STUDIES OF THE PATHOGEN, CLADOSPORIUM EFFUSUM (WINT.) DEMAREE, AND HOST-PARASITE RELATIONS IN PECAN SCAB

By

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INTRODUCTION

Pecan scab incited by Cladosporium effusum (Wint.) Demaree is recognized as the most important disease of pecans, especially in the more humid areas. With favorable environmental conditions for disease development, a crop loss of 100 percent is not uncommon on highly susceptible pecan varieties. While the most obvious loss occurs through reductions in quality and quantity of the nuts, further, unmeasured damage undoubtedly results from premature defoliation and twig infections.

Previous work on pecan scab (7,9,11,12,13,15,17,19,29) has dealt primarily with the morphology of the causal organism, variation in host susceptibility, and the application of fungicides for control of the disease. In past studies little consideration has been given the phenomena of parasitism and pathogenesis in the various stages of host-parasite relations.

At present there is need for a more adequate knowledge of control of this disease. Control measures, however, are conditioned by a knowledge of the life history of the pathogen and an understanding of the relation of environment to disease development.

The present investigation was undertaken with the objective of contributing to the knowledge of certain phases of the life history of the pecan scab organism through an investigation of the pathological histology of the disease. Soon after these studies were begun, however, it became apparent that our knowledge of how to handle the fungus itself under laboratory conditions was inadequate. Consequently, considerable effort has of necessity been directed toward culturing and inducing sporulation in cultures of the pecan scab fungus.

LITERATURE REVIEW

According to Demaree (17), the pecan scab fungus was first collected by F. S. Earle on leaves of *Hicoria alba* (L.) Brit. in Illinois in 1882. The organism was described as a new species, *Fusicladium effusum*, by Winter (36). In 1926 Demaree (16) transferred this fungus to the genus *Cladosporium* primarily on the basis that the conidia were borne in chains rather than singly as in the genus *Fusicladium*.

With the spread of commercial pecan production to the southeastern United States, pecan scab was noted as becoming of increasing importance. Striking differences in resistance or susceptibility of pecan varieties to scab became more and more evident with the continued increase in commercial plantings. It was soon evident, however, that varieties resistant in one locality were not necessarily resistant in others (19,35). The history of resistant varieties has in nearly every case been such that after a few to several years such varieties are no longer resistant. To account for these changes in susceptibility of pecan varieties the existence of physiologic races of the causal organism has been postulated. The only attempts to determine experimentally if races exist, however, have been those of Nolen (29) and Demaree and Cole (19). Nolen attempted to separate isolates of the pecan scab fungus on the basis of differences in conidial measurements. Except in one doubtful case, the organism collected from several different pecan varieties and from several localities was not separable into races. It is doubtful if the criterion used by Nolen for identifying races was sufficient for this purpose. Demaree and Cole (19) approached the same problem by inoculating each of four susceptible pecan varieties with conidia obtained from natural infections on each of the four varieties. Generally, heavy infection resulted when conidia obtained from a given variety were put back onto the same variety;

the same conidia placed on each of the other three varieties produced little or no infection. Pure cultures or cultures stemming from single conidia were not used. They considered their results suggestive or preliminary evidence of the existence of physiologic races of the scab organism.

While such preliminary studies and observations strongly indicate the probability of the existence of physiologic races of _Cladosporium effusum_ on pecan, more evidence is needed to establish this point firmly.

It should, perhaps, be pointed out that the question of races has arisen in a very similar fungus, _Cladosporium fulvum_ Cooke, the causal organism of tomato leaf mold. According to Bailey (3), eight strains have been distinguished within this species. These strains were identified according to their reaction on seven tomato species and varieties. The situation with regard to resistant tomato varieties somewhat parallels the experience with pecan varieties. Tomato varieties selected or bred for resistance to leaf mold have been in every case after exposure to natural infection for a year or two found susceptible (1,2,25,21). This behavior is explained on the basis of new or different races of the causal organism (3,21,22,24,25).

While some differences in varietal susceptibility have been noted among peach varieties to peach scab (_Cladosporium carpophilum_ Thum), the question of races of the causal organism has not been investigated.

Life history studies of pecan scab have been restricted to studies of the seasonal development of the disease, sources of inoculum, and overwintering of the fungus. Demaree (14) reported the disease occurring on leaves, twigs, shucks, catkins, and outer bud scales of the pecan. So far as he was able to determine, the fungus survived from one season to the next only as stromatal masses on late-formed twig lesions, late shuck lesions, and lesions on pinnae, rachises and petioles of leaves. With favorable temperature and moisture

conditions in the spring these stromata produced conidia which served as primary inoculum for new susceptible pecan tissue. On leaves, lesions were noted as appearing first on the larger veins or rachises and petioles. Some infection occurred later on leaf tissue between the veins. As they reached maturity, leaves became more resistant to attacks by the fungus. Shuck lesions were described as superficial; extension of such lesions beyond a few cell layers beneath the epidermis was considered as being due to secondary organisms. Only current season twigs were observed to be attacked and then only when young and growing rapidly. Conditions for optimum disease development, as noted by Nolen, were a temperature of about 77° F and abundant rainfall. _C. effusum_ has been obtained in pure culture on laboratory media and studied by Nolen (29) and Demaree (17). Nolen obtained the fungus in culture by the dilution plate method using cornmeal agar as the medium. Demaree[1] was able to obtain the organism in culture only through monoconidial isolates. Nolen concluded that nutrient cornmeal agar, of the several media tried, was best for growth of this fungus. Nolen notes the organism as sporulating freely in culture while Demaree observed only sparse sporulation. Optimum temperature for growth of the organism in culture as determined by Nolen was 68° to 77° F. The same author reported visible growth of the scab fungus in four to nine days and sporulation of the cultures in nine to twenty days. Demaree reported a period of two to three weeks for monosporous cultures to become macroscopically visible. Nolen was unable to obtain cultures of _C. effusum_ during the fall or winter and further observed that conidia available from natural sources during this period did not germinate; conidia obtained during the growing season usually germinated nearly 100 percent. Cole[2] has stated that he has

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[1] Personal communication from J. R. Cole, associate of J. B. Demaree.

[2] Personal communication from J. R. Cole.

been able to obtain cultures through single conidia from natural lesions throughout the year.

The only report of how this fungus gains entry to pecan tissue is one by Waite (35) who states that, " . . . punctures of the plant louse are used as points of entrance by the Fusicladium. The fungus can also enter in the direct way and such diseased spots are irregularly located over the leaves, fruit, and twigs. . ." No experimental evidence is suggested or presented to substantiate these statements.

Pecan scab has been recognized as a disease of considerable economic importance to the pecan industry for nearly half a century. Essentially, control measures consist of orchard sanitation and the application of protectant fungicides (5,6,7,8,9,10,11,12,13,15,18,20,27,28). Cole (10) recommends Bordeaux mixture as still the best fungicide after 30 years of trials. Ziram is recommended by the same author as being comparable to Bordeaux. A standard spray schedule of four applications beginning about the time the leaves are one-fourth to one-half grown does not always give satisfactory disease control, especially during wet years (11).

"Drought" or "spray" injury attributed by earlier workers to Bordeaux mixture sprays was considered by Cole (7) as being due to the method of application which resulted in over-spraying rather than to the material used. The same author (9) reported that Bordeaux gave a greater increase in yield through scab control than did such fungicides as wettable sulfur, the so-called insoluble coppers, Fermate, Dithane, Phygon, Purtized N5-E, Isothane Q-15, compound 341, compound 337, and Omilite.

Dormant or eradicant applications of Bordeaux mixture, Bordeaux mixture plus summer oil, lime-sulfur or dry lime-sulfur have not proven effective in pecan scab control (15,26).

MATERIALS AND METHODS

In order to have a constant and uniform source of inoculum for use in certain phases of this investigation it was deemed necessary to obtain the pecan scab fungus in culture. Collections of diseased twigs, leaves and nuts were obtained in late September and early October, 1949, from several different pecan varieties in various localities in Oklahoma.

Repeated attempts to isolate C. effusum from this material by plating out surface sterilized pieces of lesions proved of no avail. Secondary organisms, predominantly Alternaria spp., were the only fungi obtained. Inasmuch as the scab organism is so slow-growing, it is possible that it was present in a viable state in the tissue cultured but was not noted because of overgrowth by the secondary organisms.

Further trials at obtaining the organism in culture at this time were limited to dilution plates and monoconidial cultures. Dilution plates were not satisfactory, again because of contaminants. Monoconidial cultures failed in every case because the conidia did not germinate.

In January 1950 heavily infected twigs of the pecan varieties Burkett, Halbert and Squirrel's Delight were brought into the laboratory, washed to remove old spores, placed in jars of water and covered with clear plastic bags. Conidia were sparingly produced on lesions of these twigs. Using these conidia, monosporous cultures of the scab fungus were obtained from the varieties Halbert and Squirrel's Delight. In each case the percentage of spores germinating was low.

To determine the medium on which the cultures obtained grew and sporulated best, transfers were made to slants of potato-dextrose agar, cornmeal agar, nutrient cornmeal agar and malt agar. These media were made according to formulae as given by Riker and Riker (32). Although growth occurred on all

these media, conidia were produced sparsely if at all.

