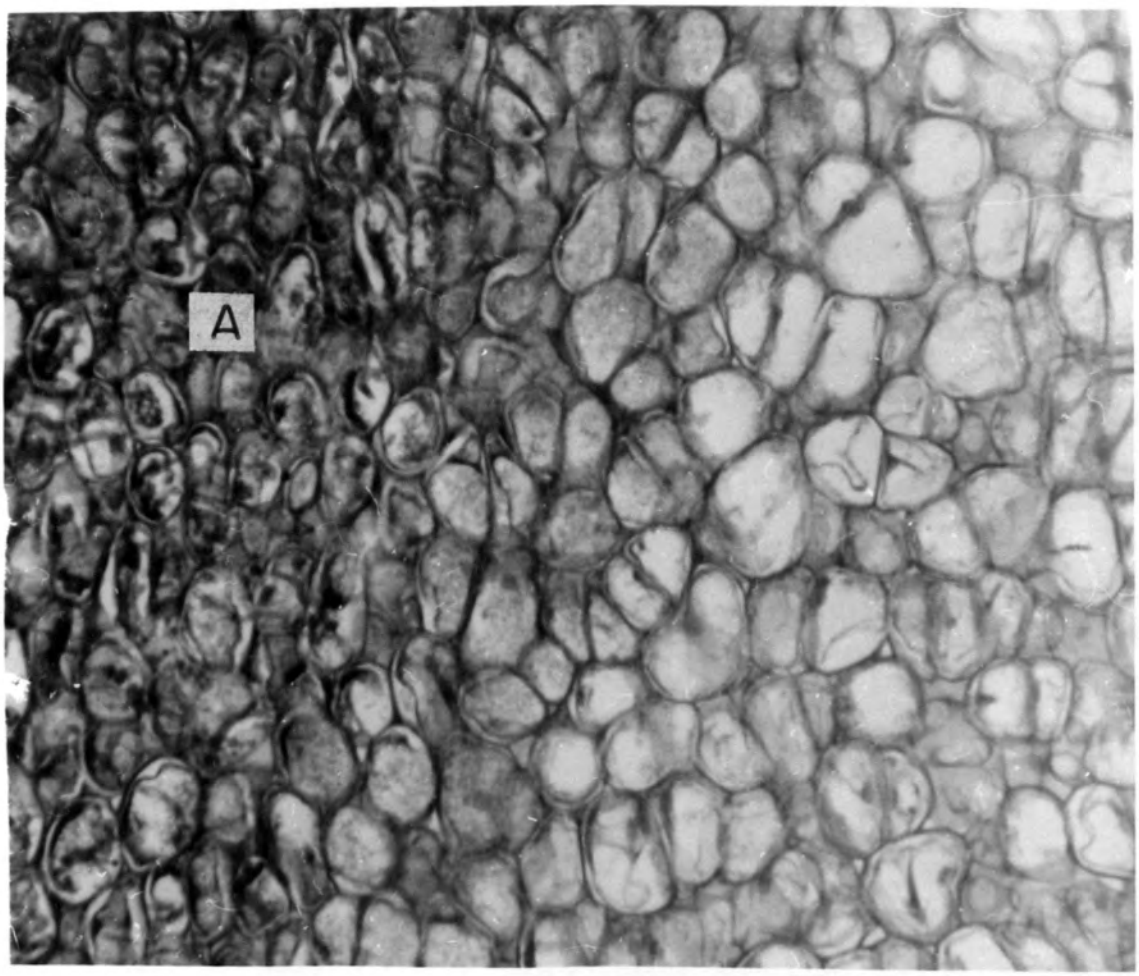
Fig. 7.--A tangential section of an infected carnation cutting showing the demarcation of the lesion incited by F. roseum