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THE ULTRASTRUCTURE AND HISTOCHEMISTRY OF INFECTION BY COLLETOTRICHUM LAGENARIUM IN CUCUMBERS INDUCED FOR RESISTANCE

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THE ULTRASTRUCTURE AND HISTOCHEMISTRY OF INFECTION BY COLLETOTRICHUM LAGENARIUM IN CUCUMBERS INDUCED FOR RESISTANCE

By

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ABSTRACT

THE ULTRASTRUCTURE AND HISTOCHEMISTRY OF INFECTION BY COLLETOTRICHUM LAGENARIUM IN CUCUMBERS INDUCED FOR RESISTANCE

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Induced resistance in cucumbers seems to be a function of the epidermis. Since induced resistance is nonspecific, it has been hypothesized that the inhibition of penetration into cells is one of the primary mechanisms. Whether inhibition is a function of cell walls or cytoplasm is not known. To determine the possible location of induced resistance, cucumber plants induced for resistance by injection with Pseudomonas syringae pv syringae were compared to control plants by electron microscopy, ultrastructural histochemistry, and energy dispersive X-ray microanalysis. Plants were also assayed for peroxidase, phenolics, and extensin in the petiolar epidermis and bulk petiole of induced and control cucumber plants. Leaves of induced plants, which were infected by Colletotrichum lagenarium, were observed to have electron-dense modifications of the walls of infected cells. Energy dispersive X-ray microanalysis (EDS) of these cell walls showed that silicon accumulation accounted for much of the electron-density. Silicon was found in cells prepared by standard glutaraldehyde/osmium tetroxide fixation and by the

bromine procedure for lignin localization. These cell wall areas also stained with potassium permanganate indicating the presence of lignin. The binding of potassium permanganate to the electron-dense areas was corroborated by EDS for Mn. Papillae developed where the fungus started to penetrate into epidermal cells. Papillae also stained with potassium permanganate; this was corroborated by EDS. Hydroxyproline, a component of the cell wall protein extensin, was not found to vary in concentration as a result of cucumber plants being induced for resistance. Peroxidase, which is thought to be responsible for polymerizing extensin and lignin, was found to occur in increased concentrations in the epidermis and bulk tissue of the petioles of induced cucumber plants. The epidermis of induced plants appeared to have higher concentrations of lignin than the petiole, a further indication that the epidermis is important in induced resistance. Carbazole staining of peroxidase for light microscopy also showed higher concentrations of peroxidase in induced cucumber leaves, though localization to a cell type was not possible. Diaminobenzidine staining for the electron microscopic localization of peroxidase suggested that peroxidase was concentrated between the electron-dense cell walls and the plasmalemma.

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LITERATURE REVIEW

LITERATURE REVIEW

Historic review of induced resistance

Acquired, systemic resistance in plants, which is induced by prior infection, occurs in a number of plant/pathogen interactions. Early studies were extensively reviewed by Chester (1933), who gave abundant evidence for the existence of acquired resistance but attributed the cause to a plant immune system based on antibodies. Much of the recent progress in determining the nature of this phenomenon dates from the studies of Gilpatrik and Weintraub (1952). They reported that viral infection of Dianthus barbatus with carnation mosaic virus caused the development of systemic protection in uninfected and apparently virus-free leaves against further virus infection. If this effect had been due to cross protection of one virus strain against another, the protected leaves would also have been systemically infected with the original virus.

Further studies by Ross (1961) showed that infection of the three lower leaves, of trimmed five leaf tobacco plants with tobacco mosaic virus (TMV), induced resistance against challenge inoculation with TMV in the upper two leaves. Lesion size on the induced leaves was reduced and generally there were fewer lesions. No TMV could be recovered from

the leaves to be challenged before the challenge inoculation. Resistance could not be induced by wounding or by chemical injury. Ross (1961) further investigated the time course of the development of resistance and reported that resistance to TMV developed after a lag of two days, reached a maximum at 7-10 days and persisted for at least 20 days. Either potato virus X (PVX) or tobacco ringspot virus was also effective in inducing resistance against TMV challenge. Plants induced by TMV were resistant against turnip mosaic virus, another lesion producing virus. Viruses that did not produce lesions did not induce resistance. Ross (1961) concluded that any leaf necrosis producing virus was as equally effective as TMV in inducing resistance against further virus infection.

