

## Haustorial Sheath Formation in Cowpea Leaves Immune to Rust Infection

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### ABSTRACT

One immune cowpea cultivar, Queen Anne, showed a unique dimorphic reaction to infection by the rust *Uromyces phaseoli vignae*; haustorial formation induced either rapid host cell necrosis or the formation of a calloselike sheath which grew up from the host cell wall to completely enclose the haustorium. In 14 other immune cultivars examined, resistance was expressed only by the more typical resistant response of hypersensitive death of invaded host cells. Although sheathed haustoria did not die immediately as did unsheathed haustoria in necrotic cells, the majority of infection hyphae in Queen Anne, like those in all the immune cultivars, ceased

to grow after the formation of the first haustorial mother cell. The formation of a haustorium appeared to be essential for necrosis of either host or parasite in all immune cultivars, including Queen Anne.

The unusual dimorphic reaction of Queen Anne may result from a greater tolerance of this variety to the toxic effects of the haustorium. The fact that the host remains immune in the absence of host cell necrosis suggests that the hypersensitive response is not the only effective means of limiting fungal growth in rust-infected tissues. *Phytopathology* 61:383-388.

During an investigation of aspects of host-parasite specificity using races of bean rust (*Uromyces phaseoli typica* Arth.) and the related cowpea rust (*U. phaseoli vignae* [Barcl.] Arth.), it was noticed that one strain of the latter could induce two apparently distinct types of host reaction in a single immune cultivar of cowpea (*Vigna sinensis* [Toner] Savi 'Queen Anne'). In the first type of reaction, invaded host cells died soon after haustorial formation. This response to infection was the only reaction observed in all other immune cultivars of cowpea examined, and has commonly been reported for other plants resistant to rust infection (12, 23). In the second type of reaction, the fully formed haustorium became enveloped by a thick sheath which appeared to grow up from the host cell wall. Host cell death was either delayed or did not occur at all. No similar dimorphic reaction has been reported in the literature. Although completely sheathed haustoria have been reported in a few resistant rust reactions (2, 10, 16, 29), stages in sheath formation have not been described. The relationship between sheath formation and host cell death has never been investigated.

The objectives of this investigation were to compare histologically the two reaction types, and to contrast the reaction of this cowpea cultivar to that of other cultivars immune and susceptible to cowpea rust infection.

**MATERIALS AND METHODS.**—Uredospores of cowpea rust were first obtained from field-infected plants. A single-pustule isolate was used for all subsequent work. Spores were produced in bulk on the susceptible cultivar Early Ramshorn and stored at  $-34^{\circ}\text{C}$ . Before use, 80 mg of spores were shaken for 3 min in 80 ml 0.01% Tween 20 (polyoxyethylene sorbitan mono-laurate) at room temperature ( $24^{\circ}\text{C}$ ) to remove self-

inhibitors of germination. Spores were collected by filtering through sintered glass, and were allowed to air-dry before the inoculation of plants.

Cowpea plants were grown in sterile soil in the greenhouse at  $30 \pm 5^{\circ}\text{C}$  and kept at the primary leaf stage by pinching out the growing tips. When 8 days old, leaves of intact plants were washed with double-distilled water and a settling tower was used to spread the spores evenly over the upper surfaces at a density of about 2,000-3,000 spores/cm<sup>2</sup>. After atomizing with double-distilled water, plants were kept in the dark at 100% relative humidity for 24 hr at  $20^{\circ}\text{C}$ . For the rest of the experimental period, plants were illuminated for 16 hr/day at an incident light intensity of 1,600 ft-c in a controlled environment chamber maintained at a day temperature of  $20 \pm 1^{\circ}\text{C}$  and a night temperature of  $18 \pm 1^{\circ}\text{C}$ .

At intervals after inoculation, whole leaf pieces were stained and cleared by the technique described by Shipton & Brown (24), using trypan blue instead of cotton blue to stain the fungus and increasing the clearing time in chloral hydrate to 2-3 days. Measurements of fungal growth and host reaction were made from over 100 infection hyphae distributed between leaves of 4 plants of each variety. A micrometer eye piece was used to measure total hyphal length. The course of pathogenesis in each variety was examined several times. In each case, results were similar; therefore, figures for only one of these experiments are given below.

