THE GROWTH OF *DIPLODIA ZEAE* (SCHW.) LEV. IN CORN STALK TISSUES

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INTRODUCTION

Many fungi and bacteria have been reported to be the cause of stalk rot of corn, either individually or together in the form of a complex. Among these organisms Diplodia zeae (Schw.) Lev. is one of the most virulent and prevalent, especially in Oklahoma. Stalk rot is one of the most serious diseases of corn wherever the crop is grown, particularly since the advent of machine harvesting. The lodged stalks resulting from stalk rot cannot be picked up by harvesting machines, and the ears produced on those stalks are usually lost. The introduction of the so-called stiff stalked hybrids off-set this loss to a certain extent, and also introduced a certain barrier to penetration by the stalk rotting fungi.

The European corn borer (Pyrausta nubilalis Hbn.) and the southwestern borer (Diatraea grandiosella Dyar), however, have provided these pathogens with a new avenue of entrance. The stalks do not lodge due to their stiffness, but chaffy ears produced on internally rotted stalks are still a factor in yield reduction.

Sanitary measures are helpful in many cases, but most investigators agree that resistance to the disease is the most feasible method of control (7,11). Basic to the search for sources of resistance is the knowledge of how the pathogen spreads and grows within the host tissue, and the effect of the presence of the parasite on these tissues.

The purpose of this investigation was to study the host-parasite relationships of the stalk rot disease of corn caused by D. zeae. The principle objectives were: (1) to learn how the organism progressed within the stalk after penetration; (2) to determine in which tissues the fungus moved most rapidly; (3) to determine which tissues, if any, served as barriers to growth of the parasite; and (4) to determine if radioactivity could be used as an aid in host-parasite relationship studies.

REVIEW OF LITERATURE

Diplodia zeae was first reported as a parasite of corn by Heald, Wilcox and Pool in 1909 (5). They reported the infection as a localized ear rot disease. The presence of fruiting bodies of the parasite on the stalks was regarded as a result of saprophytic growth. Burrill and Barrett in 1909 (2) reported a slight growth of the fungus following artificial inoculation of the stalks, but they regarded infection of stalks in the field only as a disease of senility.

Durrell, in 1923, (3) reported invasion of corn stalks by D. zeae, and he believed the infection to be limited to the internode where invasion occurred. He reported that the fungus gained entrance through the axillary bud at the base of each internode. He illustrated discolored vascular bundles in infected stalks and shanks.

Raleigh, in 1930, (14) reported this organism as a cause of seedling blight of corn and stated that, after penetration, the organism was usually restricted to the mesocotyl. McNew (13) reported that D. zeae would infect seedlings and later spread from the infected mesocotyl up into the crown of the plant. Penetration of the crown by the organism apparently occurred slowly, but spreading in the lower internodes was much more rapid. This was the first report encountered in which D. zeae infection was regarded as even semi-systemic in nature. It was also the first report which indicated any relationship between the seedling blight and stalk rot diseases caused by this organism.

Resistance to stalk rot in certain inbred lines of corn has been demonstrated (7,11,15,16). Holbert et al (6) state that corn resistant to root and stalk rot also was resistant to ear rot. Others (11,16) have concluded that resistance to D. zeae in one set of tissues does not insure resistance to this fungus in other parts of the plant.

Conflicting reports also exist regarding the nature of resistance. Durrell (4) indicated that resistant lines contained more lignified tissue, particularly in the lower nodes, than susceptible lines. Smith, Hoppe and Holbert (16) reported resistance to be due to an increased amount of sclerenchyma tissue and to an increased thickness of the cell walls in the parenchymatous tissue. Hunter and Dalbey (8) reported correlation between anatomical structure of inbred lines and resistance to lodging. Conversely Magee (12), in an histological study, reported no correlation between anatomical structures and stalk stiffness. She summarized that stiffness of stalk is probably determined by a combination of morphological characters interacting with pathological factors. Johann and Dickson (10) isolated a substance from corn stalk tissue which would retard the growth of Diplodia zeae in culture. They postulated that resistance to stalk rot may be due to the presence of such a substance in the stalks of corn.

Radioactive isotopes have not been utilized to any extent in plant pathological investigations. Warren (17) was able to tag a culture of Bacterium stewartii with readioactive phosphorus. He inoculated corn plants with this culture and was able to study the distribution of the bacterium within the host by radioactivity. Wheeler (18) labeled a culture of Glomerella with radioactive carbon 14 and used this culture to study sexual relationships between strains of this

fungus. In 1952, he (19) published a procedure which could be used for tagging fungi with radioactive isotopes.

MATERIALS AND METHODS

The culture of D. zeae used throughout these experiments was originally isolated from a diseased stalk of corn from a field near Perkins, Oklahoma. This particular culture is a transfer from the monosporidial isolation which has been used for three years in the corn stalk rot resistance tests of the Department of Botany and Plant Pathology, Oklahoma Agricultural and Mechanical College. In these tests the culture has proven to be extremely virulent.

The toothpick method (20), or slight modifications of it, was used in preparing inoculum and making the inoculations in these tests. This method was the most desirable principally because the point of inoculation can be readily detected. Fresh inoculum was prepared for each series of inoculations, so that the actual toothpick cultures were of the same age when the inoculations were made. The stalks always were inoculated in the second internode above the ground level.

The first series of stalks were inoculated on July 16, 1952, and similar series of inoculations were made at weekly intervals for eight weeks. Sufficient plants were inoculated each week so that ten plants could be cut and examined at weekly intervals for from four to eight weeks after each inoculation date.

The seed of the inbred lines K4, K44, and 87A used in the greenhouse, and the plot of the single cross K4 X K201 used in the field, were furnished by the Agronomy Department, Oklahoma Agricultural and

Mechanical College. The field plot was designed so that all the plants which were to be cut each week were grouped together. With suitable border and spacer rows, that type of design did not subject the remaining plants to unusual conditions of wind and light.

The stalks were cut at the ground level and in the fifth internode above the inoculated internode. After cutting, each series was bound and brought to the laboratory either for immediate examination, or stored under refrigeration until examination could be made. In all cases the examinations were made within two days after the material was cut.

