

PROCESSES IN THE INFECTION OF BARLEY LEAVES BY *RHYNCHOSPORIUM SECALIS*

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[Manuscript received October 16, 1969]

Summary

Conidia of *R. secalis* (Oud.) Davis germinated on barley leaves to produce short germ tubes and appressoria. Hyphae below the appressoria penetrated the cuticle and formed extensive mycelial mats between the cuticle and the outer epidermal cell walls. Epidermal cell walls beneath the subcuticular hyphae became swollen, lamellate, and collapsed so that the inner and outer walls of the epidermis came together. Mesophyll cells, beneath the subcuticular hyphae, collapsed and died before being reached or entered by hyphae which penetrated between epidermal cells and between the ends of guard cells and adjacent epidermal cells. Only areas of dead and dying cells were colonized by the hyphae.

Penetration of the cuticle, and collapse of the epidermal cell walls and mesophyll cells, appeared to be due to the activity of materials excreted by the fungus.

Subcuticular stromata formed below inoculated surfaces. Hyphae grew from these areas, through the mesophyll, to the opposite side of the leaf where they formed stromata in substomatal cavities. Conidia, which were formed on both types of stromata, protruded through the cuticle above subcuticular stromata and were extruded through stomatal pores from substomatal stromata.

I. INTRODUCTION

Leaf scald of barley, caused by the fungus *Rhynchosporium secalis* (Oud.) Davis, occurs in many barley-growing areas throughout the world. Its incidence and economic importance appear to be increasing, particularly under conditions of low temperature and high rainfall (Skoropad 1959, 1963).

The existence of physiological races of the pathogen specialized to particular barley cultivars has been amply demonstrated (Sarasola and Campi 1947; Riddle and Briggs 1950; Schein 1957; Kajiwarra and Iwata 1963; Owen 1963). It is of considerable importance to plant breeders and plant pathologists to understand the basis of resistance in resistant barley varieties. However, very little is known about this aspect of the host-parasite interaction and the reports of the manner in which the fungus enters its host, grows within the host, and sporulates are few and, in important respects, contradictory. Bartels (1928) and Mackie (1929) reported that germ tubes entered stomatal pores and Caldwell (1937) stated that the fungus entered the host by direct penetration of the cuticle and epidermis. Davis (1922) reported

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that conidia were produced on hyphae protruding from stomata, whilst Caldwell (1937) claimed that conidia were produced only on subcuticular stomata developed at points of infection.

A thorough knowledge of the mode of penetration and further development in host tissue is essential in investigations of resistance mechanisms. Since no such detailed investigation of *R. secalis* on barley has been reported since Caldwell's work in 1937 a re-examination of these processes was undertaken. This paper describes the germination of *R. secalis* conidia, and penetration and post-penetration stages of the fungus in barley leaves.

II. MATERIALS AND METHODS

Clipper (AC 2200), a barley variety susceptible to *R. secalis*, was used throughout the investigations.

Isolates of *R. secalis* used and their South Australian sources were: SA-1, collected from Two Wells; SA-5, collected from Aldinga; SA-13, collected from Ashbourne. Single-spore cultures were maintained on PSP agar (22 g Deb dehydrated potato, 10 g sucrose, 10 g peptone, 10 g agar in 1 litre of water). Inoculum was obtained from 14-day-old cultures grown on PSP agar at 15°C in the dark.

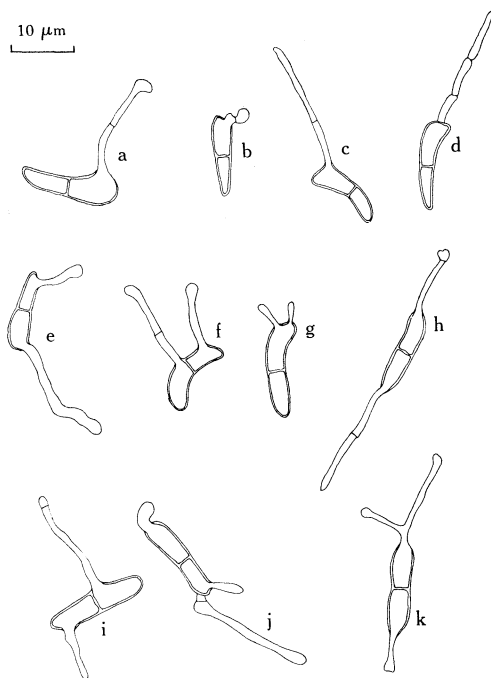


Fig. 1.—Germinating conidia after incubation for 24 hr in liquid water on leaf at 15°C.