Host reaction to infection was examined in more detail in sections cut for the light microscope on the Lab-line/Hooker Plant Microtome (Lab-line Instruments Inc, Melrose Park, Ill.) after infiltrating the tissue with water or 2.5% glutaraldehyde in 0.07 M phosphate buffer (pH 7.0) containing 0.1 M sucrose.

The ninhydrin-Schiff's reaction of protein, the hydroxylamine-ferric chloride reaction of pectic substances, and the zinc-chlor-iodide and IKI- $H_2SO_4$  reactions of cellulose were examined on unfixed material, as described by Jensen (13).

**RESULTS.**—Fifteen cowpea cultivars showed no visible symptoms after inoculation with cowpea rust, and were therefore regarded as showing an immune type of response to infection. In all of these varieties, except Queen Anne, the pattern of pathogenesis was superficially similar. Thus, just one cultivar, Calico Crowder, was chosen for detailed comparison with the unusual dimorphic reaction observed in Queen Anne. Early Ramshorn was used as the susceptible cultivar.

Cleared leaves showed that the processes of germination and appressorial formation were similar on the three cultivars tested. Penetration through stomata occurred at about 6 hr after inoculation. Comparisons of growth of infection hyphae during the first 3 days after inoculation are shown in Fig. 1. Differences in total length of mycelium in immune and susceptible varieties were apparent 24 hr after inoculation, and were pronounced by 36 hr. By 48 hr after inoculation, growth in both immune varieties had ceased. During these 2 days, the total length of mycelium formed in the immune varieties did not exceed  $80 \mu$  as compared with the average length of  $130 \mu$  formed in the susceptible host during the same period.

*Queen Anne (dimorphic immune reaction).*—As in all three varieties examined, the first haustorium was produced in Queen Anne between 12 and 24 hr after inoculation. Only 8% of haustoria seen 24 hr after inoculation had induced any necrosis of invaded host cells. Fresh and glutaraldehyde-fixed sections showed the majority of cells to be healthy in appearance apart from the accumulation of cytoplasm around the haustorium. Nearly all haustoria appeared fully formed at this time, and the majority had induced only slight thickening of the host cell wall around the point of entry (Fig. 4-B). In a few cases, the haustorial neck was surrounded by a short, collarlike structure apparently continuous with the host cell wall. This collar sometimes flared out just beneath the haustorial body (Fig. 4-D). Between 24 and 27 hr after inoculation, the number of such flared structures markedly increased, as did the degree to which the haustorial body was enveloped. Between 27 and 36 hr after inoculation, there was no significant increase in the proportion of infection hyphae with haustoria. However, the percentage of haustoria with complete sheaths increased while there was a comparable decrease in the percentage of incompletely sheathed haustoria observed (Fig. 2). Since the total percentage of haustoria with any type of sheathing material around the haustorial body stayed constant during this period, the complete sheaths must have been formed by continued growth

