

## Infection of Tobacco Callus by *Phytophthora parasitica* var. *nicotianae*

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### ABSTRACT

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Transmission and scanning electron microscopy was used to determine the response of tobacco callus tissue to inoculation with *Phytophthora parasitica* var. *nicotianae*. Callus resistant to Race 0 and susceptible to Race 1, or susceptible to Race 0 and Race 1, was used. Within 3 hr after inoculation, zoospores from compatible and incompatible races had encysted and germinated; then germ tubes of both races penetrated callus cells. Penetration often occurred in cells other than those on which the

spores germinated. By 24 hr, when a hypersensitive reaction (HR) was clearly evident in the incompatible combination, almost all of the cells in the HR area had collapsed, whereas most cells in infected areas on callus showing the compatible reaction were still turgid. After 48 hr, hyphae of the compatible fungus had penetrated <50 cell layers, whereas those of the incompatible fungus were limited to 5-8 cell layers.

It was shown earlier that the gene for resistance to *Phytophthora parasitica* Dast. var. *nicotianae* (Breda de Haan) Tucker (Ppn) in tobacco (*Nicotiana tabacum* L.) cultivars is expressed in callus cultures derived from these cultivars (7). This host-pathogen system exhibits race specificity (7), hypersensitivity (HR) (6), and phytoalexin production (1), features common to many host-parasite combinations.

This research was undertaken to provide information about the sequence and the site of events in pathogenesis of inoculated tobacco calli. Furthermore, a visual image of the infection process in resistant and susceptible callus tissues by Ppn was needed to establish further similarities between callus tissue and the intact plant.

### MATERIALS AND METHODS

**Callus tissue.** Two plant lines that had been used as parental material for our previous studies (4) and since maintained clonally, served as sources of pith callus tissues. Line 46-8 is susceptible to Race 1 (R<sub>1</sub>) but resistant to Race 0 (R<sub>0</sub>); line 49-10 is susceptible to both R<sub>1</sub> and R<sub>0</sub>. Prior to inoculation, callus tissues were grown in the dark at 25 C for 4-5 wk on Linsmaier and Skoog's medium (8) supplemented with 1 μM kinetin and 11.5 μM IAA.

**Fungus.** The fungus was grown on oatmeal agar in the dark at room temperature (20-23 C). Pieces of the mycelium (2-3 wk after transfer) were stripped off the plates aseptically and floated in 2 ml of sterile, distilled water in a 60 × 15-mm petri plate. Zoospores were obtained 3-5 days later by chilling the plate (4 C, 30 min) and then returning it to room temperature. After 30-45 min at room temperature, the concentration of zoospores was estimated with a hemacytometer and adjusted to 10<sup>5</sup> or 2 × 10<sup>5</sup>/ml as desired by adding sterile water. Then tissues were inoculated with 0.2 ml of spore suspension as previously described (3,6,7). The absence of contaminating microorganisms was confirmed by inoculating flasks containing nutrient broth with 0.1-ml portions of the inoculum.

**Electron microscopy.** Calli of line 46-8 were inoculated with R<sub>0</sub> and R<sub>1</sub> of Ppn, and at 20 min and 2, 6, and 24 hr after inoculation;

pieces of tissue were fixed for 2 hr in 5% glutaraldehyde in 0.08 M cacodylate buffer (pH 7.4), rinsed twice in cacodylate buffer, and postfixed in 1% osmium tetroxide in veronal acetate buffer, pH 7.4 (2). The tissue pieces were dehydrated in a graded series of acetone and embedded in Spurr's embedding medium. Uranyl acetate staining was done during the 70% dehydration step (70% acetone saturated with uranyl acetate for 4 hr), and lead citrate was used as a postsection stain. Four grids with at least 10 sections each from at least three tissue blocks per time period were viewed in a JEM7 electron microscope.

For scanning electron microscopy (SEM), calli were harvested 24 and 48 hr after inoculation. The calli were trimmed, fixed in 5% glutaraldehyde in cacodylate buffer (0.08 M, pH 7.4), dehydrated in a graded series of ethyl alcohol, frozen in liquid nitrogen, and then split perpendicular to the inoculated surface. The split pieces were then critical-point dried and glued to specimen stubs with the split surface facing the viewer. Metal (gold) was used to coat (200-300 Å thickness) the specimen, which was then viewed (50 mm<sup>2</sup> of tissue) in a model JSM-43 scanning electron microscope. In some of the earlier experiments, tissues (3 hr after inoculation) were not split, but the calli were mounted with the inoculated side facing the viewer (75 mm<sup>2</sup> were viewed).