Induced resistance was also reported in plants that were infected by fungi (Chester, 1933). Hecht and Bateman (1964) reported that tobacco, inoculated with the fungus Thielaviopsis basicola, developed necrotic lesions and afterwards were resistant to TMV and TNV infection. Yarwood (1954) reported that pinto bean leaves, previously infected with bean rust, Uromyces phaseoli, were comparatively resistant to further infection by bean rust. He also noted that germination of rust uredospores on bean leaves could be inhibited by the presence of either rust spores or by the close proximity of rust-infected leaves. He concluded that the inhibitor was gaseous in nature and was given off by the

fungus. Yarwood (1956) duplicated this experiment using U. phaseoli to induce sunflowers which were challenged with Puccinia helianthi and also used P. helianthi to induce beans for resistance against U. phaseoli. Both fungi therefore induced resistance against the other fungus.

The systemic nature of induced resistance in plants infected by fungi was demonstrated by Cruikshank and Mandryk (1960). Their research used tobacco plants that were inoculated with Peronospora tabacina, which causes a stem infection. The plants became more resistant to subsequent infection by P. tabacina than plants which were not previously infected. The effect was small on the mature portions of the plant and was greatest on those parts which developed after the infection.

Bell and Presley (1969) reported that attenuated or heat killed conidia of Verticillum albo-atrum were able to induce resistance in cotton, demonstrating that live pathogens were not necessary to induce resistance in certain cases.

Kuć et al. (1975) determined that Cladosporium cucumerinum or Colletotrichum lagenarium could be used to systemically protect against further infection by C. lagenarium in eight cultivars of cucumbers susceptible to infection by C. lagenarium. The size and number of lesions on protected plants were smaller than those on the control plants. Protection could be achieved by low levels of C. lagenarium such that only a single lesion of the protective

organism on a leaf could confer resistance. Damage of leaves by dry ice did not produce protection. Cucumber plants susceptible to infection by C. cucumerinum were also protected by infection with Colletotrichum lindemuthianum, a bean pathogen (Hammerschmidt et al., 1976).

Richmond et al. (1979) determined that induced resistance to C. lagenarium in cucumbers induced by C. lagenarium was caused by reduced penetration of appressoria on induced resistant plants compared to the number of appressoria that penetrated into uninduced plants. The number of conidia that germinated to form appressoria on induced and uninduced plants did not differ (Richmond et al., 1979; Hammerschmidt and Kuć, 1982). In an earlier study, Jenns and Kuć (1977) reported that the size of the lesions produced by C. lagenarium on cucumber plants, induced by tobacco necrosis virus, was also smaller than those on uninduced plants. When the epidermises of induced resistant and genetically resistant leaves were removed and the remainder of the leaf challenged by C. lagenarium, the induced resistant leaves were found to be susceptible to infection, but the leaves of plants with constitutive resistance remained resistant to infection (Richmond et al., 1979). This indicated that induced resistance in cucumbers was mediated by the epidermis.

Hammerschmidt and Kuć (1982) noted the lack of penetration by appressoria of C. lagenarium into protected

epidermis and found that lignin formed in petiolar epidermal cells, directly under appressoria. Lignin, though, was associated only with some of the appressoria which failed to penetrate. In other cases, penetration or failure of penetration occurred with no signs of lignification and, in a few of the cases lignification and penetration occurred together. A large percentage (91.7%) of the penetrations by Cladosporium cucumerinum into protected tissue were lignified, compared to the small percentage in unprotected tissues (6.2%) (Hammerschmidt and Kuć, 1982). Increases of lignin content in cucumber apical tissue occurred in protected plants challenged by C. cucumerinum. When lignin precursors were introduced into cucumber leaf disks from plants which had been protected by prior infection with C. lagenarium, the precursors were incorporated into lignins faster in the protected, compared with unprotected, tissues (Dean and Kuć, 1987). Lignin deposition was postulated, therefore, to have a role in the resistance found in cucumber plants.