Liquid cultures using the cheesecloth "wick" method as described by Nusbaum and Keitt (30) and Keitt and Palmiter (23) were attempted to try to induce sporulation in the pecan scab fungus. Six- or eight-ounce perscription bottles with a cheesecloth wick adherent to one of the inner walls were used. Liquid nutrient media were added to each bottle in 25 ml. amounts. After sterilization of the bottles, the wicks were seeded by smearing with small pieces of the pecan scab culture from cornmeal agar and incubated at about 75° F in the dark or at room temperature in indirect sunlight.

The liquid nutrient media used in the bottles and the composition of each were as follows: potato extract; decoction from 100 gm. cooked potatoes, 10 gm. dextrose, water to make one liter; malt extract; 25 gm. Difco dessicated malt extract, water to make one liter; cornmeal extract; decoction from 20 gm. cooked cornmeal, 20 gm. dextrose, water to make one liter; basal mineral salts medium plus yeast extract; KNO_3 3.12 gm., $KH_2PO_4 \cdot 7H_2O$ 0.5 gm., dextrose 5.0 gm., yeast extract 1 gm., water to make one liter; dry pecan leaf extract; decoction from 200 gm. cooked dried Burkett leaves, 20 gm. dextrose, water to make one liter; green pecan leaf extract; 200 gm. green Squirrel's Delight or Burkett leaves chopped 15 minutes in a Waring blender, juice filtered through cheesecloth, 20 gm. dextrose, water to make one liter.

In order to have pecan tissue for studies on penetration of the host by the pecan scab fungus, two-foot nursery trees of the varieties Burkett and Squirrel's Delight were obtained in early February 1950 to be planted in the greenhouse. It has long been known that pecan trees are difficult to transplant and that root and top development is rather limited the first year after transplenting. Smith (34) reported that ". . . a relatively small quantity of indole-3-butyric acid, when applied to taproots of transplanted pecan trees,

stimulates the processes of initiation and growth of new roots and of top growth." Before planting, each tree was treated by inserting three toothpicks soaked in indole-3-butyric acid at eight-inch intervals along the taproot as suggested by Smith. One tree of each variety was placed out-of-doors; the other trees were kept in the greenhouse where the temperature was maintained at about 70° F. At the end of three weeks the trees from outdoors were removed to the greenhouse. Buds on the trees kept in the greenhouse were bursting March 4, 28 days after planting. The trees left out-of-doors for three weeks did not show signs of growth until March 28.

Since sporulation of C. effusum on artificial media was never sufficient for inoculation studies, it was necessary to resort to lesions occurring in nature for inoculum. A conidial suspension was obtained by washing, in water, lesions from infected leaves. The resulting conidial suspension was standardized to contain approximately 100 conidia per low power field using a microscope with 10X eyepieces and a 10X objective. A camel's hair brush was used for applying inoculum to both upper and lower leaf surfaces. After inoculation, the leaves were bagged with plastic bags for 24 hours. Inoculated leaves were collected at five hour intervals for the first 40 hours; then collections were made at 72 hours, 96 hours and nine days. Samples for clearing were fixed in equal parts acetic acid and 95 percent ethyl alcohol; samples for sectioning were fixed in FAA (formalin 10 ml., glacial acetic acid 5 ml., 95% ethyl alcohol 50 ml., and water 35 ml.).

Peace's (31) cleared-leaf method, with some minor modifications, was used in attempting to observe spore germination and penetration on the leaf surface. Pieces of leaf were successfully cleared in a saturated solution of chloral hydrate for twelve hours followed by a five percent solution of potassium hydroxide for twelve hours. After clearing, the leaf tissue was

heated gently in lactophenol containing a few drops of acid fuchsin until a vapor was given off; the lactophenol was drained and clear lactophenol used for mounting.

It was thought that if the pecan leaf epidermis could be successfully stripped the investigations of fungus penetration would be greatly facilitated. The cleared leaves required at least 24 hours of processing and at best were cloudy and difficult to examine. Early attempts at stripping the epidermis of green pecan leaves by boiling in a 10 percent solution of potassium hydroxide failed. However, after several trials it was discovered that the epidermis could be stripped after boiling the leaves for about ten minutes in a five percent solution of potassium hydroxide. The upper epidermis of leaves three weeks old or older was easily stripped in this manner, but the lower epidermis and that of younger leaves was more difficult to remove.

For investigations of the relation of the scab fungus to host tissue in older lesions, leaves, nut husks and twigs bearing lesions were collected in October 1949 and fixed in FAA. In preliminary trials at dehydrating pecan tissue, alcohol-xylol, N-butyl alcohol and dioxan were used as described by Sass (33). The best method found for infiltrating and embedding mature leaves and husks in paraffin was the ethyl alcohol-xylol method. For young leaves and petioles the N-butyl alcohol method was more rapid and quite satisfactory. Sections of one-year-old pecan stems tended to roll when cut from unembedded stems or stems embedded in paraffin and could not be readily unrolled for staining and mounting. For this reason, it was necessary to embed the stems in celloidin according to the method given by Sass (33).

A combination of safranin-O dissolved in 50 percent ethyl alcohol and fast green dissolved in 95 percent ethyl alcohol as a counter stain proved to be a good differential stain to distinguish diseased and healthy tissue and to show

the fungus tissue in the host. This stain was used in all cases except for a few of the mature stem sections which were stained with fast green in clove oil. The celloidin was removed from the sections by the clove oil thus making it unnecessary to go through the steps of ethyl-alcohol and further dehydration. The cells of the cortical tissue of the pecan stem retained the reddish brown natural stain, while the fungus mycelium stained green.

EXPERIMENTAL RESULTS

Studies of the Fungus

Conidia of the pecan scab fungus, obtained from natural infections brought into the laboratory during the winter, were never observed to germinate in numbers greater than ten percent of the total conidia observed when planted on a nutrient medium such as cornmeal agar. On this medium, germination of conidia from scab lesions obtained during the growing season ranged up to fifty percent.

As observed in these investigations, colonies of the scab fungus from single spores were macroscopically visible in eight to ten days. Growth of the fungus on culture media was very slow; even after one month colonies were still only about 1 cm. in diameter. Monoconidial cultures of this organism were round, with a regular margin and had a nipple-like projection in the center (Fig. 1,2). Only a moderate amount of aerial mycelium was produced and this usually occurred only over the central portion of each colony. Appressed or submerged growth of the colonies was black and the aerial mycelium varied from gray to brown. During the course of this investigation, 16 monoconidial isolates of the fungus were obtained from naturally occurring infections on pecan tissue. Nine isolates were from the pecan variety Halbert, four isolates were from the variety Squirrel's Delight and three isolates were from the variety Texas Prolific. One Halbert isolate and the four Squirrel's Delight cultures were from old twig lesions brought into the laboratory in January 1950; all other isolates were from leaf lesions produced during the growing season of 1950. The only variation noted in the 16 isolates obtained was in the growth rate on cornmeal agar at a temperature of 75° to 80° F. Under these conditions three isolates from Squirrel's Delight produced colonies

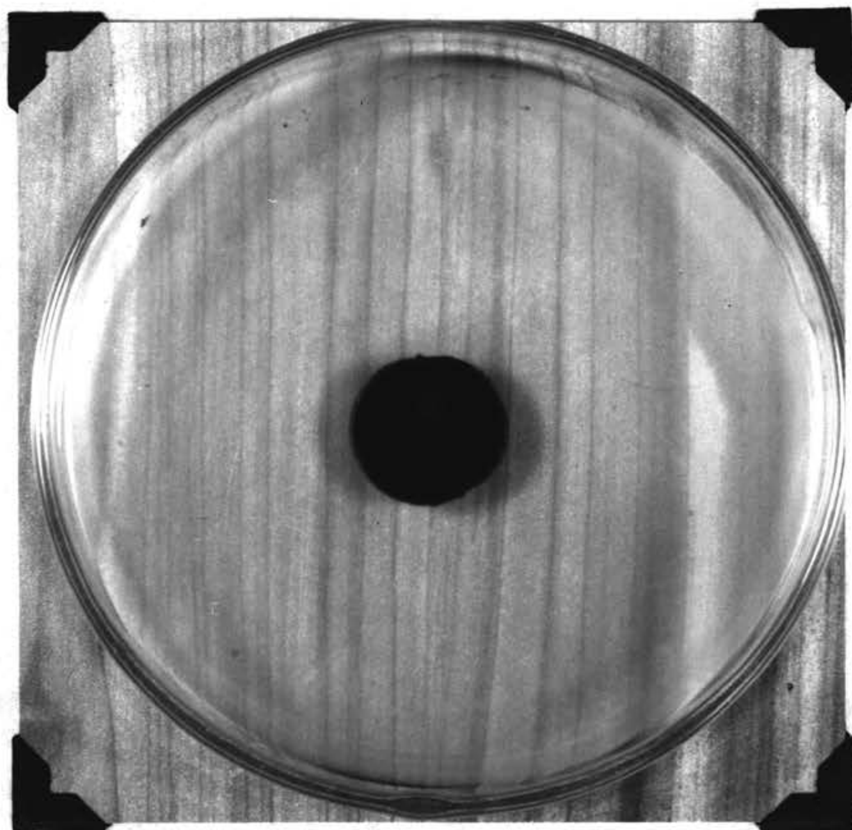

Fig. 1. Colony of C. effusum from Squirrel's Delight pecan, grown on cornmeal agar 28 days at 75° F.

