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Reactions of Resistant and Susceptible Hevea *Clones to* Colletotrichum gloeosporioides

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Pre- and post-penetration behaviour of Colletotrichum gloeosporioides *in susceptible and resistant* Hevea *clones were studied. Growth of the fungus on the leaf surface of susceptible clones was promoted while appressorium formation was promoted on resistant leaves. The movement of nuclei in both epidermal and palisade cells was seen as an early response to infection. After the entry of the pathogen, the host cells first showed a discolouration followed by an extensive disorganisation of epidermal and mesophyll cells resulting in the arrest of fungal development. In tissues of susceptible clones, this reaction was less intense and was restricted to a few cells. Extensive ramification of the pathogen was observed within the leaf tissues and acervuli were formed 72 h after inoculation.*

Field observations[1,2] have confirmed the existence of *Hevea* clones with different levels of susceptibility to *Colletotrichum gloeosporioides* (Penz.) Sacc. In preliminary studies, Liyanage and De Alwis[3] showed that there were differences in the pre-penetration behaviour of the pathogen on the leaves of susceptible and resistant clones. They also examined the post-penetration events in a susceptible reaction. However, for a complete understanding of the resistant mechanisms of the host-pathogen interactions, histopathology of both resistant and susceptible clones should be studied.

This paper examines in detail the development of *C. gloeosporioides* on leaves of resistant and susceptible clones and determines the stages at which resistant mechanisms were apparent.

MATERIALS AND METHODS

Inoculation of Leaves

Unopened buds on two-year-old resistant (R) and susceptible (S) clones, RRIC 52(R), RRIC 100(R), RRIC 103(S), RRIC 104(R), RRIC 105(R), RRIC 117(R), PB 86(S), IAN 873(R), FX 4098(S) and Tjir 1(S), grown in a budwood nursery at Dartonfield, Agalawatta were enclosed in polyethylene bags to preclude chances of infection. Nine days later, leaves formed from these buds were picked and taken to the laboratory in sealed polyethylene bags. An isolate of *C. gloeosporioides* obtained from infected leaves of clone PB 86 was grown on PDA at 28°C for four days and a suspension containing 2×10^5 spores per millilitre was then prepared from it as described by Wimalajeewa[4]. Middle leaflets (approx. leaf area - 45 cm^2) of four leaves were selected and twenty-five drops of the inoculum each of 0.01 ml were placed 2 - 3 cm apart on the adaxial surface on both sides of the midrib of leaflets of one clone using the 'Arnold' hand micro-applicator. The leaves were kept in a moist chamber at 28°C ± 2°C and 100% RH.

Leaf Clearance, Germination Assessment and Growth Measurements

Including the inoculum drops four leaf discs of 1.5 cm of diameter were cut at random from the leaves of each clone at 3, 4, 8, 12, 16 and 24 h after inoculation. Each leaf disc was treated as a replicate. They were cleared and stained by the method of Shipton and Brown[5]. Germination was assessed on counts of 400 conidia; the criterion for germination being a

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germ-tube as long as the width of the conidium. In addition, appressorium formation was assessed on 200 germinated conidia and the length of forty germ-tubes was measured.

Fixation and Staining of Tissues

Leaves of clones RRIC 52, RRIC 105, PB 86 and Tjir 1 sampled from protected buds were inoculated and leaf discs were removed as described above, but at 12, 24, 36, 48, 60, 72 and 96 h after inoculation. Leaf discs were dehydrated according to the procedure of Johansen[6] and embedded in paraffin wax (M.P. 63°C). Sections 8–10 μm thick were cut with a rotary microtome and stained with safranin and fast-green. Other sections were stained with Sudan III to examine the cuticle.

RESULTS

Conidia germinated on leaves within 3 – 4 h *(Table 1)*. At 3 h after inoculation generally more conidia had germinated on susceptible clones than resistant ones; but the differences were relatively small and not always significant (at P = 0.05). After 3 h, conidia germinated equally well on all the leaves irrespective of the degree of field resistance shown by the clones. At 12 h and 16 h after inoculation, compared to water control, germ-tubes were significantly longer on susceptible clones *(Figure 1)* and by 16 h they had grown to such an extent that they intermingled and were difficult to measure.