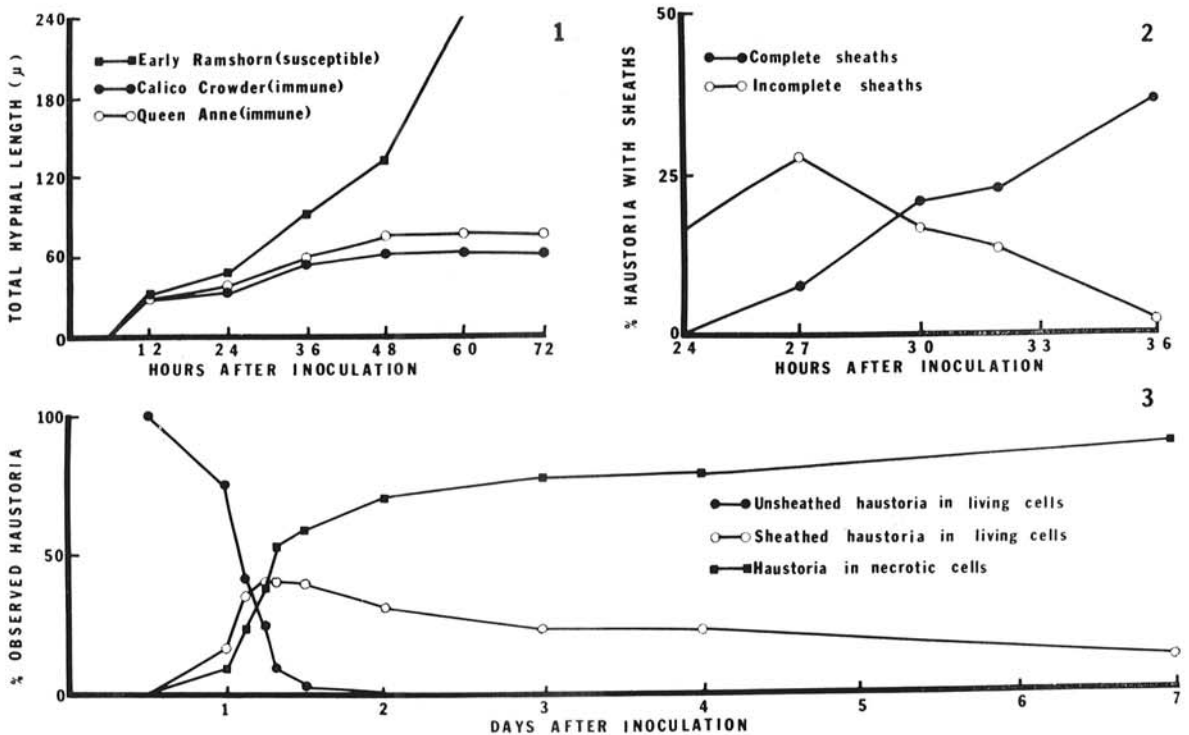
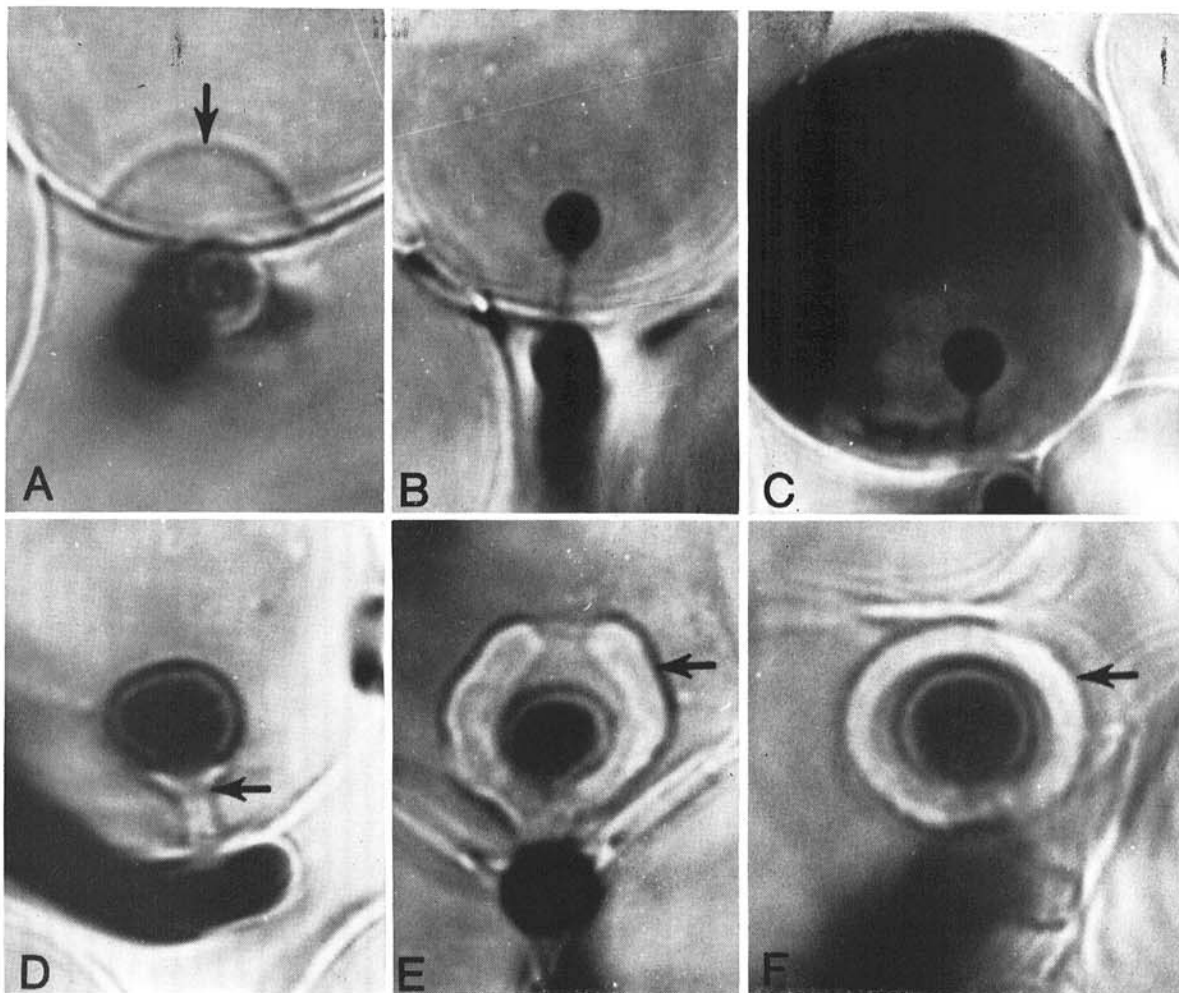