Spore germination, hyphal penetration, and subsequent development within barley leaves were examined on both surfaces of the leaf using detached leaf pieces and intact leaves on potted plants. Leaf pieces (0.5 cm²) were placed on drops of spore suspension contained in 4-cm diameter Petri dishes. These were then placed in larger dishes with water at the bottom to maintain saturated conditions. They were incubated at 15°C in the dark. Marked areas on leaves of potted plants were gently brought into contact with disks cut from fresh cultures so that some conidia adhered

to the leaves. The plants were then placed in a humid chamber at 15°C and illuminated for 12 hr a day at 1000 f.c. At the end of the incubation period, the inoculated leaf portions were cleared in a mixture of equal parts (v/v) of glacial acetic acid and absolute alcohol, stained with periodic acid-Schiff reagent (Preece 1959), and mounted in dilute glycerine or stored in 70% alcohol. Transverse sections 25 μ m thick were cut with the freezing microtome from stained leaf pieces.

Other leaf pieces were fixed in formalin-acetic acid-alcohol, embedded in wax, sectioned (Johansen 1940), and stained with periodic acid-Schiff reagent.

III. RESULTS

Conidial germination patterns, penetration, and development within the host tissues were similar in all cases investigated. Percentage germination and rapidity of penetration and infection were greater on leaf pieces placed on spore suspensions.

(a) Germination of Conidia

At 15°C and in the presence of liquid water, most germinable conidia produced germ tubes within 12 hr. One or two germ tubes were produced by most conidia (Fig. 1, *a-i*) but a few produced three germ tubes (Fig. 1, *j*). A germ tube may emerge from any free surface of the two cells of the conidium. The first germ tube usually arose from the bigger cell; where two germ tubes were formed the second usually came from the other cell, but in a few cases both developed from a single cell (Fig. 1, *g*). Branching of germ tubes on leaves was infrequent (Fig. 1, *k*) and never elaborate. The germ tubes were septate, usually about 0.8 μ m in diameter, and reached a maximum length of 20-30 μ m in 24 hr.

Soon after their formation some germ tubes enlarged slightly at the apices to form appressoria (Fig. 1, *a*) which in a few cases were cut off by septa (Fig. 1, *h* and *i*); a few were sessile on the conidia (Fig. 1, *b*).

(b) Penetration by Hyphae

(i) Penetration of the Cuticle

Below each appressorium an infection hypha formed and penetrated the cuticle within 24 hr after inoculation. In a few cases penetration was initiated from the tips of germ tubes without discernible appressoria. After penetrating, the infection hyphae enlarged and grew between the cuticle and the epidermis (Fig. 2). The first cell of the subcuticular hypha was commonly either rounded or a short rectangular segment with a rounded free end (Fig. 8, *FC*). Subcuticular hyphae grew along the grooves between epidermal cells and branched profusely (Fig. 3). The subcuticular hyphae were broad with short segments about 10 μ m long and 1.5-3.5 μ m in diameter. Subcuticular hyphae stained well only if the method described by Preece (1959) was modified so that leaf pieces and sections were placed in 10% aqueous potassium metabisulphite solution, rather than sulphurous acid solution, after being removed from basic fuchsin solution.

Often haloes, which stained deep red with periodic acid-Schiff reagent, appeared in the cuticle around the appressoria or at the tips of some germ tubes without appressoria. Haloes were associated with both successful and unsuccessful penetrations but were more pronounced around some appressoria from which penetration had not taken place. In Figure 4, the appressorium [which is normally a small swelling at the tip of the germ tube (Fig. 2)] is completely masked by such a dark

halo (H_1) which has a diffuse margin extending well beyond the deeply stained area. Figure 4 also shows a halo (H_2) after the appressorium had become detached.