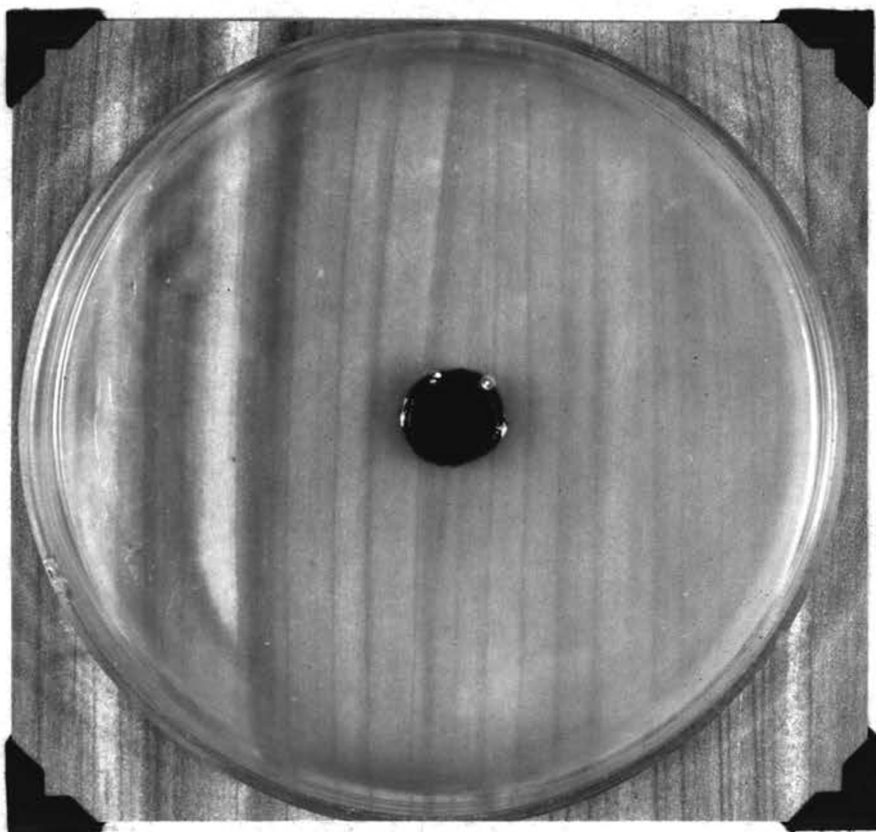

Fig. 2. Colony of C. effusum from Halbert pecan, grown on cornmeal agar 28 days at 75° F.

about one-third larger than any of the other isolates. Decreasing amounts of growth were noted on cultures in the following order: two isolates from Squirrel's Delight and two isolates from Halbert were about equal, six isolates from Halbert, and two isolates from Squirrel's Delight and the original Halbert isolate.

The two cultures selected for attempts to induce sporulation, the original Halbert isolate and one of the Squirrel's Delight isolates, grew about equally well on nutrient cornmeal agar, potato-dextrose agar, and malt agar. On cornmeal agar these cultures grew slightly better so this medium was the choice in all subsequent work involving a solid medium. Only minor variations in growth were noted when these two cultures were grown on cheesecloth wicks in the various liquid nutrient solutions.

Conidia of the cultures tested were produced only in trace quantities on any of the media or under any of the experimental conditions tried. This is in line with results obtained by Demaree (14) but is in contrast to the report by Nolen (29) to the effect that his isolates sporulated freely in culture. Inasmuch as this was to be primarily an investigation of the pathological histology of pecan scab, further attempts to induce sporulation of the organism in culture were reserved for future studies.

Pathological Histology

Through observations it was noted that scab lesions may develop on either the dorsal or ventral surface of the pecan leaf. However, lesions on the dorsal surface of the leaf far outnumber those on the ventral surface. The mature scab lesions were round to oval in shape, those on the veins being elongated parallel with the vein. Scab lesions first appeared as slightly darker areas in the surrounding tissue and became a dense velvety black as the fungus developed. The large number of conidiophores and conidia gave the older lesions a soft gray appearance. Secondary infections, observed after a prolonged rainy period in July, appeared as concentrated areas of small lesions on the leaf surface. The resulting lesions were not necessarily concentrated on the leaf vein as was observed in primary infections. The area covered and location of these secondary lesions indicated that they had resulted from conidia washed from primary lesions onto healthy tissue.

Examination of cleared samples from inoculated areas of Burkett and Squirrel's Delight leaves in the greenhouse showed that an extremely low percentage of the spores germinated on the leaves. This difficulty was considered at least partially due to the fact that the greenhouse temperature could not be carefully regulated as the glass in the greenhouse had been destroyed in a hail storm April 2. Field observations of natural infection during the spring and summer of 1950 tended to parallel the observations in the greenhouse and indicated that temperature and moisture were important factors in the development of scab infection. Spores that did germinate on leaves in the greenhouse produced either one or both of two types of germ tubes. One type of germ tube was short and heavy, often divided by one or more septa, and apparently had an appressorium at the tip. The other type of germ tube was long and slender with no septa and no appressorium (Fig. 3,4,5). Several instances

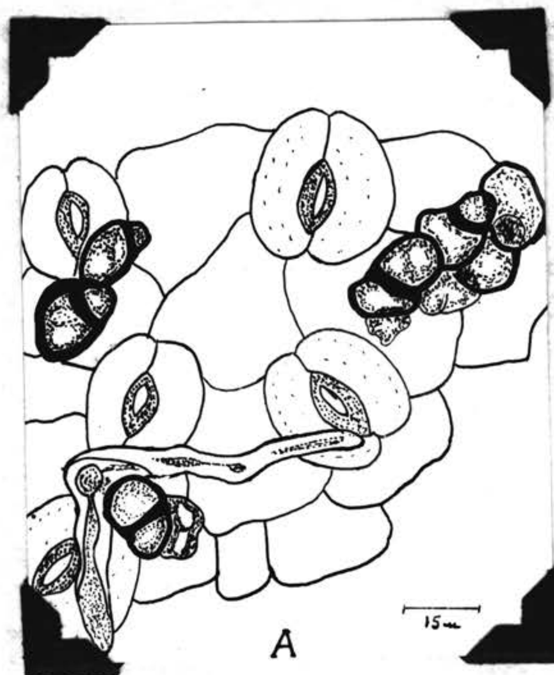

Fig. 3. Lower epidermis of Burkett leaf 9 days after inoculation. Note pads of heavy-walled mycelial cells with shrunken spores attached.

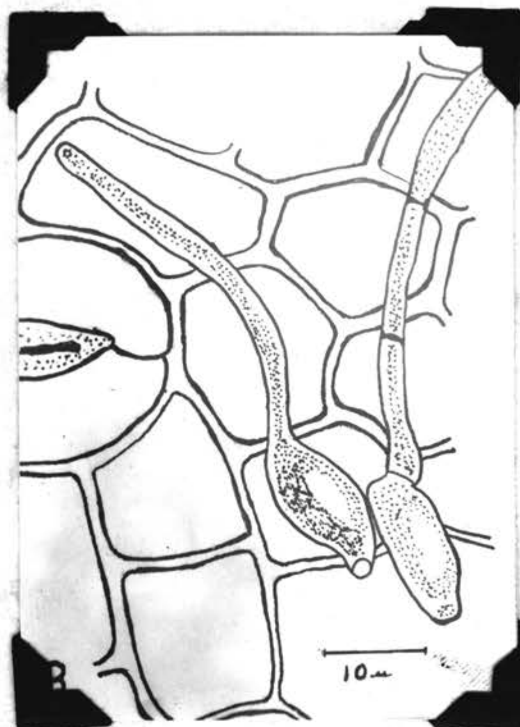

Fig. 4. Germinating spores of C. effusum on lower epidermis of Squirrel's Delight leaf 24 hours after inoculation.

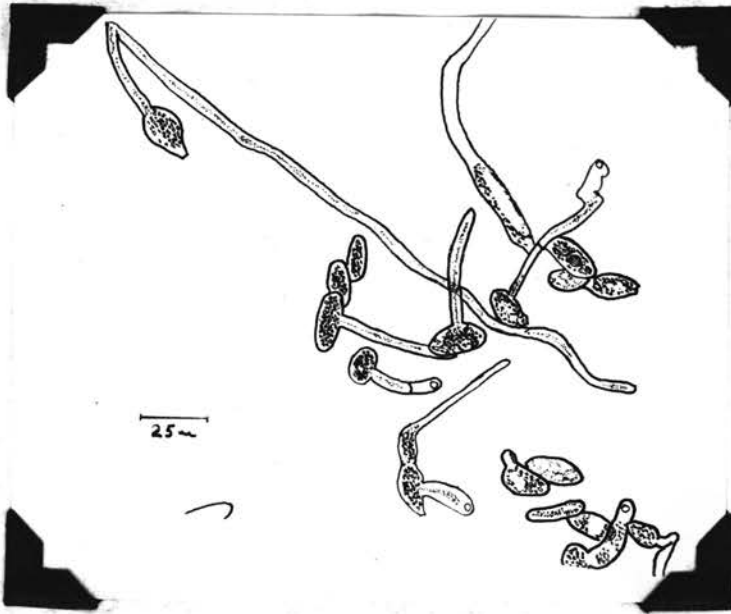

Fig. 5. Germinating spores of C. effusum from Burkett pecan after 36 hours incubation in distilled water at room temperature. Note two types of germ tubes.

were observed where both types of germ tubes had grown across the apertures of stomata, but in no case were germ tubes seen to enter the host through stomata. This suggests that stomata are probably not important in the phenomenon of penetration by C. effusum.