Fig. 1-3. 1) Hyphal growth in immune and susceptible cultivars of cowpea during the first 3 days after inoculation. 2) Changes in the proportion of haustoria with incomplete and complete sheaths during the first 36 hr after inoculation of the immune cultivar Queen Anne. Measurements apply only to the first haustorium formed by each infection hypha. 3) Host cell reaction after haustorial formation in immune Queen Anne. Measurements apply only to the first haustorium formed by each infection hypha.



**Fig. 4.** Whole-leaf preparations of the immune cowpea cultivar Queen Anne 24-28 hr after inoculation; **A**) 28 hr after inoculation. Pronounced thickening of host cell wall (arrow) adjacent to haustorial mother cell. No haustoria are formed in such cases; ( $\times 2,060$ ) **B**) 24 hr after inoculation. Apparently fully formed haustorium in healthy host cell. No signs of sheathing material around the body of the haustorium; ( $\times 1,710$ ) **C**) 28 hr after inoculation. Haustorium in necrotic, slightly plasmolyzed host cell. No signs of sheathing material around haustorium. The necrotic host cell has retained the trypan blue stain while its healthy neighbors have remained colorless. ( $\times 1,710$ ) **D, E, F**) Possible stages in sheath (arrowed) formation. No opening in the sheath could be seen at any plane of focus in **F**. (all  $\times 2,060$ )

of incomplete sheaths. Thus, Fig. 4-D, E, F presumably illustrates stages in sheath formation. All sheaths were complete by 48 hr after inoculation. Sheathed haustoria amounted to about 40% of all haustoria produced by this stage of infection.

Figure 3 shows that during the period of sheath formation there was also an increase in the percentage of haustoria inducing host cell necrosis. Sections of fresh and glutaraldehyde-fixed material showed that chloroplasts had disintegrated and protoplasts were plasmolyzed. Walls and contents of invaded cells turned brown, and host cell collapse followed rapidly. Up to 36 hr after inoculation, it was still possible to detect a haustorium within each dying cell and almost all (over 94%) had no detectable sheath (Fig. 4-C). Only rudimentary sheaths were observed in the remainder. Thus, cell death during the first 36 hr after

inoculation was induced almost exclusively by un-sheathed haustoria. This explains why the proportion of healthy cells containing un-sheathed haustoria decreased rapidly during this period of cell necrosis, so that by 48 hr after inoculation no more were observed (Fig. 3).

Although growth of infection hyphae had already ceased, from 2 until 7 days after inoculation an increasing number of cells containing sheathed haustoria became necrotic (Fig. 3). The process, however, was much slower than that observed earlier for un-sheathed haustoria, and even after 7 days, 33% of the sheathed haustoria originally produced could still be found in apparently healthy cells. The only change observed in some was that the sheath had turned from its original colorless condition to a deep reddish brown.

Collapse of necrotic cells containing haustoria was

accompanied by collapse of haustorial mother cells. Such rapid collapse of mother cells was not observed accompanying the sheathing reaction.