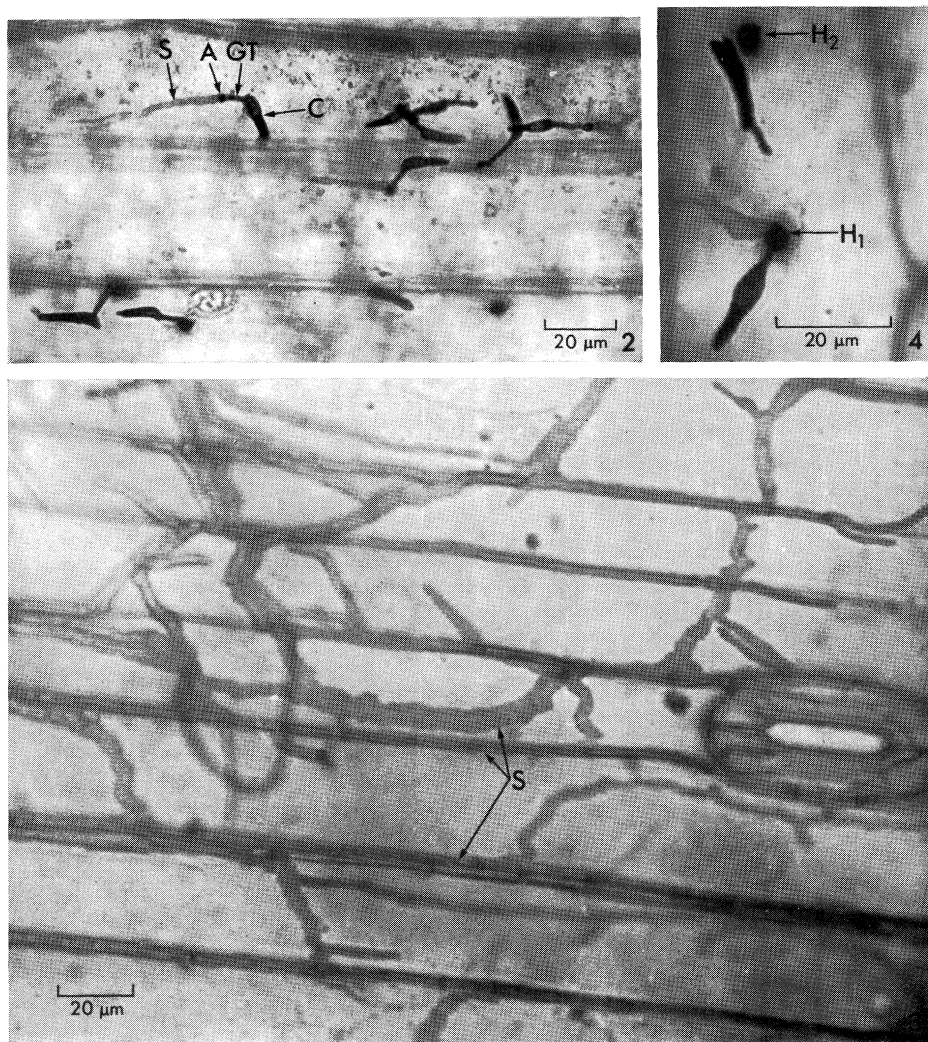


Fig. 2.—Conidia germinating and penetrating the cuticle of leaf after incubation for 24 hr at 15°C. A, appressorium; C, conidium; GT, germ tube; S, subcuticular hypha.

Fig. 3.—Subcuticular hypha (S) after incubation for 4 days at 15°C.

Fig. 4.—Haloes (H_1 and H_2) in the cuticle of a leaf inoculated with conidia, incubated at 15°C, and stained with periodic acid-Schiff reagent 24 hr after inoculation.

The epidermal walls swelled and became lamellate in the vicinity of subcuticular hyphae (Figs. 5 and 6), the anticlinal walls of the epidermis collapsed and, consequently, the outer periclinal walls fell against the inner walls (Fig. 6) forming a composite layer which persisted beneath the subcuticular mycelium (Fig. 9). The collapse of mesophyll cells just beneath the epidermis followed; cell walls retracted