To obtain information on the length of time required for conidia to germinate and to have some idea of what might be expected when leaves were inoculated, spore germination was studied in hanging drop preparations and in free drops of water on slides. For this purpose the spore suspension obtained from naturally occurring lesions and used to inoculate Burkett and Squirrel's Delight leaves was used. Under these conditions approximately 30 to 40 percent of the spores germinated in 48 hours; a few had developed a rupture in the spore wall, preparatory to germination, after two hours. The germinating spores produced from one to three germ tubes each. Germ tubes were produced from either end or side of the spore. Both the long slender germ tube and the short heavy germ tube with what appeared to be an appressorium were observed (Fig. 5). Many of the ungerminated spores and the germinated spores attached themselves to the slides as demonstrated by the fact that it was not possible to detach them in running water.

Actual penetration of pecan tissue by the scab fungus was observed in only one questionable instance. In this instance what was interpreted as a slender infection hypha was seen penetrating the cuticle in a cross section of a leaf; the infection hypha appeared to originate from an appressorium. Repeated observations on cleared leaves, stripped epidermis, and stained leaf cross sections failed to give conclusive information on the mode of penetration. Leaves used in the observations on penetration were collected from both the field and from inoculated trees in the greenhouse.

As observed in this investigation the epidermis of the dorsal and ventral

leaf surfaces is quite different in character. The dorsal epidermal cells are somewhat smaller than the ventral epidermal cells. There are in the dorsal epidermis a large number of stomata, approximately one for every three epidermal cells on the mature leaf (Fig. 3). The ventral epidermis appears to be entirely free of stomata. Well developed stomata were observed in the dorsal epidermis of the tooth-like serations of young leaves before they had unfolded from the bud. The epidermis of young leaves was difficult to examine because of the great number of close-set epidermal hairs. These hairs were of two types: bulb-like glandular hairs which overlapped and completely covered the tissue of the leaf blade and peltate hairs which occurred almost exclusively on the veins. With respect to type and distribution of these hairs the epidermis of the upper and lower leaf surfaces was similar. The glandular hairs secrete a clear fluid which makes the young leaf sticky to the touch. At times this fluid collects and gives the appearance of honey dew produced by aphids. As the pecan leaf matures both types of hairs appear to disintegrate (Fig.6) until the mature leaf is practically smooth and glossy. There were no apparent differences in the number and distribution of stomata, prevelance of epidermal hairs, or thickness of cuticle in the varieties Halbert, Burkett, Squirrel's Delight, Stuart and Texas Prolific.

Examination of cleared leaves and cross sections of leaves taken at intervals during the early development of the scab fungus revealed that approximately nine days following inoculation, "pads" of fungus tissue consisting of two to many heavy-walled mycelial cells were produced on the epidermis and beneath the cuticle (Fig. 5,7). That these pads were subcuticular in position was clearly shown in cross sections of inoculated pecan leaves. These pads somewhat resemble the fungal stromata or pads described by Nusbaum and Keitt (30) formed by <u>Venturia inaequalis</u> in a subcuticular position beginning 36

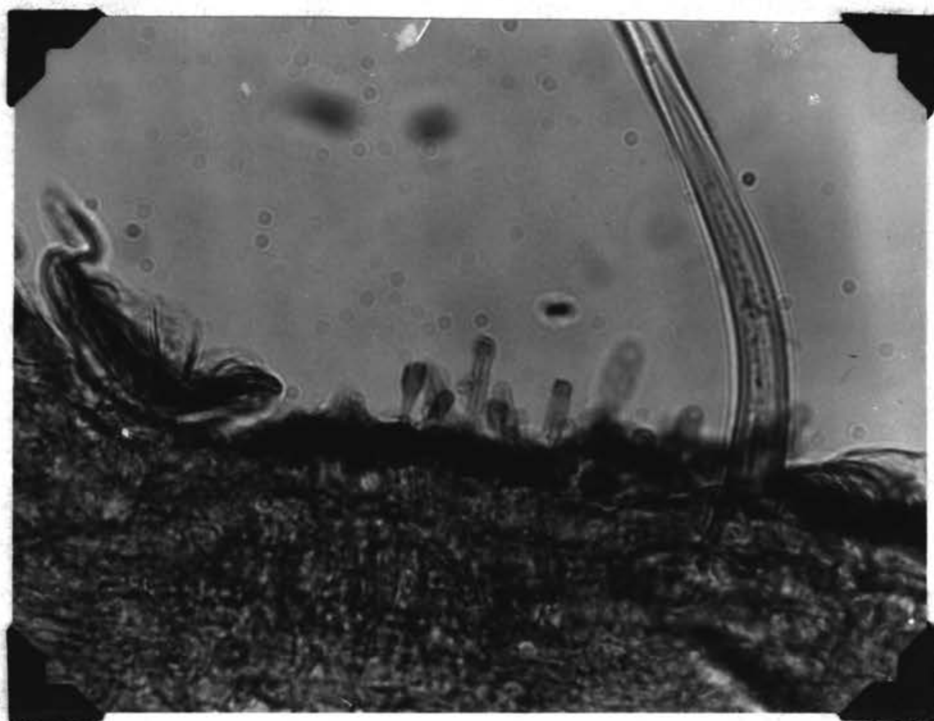

Fig. 6. Cross section of infected young Burkett leaf showing collapsed glandular hair left, and filamentous epidermal hair right. Conidiophores of C. effusum shown on the black fungus tissue center. (X 112)

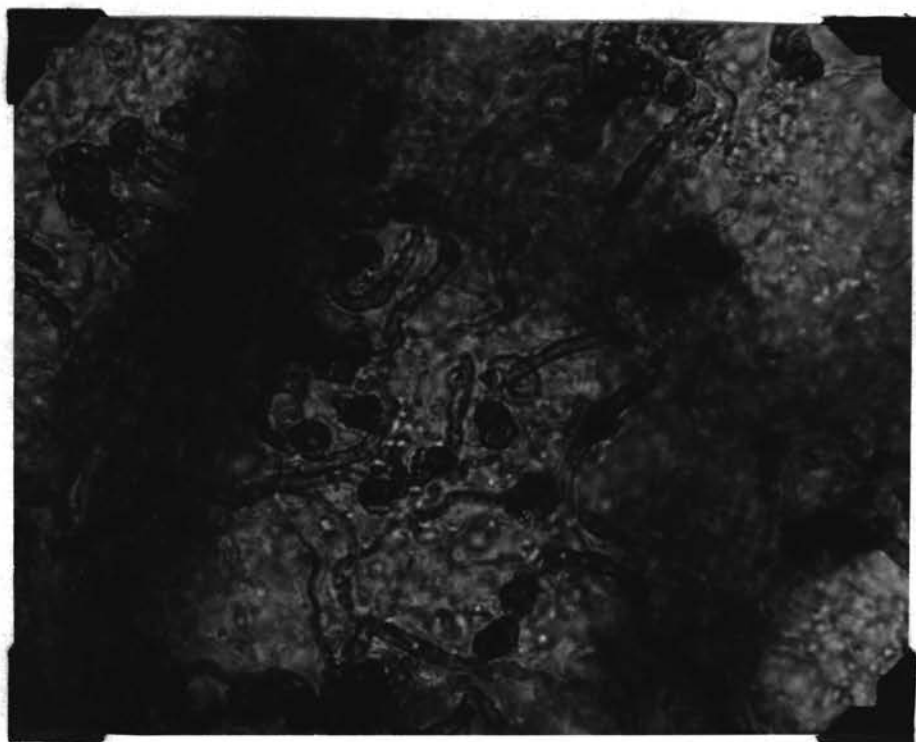

Fig. 7. Cleared Burkett leaf showing early development of C. effusum on upper epidermis. Pad-like fungus tissue with germ tubes attached; 9 days after inoculation. (X 168)

hours after inoculation of apple leaves. Further development of the pecan scab fungus resulted in a dense mass of the heavy-walled cells which form the black lesion observed on the pecan leaf.

Naturally infected leaves from Burkett and Squirrel's Delight trees with infections approximately ten days old were collected and cleared to observe the development of the pecan scab fungus. The leaves bore many secondary lesions that were barely visible macroscopically. These new lesions consisted of many pads or small groups of the dark fungus cells mentioned previously. Around the periphery of each lesion the pads were somewhat scattered and there was no apparent connection between pads or between pads and the main lesion. The larger pads of cells appeared to produce hyphae that grew over the subcuticular surface of the leaf (Fig. 7). A shrunken light brown mass resembling a collapsed spore was attached to many of the pads. Older scab lesions consisted of a solid mat of these fungus pads.