On susceptible clones, appressoria were generally first observed 4 – 6 h after inoculation and their number increased progressively *(Figure 2)*. However, some germ-tubes continued to elongate without forming appressoria and produced secondary conidia. On resistant clones, some appressoria formed as early as 3 h after inoculation, but these were senile or developed at the end of a short germ-tube. More appressoria formed on resistant clones than susceptible clones. On both resistant and susceptible clones, most appressoria

TABLE 1. MEAN PERCENTAGE GERMINATION OF CONIDIA OF *COLLETOTRICHUM GLOEOSPORIOIDES* ON LEAVES OF TEN *HEVEA* CLONES AT DIFFERENT TIMES AFTER INOCULATION

Clone	Mean percentage germination[a]			
	3 h	4 h	8 h	12 h
Susceptible[b]				
RRIC 103	60.0	99.2	99.3	99.7
FX 4098	58.0	95.7	99.0	100.0
PB 86	62.0	98.9	99.2	99.7
Tjir 1	60.9	98.0	100.0	100.0
Resistant[b]				
RRIC 52	54.6	99.0	99.8	100.0
RRIC 100	57.0	92.3	99.3	100.0
RRIC 104	54.0	98.6	100.0	100.0
RRIC 105	55.5	95.4	99.3	99.0
RRIC 117	56.2	97.6	100.0	100.0
IAN 873	59.0	96.1	100.0	100.0
Water control	49.8	94.5	98.6	100.0
L.S.D. (P = 0.05)	5.46	N.S.	N.S.	N.S.

[a]Assessed on 400 conidia

[b]Based on field observations

N.S. = Not significant

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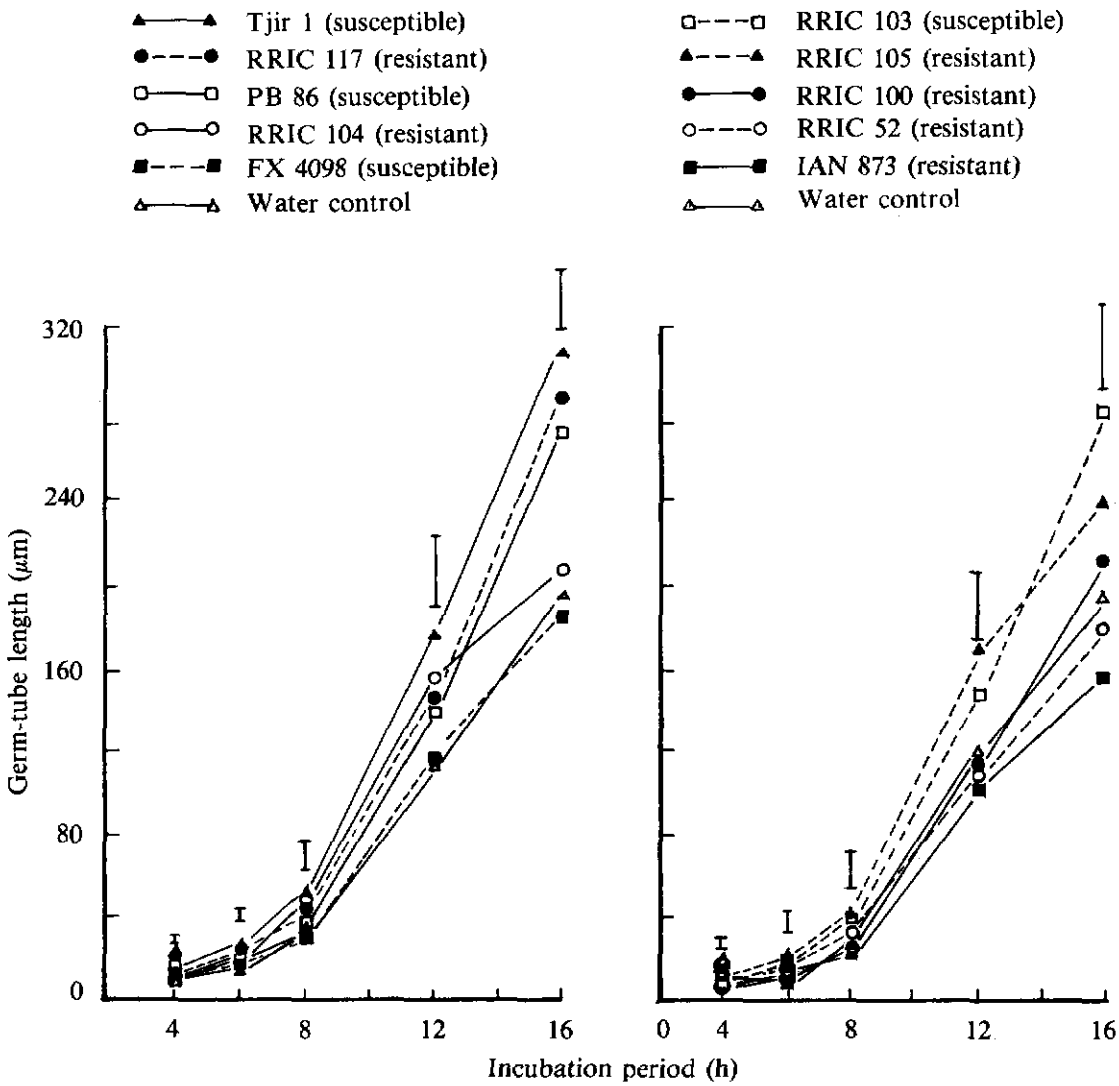