### RESULTS

The HR of resistant tissue was discernible within 6 hr of inoculation. By 24 hr after inoculation, necrosis (darkened cells) had occurred in the resistant, but not in the susceptible, tissues. Necrosis was judged by changes in ultrastructure of the cells either in direct contact with, or at some distance from, penetrating hyphae or zoospores (Figs. 1 and 2). Necrosis was observed six to nine cells from the actively growing hyphae in the incompatible combinations. There was disorganization of the cytoplasm, vesiculation, loss of tonoplast integrity, and breakdown of mitochondria (Figs. 1 and 2). Although many mitochondria in adjoining cells appeared to be intact, membrane invaginations often resulted in doughnut-shaped mitochondria when they were viewed in cross section. Onset of necrosis was manifested by the formation of osmophilic globules in cells (top of Fig. 2) adjoining those that showed advanced necrosis (bottom of Fig. 2).

The contrast between the ultrastructure of cells from incompatible and compatible combinations was striking because of the complete lack of necrosis in the compatible combination (Fig. 3). Zoospores germinated and penetrated the susceptible cells

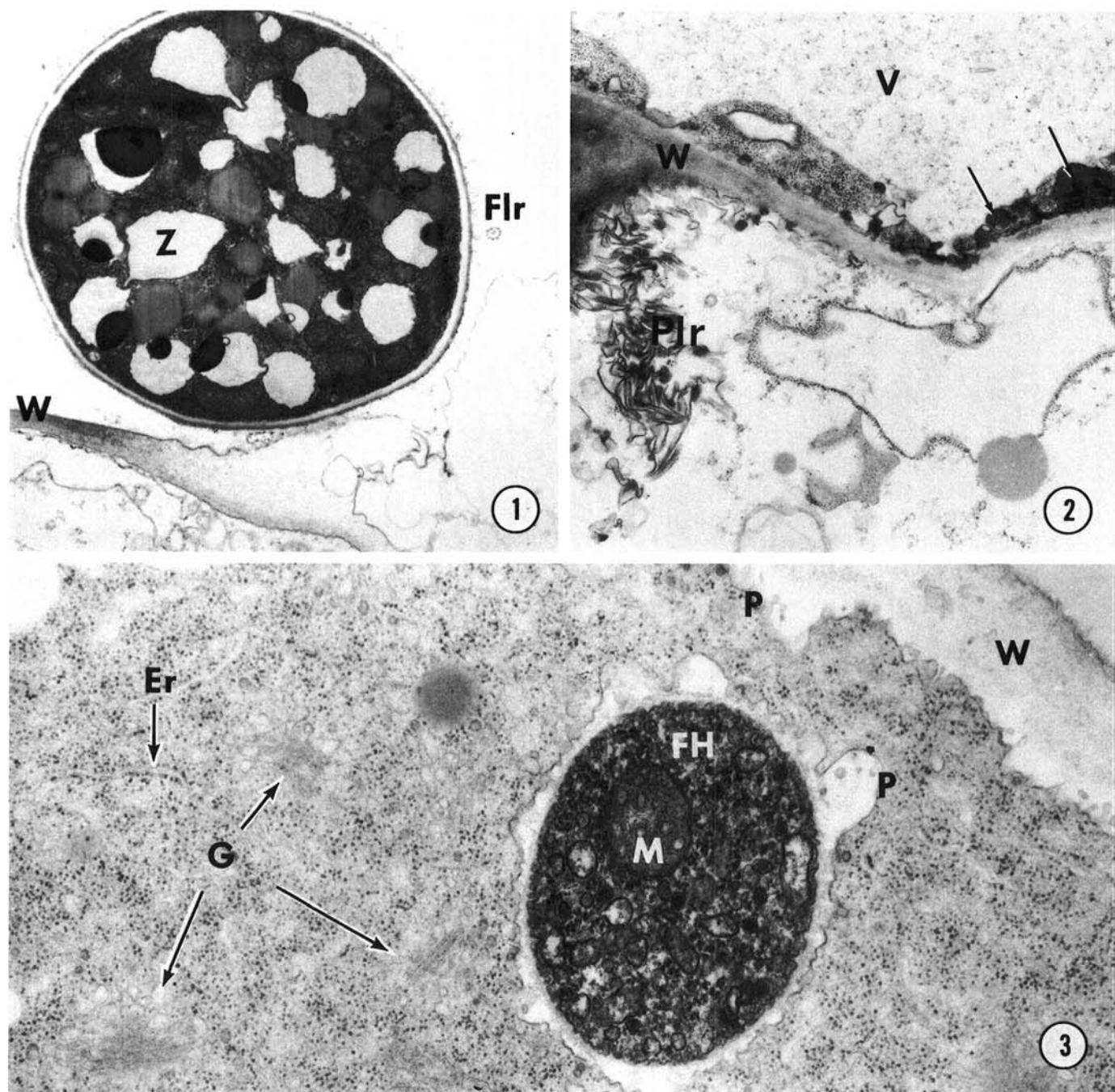
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without perceptible changes in the ultrastructure of host cells except for a wall lesion, similar to that described by Hanchey and Wheeler (5). The number of Golgi bodies increased in the immediate vicinity of the intracellular hyphae. The concentration, ultrastructure, size, and shape of mitochondria, peroxisomes, endoplasmic reticulum, and ribosomes in the cytoplasm appeared to be unaltered by the presence of the fungus for at least 48 hr after inoculation.