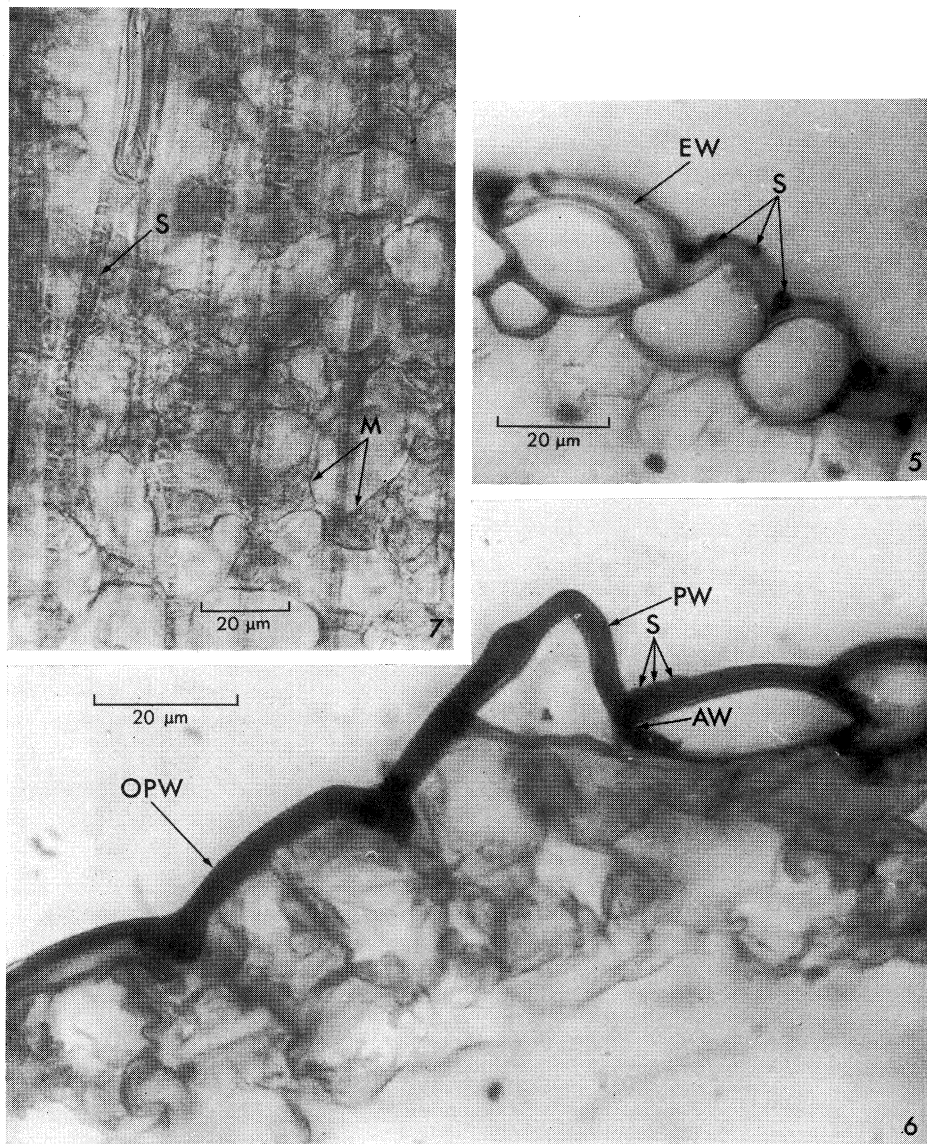


Fig. 5.—Transverse section of infected leaf showing swollen, lamellate epidermal walls (*EW*) and subcuticular hyphae (*S*).

Fig. 6.—Transverse section of infected leaf showing stages in the collapse of anticlinal cell walls of the epidermis. Mesophyll cells beneath the epidermis are also collapsing. Note the swollen lamellated cell walls. *AW*, collapsing anticlinal cell wall; *OPW*, outer periclinal wall collapsed against inner wall; *PW*, periclinal cell wall; *S*, subcuticular hyphae.

Fig. 7.—Surface view of cleared leaf showing collapsing mesophyll cells (*M*) and subcuticular hyphae (*S*).

and adjacent walls remained in contact only at the positions of the plasmodesmata (Fig. 7) and chloroplasts aggregated in each of the affected cells (Fig. 9). All these changes took place while the fungus was confined between the cuticle and the epidermis.

(ii) Penetration of the Epidermis

Ten days after inoculation the subcuticular mycelium was extensive. Hyphae encircled stomatal pores, anastomosed, and covered guard cells, but did not penetrate stomatal pores (Fig. 3). Branches grew from the large hyphae in the depressions between epidermal cells, penetrated between the side junctions of these cells, and entered the mesophyll. Penetration was most frequent at the end junctions between guard cells and contiguous epidermal cells. At this position penetration was commonly initiated by hyphal tips.

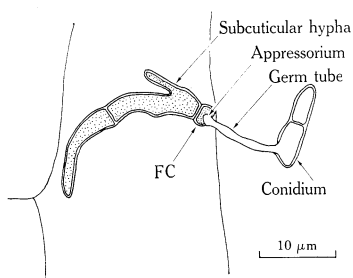


Fig. 8.—Penetration of leaf cuticle by germinating conidium 24 hr after inoculation. *FC*, first cell of subcuticular mycelium which is generally small and rounded.

