**Rhynchosporium** leaf blotch of barley studied during the subcuticular phase by electron microscopy

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The subcuticular phase in the development of *Rhynchosporium* leaf blotch of barley was studied by scanning and transmission electron microscopy. After direct penetration of the cuticle, hyphae grow beneath the cuticle in the region which is rich in pectic substances, often growing along the line where adjacent epidermal cells meet. The cuticle over the hyphae is stretched but not ruptured and there is only localized disruption of the cell wall. Appositions of cell wall material occur on the inner surface of the epidermal cell wall. Close to subcuticular hyphae, short lengths of the plasmalemma of epidermal cells may be separated from the cell wall. The epidermal cells collapse and, in the underlying mesophyll cells, the chloroplasts show degenerative changes.

**INTRODUCTION**

In only a few host-parasite combinations do phytopathogenic fungi establish a subcuticular mycelium in the leaf of the host, prior to a more comprehensive invasion. Thus, *Spilocaea oleagina* (Cast) Hughes grows in the outer layer of the epidermal cell wall [11] and *Diplocarpon rosae* Wolf. [3] and *Venturia inaequalis* (Cooke) Wint [20] grow between the cuticle and the outer wall of the epidermal cells.

In this laboratory we have been interested in the growth of another subcuticular parasite, *Rhynchosporium secalis* (Oudem) J. J. Davis, which causes barley leaf blotch. Previous investigations into the development of the pathogen, which had described the first mycelium as subcuticular, were made with the limited resolving power of the light microscope [4, 8]. Since the term “subcuticular” describes a region that is comprised of a number of thin, yet distinct, layers, in which different substances may predominate, it was the first object of this work to use the greater resolving power of the electron microscope to define which of those layers was colonized by *R. secalis*.

We reported previously [12] that as early as the seventh day after infection the subcuticular mycelium of *R. secalis* caused permeability changes in the underlying host cells, with the result that a greater supply of nutrients became available to the parasite. Our second object in this work was to examine whether ultrastructural changes occurred that could be responsible for the physiological changes that we had detected.

Some other subcuticular parasites, e.g. *D. rosae*, and *V. inaequalis* produce haustoria during their subcuticular phase. There is no evidence from light microscopy that

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*R. secalis* produces haustoria during this phase and we have argued [12] that the absence of such organs, whose probable function is to absorb foodstuffs from inside host cells, adds to the importance of the permeability changes caused by *R. secalis*. In this study we re-examined whether *R. secalis* produced any intracellular process resembling an haustorium.

**MATERIALS AND METHODS**

The growth of barley plants (var. Deba Abed) and fungus cultures, and the method by which the second seedling leaves were inoculated and maintained, have been described previously [12]. Only susceptible host-variety fungus-isolate combinations were studied. For transmission electron microscopy (TEM), small pieces of leaf tissue were fixed for 5 h in 2.5% glutaraldehyde buffered with 0.025 M-phosphate at pH 7.0. The leaves were washed twice in buffer, post fixed in osmium tetroxide overnight and dehydrated in a graded ethanol series. Tissue for examination in the TEM was embedded in either Epon 812 or the low viscosity resin of Spurr [19]. Thin transverse sections were cut and post stained on the grid using uranyl acetate and lead citrate [17]. The sections were examined in an AEI 801 electron microscope at 80 kV.

Leaves to be studied in the scanning electron microscope (SEM) were taken from 100% ethanol through a series of ethanol amyl acetate mixtures [18] and dried in a critical point dryer similar to that used by Boyde & Wood [7]. The dried material was mounted on stubs and coated with a 20-nm layer of gold prior to examination in a scanning electron microscope (Cambridge Instruments Ltd.).

**RESULTS AND DISCUSSION**

In a healthy barley leaf the epidermal cells are turgid and have a convex outer surface. The stomata occur in rows and lie somewhat below the surface of the other epidermal cells [Plate 1(a)]. In infected leaves the epidermal cells had all collapsed on to the underlying mesophyll cells by the tenth day after inoculation. The outline of the mesophyll cells could then sometimes be seen in surface view [Plate 1(d)].