The observations were made by splitting the stalks longitudinally through the hole where the toothpick had been inserted. The following data were recorded: (1) the percent of the inoculated internode which was necrotic and discolored; (2) the percent of the area of any other internode which was necrotic or discolored; (3) the approximate number of discolored bundles observed in the internodes above and below the inoculated internode, and the length of the discoloration expressed in internodes and/or centimeters; (4) the percent of necrosis and chlorosis of the external rind.

The migration of the fungus also was studied by isolations from necrotic and discolored tissues, and by histological sections made from such tissues. The isolations were made in petri dishes containing approximately 20 cc of potato-dextrose agar.

Some of the histological slides were prepared by hand sectioning of fresh material. Others were made with the sliding or rotary microtome, either from fresh or from paraffin-embedded material.

In preliminary studies iron hematoxin, and safranin and fast green were used for staining the histological sections. These stains did not prove satisfactory in differentiating host and pathogen in these sections, and were discontinued. Clear, unstained sections were found to be quite satisfactory for examination; consequently no other stains were tested.

The materials and methods used in the studies with the radioactive substance were specialized and directly connected with the results obtained. They will be described later in connection with the discussion of the results of these experiments.

RESULTS

It was found that infections by D. zeae progressed similarly regardless of the date of inoculation, except that the progress within the stalk was somewhat slower after the later dates on inoculation. Consequently, the description of the progress of infection of D. zeae in corn stalk tissue that follows was derived from the stalks which were inoculated July 16, 1952; some of which were cut at weekly intervals until September 10, 1952. Each stalk was cut longitudinally, and examined for necrosis and other symptoms of infection.

On week after inoculation approximately fifty percent of the inoculated internode was rotted. The necrotic area extended upwards from the point of inoculation to just below the first nodal plate and downward to the meristematic region just above the lower node (Fig. 1). The intensity of coloration of bundle and pith tissue varied in the affected protion of the internode from a dark brown to black around the point of inoculation to a light tan at the extremes of the area involved. The pith tissue surrounding the discolored vascular bundles also had a very dark brown color. Necrosis often had passed through the nodal plate in one or more bundles and extended into the internode above the inoculation for a distance of as much as 1.5 centimeters. In the other direction, necrotic bundles were found which progressed through the entire internode below the one which was inoculated. The tissue surrounding the discolored bundles where they passed through the node had a water soaked appearance. D. zeae was

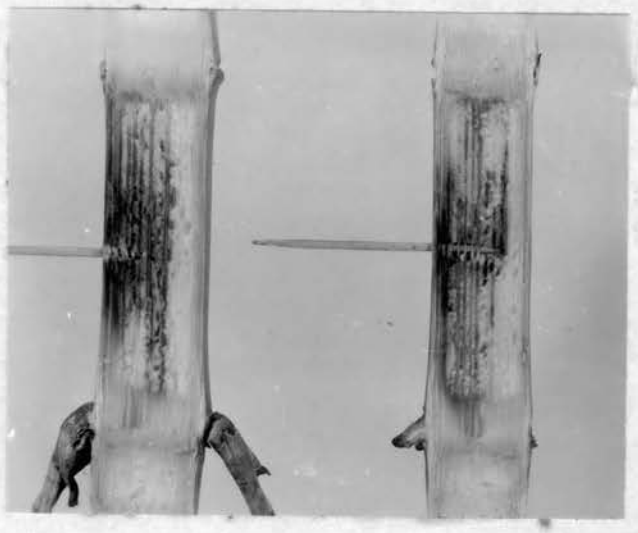

Fig. 1. The development of infection by Diplodia zeae in corn stalk tissue one week after inoculation.

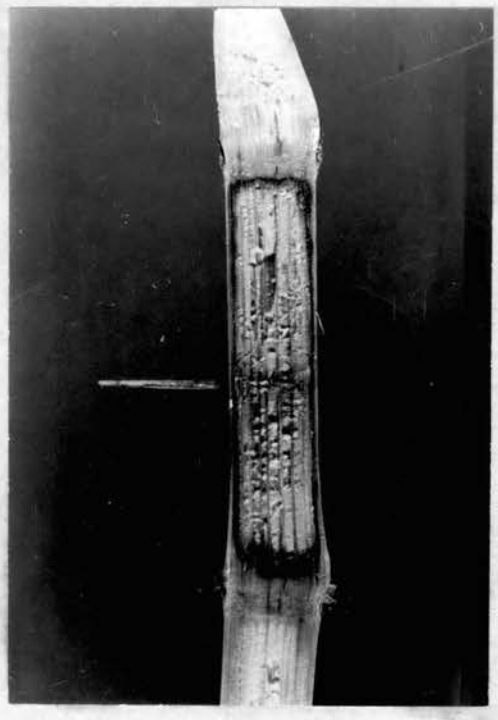

Fig. 2. The development of infection by Diplodia zeae in corn stalk tissue two weeks after inoculation. Note the black ring surrounding the inoculated internode.

isolated from all necrotic tissues of the inoculated internode and from most of the necrotic bundles found in the internode above the inoculation. However, water-soaked nodal tissue surrounding these bundles was always sterile.

After two weeks, the entire inoculated internode was necrotic, and many stalks were found in which this internode was encircled by a ring of blackened tissue (Fig. 2). The ring lay around the inside of the rind and inside the upper and lower nodal plates.

There was an increase in the number of discolored bundles which could be found in the first internode above the inoculated internode. A number of such bundles extended upwards into the second internode above the inoculation. When these bundles were excised and plated on potato-dextrose agar, cultures of D. zeae were obtained from most of the bundles extracted from the first internode, but all those bundles extracted from the second internodal region above the inoculation were sterile.

Two weeks after inoculation necrosis of tissue other than vascular bundles first appeared in the nodal regions above the inoculated internode. A portion of the stalk containing the necrotic tissue, extending from the inoculated internode through the node and into the internode above, was killed, fixed and sectioned for microscopic examination.