The invading fungus first established intercellular mycelium between the collapsing cells of the mesophyll. Soon the protoplasts and chloroplasts of the cells disintegrated leaving mainly the cell walls. Following death of the cells, the hyphae began to penetrate the mesophyll cell walls thus becoming intracellular (Fig. 10).

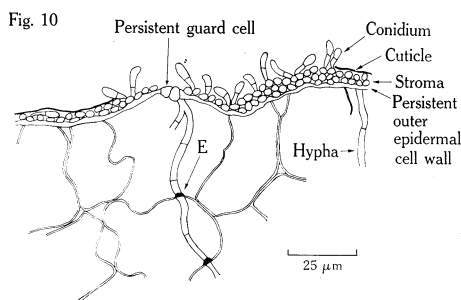
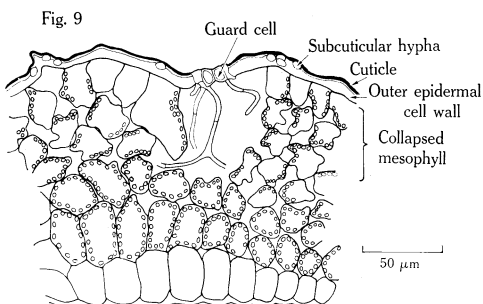


Fig. 9.—Transverse section of infected leaf showing infection of mesophyll. The epidermis and mesophyll have collapsed and chloroplasts in each affected mesophyll cell have clustered together. Fig. 10.—Transverse section of infected leaf showing structure of the subcuticular stroma. *E*, point of entry of hypha into cell wall is marked by a deep staining reaction with the periodic acid-Schiff reagent.

Characteristic deep-staining areas were found at the positions where the hyphae had penetrated the cell wall (Fig. 10, *E*). The average width of hyphae growing in the mesophyll was about $1.5\ \mu\text{m}$.

(c) Formation of Stromata

Subcuticular mycelium continued to grow forming a closely packed hyphal mat, the subcuticular stroma, on the inoculated side of a leaf. The stroma varied in thickness from one to several layers of cells in any one infected area and it gradually separated the cuticle from the epidermis (Fig. 10).