The way in which *R. secalis* penetrates the barley leaf has been the subject of some dispute. Earlier workers [6, 15] had concluded that the germ tube entered the host through stomatal pores, but, more recently, Caldwell [8] and Ayesu-Offei & Clare [9] have opposed this idea and said that all penetrations were directly through the cuticle. We found that germinating spores were capable of establishing a direct penetration of the leaf [Plates 1(d), (f)]. We also saw a few surface hyphae which grew directly into stomatal pores, e.g. Plate 1(e). When leaves are inoculated in the laboratory with a spore suspension, the suspension unavoidably contains a few mycelial fragments which give rise to some wholly superficial growth of the fungus. However, subcuticular hyphae could be seen in the SEM mode and were easily distinguished from superficial hyphae (Plate 1), and it was a superficial hypha that was seen growing into a stomatal pore. These stomatal penetrations were rare and there is no evidence that infection can become established in this way.
A study by scanning (Plate 1) and transmission (Plates 2–4) electron microscopy of the development of *R. securis* on barley (leaf blotch). Unless stated otherwise, all material was infected 10 days before examination.

**Plate 1.** (a) Upper surface of healthy leaf showing convex nature of epidermal cells. (b) Germinated spores (G) on the upper surface of the leaf, 7 days after inoculation. The epidermal cells have begun to collapse. (c) Old subcuticular hyphae showing “tram line” effect. (d) A germinated spore gives rise to a direct penetration of the cuticle. A superficial hypha grows across an open stomata. (e) Young subcuticular hyphae (H) raise up the cuticle. Superficial hyphae (S) are seen and one of these is growing into the stomatal pore. (f) Detail of the cuticular penetration in 1(d). The appressorium (A) has collapsed and is surrounded by a circular ridge in the leaf.

**Plate 2.** (a) Junction of anticlinal walls of healthy epidermal cells after staining in Albersheim’s hydroxylamine/HCl reagent. Pectic substances, which appear electron dense, are concentrated above the anticlinal walls, and spread out in a narrow band over the epidermal cells. (b) T.S. of a young subcuticular hypha growing in the pectin rich region above the anticlinal walls of adjacent epidermal cells. Seven days after inoculation. (c) T.S. of a subcuticular hypha growing above an epidermal cell. The cuticle (CU) is raised up but not broken. To the left is seen a second subcuticular hypha. Pectic (PE) and cellulosic (C) layers are disrupted. (V) vacuole. (d) T.S. of an old subcuticular hypha which has collapsed leaving two lateral ridges. Seven days after inoculation. (e) L.S. of a young subcuticular hypha growing above the cellulosic layer (C) of the host wall. Seven days after inoculation.

**Plate 3.** (a) Chloroplast from an infected leaf in which the epidermis has collapsed. Numerous large plastoglobuli (PG) evident. (b) Chloroplast from a healthy leaf. (c) L.S. of tip of subcuticular hypha. The epidermal cell has collapsed and has become thickened beneath the cellulose layer (C) by appositions (AP) on the inner surface. (d) T.S. of two subcuticular hyphae at the junction of two collapsing epidermal cells. Host cell wall shows localized thickenings (AP).

**Plate 4.** (a) T.S. of an epidermal cell from an infected leaf. To the right, and close to a subcuticular hypha, a length of the plasmalemma is widely separated from the cell wall. The fibrillar appearance of the wall shows some disruption in this area. To the left the cell wall and plasmalemma show a more normal association. (b) T.S. of an epidermal cell from a healthy leaf. The plasmalemma is closely associated with the cell wall throughout its length.
Plate 4
When the germ tube penetrated the cuticle a ridge appeared around the point of penetration [Plate 1(f)]. This ridge is probably the cause of the haloes seen around penetration points by Ayesu-Offei & Clare [4]. A penetration is a comparatively rare event [7] and we were unable to find a penetration site to study in section by TEM. In the SEM mode some subcuticular hyphae appeared as double ridges, producing a “tram line” effect [Plate 1(c)]. The effect was explained when leaf sections were examined in the TEM, for it was found that older hyphae, with large vacuoles, had each collapsed centrally, leaving two lateral ridges [Plate 2(d)].