Cross sections of pecan scab lesions on leaves showed the fungus mycelium developed extensively between the cuticle and epidermis (Fig. 8, 10, 11). At the edge of the lesion the epidermal cells appear to be normal, but as the center of the lesion is approached the epidermal cells are killed and progressively collapsed until their identity is lost (Fig. 9,10,12). Conidiophores develop from a stroma-like layer composed of heavy-walled fungus tissue on the surface of the scab lesion (Fig. 6). In approximately 250 sections of scab lesions on pecan leaves intercellular fungus mycelium was observed, but haustoria or intracellular mycelium were never observed. Epidermal cells at the edge of the expanding lesion appeared to be normal. As the center of the scab lesion was approached the epidermal cells were dead and collapsed and diseased tissue was observed deeper and deeper in the leaf tissue (Fig. 8). The protoplasm of the mesophyll and palisade cells, in the infected area,

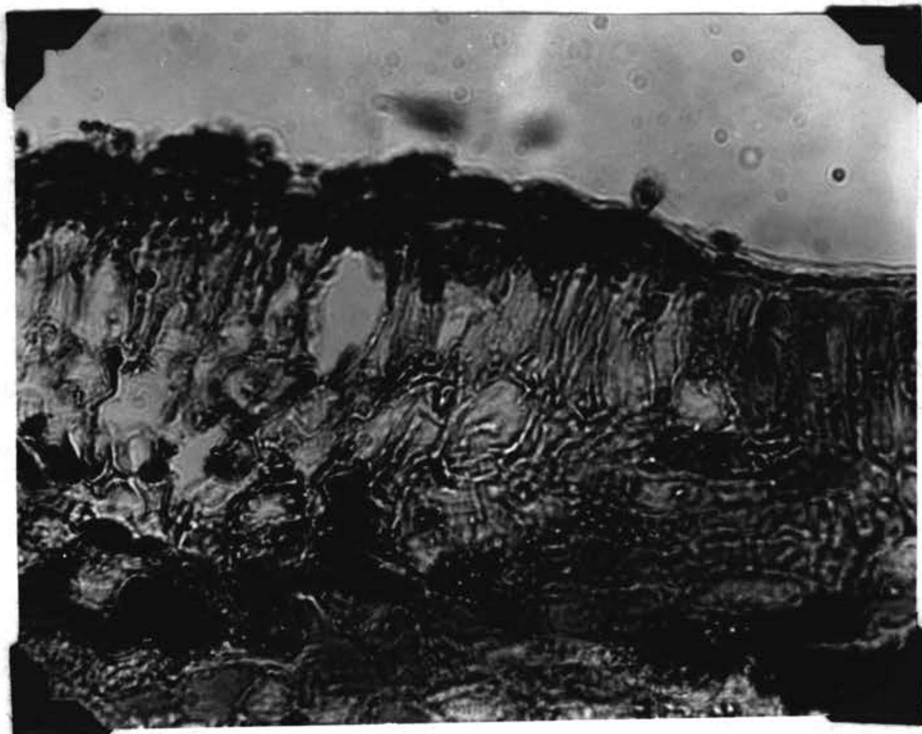

Fig. 8. Cross section of old scab lesion on mature Squirrel's Delight leaf. Compare collapsed palisade and mesophyll tissue upper left with healthy mesophyll lower right; cuticle extending over dark colored fungus, top center. (X 168)

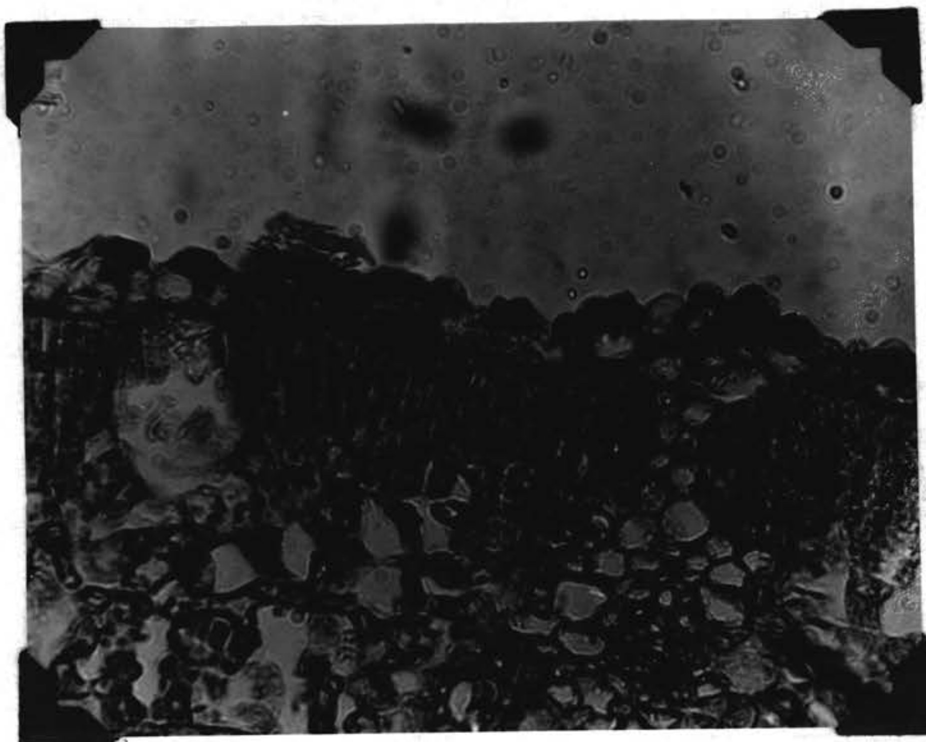

Fig. 9. Cross section of infected young Squirrel's Delight leaf 14 days after inoculation. Dark fungus tissue on surface of collapsing upper epidermis. (X 168)

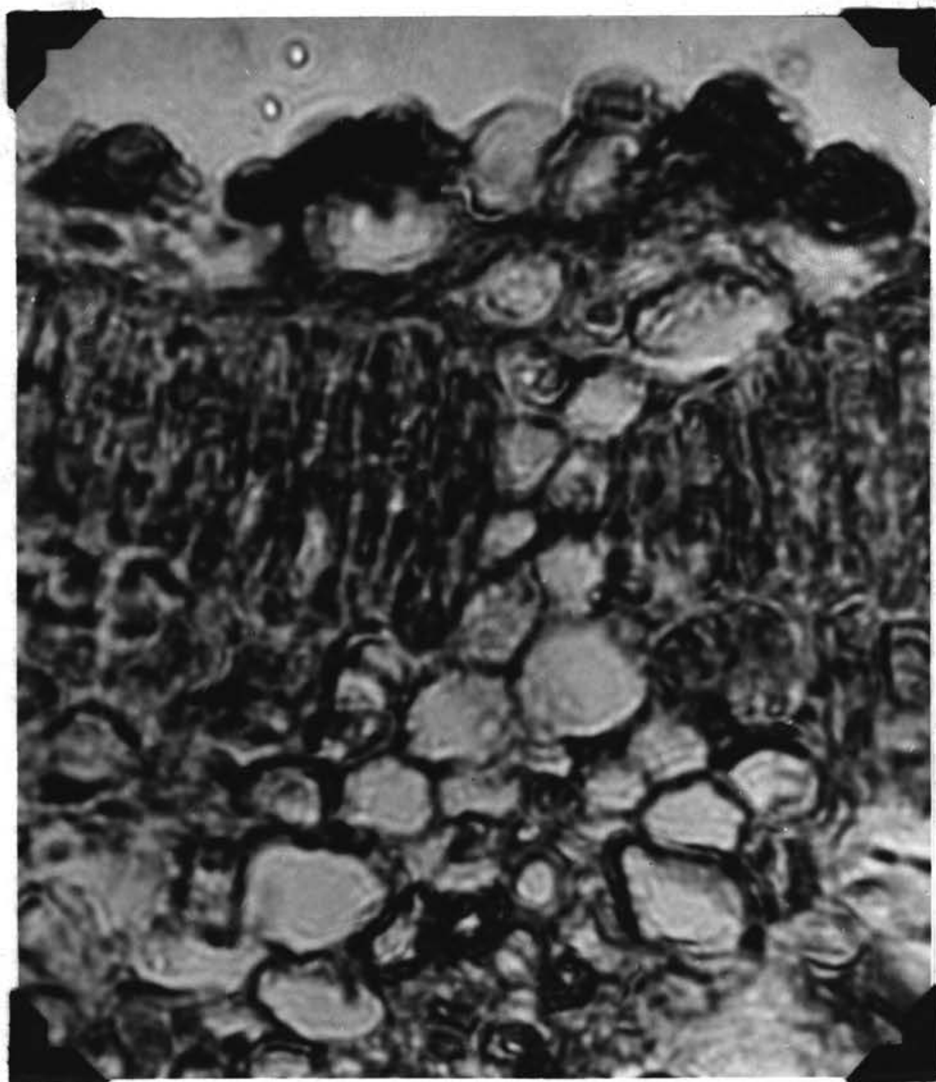

Fig. 10. Cross section of young Squirrel's Delight leaf, showing fungus tissue on collapsing upper epidermis. Note vascular bundle sheath extending to upper epidermis of the leaf. (X 350)