A systemic increase in peroxidase activity was found in the second true leaf of cucumber plants that were protected by C. lagenarium injection in the first true leaf (Hammerschmidt et al., 1982). As in earlier reports, injury with dry ice did not produce resistance and did not cause the systemic increase of peroxidase levels above that of uninjured plants. Smith and Hammerschmidt (1988) compared

the total activity of the soluble acidic peroxidases of cucumber, muskmelon and watermelon and reported that systemically induced leaves had a two-fold or greater activity compared to control leaves. Peroxidases in these species were found to migrate as a cluster of three bands on high pH native polyacrylamide gels (Smith and Hammerschmidt, 1988).

Research on the source and movement of the signal that causes systemic protection was done in cucumber plants by grafting and removing the first leaves, which were infected by C. lagenarium (Dean and Kuć, 1986). Leaves grafted on an infected plant became resistant, as long as the induced leaf remained on the plant. Induced plants, where the infected leaf was removed seven days after the initial infection and challenged ten days later, were characterized by a progressive lessening of the level of resistance, in newer leaves, to challenge by C. lagenarium. This demonstrated that the inducing signal comes from only the infected leaf (Dean and Kuć, 1986). Salicylic acid was shown to increase in concentration in cucumber plants induced to become resistant with both C. lagenarium and tobacco necrosis virus and to also induce resistance (Métraux et al., 1990). In work with tobacco cultivars infected with tobacco mosaic virus, it was found that salicylic acid levels increased in resistant but not in susceptible cultivars and that induction of proteins related to resistance rose in parallel

to salicylic acid (Malamy et al., 1990). These two studies suggested that the inducing signal for induced resistance may be salicylic acid.

Xuei et al. (1988) examined the ultrastructural changes that occurred in the leaves of control and induced resistant cucumber plants during infection. Plants were induced by inoculation on the first leaf with C. lagenarium and challenged with C. lagenarium on the second leaf. Few appressorial penetrations into induced leaves at 48 hours were observed to occur whereas approximately 70% of the appressoria on control leaves had penetrated host epidermal cells. They found no ultrastructural differences between induced and control leaves. Conidia on control leaves germinated to produce appressoria by 24 hours after challenge. Appressoria had electron-opaque inner walls, electron-lucent outer walls, and "associated cone-like structures". In C. lindemuthianum a similar structure was termed an 'internal flange', attached to which were 'cone-like structures' (Mercer et al., 1975). Xuei et al. (1988) did not differentiate between internal flanges and cone-like structures. Penetration into epidermal cells by penetration pegs occurred after 48 hours. Papillae occurred around the sites of penetration between the plasmalemma and plant cell wall. By 120 hours infected cells were dead. In some cases infection by fungi into induced leaves were compatible as happened in control leaves. The epidermal cell walls under

some appressoria were more electron-dense than was found in control leaves. Fewer appressoria were seen to have penetrated epidermal cells by 48 hours than were seen in the control.

Ultrastructure of fungal infection

The infection of plants by other members of the genus Colletotrichum has been studied ultrastructurally. Politis and Wheeler (1973) examined the infection of maize leaves by C. graminicola. Compatible (Mercer et al., 1975) and incompatible interactions were studied in Phaseolus vulgaris infected by C. lindemuthianum (O'Connell et al., 1985). Both C. graminicola and C. lindemuthianum infected in the same manner as C. lagenarium by directly penetrating through the epidermal wall by means of melanized appressoria from which penetration pegs develop. C. graminicola only took 9 hours to begin penetration after inoculation with conidia (Politis and Wheeler, 1973). Penetration by C. lindemuthianum into Phaseolus vulgaris was reported to begin 40 to 50 hours after inoculation (Mercer et al., 1975). Papillae formed in maize infected by C. graminicola (Politis and Wheeler, 1973). The localization of hydroxyproline-rich glycoproteins (HRGPs) in papillae, and of chitin by immunocytochemistry were described for Phaseolus vulgaris cultivars infected by races of C. lindemuthianum (O'Connell et al., 1990; O'Connell and Ride, 1990). HRGPs were found in the cell walls of living cells next to dead

hypersensitive cells, which resulted from the incompatible reaction to infection of the hypocotyl of Phaseolus vulgaris by Colletotrichum lindemuthianum (O'Connell et al., 1990). HRGPs were suggested to act as a mechanical barrier to further fungal penetration and as a place for deposition of lignin (O'Connell et al., 1990).