Between the point of inoculation and the first node above most of the vascular and pith tissue was necrotic. Gross sections in this region disclosed dark-colored pith cells which formed a line or band, one to several cells in width. These discolored cells encircled the bundles and extended from one bundle to another. The cells comprising this band, had collapsed and were irregular in outline. The parenchyma

cells encircled by the band, by contrast, were only slightly colored. In sections taken from tissue closer to the nodal plate not all of the pith tissue was necrotic. The bands of dark discoloration had disappeared, and the discoloration of all affected tissue was less intense.

Hyphae of D. zeae were observed intracellularly in the cells in and adjacent to this darkened band of pith tissue. Hyphae also were found to a limited extent in cells which were not highly colored. In most cases these hyphal strands were not found in any organized arrangement, but occasionally the hyphae were "balled" or in a circular position within the lumen of the cell. The number of hyphae varied from a few strands to tight balls of mycelium. A dark colored substance also was found in these cells which increased in intensity as the amount of mycelium increased.

In all instances where even a slight discoloration of the pith cells was observed, the intercellular spaces were filled with a dark colored substance. The pith cells surrounding the bundles and adjacent to the fibers forming the vascular sheath were usually necrotic, while the sheath itself was either free of discoloration or only slightly affected. Necrosis within the bundle was usually limited to xylem and phloem tissues. The phloem tissue was more often and more intensely discolored than the xylem. In the center of the infected area many sieve tubes in the phloem and vessels in the xylem were plugged with a dark brown substance which appeared to be simply a mass of fungal hyphae. Less plugging was noted toward the edges of the infected region. Hyphae were often observed in the lumen of sieve tubes, and along the inner cell walls of the vessels, but the hyphae found in the phloem appeared much smaller than that found either in the pith or in the xylem.

After the stalks were parasitized for two weeks, discolored pith cells could be found occasionally in the nodal region. This necrotic tissue had the appearance of a wedge, the point of which terminated at or near the nodal plate (Fig. 3).

Necrosis of the bundle tissue did not decrease as that bundle passed through the node. Where infected bundles had branched in the node, the infection followed both branches.

Three weeks after infection the pith tissue of the inoculated internode began to deteriorate, giving rise to the "dry rot" symptom so characteristic of infection by D. zeae. Necrotic bundles could be found as far as the fourth internode above the inoculation point. D. zeae was isolated from bundles in the first and second internode above the inoculated internode, and from the pith tissue in the first internode above the inoculation.

Necrotic parenchymatous tissue surrounding heavily infected bundles was traced through the node into the internode above the inoculated internode (Fig. 4). Stalks also were found in which the upper internode was encircled by a ring of blackened cells.

At the end of three weeks chlorosis and necrosis of the external rind tissues was evident. From the point of inoculation, a narrow strip of brown to gray color extended beyond both the upper and lower nodes.

After four weeks, deterioration of the pith had usually started in the internode above the inoculation. A mycelia mat was often found in the base of this internode. There was an increase in the number of necrotic bundles found in the fourth internode above the inoculated internode. D. zeae was isolated from the necrotic bundles in the third internode above the inoculation, but could not be isolated from the pith

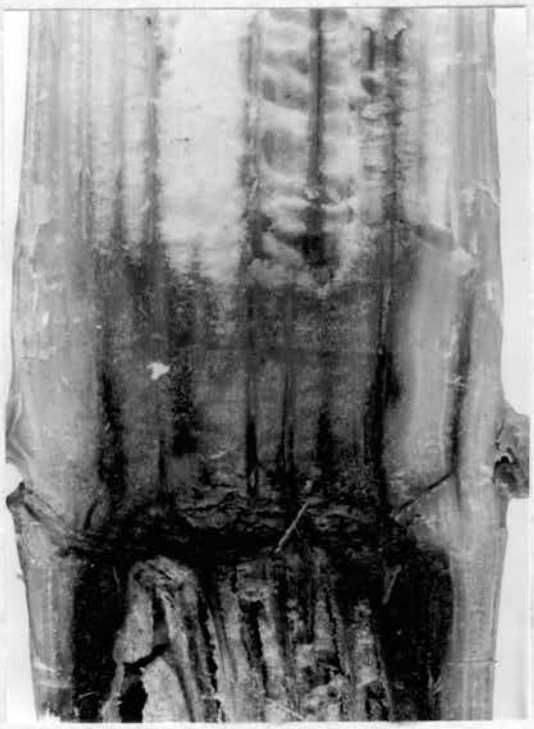

Fig. 4. A section through the node at the upper end of the inoculated internode showing that infection had passed through the nodal plate three weeks after inoculation. Both bundle and pith tissue were necrotic.

Fig. 3. Infection of corn stalk tissue by Diplodia zeae two weeks after inoculation. Note necrotic bundles extending through the node and the wedge of necrotic pith tissue extending into the region of the nodal plate.

tissue of the second, third, or fourth internodes. Most of the bundles in the upper internodes were necrotic at the end of four weeks.

Paths of necrotic tissue extending from the inoculated internode, through the node and into the internode above were again observed (Fig. 5 and 6). From the standpoint of external symptomatology, it is interesting to note that these "paths" of necrotic tissue which extended through the node were closely associated with the chlorotic and necrotic streaks in the rind tissue (Fig. 7 and 8). Discoloration in the nodes and internodes below the inoculation also was prevalent (Fig. 9 and 10).

Pycnidial formation had started in the necrotic area of the rind at the end of four weeks and spore liberation by the end of the fifth week.

After five weeks, the internode of inoculation was in advanced stages of decay. Necrosis and decay of both the nodal and internodal tissues above and below the inoculated internode was extensive, particularly below the inoculation. The rind of the inoculated internode was almost completely necrotic and there was a loss of rigidity.

The most severely diseased stalks were dead at the end of six weeks. The inoculated internodes, the internode below and the two internodes above the inoculation were necrotic and wholly or partially decayed. Each week when the stalks were cut at least one was found where the infection was limited to the inoculated internode and to a few necrotic bundles running above and below the inoculation. Even after six weeks a few of the stalks were still green and when these were cut it was found that the infection was limited by the nodes above and below the inoculation. These, however, were the exception rather than the rule.