Figure 1. Germ-tube growth of C. gloeosporioides *on the leaf surface of ten* Hevea *clones at different times after inoculation.*

(about 70%) formed between the anticlinal walls of two adjacent epidermal cells. Few appressoria formed on the glass slides.

In cleared leaf discs of clones RRIC 52(R), RRIC 105(R), PB 86(S) and Tjir 1(S), the infected epidermal cells showed granulation within 8 - 12 h of inoculation. This was always followed by deep discolouration resulting in the necrosis of epidermal cells. This process intensified with time. In resistant clones, cell discolouration occurred rapidly and affected a large number of cells around the infected site *(Figure 3)*. However, the brown discolouration that occurred in susceptible tissues was less intense and was confined to a few infected cells. A darkly stained substance also accumulated in the intercellular spaces around the penetration

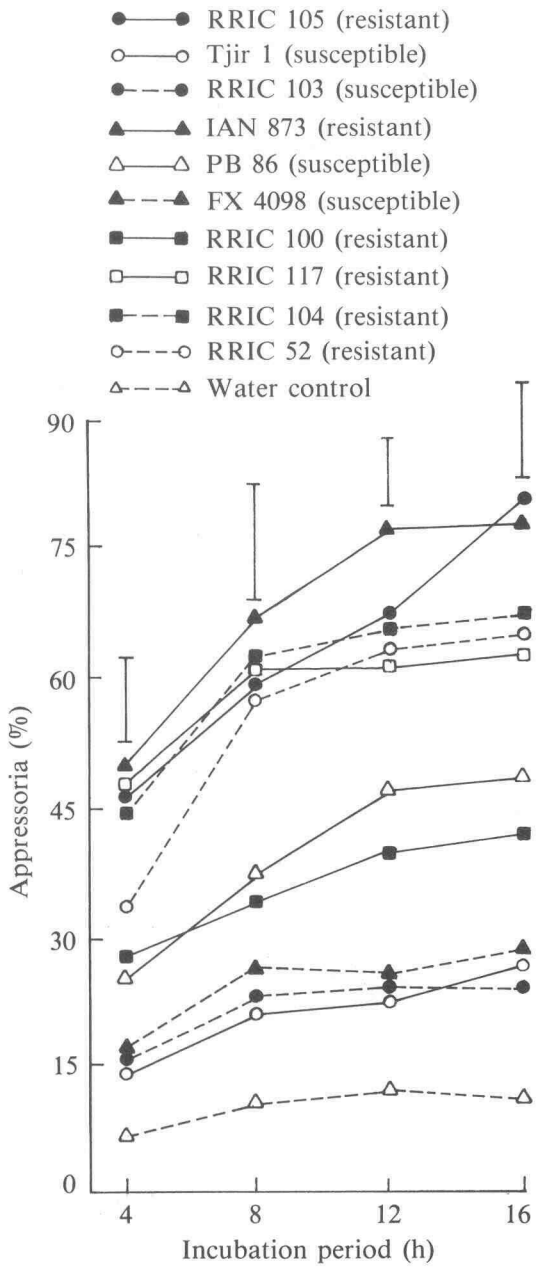

Figure 2. Appressorium formation by C. gloeosporioides *on the leaf surface of ten* Hevea *clones at different times after inoculation.*

sites. However, no distinction could be made between resistant and susceptible clones on the basis of this darkening.