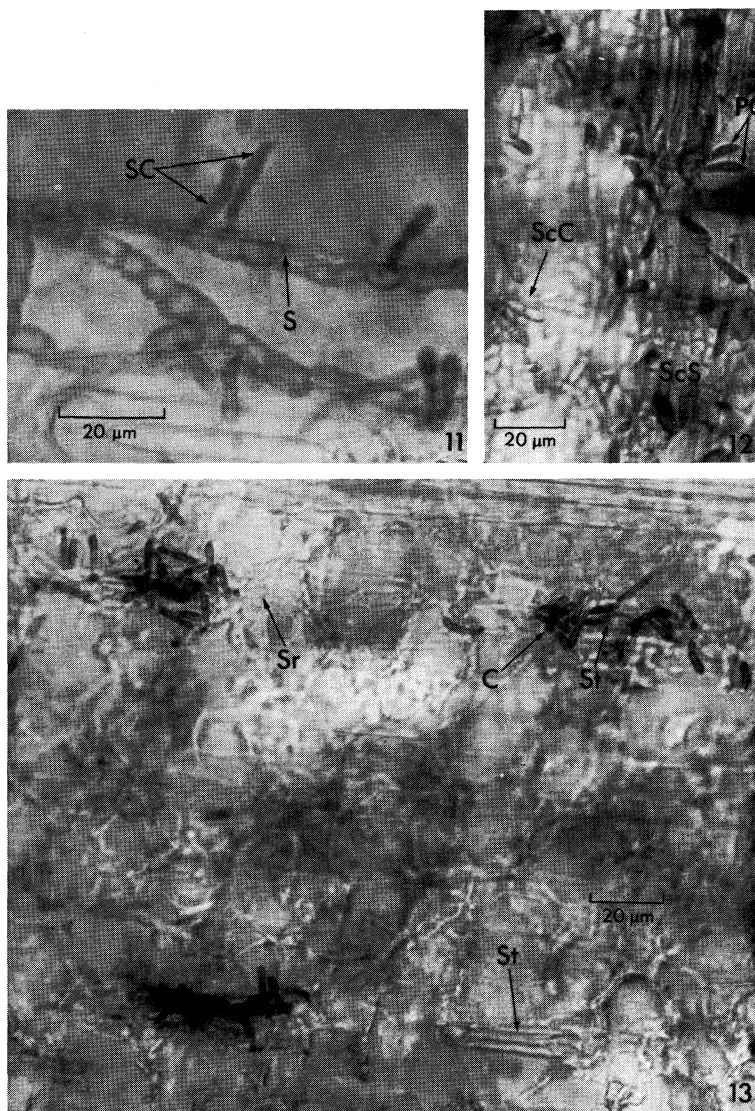


Fig. 11.—Sessile conidia (*SC*) on subcuticular hyphae (*S*) produced 10 days after inoculation.

Fig. 12.—Subcuticular stroma (*ScS*) producing conidia. Subcuticular conidia (*ScC*) stain lightly; protruding conidia (*PC*) stain darkly.

Fig. 13.—Conidia (*C*) produced by substomatal stomata (*Sr*) being extruded through stomata (*St*).

Hyphae grew from the inoculated sides of leaves, through the mesophyll, and developed substomatal stomata in the cavities beneath stomata (Fig. 12).

(d) Sporulation

Conidial production began while the mycelium was still subcuticular. Sessile conidia were formed on short segments of the hyphae, each segment producing one or several conidia (Figs. 11 and 12). Mature conidia tended to become erect and forced their way through the cuticle and could then be seen protruding through the surface (Fig. 12). After a build-up of conidia, the cuticle cracked in various places while remaining intact in others (Fig. 10).

The substomatal stomata also produced conidia, which extruded through the stomatal pores (Fig. 13).

IV. DISCUSSION

The isolates used in the present study penetrated leaves in the way described by Caldwell (1937): germ tubes penetrated the cuticle, and branches from the subcuticular hyphae penetrated the epidermis and mesophyll. Direct penetration of stomatal pores was not observed in the present study but hyphae did aggregate above guard cells and penetration was effected between the end walls of guard cells and contiguous epidermal cells. This may have given the impression of direct stomatal penetration as reported by Bartels (1928) and Mackie (1929).

The signs of chemical modification of the cuticle at points of penetration suggest that cuticular penetration may not be entirely mechanical but may involve enzymic degradation of the cuticle. Cutinases have been recorded for *Penicillium spinulosum* (Heinen and Linskens 1960) and Kunoh and Akai (1969) concluded that *Erysiphe graminis* produced cutinases which modified the cuticle of barley leaves during infection. The extensive modifications of epidermal cell walls suggest that the fungus may excrete enzymes which degrade them. Enzymes capable of degrading plant cell walls are produced by very many plant pathogenic fungi.

The positional relationship of the end walls of guard cells and adjacent epidermal cells may explain the frequency of penetration around stomata. At these junctions the ends of epidermal cells overhang the sunken guard cells forming concavities at the level of the upper surface of the guard cells. It appears that hyphae entering these stomatal concavities force their way between the guard and epidermal cells.

Preliminary results suggest that the collapse of mesophyll cells beneath subcuticular hyphae is caused by toxic substance(s) produced by the hyphae. Toxic materials having similar effects have been reported for a number of plant pathogens (Wheeler and Luke 1963). Work is in progress to attempt to verify the presence of such toxin(s) in barley infected by *R. secalis*.

Hyphae in the mesophyll did not spread laterally or longitudinally within leaves, thus lesions increased in diameter as a result of the growth of subcuticular mycelium. Similar observations were made by Caldwell (1937). However, hyphae did grow through the mesophyll from inoculated leaf surfaces to form substomatal stomata beneath the uninoculated surfaces. This contrasts with Caldwell's (1937) observations as does our observation of conidia being extruded through stomatal

pores from substomatal stomata. Davis (1922) also reported observing conidia protruding through stomata.

The cuticle above subcuticular stomata remained intact until masses of mature conidia were produced, protruding through the cuticle and cracking it. This has considerable epidemiological significance since stomata are effectively protected until conditions become favourable for conidial production. Low temperature and free water, which are necessary for sporulation, also predispose susceptible hosts to infection (Caldwell 1937; Skoropad 1966).

V. ACKNOWLEDGMENT

The generous provision by Mr. D. H. B. Sparrow of seed of the barley variety Clipper (AC 2200) is gratefully acknowledged.

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