f. cerealis in the pith parenchyma at a node. A) Infected area (X100).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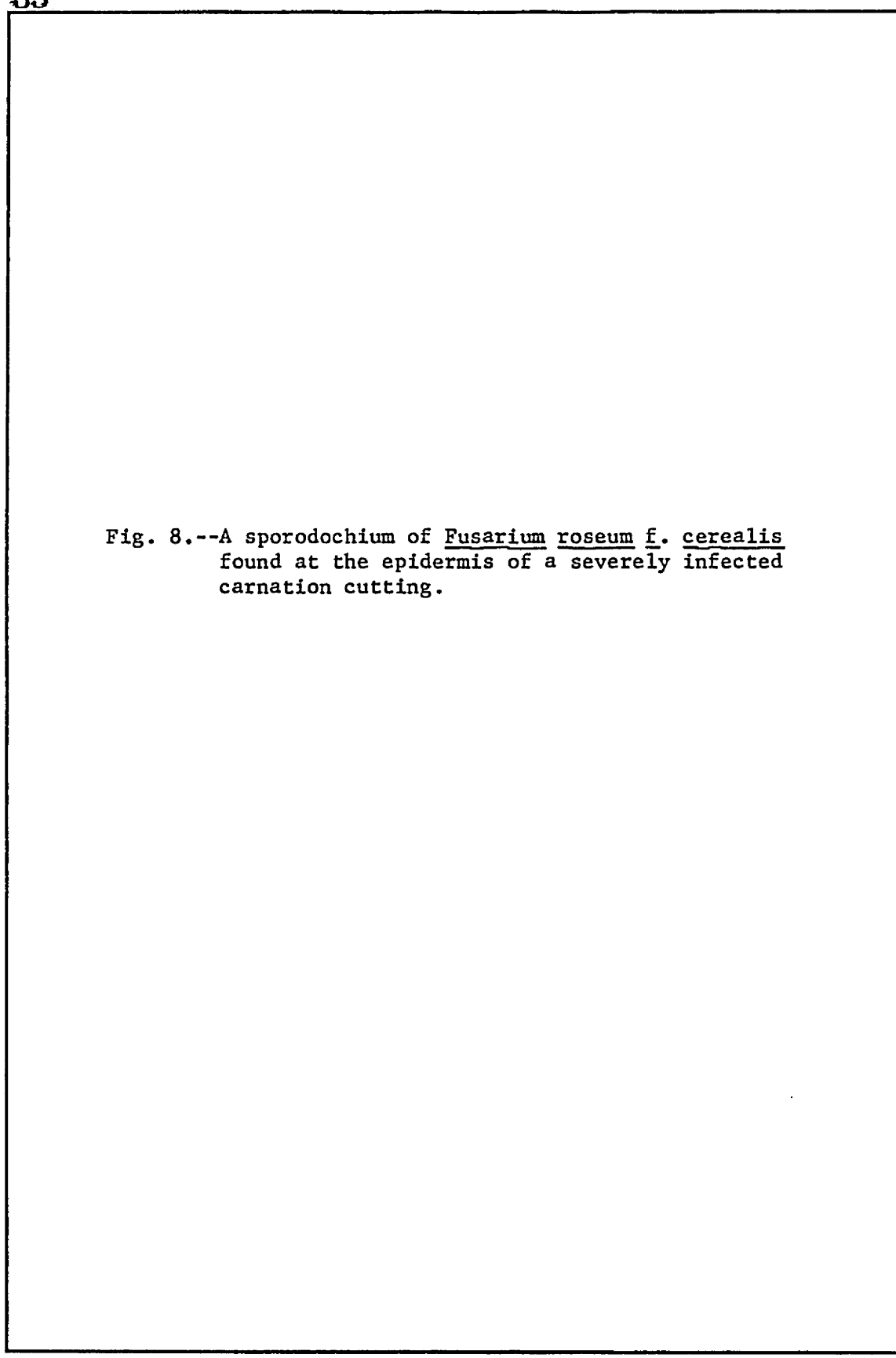