Wolkow et al. (1983) examined the effects of melanin biosynthesis inhibitors on the ultrastructure and pathogenicity of C. lindemuthianum. Comparisons of melanin minus mutants and melanin biosynthesis inhibitor treated conidia with normally melanized controls showed that melanin is necessary for penetration of epidermal cell walls. The ability of Magnaporthe grisea, the rice blast pathogen, to penetrate through the epidermal wall was also found to depend on the melanization of the appressoria (Howard and Ferrari, 1989). Melanin was found to allow appressoria to resist plasmolysis when placed in higher solute concentrations than could unmelanized appressoria. It was hypothesized that melaninization creates a permeability barrier allowing for a high internal hydrostatic pressure in the appressoria which enabled the penetration peg to pierce the plant cell wall (Howard and Ferrari, 1989).

Role of lignin and peroxidase in resistance

Hammerschmidt et al. (1985) examined the nature of constitutive resistance in cucurbits. They found that epidermal peels from cucurbit hypocotyls inoculated with C.

lagenarium, C. atramentarium and Helminthosporium carbonum, which were stained by phloroglucinol-HCl, were also stained by $KMnO_4$. Treatment of epidermal peels with hot (80°C) aminoethanol, which is a delignifying agent, removed the material which reacted with phloroglucinol-HCl and $KMnO_4$. Hammerschmidt et al. (1984) determined that increases in hydroxyproline, a constituent of extensin, and lignin occurred in cucumber cultivars resistant to Cladosporium cucumerinum but not in susceptible cultivars. Earlier, Esquerré-Tugayé et al. (1979) found that the resistance of muskmelon (Cucumis melo L.) to C. lagenarium could be enhanced by treating seedlings with hydroxyproline or by treatment with ethylene which increased levels of extensin in plants.

Lignin has been localized, using phloroglucinol staining for light microscopy, around the sites of appressoria on the petiolar epidermis of cucumbers with induced and cucurbits with non-host resistance, where it appeared to cause the inhibition of fungal penetration (Hammerschmidt and Kuć, 1982; Hammerschmidt et al., 1985). Lignins were shown to be polymerized by peroxidase (Negrel and Lherminier, 1987). Increased peroxidase activity occurs in induced plants, and is the result of the enhancement of the levels of acidic isozymes, which are thought to be the type of peroxidase involved in the polymerization of lignin (Negrel and Lherminier, 1987). However, peroxidase in induced plants

has not been localized using any form of microscopy (Smith and Hammerschmidt, 1988). Ultrastructural localization of peroxidase and lignin around the sites of infection could provide additional evidence supporting the role of both compounds in induced resistance.

Ultrastructural localization of lignin

Lignin has been localized at the ultrastructural level with the $KMnO_4$ staining procedure (Hepler et al., 1970; Hayat, 1986). Phloroglucinol, like $KMnO_4$, will combine with many cellular components. Both, however, stain lignin and not the other cell wall constituents, such as cellulose, hemicellulose, pectins (Hepler et al., 1970). Tests on the reactions of $KMnO_4$ with a range of substances further substantiated the lack of reactivity of $KMnO_4$ with most cell wall substances, except for polyphenolics such as lignin (Bland et al., 1971). Both phloroglucinol and $KMnO_4$ staining for light microscopy gave equivalent results on the lignification in infected cucumbers (Hammerschmidt et al., 1985). The usefulness of $KMnO_4$ staining might therefore be limited to structures, such as the cell wall, where the constituents are limited in number and the material of interest is the only one which reacts with $KMnO_4$.