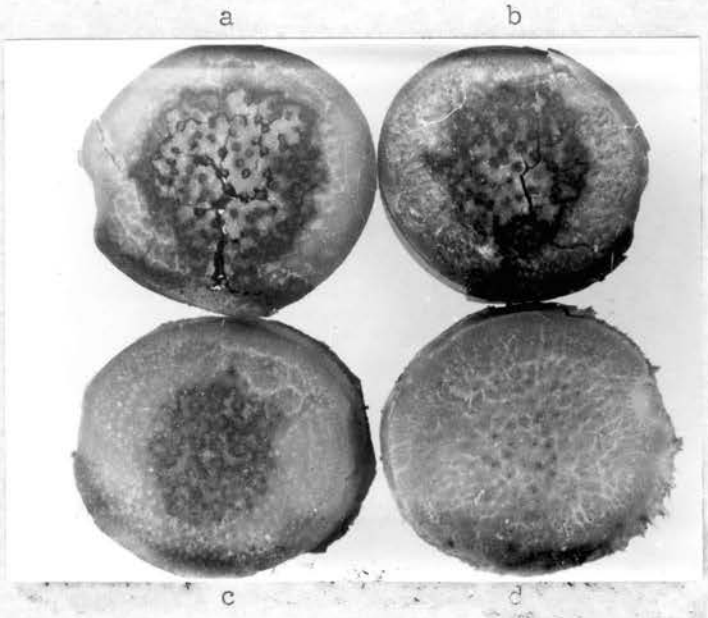

Fig. 5. The extent of necrosis of pith tissue three weeks after inoculation; (a) 6 cm. below upper nodal plate
(b) 4 cm. below upper nodal plate
(c) 2 cm. below upper nodal plate
(d) Through the upper nodal plate
Note the necrotic areas in the rind tissue. These necrotic areas were associated with external symptoms in the rind.

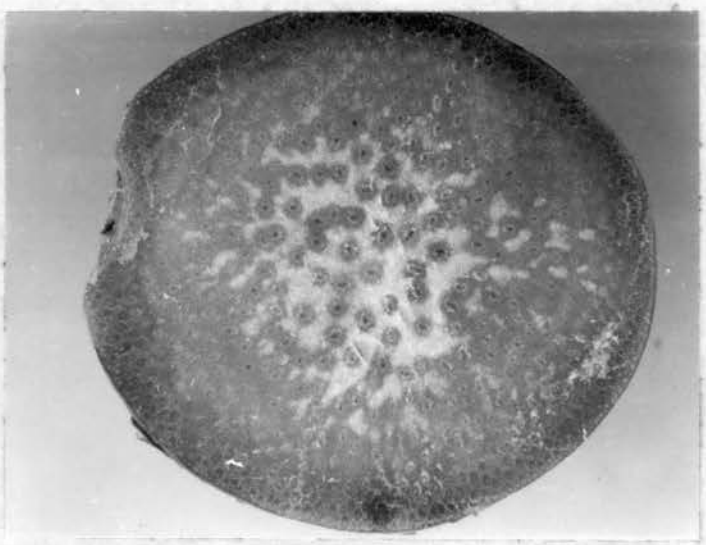

Fig. 6. A section of a corn stalk 2 cm. above the nodal plate at the upper end of the inoculated internode, showing the upper limit of necrosis in the pith tissue three weeks after inoculation.

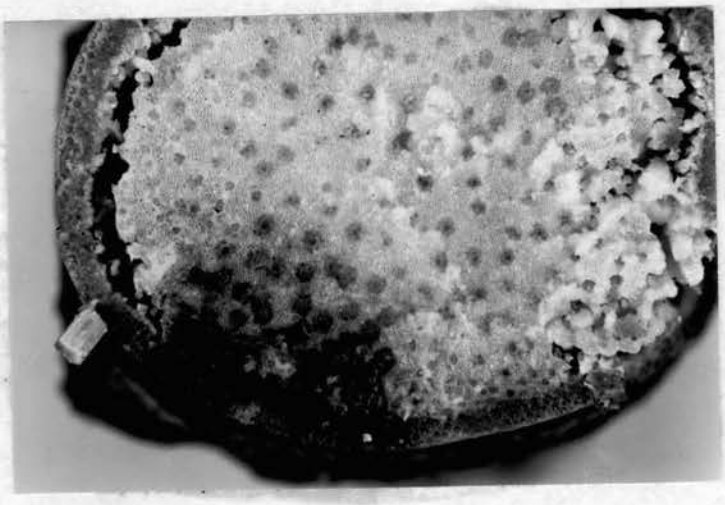

Fig. 7. A cross section through the first internode above the inoculated internode showing necrosis of the rind and adjacent pith tissue.

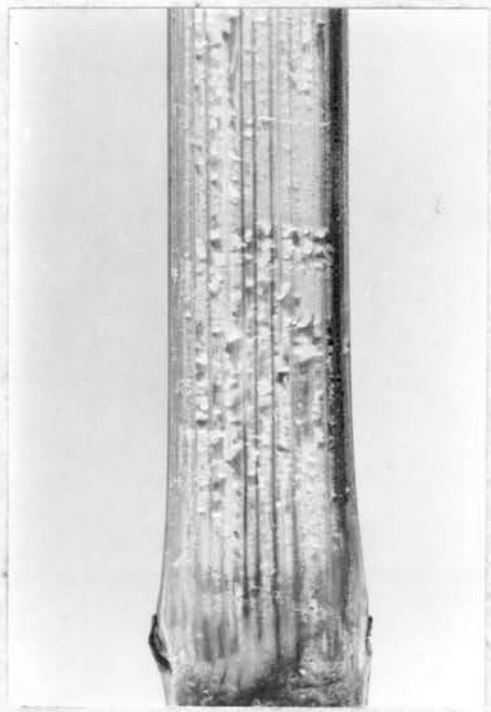

Fig. 8. Longitudinal section of the first internode above the inoculated internode showing the association of chlorisis of the rind with necrosis in the pith tissue.

Fig. 9. Infection of the nodal region at the lower end of the inoculated internode four weeks after inoculation.