With scanning electron microscopy, the surfaces of resistant (46-8) and susceptible (49-10) calli appeared to be identical prior to

inoculation. Most zoospores had encysted and germinated on both types of callus 3 hr after inoculation, but penetration was not limited to that cell on which encystment occurred (Fig. 4).

By 24 hr after inoculation, the surface cells of the compatible combination showed little cell collapse (Fig. 5), which was mainly related to preparative procedures. In the incompatible combination, most of the surface cells had collapsed (Fig. 6). By 48 hr after inoculation, the fungus had penetrated more than 50 cell layers into the susceptible calli (Fig. 7), but only five to eight cell layers had been penetrated in the incompatible combination.



**Fig. 1-3.** Transmission electron microscopy of resistant (line 46-8) and susceptible (line 49-10) tobacco callus tissue after inoculation with *Phytophthora parasitica* var. *nicotianae*. **1,** An encysted zoospore (Z) of Race 0 of Ppn on resistant (46-8) tissue, 6 hr after inoculation. Note the disarray of the parietal cytoplasm in the cell immediately beneath the spores. Note also the flagellar remains (Flr). (Wall = W) ( $\times 10,200$ ). **2,** Resistant tissue (46-8) necrosis in advance of the fungus, 6 hr after inoculation. Note that tissue collapse has progressed to complete disintegration and disorganization of the membranes and the cytoplasm in the lower cell. In the upper cell, an earlier stage of necrosis can be observed. Note the intact vacuole (V), and the cytoplasm with ribosomes. Osmiophilic globules (arrows) indicate necrosis ( $\times 17,500$ ). **3,** A hypha (FH) of Ppn penetrating a susceptible cell (49-10), 24 hr after inoculation. Note both the intact plasmalemma (P) of the host near the wall (W) and the penetrating hypha (FH). The cytoplasm does not differ structurally from that of uninfected cells. (Er = endoplasmic reticulum, G = Golgi zones, and Plr = plastid remains) ( $\times 35,000$ ).