The results given above describe the events following the formation of the first haustorium by each infection hypha. After the initial reaction by the host (regardless of which type), about 15-20% of infection hyphae branched above the haustorial mother cell and a second haustorium was formed in a neighboring mesophyll cell. In 75% of these cases, the host cell reaction to invasion was of the sheathing type. No further growth of infection hyphae was observed. Even when only one haustorium was formed, infection hyphae appeared to remain alive for several days after growth had ceased.

About 10% of infection hyphae produced no haustoria or host cell necrosis, and a marked thickening of host cell walls adjacent to the haustorial mother cell was commonly observed (Fig. 4-A). Walls of healthy cells adjacent to collapsed cells containing haustoria also thickened. In a few instances, two or three of these adjacent cells became necrotic. No signs of necrosis were seen in fresh or glutaraldehyde-fixed sections before the formation of the first haustorium.

*Calico Crowder (isomorphic immune reaction).*—As in the other immune varieties, slight thickening of host cell walls could usually be detected around the point of entry of haustoria. Invaded cells of this immune variety appeared necrotic soon after haustorial penetration. As in Queen Anne, host cell collapse was associated with collapse of the haustorial mother cell appressed to it. By 24 hr after inoculation, every haustorium observed had started to induce cell necrosis. Usually each infection hypha produced only one haustorium before growth ceased, although the mycelium appeared healthy for several days. After the death of the invaded cell, 10-15% of infection hyphae formed a branch above the original haustorial mother cell. Commonly, such branching occurred near the substomatal vesicle, and the second haustorium was formed in an epidermal cell. Although the cell cytoplasm became brown and granular, infected epidermal cells did not collapse as did similarly invaded cells of the mesophyll. No infection hypha was observed to produce more than two haustoria.

No signs of necrosis of host cells were observed before the formation of the first haustorium. As in Queen Anne, collapse of haustorium-containing cells was often followed by localized thickening and browning of adjacent walls of neighboring cells. In a few cases, three or four of these neighboring cells became necrotic. Approximately 10% of all infection hyphae formed in this variety did not form a haustorium, and a pronounced thickening of the host cell wall adjacent to the haustorial mother cell was commonly observed. No signs of host cell necrosis were observed in such cases.

*Early Ramshorn (susceptible reaction).*—The production of haustoria in the susceptible cowpea cultivar Early Ramshorn was accompanied by no observable thickening of host cell walls around the point of entry.

During the first 3 days after inoculation, host cells remained apparently healthy apart from an accumulation of cytoplasm around each haustorium. Necrosis of host cells was sometimes observed in older infections, but amounted to no more than two or three cells adjacent to older parts of the mycelium. Less than 1% of all infection hyphae produced no haustoria, and a localized thickening of the host cell wall, similar to that described for immune cultivars, was observed in these cases. No further growth of such infection hyphae occurred.

*Histochemical reactions of sheaths and wall thickenings.*—The ninhydrin-Schiff's reaction for protein and the zinc-chlor-iodide and IKI-H<sub>2</sub>SO<sub>4</sub> tests for cellulose failed to stain the sheath material; in the latter reaction it disappeared immediately after the 75% acid was added. In all three cultivars, there was no detectable increase in coloration of thickened areas of the host cell wall after these tests. The hydroxylamine-ferric chloride reaction for pectic substances also did not show any increase in coloration of cell walls in infected areas of each cultivar, nor did it stain the haustorial sheath in Queen Anne. These sheaths, however, did stain strongly with dilute aqueous aniline blue and swelled rapidly in ammonium hydroxide. Commonly, the entire sheath disappeared in 1% sodium hydroxide. These latter reactions are all characteristic of callose (14). In a few instances, only the main body of the sheath dissolved in 1% sodium hydroxide, and a collar of material was left around the haustorial neck. The areas of thickened cell wall associated with haustorium entry in the immune varieties also stained with aniline blue. The wall thickenings formed in response to adjacent necrotic tissue usually stained with this dye, but any blue coloration was sometimes obscured by the brown pigmentation of the cell wall.