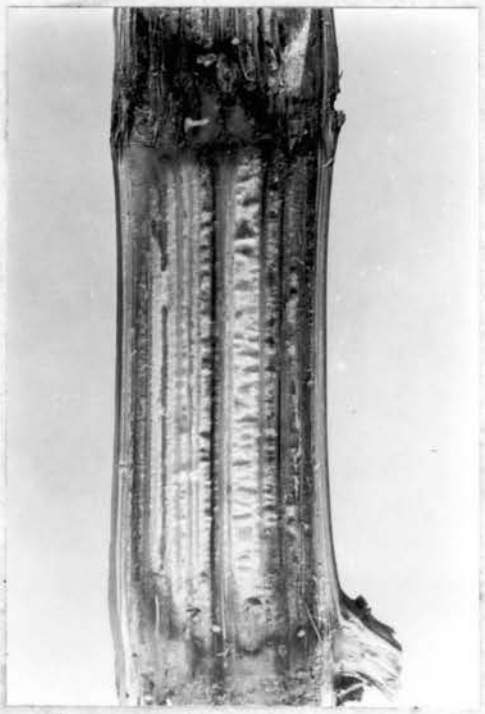

Fig. 10. Infection of the first internode below the inoculated internode four weeks after inoculation.

All observations made on stalks cut during the fourth through the eighth week after inoculation were hampered by an infestation of the southwestern corn borer. Seven weeks after inoculation one hundred per cent of the stalks were infested, rendering the material useless for these studies. The borer tunnels made by these insects provided other interesting observations. Isolations made from the frass of the borer tunnels yielded cultures of many fungi and bacteria, but isolates of D. zeze were dominant. Evidence that the borers facilitated the movement of D. zeae through the stalks was observed from a stalk cut two weeks after inoculation. In this case, a borer tunnel extended from below the point of inoculation through the inoculated internode, and two internodes above. D. zeae could not be isolated from tissue surrounding the tunnel in the internode below the innoculation, but was readily recovered from the tissues near the tunnel in the upper internodes. Tissues surrounding the borer tunnel in these upper internodes were definitely necrotic, but below the point of inoculation there was very little necrosis of the tunnel wall. It was evident that the borer had become infested with D. zeae as it passed upward through the inoculated internode and had carried the fungus into the upper internode.

EXPERIMENTS INVOLVING THE USE OF A RADIOACTIVE SUBSTANCE

In addition to the usual histological studies, an attempt was made to utilize a radioautographic technique in the determination of the host parasite relationship. Radioactive sulfur 35 was used in these tests for several reasons: (1) sulfur 35 is relatively

inexpensive; (2) it is readily available; (3) the half-life is sufficiently long to cover the period from original tagging of the pathogenic culture, the passage through the host, to the final exposure of the radioautographs; (4) the material emits only beta rays which are less hazardous to personnel.

It was necessary to determine, first, whether or not D. zeae would assimilate sulfur 35 from a culture medium. Sulfur 35 with an activity of 7,332 cpm/lambda (counts per minute per lambda), as determined on the fifth shelf of a Berkely Decimal Counter and Scaler, model 2000, was used in preliminary studies. To a series of four sterile petri dishes each containing 20 cc. of warm potato-dextrose agar, 20, 40, 60, and 100 lambdas of sulfur 35 was added by means of a Misco micropipette and a Tuberclin syringe. The isotope was throughly mixed with the agar with a small glass stirring rod. The media in this series of plates was solidified and inoculated with a small bit of aerial mycelium from a four-day-old culture of D. zeae. Care was taken not to touch the media with the inoculating needle when these transfers were made. The radioactive plates were incubated at room temperature for four days. Several samples of mycelium from the edges of each of the resulting colonies were then taken and placed in monitorial flanchets. Care again was exercised to avoid transferring any of the medium along with the mycelium. These samples were ashed and radioactivity evaluated on the counter. All of the samples indicated radioactivity, particularly those from the plates containing 60 and 100 lambdas of sulfur 35 (Table 1), which indicated that the fungus had taken up this radioactive material. At the same time that the samples were collected for radioactive counts, transfers of mycelium were made to microscopic glass

Table 1. The radioactivity of mycelial fragments from cultures of Diplodia zeae grown on a radioactive medium.

Volume of sulfur 35 per 20 cc. of PDA[1]	Radioactivity in counts per minute above background[2]
20	4
40	7
60	32
100	62

[1] In lambdas.
[2] Counts made on second shelf of the Gieger counter.

slides for the preparation of radioautographs. All of the materials used for radioautographs were prepared in accordance with the procedures given in Table 2 which were developed by Steffey[1].

The slides so prepared were then coated with Ansco Radioautographic Emulsion A following the procedure outlined by Bélanger and Lebland (1).

The slides containing the mycelial fragments were exposed to the radiation for 8 days in a bakolite slide box. This container was kept dry and cool by calcium chloride and refrigeration. All the radioautographs were developed and dehydrated in accordance with the following procedures which also were developed by Steffey.

[1] O. D. Steffey. Unpublished data, Department of Botany and Plant Pathology, Oklahoma Agricultural and Mechanical College, Stillwater, Oklahoma.

Table 2. Procedure for preparing material for radioautographs.

Preparation of Slides for Coating:

1. Mark slides, clean in chrome sulfuric acid cleaning solution, rinse thoroughly and place in distilled water.

2. Dip slides in a subbing solution[1] and place in glass staining racks to dry.

3. Dip slides in 4% formalin and float the plant material onto the slide and attach according to accepted practices. Then place these slides in glass staining racks to dry.

Coating sections with celloidin:

1. After slides are sufficiently dry process in the following solutions:

Toluene I	3 min.
Toluene II	3 min.
Toluene III	3 min.
100% EtOH I	1 min.
100% EtOH II	1 min.
95% EtOH	1 min.
Safranin O in 95% EtOH	3 to 15 min.
95% EtOH Destain	1 min.
100% EtOH	1 min.
Celloidin (1%)	1 min.
Drain	10 to 15 sec.
70% EtOH	1 min.

2. Remove slides from 70% alcohol and place in a glass staining rack and allow to dry thoroughly.

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[1] Formula for subbing solution:

Gelatin	5.0 gms.
Chrome Alum	0.5 gms.
Water to make	1000 cc.

Developing:

After exposure, develop slides as follows:

D-72----------1 to 2 min.

Acid Stop (1 1/2 oz. of 28% solution of glacial acetic acid in 32 oz. water)----------1 min.