Fig. 8.--A sporodochium of *Fusarium roseum f. cerealis* found at the epidermis of a severely infected carnation cutting.

a stroma-like structure within the infected stem (Figs. 9 and 10). These masses appeared to be distinct structures composed of isodiametric hyphal cells. No differentiation was observed within these masses.

The infection caused cellular contents to be disorganized. Commonly a gum-like projection occurred on the walls of cells in the infected area. The characteristic staining of parenchyma walls changed within invaded tissue and slightly ahead of the advancing mycelium. Proliferation of cells in response to the infection was not observed.

No structural differences between the two carnation varieties were found which could account for the relative resistance of Pink Sim and the susceptibility of Miller's Yellow.

Histochemical tests

Ruthenium red stain for pectin.--The middle lamella of killed and fixed cells was stained a typical red indicating the presence of pectin. No detectable difference in the amount of pectin was found between infected and non-infected stems. Fresh sections cut with a freezing microtome provided similar results.

Zinc-chloro-iodine cellulose test.--The walls of killed and fixed non-infected parenchyma cells stained a typical blue indicating the presence of cellulose. Xylem elements, phloem fibers and cells near the cut surface stained light yellow. No reaction for cellulose was observed within the infection area or several microns in advance of the mycelium. The infected area, including

Fig. 9.--A tangential section of a carnation cutting severely infected with F. roseum f. cerealis showing a stroma-like mass of hyphae near a sporodochium. A) Stroma-like mass. B) Sporodochium (X100).

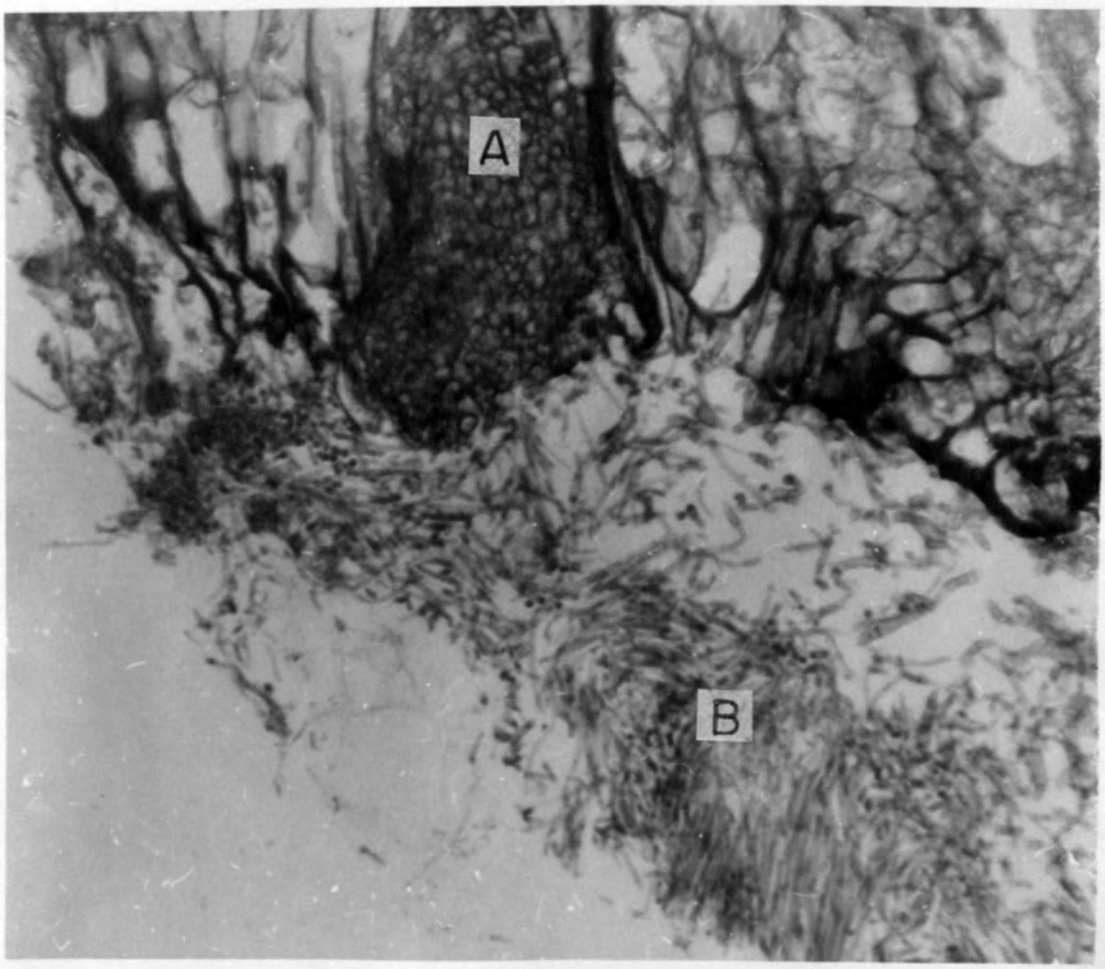
A
B

Fig. 10.--A tangential section of a carnation cutting severely infected with F. roseum f. cerealis showing a stroma-like mass of hyphae within the stem. A) Stroma-like mass (X100).


A

host tissue, hyphae and gum-like deposits within the lesion stained yellow-orange. The same results were noted with fresh sections cut with a freezing microtome.

KI-I and sulfuric acid test for cellulose and lignin.--Walls of non-infected parenchyma cells stained blue and xylem element yellow, indicating cellulose and lignin respectively. A yellow staining area encircled the wounded areas. The phloem fibers stained green indicating the presence of a lignified primary wall covering the inner cellulose secondary wall. The cellulose test within the lesion was essentially negative. The host tissue, hyphae, and gum-like deposits in the infection stained yellow.