## DISCUSSION

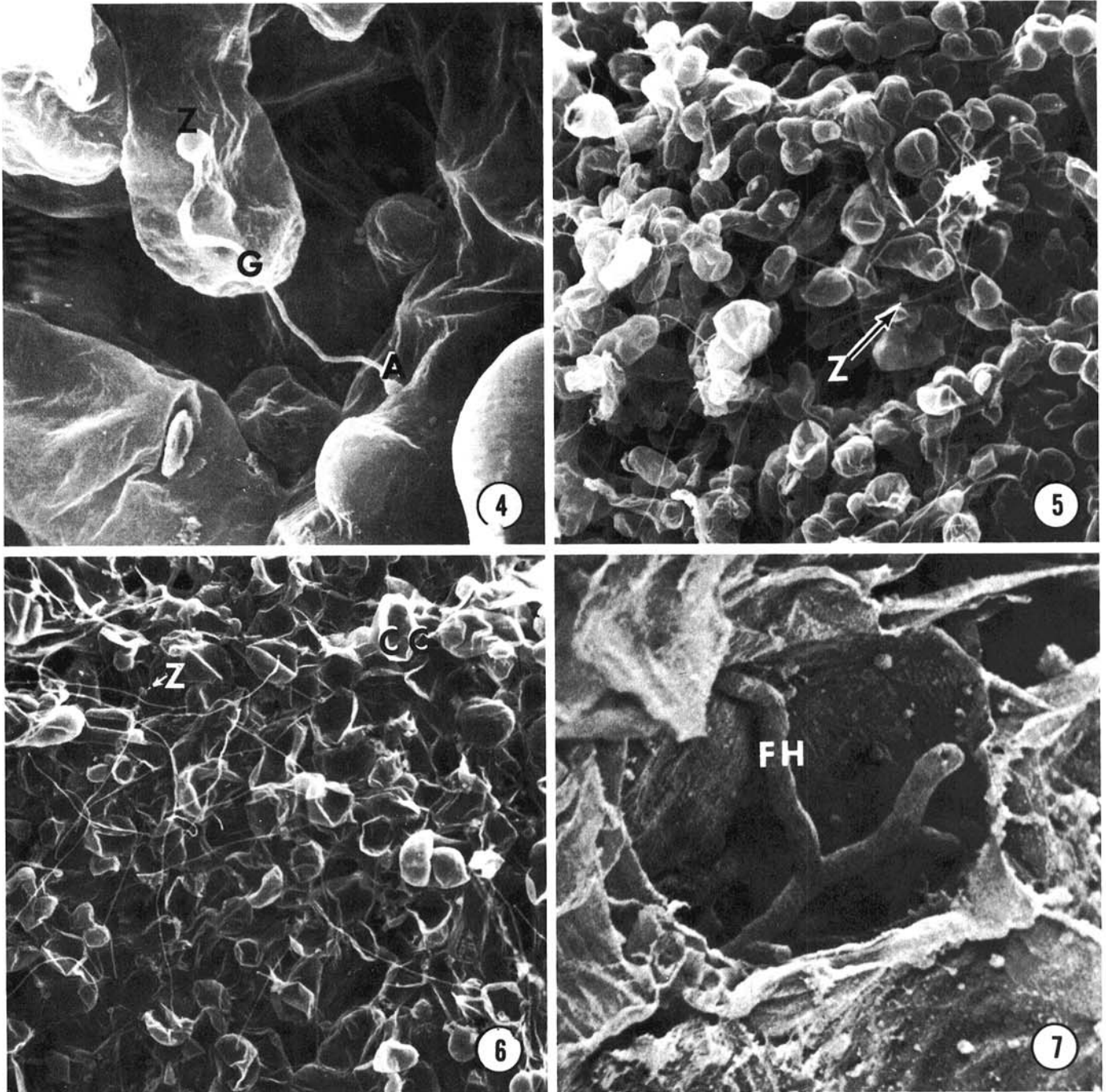
We have contended in the past that plant tissue culture provides an opportunity to study the expressions of resistance and susceptibility under well-defined conditions (3,6). It was shown previously (3,6) that the host-pathogen system used in this study allows for the expression of genes for resistance to *Phytophthora parasitica* var. *nicotianae*.

In this study, we have shown that the ultrastructural changes occurring in the resistant and susceptible callus tissues inoculated with Ppn zoospores are strikingly similar to those that occur in

intact plants roots invaded by Ppn as described by Hanchey and Wheeler (5).

The HR of cells of calli in the incompatible combination is first evident by the early necrosis of invaded cells and of those in the immediate vicinity. The convoluted nature of the callus surface made it difficult to determine exactly how far in advance of the fungus necrosis had occurred. Within the time frame in which these phenomena were studied (48 hr), we did not see an HR of susceptible tissue. These results were identical to those reported earlier (4).

By using scanning electron microscopy, we have shown that



**Fig. 4-7.** Scanning electron microscopy of resistant (line 46-8) and susceptible (line 49-10) tobacco callus tissue after inoculation with *Phytophthora parasitica* var. *nicotianae*. **4.** An encysted zoospores (Z) on a callus cell at the inoculated surface, 3 hr after inoculation. A germ tube (G) and an appressorium (A) have been formed. The latter is formed on a cell spatially removed from the cell where germination was initiated ( $\times 400$ ). **5.** Germinating zoospores (ZX) and hyphal development, 24 hr after inoculation. Note that callus tissue (susceptible) has remained turgid as compared with that in Fig. 6 ( $\times 100$ ). **6.** Resistant tissue, 24 hr after inoculation. Zoospores (Z) germinated and hyphae developed. Note the general loss of turgidity of this tissue and the many collapsed cells (CC) ( $\times 100$ ). **7.** A freeze-fractured cell in the 45-50th cell layer from the surface of this susceptible callus, 40 hr after inoculation. Note the branching fungal hypha (FH) ( $\times 1,600$ ).

germination and penetration by Ppn in compatible and incompatible combinations are quite similar. It is apparent that loss of turgor and collapse of surface cells of these calli occur only during the incompatible reaction. Coincidentally, but perhaps not causally related, penetration of resistant calli did not extend deeper than five to eight cell layers from the surface. In contrast, when no HR occurred in the compatible combination, penetration by the fungus continued unabated.

This study provides additional evidence for the validity of using tissue culture for the study of molecular events involved in expression of resistance. Clearly, within 24 hr, the important events for resistance are established. Thus, this study provides the time framework of pathogenesis for further physiological studies.

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