Kodak Acid Fixer----------5 min.

Water wash----------30 min. to 1 hr.

Dehydration:

Carry slides horizontally through the following solutions up carbol-xylol:

95% EtOH----------1 min.

100% EtOH----------1 min.

100% EtOH plus Xylene (50-50)----------1 min.

Xylene I----------1 min.

Xylene II----------1 min.

Xylene III----------1 min.

Carbol-xylol (25 cc. Phenol to 75 cc. Xylol)-1 hr. or longer

Balsam

Add coverslips

Carbol-xylol was used as a dehydrant and clearing agent, but later results have shown that xylene alone would have been sufficient.

Fifteen seconds was found to be the optimum developing time for maximum resolution with the material used in these studies.

This experiment was repeated except that the volume of isotope was increased to 100, 300, 600 lambdas, and one milliliter of sulfur 35 to each petri plate. The Gieger count of bits of aerial mycelium from

the culture in the plate containing one milliliter ranged from 223 to 430 cpm., averaging 321 cpm. Radioautographs were prepared and developed after three days exposure. These slides indicated that the fungus mycelium was highly radioactive (Fig. 11).

Two methods were used in determining if the isotope was secreted by the fungus or diffused into a non-active medium. In the first test, sterile microscopic slides were dipped in warm potato-dextrose agar and, after the agar had solidified, were inoculated with a tagged culture of D. zeae. Formaldehyde fumes were used to kill the mycelium after 8 days of growth at room temperature and the slides then prepared for radioautography. The usual ethyl alcohol dehydrant series was substituted for the toluene series in this particular experiment. Radioactivity did not indicate any diffusion of the isotope in radioautographs developed after nine days exposure.

In the second test, five 125 ml. erlenmeyer flasks containing 10 cc. of potato-dextrose broth were autoclaved and inoculated with a tagged culture of D. zeae. After a dense mat of mycelium had formed over the surface of the broth, the remaining liquid was decanted into monitorial flanchets. The flanchets were placed on a hot plate and the liquid evaporated. Counts of radioactivity of the residue were made.

The results of this and subsequent tests were inconclusive. Some times the counts of radioactivity were above normal background counts; at other times they definitely were not. In any case, there did not seem to be any definite pattern of diffusion or secretion of the radio-isotope from the tagged fungus culture into the surrounding medium.

There were no visiable affects on the cultural characteristics of the organism at any of the concentrations used.

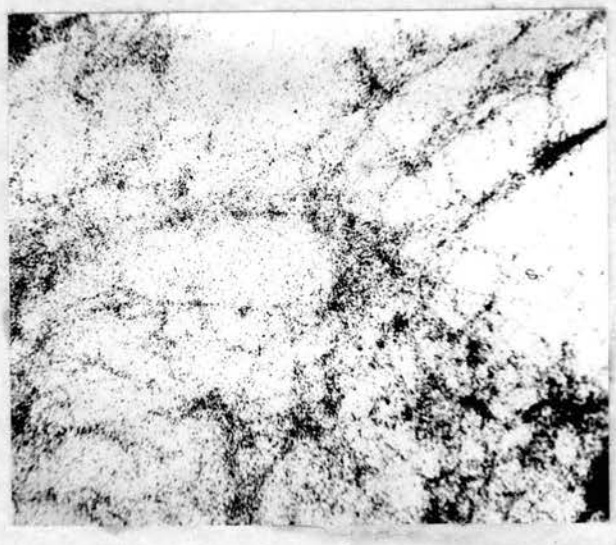


Fig. 11. The radioactivity of mycelium of Dipoldia zeae after 4 days growth on a medium containing radioactive sulfur 35.

It was believed that the more radioactivity that the fungus could acquire without radiation damage, the greater were the possibilities of obtaining radioautographs after disease development. Consequently, the inoculum used in the greenhouse and field studies was grown for four days on media which contained one milliliter of radioactive sulfur 35 with a count of 6,419 cpm/lambda recorded on the third shelf of the Gieger counter.

The inoculation technique used was a modification of the toothpick method previously mentioned. Quill type toothpicks impregnated with potato dextrose broth were used to carefully remove aerial mycelium from the surface of the radioactive plates. This peg, together with the mycelium was inserted in a hole made in the stalk with a small nail-punch. Anytime one of the toothpicks came in contact with the agar in the process of picking up the fungus mycelium it was not used, but was placed in a container for destruction. The same procedure was used with a normal culture of D. zeae for a control. After inoculation, the peg and the cite of the inoculation were wrapped with aluminum foil. All of the precautionary measures normally used in the handling, storing, and disposing of radioactive materials were utilized in these studies. All tools, equipment and personnel were constantly monitored for radioactivity.

Preliminary studies on disease development with a radioactive culture of D. zeae were made in the greenhouse during the fall and winter of 1951-1952. Three inbred lines of corn, K4, K44, and 87A, were inoculated in the manner just described in the second internode above the ground level. Four weeks after inoculation the stalks were split and the extent of necrosis and discoloration noted. From

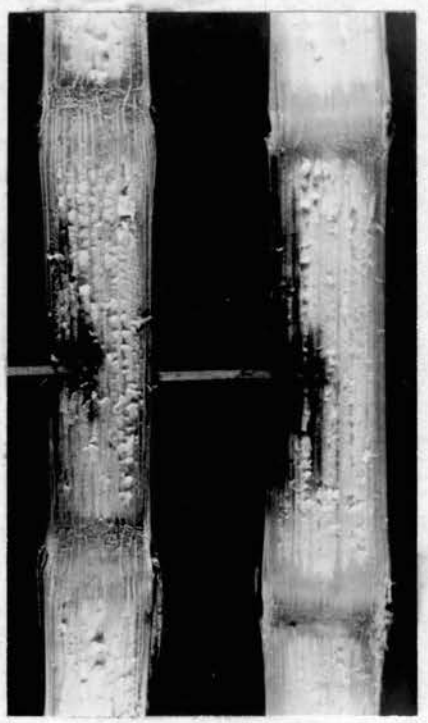
selected stalks, material was taken from each internode where necrosis and discoloration appeared and at one centimeter intervals above the point of inoculation within the inoculated internode. The material was killed and fixed in Bouin's solution, then embedded and sectioned in the usual manner. Radioautographs were prepared as described. Following an exposure of one month the only tissue exhibiting radioactivity came from the inoculated internode. Radioactivity was evident as far as four centimeters above the point of inoculation.