Lignin has also been localized ultrastructurally in woody plants by the use of bromine as a stain, which was then detectable by energy dispersive X-ray microanalysis (EDS)

(Saka et al., 1978). This technique has not been applied to research on herbaceous plants.

Energy dispersive X-ray microanalysis (EDS) is a technique by which the X-rays produced by the interaction of the electron beam and the sample, are gathered and converted to a spectrum. Different elements have characteristic X-ray peaks (Sumner, 1983). The use of EDS in histochemical studies has occurred since the development of EDS (Hale, 1962). EDS, though, has not been extensively used for this purpose (Sumner, 1983). In general, the greatest limiting factor in the use of EDS with biological specimens has been the mobility of the ions of interest during processing of samples. Where this has been a problem in samples fast freezing techniques have been used (Sumner, 1983). Histochemical staining results in the deposition of materials which are usually not mobile. In certain stains, it is possible to determine the location of the stain due to a characteristic element which is not ordinarily found in the sample (Sumner, 1983). Then it is possible to determine the localization of the stain with an electron microscope, even if the stain is difficult to distinguish in the electron microscope, as in the case of bromine and potassium permanganate.

Ultrastructural localization of silicon

Another use of EDS is to determine the number and location of elements (Sumner, 1983). Kunoh and Ishizake

(1976) used EDS to show that silicon sccumulated around the penetration sites on barley of powdery mildew, Erysiphe graminis hordei and in the papillae. Zeyen et al. (1983) also used EDS with specimens of barley to localize silicon in papillae and in epidermal cells that died after infection by powdery mildew.

Kunoh and Ishizaki (1975) used microincineration to demonstrate the presence of silicon around infection sites of E. graminis hordei on barley but did not find silicon in cucumber cotyledons infected by Colletotrichum lagenarium. They suggested that silicon accumulation was a generalized defensive response of barley.

Ultrastructural localization of peroxidase

Behnke (1969) adapted the use of the ultrastructural technique for peroxidase staining of Graham and Karnovsky (1966) to the staining of endogenous peroxidase in animal tissue after fixation with glutaraldehyde fixation. Goff (1975) used this technique to localize peroxidase in onion root tips and determined that peroxidase was associated with the cell wall, cell membrane, as well as other membranous systems in root cells.

Resistance induced by the heat shock of seedling cucumbers

Resistance can also be induced for a shorter period of time in seedling cucumbers by heat shock (Stermer and Hammerschmidt, 1984). For the first 12 hours after the heat shock, seedlings are more susceptible to infection than are

control. After this period, resistance to infection develops and this resistance can last for over two days (Stermer and Hammerschmidt, 1984). Resistance induced by both heat shock and prior infection are nonspecific and effective against a wide range of pathogens (Stermer and Hammerschmidt, 1984; Hammerschmidt et al., 1982). Also, in both systems, resistance is correlated with the enhancement of peroxidase concentrations of induced plants (Stermer and Hammerschmidt, 1984; Hammerschmidt et al., 1982). It has been postulated that the enhanced levels of extensin and peroxidase are indications of the increased deposition of cell wall material and that the increase in cell wall thickness is responsible for the nonspecifity of resistance due to the greater difficulty encountered by pathogens in penetrating host cells (Esquerré-Tugayé et al., 1979; Hammerschmidt et al., 1982).

Objectives

The objectives of this research were to determine what the role of the epidermal cell wall compared to the cytoplasm was in the defense of cucumber plants against infection by Colletotrichum lagenarium. This was to be accomplished by studying the fine structure of the initial events of infection to assess the relative importance of different structures in the cell wall and cytoplasm to the course of infection. Since lignin, extensin, and silicon are known to be important in disease resistance in plants,

the localization of these materials was compared with the various resistance structures in cells to see if there was a correspondence between these materials and the cell structures. Peroxidase is responsible for polymerization of lignin and extensin so the localization of peroxidase was determined and compared to that of lignin.