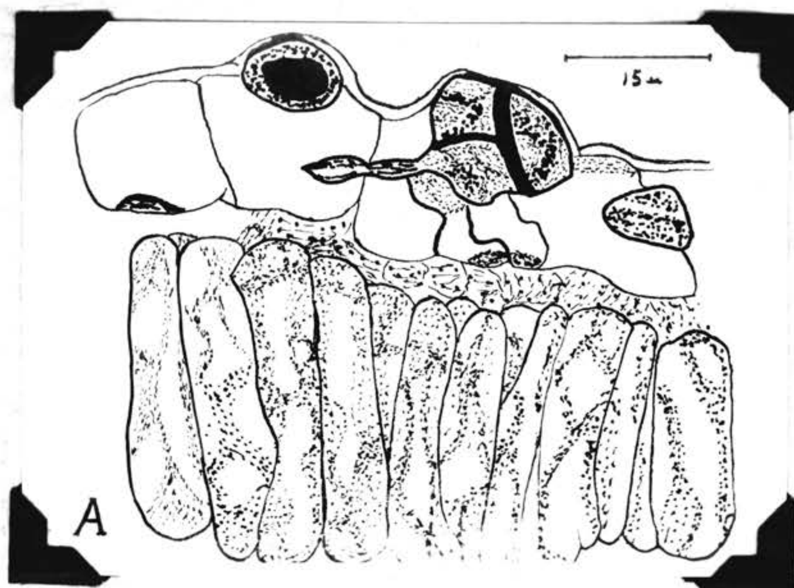

Fig. 11. Drawing showing pecan scab fungus developing between cuticle and upper epidermis of Burkett leaf; 9 days after inoculation.

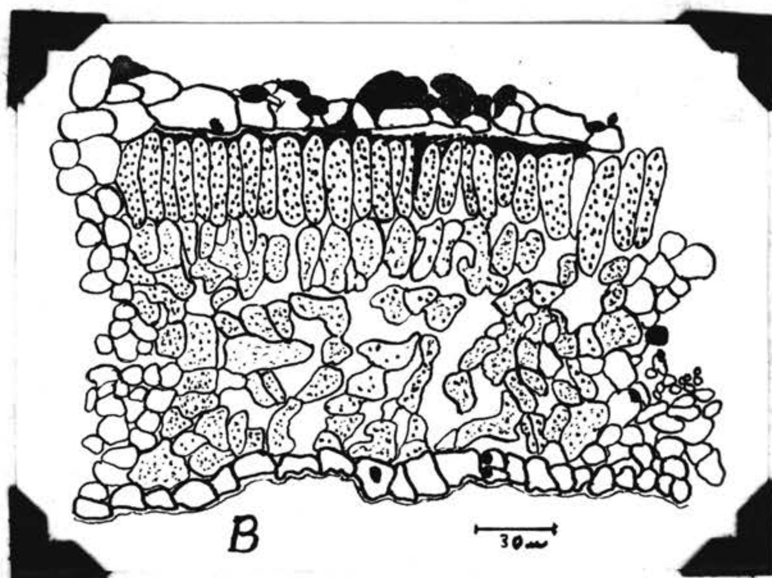

Fig. 12. Drawing of pecan scab fungus developing in intercellular spaces between upper epidermis and palisade cells of Burkett leaf. Cross section through young natural infection approximately ten days after inoculation.

appeared to become granular and disorganized. In the older portion of the lesion these cells were shrunken and finally disintegrated. In the older lesions the diseased area extended through the thickness of the leaf. A necrotic spot appeared on the leaf surface opposite the scab lesion and expanded until it was approximately the same size as the original lesion. Sporulation was never observed on the lesion opposite the site of original infection.

An examination of approximately 200 transverse sections and 100 longitudinal sections, selected from the many sections cut from husks of Burkett, Halbert and Squirrel's Delight pecan varieties collected in October, revealed no evidence that the pecan scab fungus penetrated deeper into the tissue than the vascular bundles. At first fungus mycelium apparently developed for some distance in the intercellular spaces of the subepidermal parenchyma; tissue was killed and disorganized to a progressively greater depth as the center of the lesion was approached (Fig. 13,14). The overall appearance in cross section of the diseased area is that of a cone or V with the vascular bundle at the apex. Infected tissue was not visible at a greater depth than the vascular bundle of the husk. Near the center of the lesion five to seven layers of parenchyma tissue were disorganized and shrunken beyond recognition and the intercellular spaces were filled with brick-shaped fungus cells (Fig. 13,14). The scab fungus developed below the epidermis of the nut husk forming a stromatal mass which ruptured the epidermis and produced conidiophores. Intracellular development of the scab fungus in the nut husk was not observed during this investigation.

Macroscopically the pecan scab lesion on the young stem was somewhat elongated and black. Under the microscope the stroma was several cells deep and composed of heavy-walled segments of entwined fungus hyphae. Each stroma was surrounded by a circular depression in the host tissue. In cross and

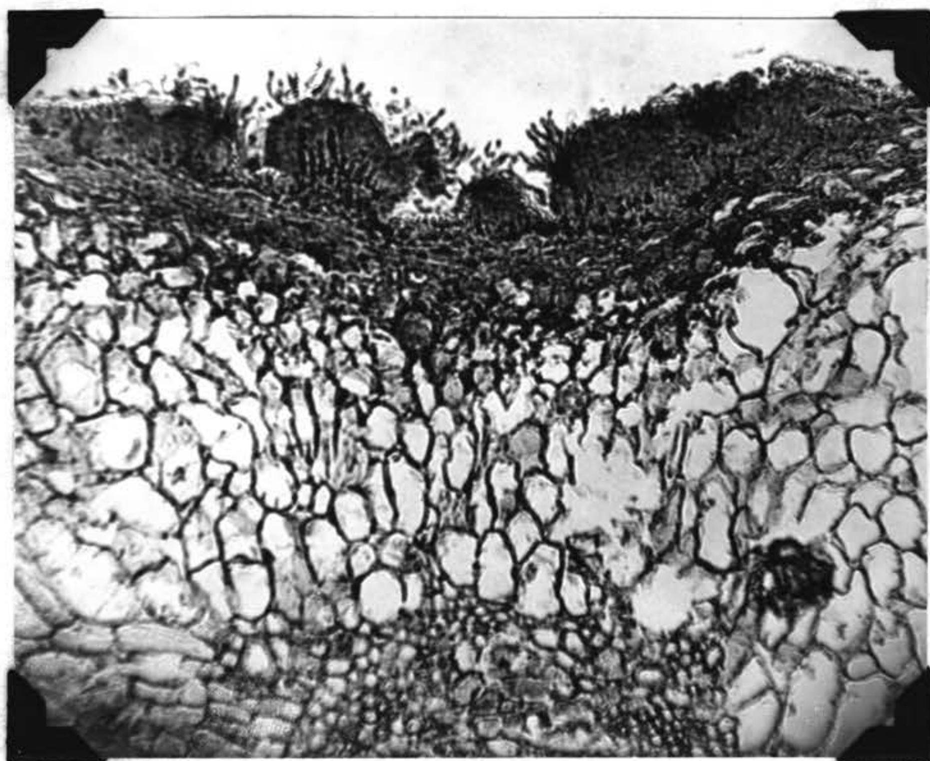

Fig. 13. Cross section of infected mature Squirrel's Delight nut husk. Dead parenchyma tissue in cone shaped center section terminating in the vascular bundle shown at bottom center; healthy host parenchyma, lower right and lower left; fungus stroma with conidiophores shown at top. (X 168)

Fig. 14. Enlargement of portion of Fig. 13, showing infected mature husk of Squirrel's Delight. Stroma and conidiophores top; collapsed parenchyma beneath stroma; region of incipient collapse of host parenchyma, center to left. (X 350)

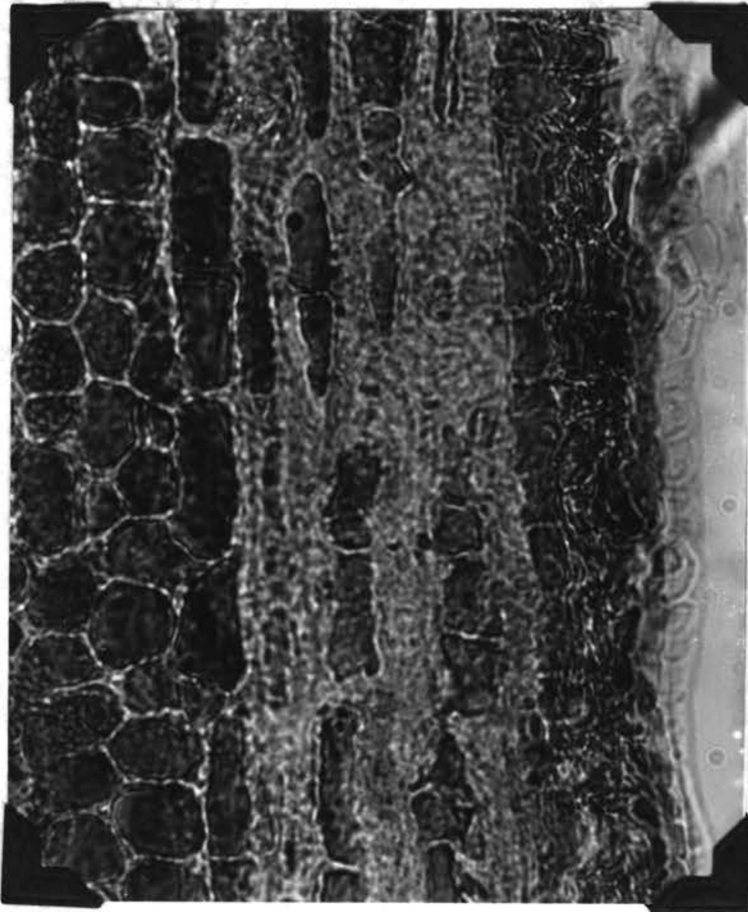

Fig. 15. Longitudinal section of infected one-year-old Burkett stem showing collapsed epidermis and cork area at right; disorganized cortical tissue center, with intercellular spaces filled with fungus mycelium. (X 168)