The following summer inoculations with a radioactive culture of D. zeae were made beginning August 6, 1952, in the single cross K4 X K201. Five plants were inoculated weekly for a period of five weeks. Similar inoculations were made with a normal culture of the fungus.

The procedures used for preparing inoculum and making the inoculations were described above. The activity of the isotope used in the culture medium was 7,288 cpm/lambda, recorded on the third shelf of the counter.

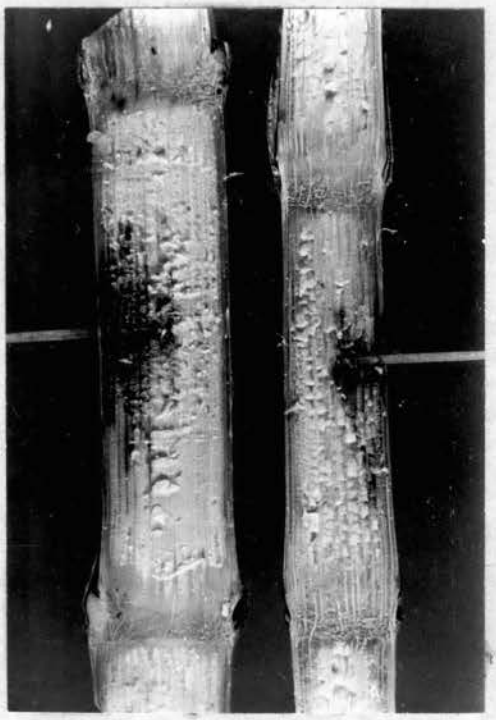
At one and two weeks after inoculation one isotopic and one control plot were cut and readings were made on the percent of necrosis and discoloration. No appreciable difference in infection was noted between the stalks inoculated with the normal culture and those inoculated with the radioactive culture (Fig. 12).

At the end of two weeks necrosis and discoloration was still limited to the inoculated internode (Fig. 13) except for a few vascular bundles. Material for embedding and radioautography was taken at one centimeter intervals above the point of inoculation within the inoculated internode. Discolored bundles above the inoculated internode also were extracted for sectioning. The remaining plots were not used because of high infestation



A B

Fig. 12. Infection of corn stalk tissue by (A) a radioactive culture of Diplodia zeae and (B) a normal culture.



A B

Fig. 13. Infection of corn stalk tissue by a radioactive culture of Diplodia zeae after (A) two weeks, and (B) one week.

of the southwestern corn borer. Radioautographs of infected bundles did not show any radioactivity, even after two months of exposure. The remaining material, consisting of sections from the inoculated internode, was allowed to expose for 119 days. When these exposures were developed considerable radioactivity was found.

It was not possible to directly identify the presence of mycelium of D. zeae by radioactivity in any of the radioautographs. Radiation could be associated with certain tissues, and with certain very large individual cells of tissues such as the pith parenchyma and xylem vessels (Fig. 14 and 15). The amount of radioactivity appeared to vary proportionately with the intensity of infection as measured by necrosis, discoloration and the presence of fungus mycelium. Radioactivity was always associated with necrosis, but necrotic areas were not always radioactive. Radiation emanated from the pith tissue, and from the phloem and xylem tissues of the bundles. The tissue forming the bundle sheath was neither necrotic nor did it exhibit radiation.

DISCUSSION

The penetration phenomenon and symptomotology of stalk rot have been rather adequately studied (3, 7, 10), but little has been reported concerning the progress of the fungus after penetration of the host. Durrell (3) has reported that the fungus is usually restricted to the internode of invasion, and that rotting of the entire stalk is a result of numerous different infections.

The experiments reported here clearly indicate that D. zeae is capable of migrating throughout the corn stalk after penetration is accomplished. When the toothpick method of inoculation is used the

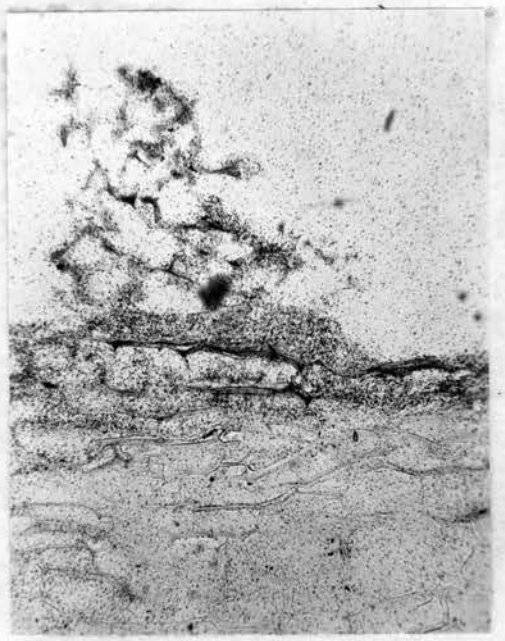


Fig. 14. Longitudinal section of pith tissue from a corn stalk inoculated with a radioactive culture of Diplodia zeae showing radiation associated with necrotic cell walls of parenchyma cells.

Fig. 15. Longitudinal section of pith and bundle tissue of a corn stalk inoculated with a radioactive culture of Diplodia zeae showing radiation associated with vessels of the xylem.

fungus is introduced directly into both the parenchymatous pith tissue and the vascular tissues. This method closely approximates the entrance of the fungus through borer insect tunnels. Spread of the fungus in the parenchymatous tissue was primarily intercellular at first, later thoroughly permeating the cells. Radioautographs indicated that the cell walls of diseased parenchyma cells were highly radioactive, which supported the microscopic observations. The mycelium spread freely through this tissue until the nodal region was reached where passage through the nodes was restricted. Spread of the fungus within the pith tissue is less rapid than in the vascular tissues. The vascular sheath forms a barrier which is difficult for the fungus to penetrate. Movement of the fungus into and out of the vascular elements, except at the point of inoculation, is restricted by this sheath tissue.