Phyloroglucinol test for lignin and wound gum.--Xylem and phloem fibers were stained a characteristic red which indicated the present of lignin. A positive test was also obtained in cells adjacent to wounds. The gum-like deposits found within infected cells did not give a positive reaction. No reaction was noted in association with other structures within the infected area.

Sudan IV stain for suberin, fats, and oils.--The cutin of the epidermis stained bright red. The dead outer most layers of cells at the base of the cutting where stained light red indicating suberization had occurred in this region. This test indicated no reaction associated with the infection.

Lacmoid stain for callose.--The sieve tube plates of the phloem stained a brilliant blue indicating the presence of callose. No wound-callose deposits were observed in the infected sections.

KI-I test for starch.--Grains which stained a characteristic blue-black were found in a layer of cells that lay at the inner boundary of the cortex and in chlorenchyma cells near the epidermous. An accumulation of grains was found to be present in the pith parenchyma at the node. The grains were 4-8 μ in diameter. There was an average of 10 grains per cell in the nodal area. There was very little starch found in the infected sections.

Chapter V

DISCUSSION

The histochemical tests indicate that suberization and lignification took place at the cut surface and the observation of proliferation of cells at the cutting base was consistent with studies which indicate that cuttings become progressively more resistant to infection after striking. There is further evidence[1/] that the resistance is lowered about the 15th day of propagation. Penetration of the fungus in the tissues damaged by the growth of adventitious roots through the stem was noted. This study indicates that this lowering of resistance is due to adventitious root emergence. Substances which diffuse from the root or from cells damaged by growth of adventitious roots through the stem could serve as nutrients for the fungus.

Moreau (14) has reported that the infection is limited by lignified tissues of the stem. This may be due to a lack of ability by the parasite to synthesize enzymes which are able to break down lignin. As the histochemical tests indicated that pectin was present in infected areas, this would suggest that there was no significant production of pectolitic enzymes by the fungus.

1/ Baker, Ralph. Unpublished data.

Histochemical tests indicated that there was very little cellulose in the area invaded by the pathogen. This may indicate that the cellulitic enzymes are important in the host-parasite relationship and that the fungus utilized cellulose as an energy source. On the other hand, the cellulose test may have been obscured by the gum-like substances which were found to develop on cell walls within and adjacent to the lesion. A second, and more obvious consideration is the fact that the fungus does penetrate the walls of parenchyma cells which are primarily composed of cellulose. Thus wall penetration indicates the activity of cellulitic enzymes.

The limitation of the growth of the pathogen in the stem at the node was observed. Since histochemical tests of the cell walls in this area indicated that they were primarily composed of cellulose, there was no obvious explanation for the suppression of invasion in this area. Thus there appears to be evidence for the production of cellulitic enzymes, but their full significance has not been established.

The accumulation of gum-like substances in the lesion and the changes in the staining characteristics of the wall are interpreted as host reactions to the infection. This is not a specific reaction of the host to the activity of the fungus, as similar but less intense reactions were observed in tissues which had been wounded mechanically. Proliferation as a response to the fungus was noted in older plant tissues by Moreau (14). Such a

response was not noticed in these studies. This lack of proliferation by the host in response to the fungus may be due to the rate of invasion through the host tissue and the lack of vigor of the cutting during propagation.

The stroma-like masses were found in only a few infected stems. As these were found in the later stages of infection, which might be complicated by secondary organisms, a critical study of their development was not possible.

Chapter VI

SUMMARY

The morphological changes of carnation cuttings infected with Fusarium roseum f. cerealis (Cke) Snyd. & Hans. were studied during the propagation period utilizing the classical paraffin method of microtechnique. Areas of damaged tissue within the non-infected stems were found associated with growth cracks, perlite particles adhering to the cut surface, and by emerging adventitious roots. Penetration by the pathogen was found in areas of damage caused by emerging roots. Infection was limited by lignified tissues and in the pith parenchyma at the node. Sporodochia and stroma-like masses were observed in late stages of infection. Histochemical tests indicated that cellulose decomposition may take place within the lesion, while the presence of pectin, callose, lignin, and suberin appeared not to be altered in the infected area. The host response to the infection consisted primarily of gum-like deposits found at the lesion. These gum-like substances were not identified by the histochemical tests.

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