*DISCUSSION.*—Except for the frequent deposition of calloselike material on cell walls of immune plants, no cytological differences were detected between immune and susceptible cowpea cultivars before the formation of the first haustorium. Thus, in this system, penetration of the host cell by the parasite is necessary for necrosis of either organism to take place. Many cytological studies are not clear on this point, but a similar situation seems to exist in a few host-parasite combinations (1, 10, 16). In contrast, in several other plants showing varietal resistance to rust infection and in certain nonhost reactions, deleterious effects on either host or parasite have been observed before haustorial formation (8, 15, 17, 22, 26, 27, 30).

Whereas haustorial-induced necrosis of host cells in all immune varieties of cowpea apparently killed the haustorium and its mother cell, the sheathing reaction peculiar to the cultivar Queen Anne did not result in immediate death of these fungal structures. But, as in the other immune varieties showing only a necrotic response to haustorial formation, growth of infection hyphae usually ceased after the formation of the first haustorial mother cell. Presumably, the sheathed but still living haustorium derived no more nourishment

from the healthy host cell than did those killed by host cell necrosis. Histological studies suggested that the main body of the sheath was composed of a callose-like material. Callose has been reported to be less permeable to small molecules than other cell wall components (9), and may therefore restrict or prohibit the passage of nutrients to the fungus. However, preliminary investigations with the electron microscope suggest that changes in the host plasmalemma surrounding the haustorium play a more important role in restricting pathogen growth.

In all the immune cowpea cultivars, including Queen Anne, the reaction of the host cell to haustorial invasion did not result in the immediate death of the whole infection hypha. Thus, the high degree of resistance of these varieties to rust infection is not related to rapid death of the fungal mycelium. Starvation may be the cause of the cessation of fungal growth (27).

Hypersensitive death of invaded host cells is the characteristic reaction of plants showing physiological varietal resistance to rust infection (12, 23, 27), and the degree of resistance of the tissue can often be related to the rapidity of the hypersensitive response (10, 16, 27). Therefore, perhaps the most interesting feature of the sheathing reaction observed in Queen Anne is that it demonstrates that the highest degree of varietal resistance (immunity) need not involve hypersensitive death. Thus, necrosis is not the only effective means of limiting fungal growth in rust infected tissue.

The fact that sheaths have only rarely been reported in other resistant reactions could be due to the preclusion of sheath formation by the rapidity of host cell necrosis once initiated. In support of this, the sheaths observed by Allen (2) around haustoria of *Puccinia graminis tritici* in resistant wheat were only found when the host necrotic reaction was less vigorous than it had been initially. Similarly, sheaths were more commonly observed in the less vigorous resistant reactions induced by races of *P. sorghi* on certain lines of corn (10). That the sheathing reaction is the one involving the less severe action on the part of the haustorium is suggested by the fact that almost twice as many sheaths were induced in Queen Anne by the second haustorium of each infection hypha than by the first. The unusual dimorphic reaction of Queen Anne may be the result of a greater tolerance of this variety to the toxic effects of the haustorium. Because of this greater tolerance, not all haustoria secrete enough toxic material to cross the "threshold level" and induce immediate cell death. Sheath formation could be a response to sublethal levels of these toxic substances or to some other aspect of the haustorium that the host recognizes as "foreign". It is interesting that a remarkably similar type of callose sheathing reaction has been induced in *Nicotiana* cells simply by the insertion of a glass needle (20).

Sheath formation does not appear to be restricted to resistant reactions, and preliminary observations with the electron microscope have shown that by 6 days after inoculation, a few sheathed haustoria can be found in the susceptible cowpea cultivar Early Rams-

horn. Partial or complete sheaths have been observed in many other compatible rust and downy mildew infections (5, 6, 7, 10, 21), and several authors have concluded callose to be a major component of these structures (6, 7, Mangin as cited by Rice [21]). In most cases such sheaths are rarely seen during the early stages of infection, and since cellular disorganization eventually occurs even in susceptible tissue (23), it is not unreasonable to assume that this sheath formation results from the gradual development of some degree of host injury or other form of incompatibility between host and parasite. In contrast, the formation of the commonly observed sheaths around powdery mildew haustoria (25) does not appear to be correlated with resistance; in fact, these sheaths have been suggested to be essential for the establishment of the compatible reaction (11). It is, therefore, not surprising that electron microscopy has shown these sheaths to be fundamentally different in nature to those detectable with the light microscope in rust and downy mildew infection (3, 4, 5, 6, 18, 20, 28).

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