It is evident, then, that the growth and spread of the fungus is restricted by lignified tissues such as the bundle sheath and the tissues of the nodal region. Durrell (4) reported that D. zeae was able to assimilate cellulose more rapidly than lignin. Further evidence is the fact that this fungus grows more rapidly and extensively in the non-lignified phloem than in the lignified xylem elements of the vascular bundles. This would indicate that varieties with greater lignification in the nodal regions and in the bundle sheath would be more resistant to parasitism by D. zeae. However, lignified tissue does not prevent penetration by D. zeae, but merely slows down the process. Further evidence of this is the ability of the fungus to make some growth in the tissues of the rind, most of which are heavily lignified.

Certain parenchyma cells in the pith and sieve elements in the phloem were found to be filled with a mass of D. zeae hyphae, which

appeared to plug these cells. Dark colored substances also were found to plug the xylem vessels, but mycelium could not be definitely detected in the vessels which were plugged.

Johann (9) obtained lesions on corn seedlings by using the staling products from the growth of the fungus Penicillium oxalicum (Currie) Thom on artificial media. McNew (13) has reported that lesions caused by D. zeae on the mesocotyl of corn seedlings extend in advance of the mycelium. Similarly in this study, discoloration appeared in the vascular tissue in advance of the mycelium, as shown by the isolations made from this tissue. However the organism usually can be isolated from discolored bundles within a week after the first discoloration appears. These facts suggest that some toxic substance is formed by the growth of D. zeae which may kill the cells in advance of penetration by the hyphae.

The results with radioactive sulfur 35 demonstrated that D. zeae can be tagged simply by growing the organism for a short period on a media containing the radioactive substance. The tagged culture of D. zeae was of value in these studies on host-parasite relationships. However, further refinement of photographic emulsions for radioactive sensitivity would be of great benefit in studies of this nature.

SUMMARY

The parasitism of Diplodia zeae (Schw) Lev. in the stalks of corn was studied.

Diplodia zeae migrated from internode to internode in three ways: (1) in the phloem and xylem tissues of vascular bundles; (2) in parenchymatous tissue through the nodal region; (3) in external rind tissues, until past the node, and then into the internal stalk tissues.

The lignified bundle sheath appears to delay lateral spread of the fungus from bundle tissue; and conversely, to delay its entry into the bundle.

The limited amount of parenchymatous tissue in the nodal region retards the rate of vertical migration of the fungus in the pith.

Isolations from infected bundles indicated that discoloration preceeds hyphal penetration.

Evidence was obtained that the southwestern corn borer, Diatraea grandiosella Dyar., may accelerate the spread of D. zeae in the stalk, principally by providing a passage through the nodes.

D. zeae was effectively tagged with radioactive sulfur 35 by assimulating the isotope from an artificial medium. The value of this culture containing a radioactive isotope in the study of host-parasite relationships was demonstrated.

LITERATURE CITED

1. Bélanger, L. F. and C. P. Leblond. A method for locating radioactive elements in tissues by covering histological sections with a photographic emulsion. Endocrinology 39: 8-13. 1946.

2. Burrill, T. J. and J. T. Barrett. Ear rots of corn. Ill. Agr. Expt. Sta. Bul. 133. 1909.

3. Durrell, L. W. Dry rot of corn. Iowa Agr. Expt. Sta. Res. Bul. 77: 347-376. 1923.

4. _____, Preliminary study of fungous action as the cause of down corn. Phytopathology 15: 146-154. 1925.

5. Heald, F. D., E. M. Wilcox, and V. W. Pool. The history and parasitism of Diplodia zeae (Schw.) Lev. Nebr. Agr. Expt. Sta. Rpt. 22: 1-7. 1909.

6. Holbert, J. R., W. L. Burlison, H. H. Bigger, B. Koehler, G. H. Dungan, and M. T. Jenkins. Early vigor of maize plants and yield of grain as influenced by the corn root, stalk, and ear rot diseases. Jour. Agr. Res. 23: 583-630. 1923.

7. _____, _____, B. Koehler, C. M. Woodworth, and G. H. Dungan. Corn root, stalk, and ear rot diseases, and their control thru seed selection and breeding. Ill. Agr. Expt. Sta. Bul. 255. 1924.

8. Hunter, J. W. and N. E. Dalbey. Histological study of stalk-breaking in maize. Am. Jour. Bot. 24: 492-494. 1937.

9. Johann, Helen. Penicillium injury to corn seedlings. Phytopathology 18: 239-242. 1928.

10. _____, and A. D. Dickson. A soluble substance in cornstalks retards growth of Diplodia zeae in culture. Jour. Agr. Res. 71: 89-110.

11. Koehler, B. and J. R. Holbert. Combating corn diseases. Ill. Agr. Ext. Circ. 484: 1-36. 1938.

12. Magee, Jean Anne. Histological structure of the stem of Zea maize in relation to stiffness of stalk. Iowa State Col. Jour. Sci. 22: 257-268. 1948.

13. McNew, G. L. Crown infection of corn by Diplodia zeae. Iowa Agr. Expt. Sta. Res. Bul. 216. 1937.

14. Raleigh, W. P. Infection studies of Diplodia zeae (Schw.) Lév. and control of seedling blight of corn. Iowa Agr. Expt. Sta. Res. Bul. 124. 1930.

15. Smith, A. L. and J. R. Holbert. Cornstalk rot and ear rot. (Abs.) Phytopathology 21: 129. 1931.

16. _____, and P. E. Hoppe, and J. R. Holbert. Development of an inoculation technique of Diplodia stalk rot of corn. Phytopathology 28: 497-504. 1938.

17. Warren, J. R. The use of radioisotopes in determining the distribution of Bacterium stewartii Erw. Smith within corn plants. Phytopathology 41: 794-800. 1951.

18. Wheeler, H. E. The use of C^{14}-labeled sucrose for "tagging" fungus mycelium. (Abs.) Phytopathology 41: 38. 1951.

19. _____. The use of radiocarbon for tagging fungi. Phytopathology 42: 431-435. 1952.

20. Young, H. C., Jr. The toothpick method of inoculating corn for ear and stalk rots. (Abs.) Phytopathology 33: 16. 1943.

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