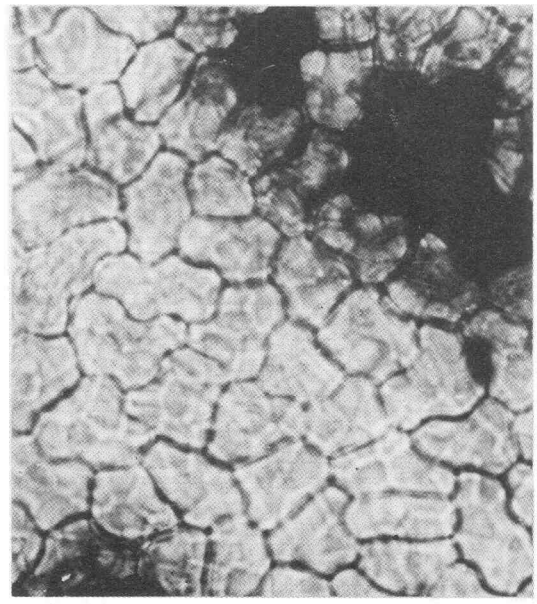

Figure 3. Necrosis of affected epidermal cells of resistant clone RRIC 105, 24 h after inoculation.

Post-penetration Behaviour

In both resistant and susceptible tissues, initial penetration occurred directly below the appressorium. No chemical change or dissolution of the cuticle was observed when it was stained with Sudan III. Also, no mechanical disruption was seen. The fungus had penetrated the tissues 24 h after inoculation and sites of penetration were indicated by disorganisation of the underlying epidermal cells. The changes that occurred were indicated by a deep discolouration.

In susceptible clones only the penetrated epidermal cells showed necrosis. Some appressoria which formed on resistant clones stayed without causing any change in the cells below them while others caused penetration resulting in intensive discolouration in the epidermal cells, commencing around the inner side of the cell wall *(Figure 4)*. This progressed rapidly causing more damage to neighbouring epidermal cells eventually leading to their death. When the fungus had gained entry through the cuticle and the cell wall, in susceptible clones, the hyphae

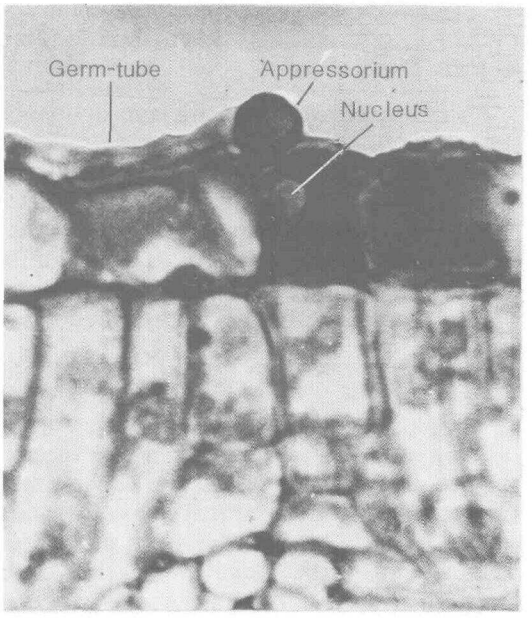

Figure 4. Host reactions in epidermal cells of resistant clone RRIC 105 following penetration of C. gloeosporioides, *24 h after inoculation. Note the movement of the nuclei towards the penetration site and the brown discolouration beginning around the cell wall.*

immediately enlarged and entered the lumen of the epidermal cells and appeared as a tube-like structure. Secondary hyphae that originated from primary hyphae continued to grow deeper into mesophyll cells in susceptible clones even when the infected epidermal cells remained discoloured. These hyphae invaded the cells intercellularly, mostly close to the cell wall. Occasionally, one or two palisade cells were discoloured. At 60 h after inoculation, hyphae had profusely branched and colonised the mesophyll cells both intercellularly and intracellularly. Some hyphae grew abundantly within the tissues below the cuticle and gave rise to acervuli over the leaf surface *(Figure 5)*. Mature acervuli were formed 96 h after inoculation.