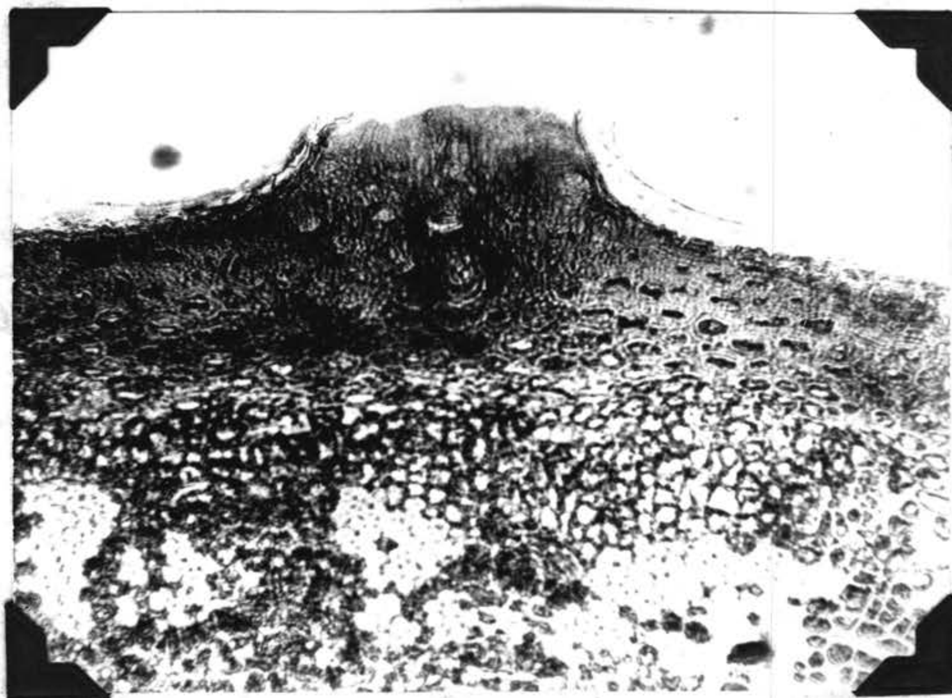

Fig. 16. Cross section of infected Burkett stem. Stroma bursting through epidermis upper center; collapsed host cortex cells right and left of stroma; intercellular spaces filled with fungus mycelium. (X 168)

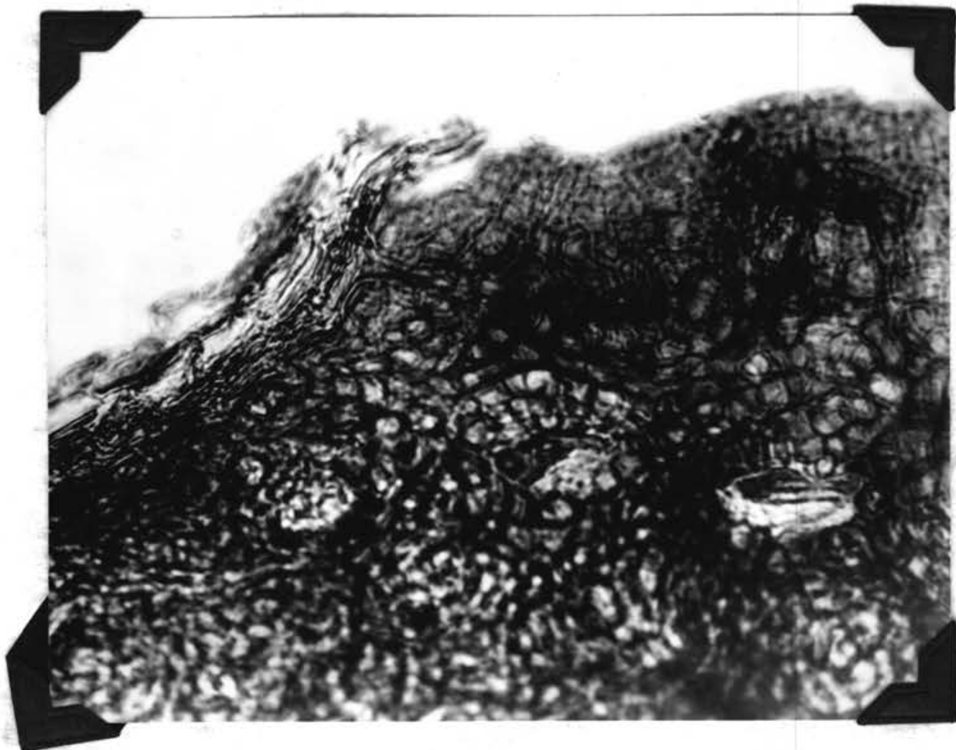

Fig. 17. Enlargement of a portion of Fig. 16, showing upper left portion of C. effusum stroma. Note broken epidermis, top center, and three collapsed host cells, lighter areas, in line across lower part of picture, surrounded by fungus mycelium. (X 350)

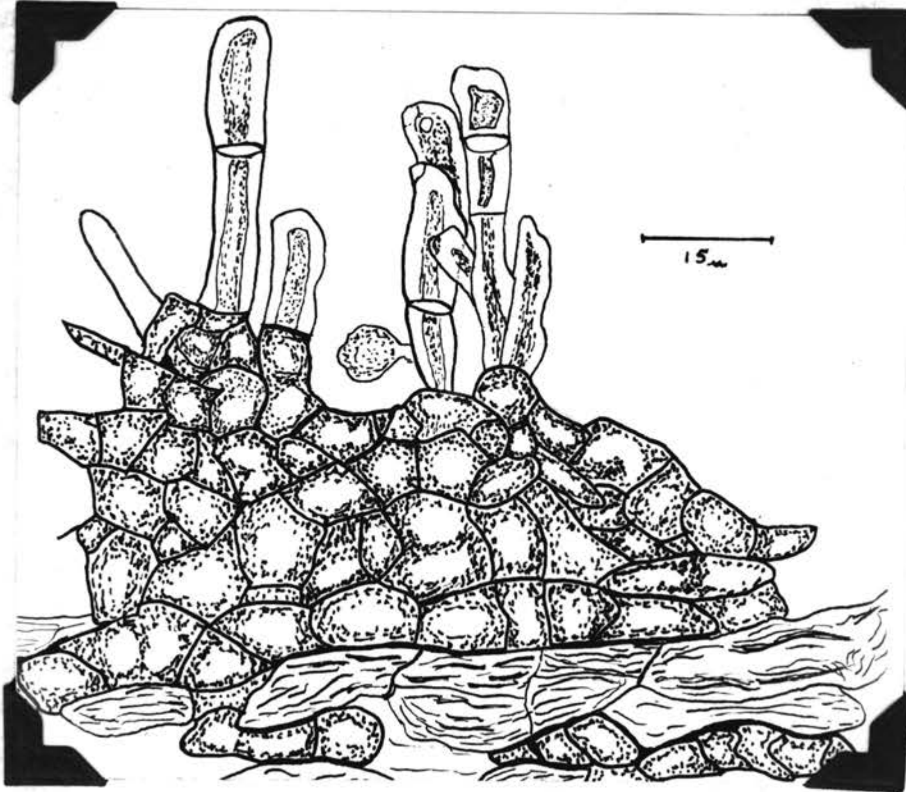

Fig. 18. Drawing of pecan scab lesion on Burkett twig. Note the development of conidiophores on the stromatal mass, upper, and the growth of fungus in the intercellular spaces of the host, lower.