After penetration, the hyphae grew externally to that region of the epidermal cell wall that is predominantly cellulosic, and were frequently found growing along the line of the junction between two epidermal cells, above the juxtaposed anticlinal walls. These regions correspond closely to those which, in many plants [16], are rich in pectic substances. The presence of pectic substances in these regions of the barley leaf was confirmed when the leaves were stained in Albersheim’s hydroxylamine/HCl reagent [2]. The carbomethoxy groups of the pectic substances react with alkaline hydroxylamine to produce pectin hydroxamic acids which react with ferric ions to form insoluble red complexes which enhance the electron dense appearance of the pectic substances [Plate 2(a)]. Unfortunately the alkaline conditions required for the formation of hydroxamic acids cause a deterioration of fine structure.

There was no widespread disruption of the cell wall caused by the first subcuticular hyphae. Lateral to each subcuticular hypha there was a broadening and slight bubbling in the band of pectic substances [Plates 2(c), (d), 3(c), (d)] suggesting that pectic enzymes were active in that region. Plates 2(c) and 3(d) show two of the rare instances where damage to the cell wall was readily apparent; in both cases two hyphae may be seen growing close together and, probably, acting together on the wall. The pectic substances appear grainy and bubbly and, interestingly, the cellulosic region of the wall also shows some disruption. Therefore, any cell wall degrading enzymes that the fungus secretes at this stage act in a very localized area. Remarkably similar changes were seen when rice cotyledons were attacked by Cochliobolus miyabeanus [1]; hyphae developed under the cuticle, raising it, the pectin developed a granular appearance around the hyphae and the cellulose layer showed almost no change. In apples and pears infected intercellularly with Sclerotinia fructigena Aderh. & Ruhl. localized degradation of the cell wall, with later structural disorganization of the plasmalemma, has been attributed to the activity of pectic enzymes [9].

Infection caused localized thickenings of the cell wall usually in a region close to a subcuticular hypha [Plates 3(c), (d)]. In apples leaves similar wall appositions also form on the inner side of host epidermal cell walls underneath subcuticular stromata of V. inaequalis in tissue exhibiting various host reactions [14].

In those regions of the host cell wall where the fungus caused recognizable disruption, that is in the proximity of subcuticular hyphae, the close association between cell wall and plasmalemma was disrupted sometimes, with the plasmalemma being widely separated from the cell wall [Plate 4(a)]. Changes in the normally close binding of the cell wall to the plasmalemma [Plate 4(b)] may be responsible for the changes in the permeability of host cells that occur soon after infection [12].
Mesophyll cells were not affected by infection until the epidermal cells had begun to collapse, an observation which agrees with the results of physiological experiments (unpublished work done in this laboratory) which show that rates of CO₂ fixation by the host are not reduced by infection until after the epidermal cells have collapsed. When changes could be observed in the mesophyll cells, chloroplasts became swollen, and the plastoglobuli became more conspicuous and more numerous [Plate 3(a)]. The intergranal lamellae became progressively more disorganized, with the spaces between them becoming inflated. A similar series of events has been reported for a number of other diseases, e.g. "sooty blotch" of white clover [10] and is characteristic of senescence in the leaves of annuals and perennials [13].

At this stage the fungus was still confined beneath the cuticle, no haustoria could be found, and the only visible symptom of disease was the silvering of the epidermis at the point of inoculation. Only when the fungus subsequently grew out into the rest of the leaf did the typical "leaf blotch" lesion develop.

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REFERENCES