In resistant clones though penetration had occurred 24 h after inoculation hyphal growth was restricted to epidermal cells. Palisade cells were disorganised below the epidermal cells where the fungal growth was arrested. This reaction intensified at 48 h after inoculation. These cells which were deformed showed high affinity to the fast-green stain. Although complete cell collapse was not observed at the early stages of infection, the cytoplasm of some cells in the infected area showed some disorganisation. When compared to susceptible clones, acervuli were rarely formed in resistant clones 72 h after inoculation and the hyphae were restricted to the subcuticular region *(Figure 6)*. The movement of nuclei in both epidermal and palisade cells in response to infection was a phenomenon frequently noted as early as 12 h after inoculation in resistant clones, even before the occurrence of granulation of the cells *(Figure 7)*. The nuclei of the infected epidermal cells moved towards the penetration point *(Figure 5)* and those which were adjacent to epidermal cells also moved towards the infected cells. The nuclei which lie normally in the middle of the palisade cells in the leaf tissues, moved towards the infected epidermal cells.

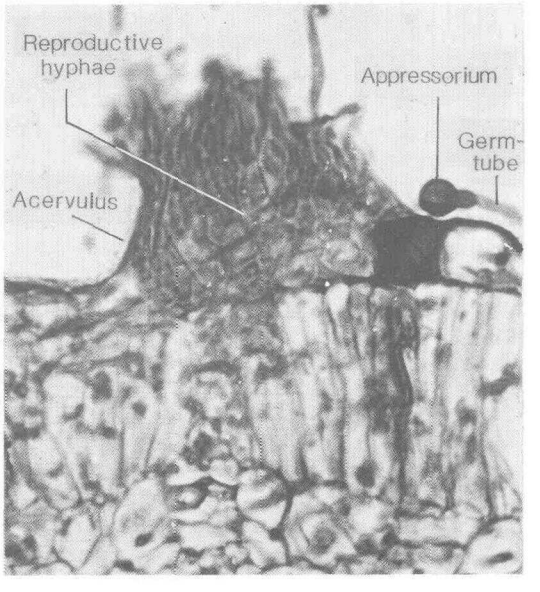

Figure 5. Formation of acervulus of C. gloeosporioides *following penetration into susceptible clone PB 86, 72 h after inoculation. Note the intercellular hypha within the discoloured epidermal cell below the appressorium.*

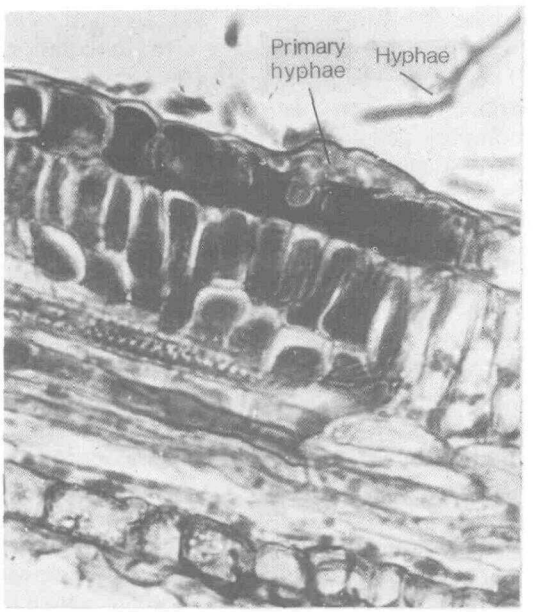

Figure 6. Arrest of fungal growth of C. gloeosporioides *in resistant clone RRIC 105, 72 h after inoculation.*

DISCUSSION

These studies indicate that spore germination occurred equally well on all the clones but stimulatory effect on germ-tube growth on susceptible clones could have been due to nutritional substances which accumulated in the inoculum drops with development of incubation period[7]. However, a similar effect was not seen on resistant clones. Presence of inhibitory substances in these clones[3] could have counteracted the nutritional effect.

The large number of appressoria formed in resistant hosts can help increase the number of penetration sites, especially in incompatible hosts. The factors that induced their development seem to be more complex and require more specific conditions then spore germination[8]. Acidic substances which are associated with *Hevea* leaf waxes stimulated appressorium formation[7]. The orientation of appressoria between two epidermal cells on the leaves may be due to the production of specific substances at these depressions. Some bacteria are known to stimulate appressorium formation of *C. gloeosporioides*[9] and these could multiply at such sites.