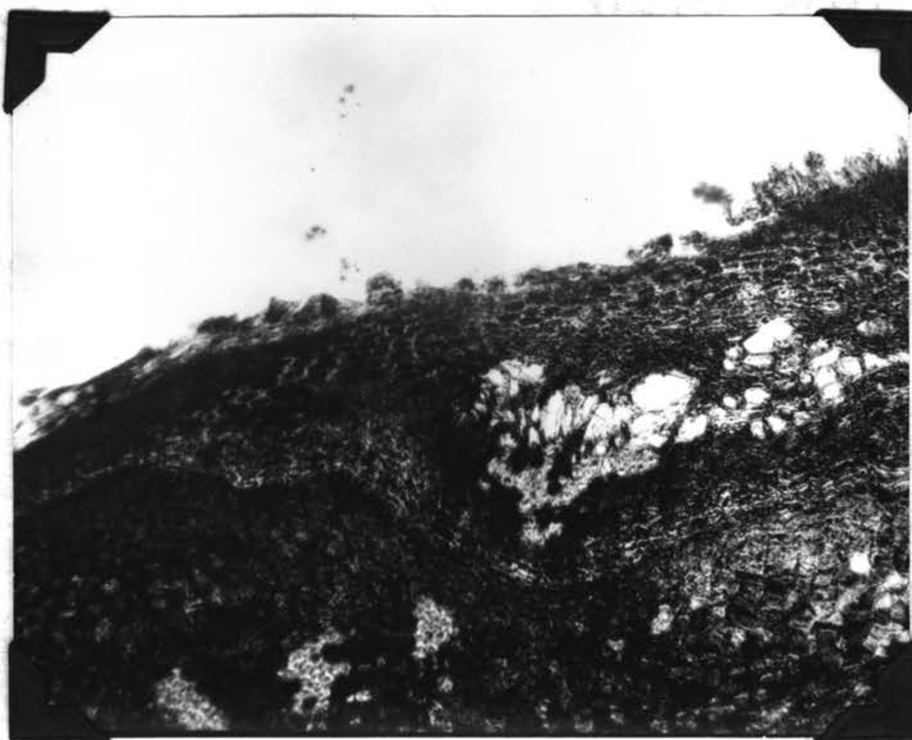

Fig. 19. Cross section of scab lesion on Burkett twig; showing layer of cork between diseased area, top, and healthy cortex at the bottom; compare disrupted bundle of cortical fibers separated by cork from normal bundles of cortical fibers, lighter areas, bottom left center. (X 168)

longitudinal section, a twig lesion was observed to consist of a mass of fungus hyphae developed in the intercellular spaces of the sub-epidermal region beneath the sunken portion of the lesion (Fig. 15,16). The epidermis remained intact over the lesion except where the stroma broke through, but the cells appeared shrunken and dead (Fig. 17,18). The cells of the subepidermal layer and cortex appeared to disintegrate gradually and were observed as small irregular dark colored islands among the fungus mycelium.

Scab lesions on mature pecan twigs collected in the fall were totally or partially isolated from healthy tissue by the formation of cork tissue. The fungus mycelium appeared to develop between the natural layer of cork and the epidermis at the outer periphery of the scab lesion. A cork cambium was initiated further and further toward the center of the twig forming a cup-like under boundary of the diseased tissue. Cell division in the cork cambium continued for some time forming a layer of cork several cells thick and forcing the bundles of cortical fibers out of their normal position. As a consequence, bundles of fibers in the diseased area appeared to be much nearer the epidermis than they were in the healthy tissue (Fig. 19). In immature stems a cork cambium may be formed only part way beneath the diseased tissue or may be absent all together, thus allowing the fungus to extend to a greater depth where the cork is not formed. In such instances the visibly diseased area often extended to the cambium layer of the twig. However, in examination of 50 serial cross sections and 25 serial longitudinal sections of Burkett and Halbert varieties, hyphae were not observed deeper than approximately mid-way through the cortex of the stem and they were entirely intercellular. The diseased tissue appears to develop in advance of the growth of the mycelium.

DISCUSSION

At the time this investigation was initiated difficulty in isolation and culture of _C. effusum_ was not anticipated. Because of the difficulties encountered it was necessary to spend more time on this phase of the problem than was expected. Pure cultures of the fungus and an abundant supply of spores for inoculum were considered necessary for an adequate study of the phenomenon of host penetration and of further stages in the development of host-parasite relations. Reasonably satisfactory methods of obtaining and handling _C. effusum_ in culture were discovered, but the cultures obtained could not be induced to sporulate under the various experimental conditions used. Since satisfactory methods of handling this fungus in culture so as to induce sporulation were not discovered, it would seem that an investigation of the physiology of the scab fungus would be fundamental to further attempts at elucidating host-parasite relations in this disease.

As has already been pointed out, there is presumptive evidence for the existence of physiologic specialization in the pecan scab fungus and but little experimental evidence on this point. In the present studies, observations on cultures of _C.effusum_ grown in the laboratory on cornmeal agar have revealed a difference in the size of fungus colonies as obtained from the varieties Squirrel's Delight and Halbert. While such differences in themselves would not be sufficient for distinguishing physiologic races in the fungus, they may be of value in supplementing possible pathogenicity differences, if such are revealed, in future investigations. Under the conditions of the present investigation there were no apparent differences in the reaction of the pecan varieties Burkett, Halbert or Squirrel's Delight to the pecan scab fungus.

Although considerable time and effort were spent in an attempt at determining the mode of penetration of pecan leaves by the pecan scab fungus,

conclusive evidence on this point was not obtained. From the evidence at hand, it appears that penetration is direct. Many observations on inoculated, cleared leaves and epidermis and cross sections through inoculated leaves failed to reveal a single instance where the fungus had entered the leaf by way of a stoma. What was interpreted as evidence for direct penetration was found in only one leaf cross section. Here a single infection hypha was observed penetrating the cuticle; detailed examination of many more sections similarly prepared failed to give further evidence on this point. Additional negative evidence for direct penetration may be adduced from the fact that infection may occur through either leaf surface. As seen in the present studies stomata were found on the lower and never on the upper leaf surface. Both direct and indirect penetration of host tissue have been reported with other _Cladosporium_ incited diseases. Bensaude and Keitt (4) reported that _C. carpophilum_, the causal organism of peach scab, developed appressoria and penetrated the host directly. Guba (22) found that _C. fulvum_, the causal organism of tomato leaf mold, enters the tomato leaf through the stomata.

In the early phases of disease development, _C. effusum_ on pecan leaves appears to behave similarly to the parasitic phase of _Venturia inaequalis_ on apple leaves. While the apple scab organism remains in a subcuticular position throughout the parasitic phase (36), the pecan scab organism apparently soon invades the deeper tissues of the pecan leaf. This is indicated both from the studies of leaf sections and observations that lesions initiated on the upper leaf surface might soon produce necrosis on the lower leaf surface.

Because of limited time and failure to obtain sufficient inoculum from the fungus cultures, penetration and early disease development on pecan twigs and husks were not studied. In older twig and shuck lesions hyphae were observed to a depth of ten to twelve cell layers. Observations at progressive

stages in the development of hyphae of _C. effusum_ indicated that the hyphae observed at all depths in the tissue were those of the pecan scab organism. However, as has already been noted, it was possible to isolate organisms other than the scab fungus from surface sterilized leaf, twig, and shuck lesions. Demaree (17) indicated that _C. effusum_ was superficial in pecan tissue and extended only slightly below the epidermis. While the findings in the present investigation are not conclusive, there is evidence that the fungus does not penetrate twig tissue beyond the cambium layer. This knowledge may have practical application in indicating the possible effectiveness of eradicant sprays. Further detailed work would be necessary to establish this point.

As observed in the present studies the mycelium of the pecan scab fungus in pecan host tissue is intercellular; haustoria or intracellular hyphae were never observed. Pecan tissue was killed or damaged in advance of the development of the fungus mycelium, thus indicating the production of a diffusable toxin by the fungus. The protoplasm of host cells in advance of actual invasion by the fungus reacted by becoming granular and disorganized. The cells appeared to become gradually shrunken and separated from the surrounding tissue as the mycelium continued to develop. In each case the infected area in the tissue was clearly differentiated from the surrounding normal tissue. An apparent affinity of the pecan scab fungus for vascular tissue was noted on husks and leaves. Lesions observed on husks had developed directly above and extended toward a vascular bundle; naturally produced leaf lesions were located predominantly on the leaf veins.

While this investigation has necessarily been of a preliminary nature and devoted primarily to the development of techniques, it does provide a basis for further research directed toward a better understanding of host-parasite relations and a more complete life history of the causal organism.

Such knowledge should contribute to an understanding of some of the problems involved in controlling pecan scab.

SUMMARY

Cladosporium effusum, the incitant of pecan scab, was obtained in pure culture from pecan tissue only through single-spore isolations. The scab fungus was not induced to sporulate on the various laboratory media tried.

Pecan leaf, petiole and husk tissues were sectioned most satisfactorily when embedded in paraffin. It was necessary to embed stems in colloidin for sectioning. Pecan leaf epidermis was most readily obtained after boiling the leaves in potassium hydroxide.

Leaves of Squirrel's Delight, Halbert, Burkett, Stuart, and Texas Prolific pecans were demonstrated to have numerous stomata in the lower epidermis and none in the upper epidermis. Evidence is presented that penetration by the pecan scab fungus on leaves is direct.

Squirrel's Delight, Burkett, and Halbert varieties of pecans responded to twig infections by producing a layer of cork cells immediately beneath affected tissues. Such a response was not observed in leaf and husk infections.

As observed in various types of pecan tissue, mycelium of the scab fungus was intercellular. Death of host tissue was in advance of the invading fungus hyphae, thus indicating possible toxin production by the fungus.

The pecan scab organism overwintered in the form of stromata on pecan twigs; these stromata were capable of producing conidia at any time during late winter when temperature and moisture conditions were favorable.

The influence of temperature and moisture on infection by *C. effusum* was indicated by the lack of infection in the field or on inoculated trees during cool, hot, or dry periods in early summer.

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STUDIES OF THE PATHOGEN, CLADOSPORUM EFFUSUM (WINT.) DEMAREE, AND HOST PARASITE RELATIONS IN PECAN SCAB

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