There was no evidence for chemical dissolution of the cuticle or mechanical penetration of the host. However, ultra-structural studies of the fungus penetrating other hosts[10] revealed that if the enzymes were involved they were restricted to the area adjacent to the tip of the infection peg, which could be small in size as in *Populus*[11] and Robinson tangerines[10].

The penetration sites in both resistant and susceptible host tissues could be seen within 12 - 24 h after inoculation, but some appressoria on resistant clones appeared to be inactive suggesting that cuticular factors could reduce the penetration into epidermal cells as explained by Wilson and Coffey[12]. Although the appressoria were formed within 4 - 5 h they could remain inactive and form infection hyphae even after 72 h , causing effective penetrations[13]. The nuclear movement, a phenomenon less frequently reported[14,15] is one of the early

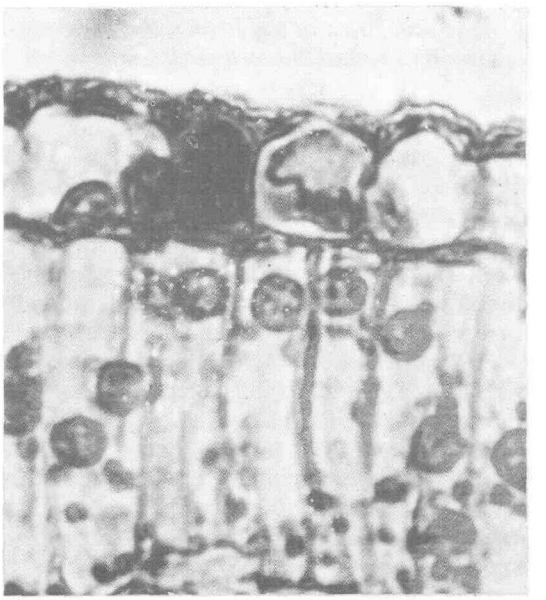

Figure 7. Migration of nuclei towards the infected site of resistant clone RRIC 105, 12 h after inoculation. Note the discoloured epidermal cells and the orientation of nuclei.

cytological reactions in response to infection. Although this was observed 12 h after inoculation, it could be the first indication of a hypersensitive reaction as described by Contreras and Boothroyd[16]. The migration of nuclei towards the infection site possibly helps the host cells to produce more proteins, and in turn enzymes required for the increased metabolic activity at these sites.

Following penetration, colonisation of the pathogen within the host tissue took place as described by Marks *et al.*[11], Liyanage and De Alwis[3], Brown[17] and Te Beest *et al.*[13] The primary hyphae grew intracellularly and secondary hyphae invaded the lumen of epidermal and palisade cells. Subsequent growth of secondary hyphae continued both intracellularly and sometimes intercellularly. It was observed that in resistant clones, growth of the fungus was restricted without the formation of acervuli which limited sporulation, resisting further spread of the pathogen[18]. This could take the form of incomplete resistance, or complete resistance[19]. The defence reaction of resistant *Hevea* clones to *C. gloeosporioides* was first seen 12 h after inoculation, at which stage there was an increased granulation of the epidermal cell surrounding the penetrated cells, followed by a brown discolouration. These changes took place more rapidly in resistant clones than in susceptible ones. These observations are consistent with those observed for *C. lindemuthianum*[20]. The formation of granules and discolouration of cells are events commonly associated with resistant reactions[21]. Muller[22] pointed out that in diseases involving necrotropic fungi, the spread of the pathogen often appears to depend on the balance between the pathogenic activity of the fungus and the speed of counteraction by the host.

Although browning indicates the death of host cells[21], in this study no complete cell collapse of the host tissues was seen at the early stages of pathogenesis; at later stages only some infrequent cytoplasmic collapse was evident. Therefore, cell collapse may not be the primary cause of post-penetration resistance as suggested by Leath and Rowall[23]. The discolouration in the cytoplasm around the inner side of the cell wall may be due to accumulation of polymerised products of polyphenols such as lignin and tannin[24]. The development of a morphological barrier by deposition of lignin was suggested as the resistant factor in cucumber to infection by *C. obicularae*[25]. Oxidative enzymes namely peroxidase which brings about polymerisation of polyphenols are synthesised in the cytoplasm itself[26] and these may be involved in initiating host-cell reaction. However, initiation of the reaction around the cell wall also suggested that enzymes responsible for it were closely associated with the cell wall.

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