CHAPTER II

ULTRASTRUCTURE AND HISTOCHEMISTRY OF THE INFECTION OF CUCUMBERS, INDUCED TO BE RESISTANT, BY *COLLETOTRICHUM LAGENARIUM*

ABSTRACT

Lignin is hypothesized to be a factor in the ability of plants to resist infection. To determine if lignin could be localized around sites of infection, control (susceptible) cucumber plants and plants with systemic resistance induced by injections of Pseudomonas syringae pv. syringae were challenge inoculated with Colletotrichum lagenarium. Genetically resistant seedlings of pumpkin were also inoculated and examined. The ultrastructure of plant and fungus was examined 24 to 72 hours after inoculation. Conidia produced germ tubes with appressoria by 24 hours. Appressoria developed internal elaborations of cell wall around the origin of penetration pegs. In cucumber, penetration of plant epidermal cell walls occurred by 48 hours with papillae formed at the site of penetration. Both elemental bromine and potassium permanganate were used to histochemically stain for lignin, which has been associated with resistance in cucumbers. Potassium permanganate, used in concert with energy dispersive X-ray microanalysis (EDS), confirmed the presence of lignin in papillae and in electron-dense areas of plant cell walls directly underneath appressoria. Using EDS, silicon was localized in the electron-dense areas of plant cells in mature cucumber

leaves, but did not occur in seedling pumpkin hypocotyls. Cell walls under appressoria in seedling pumpkin hypocotyls stained with bromine, indicating lignin deposition. The staining of hypocotyl cell walls by bromine was confirmed by EDS. This is the first report of silicon in induced cucumber tissue. There seemed to be no qualitative difference in the resistance reactions that occurred in induced and control plants.

INTRODUCTION

Inoculation of cucumber plants with a variety of different pathogens can induce systemic resistance against subsequent infection. In this study, the process of infection by C. lagenarium race 1 into cucumber with induced resistance or uninduced, was examined using electron microscopy and histochemistry to localize lignin and peroxidase and to determine if the enhancement of these putative resistance factors could be further associated with resistance.

METHODS AND MATERIALS

Plant material

Seeds of cucumber plants (Cucumis sativus L., cv SMR 58) were placed in 1% sodium hypochlorite, washed in dH_2O, and left to germinate on moistened germination paper for 6 days. Seedlings were planted in clay pots and grown in greenhouses under natural light. Resistance was induced by Pseudomonas syringae pv. syringae, cultures of which were inoculated into nutrient broth 24 hours earlier, diluted to ca. 10^8 cfu/ml, and then injected into the first true leaf. Injections were done with a 5 ml hypodermic syringe, without

a needle, through abaxial stomata. Plants received a second inducing inoculation, in the second true leaf, 7 days later. Control plants were not injected, since injury does not produce the changes associated with induced resistance (Hammerschmidt et al., 1982).

Seven days after the second inducing inoculation, the third true leaf of both induced and control plants, were removed and placed into a humid chamber at 23°C. Six 30 µl droplets with 3 x 10^6 conidia/ml of Colletotrichum lagenarium (Pass.) Ell. and Halst. race 1 were placed on the adaxial surface of the leaf. These conidia were harvested into dH_2O from 8 day old cultures which were grown on potato dextrose agar (PDA) in the dark at room temperature. The conidia were filtered through four layers of cheesecloth before being used to inoculate leaves.

Eight day old pumpkin seedlings (Curcurbita pepo cv Spookie) were also inoculated on the hypocotyl with C. lagenarium using an atomizer. Seedlings were rolled into moist paper towels and kept moist and in the dark for 48 hours. Pumpkin is genetically resistant to infection by C. lagenarium and served as an example of constitutive resistance.

Electron microscopy

At 24, 48, and 72 hours, 1 mm square samples of induced and uninduced cucumber leaves were fixed overnight in 2.7% glutaraldehyde in 0.05 M, pH 6.8 phosphate buffer at 4°C

using vacuum infiltration, washed with buffer (4x), postfixed in 1% OsO_4 in buffer at 4°C for 90 minutes, and dehydrated in a graded ethanol series. After dehydration samples were prepared for scanning electron microscopy (SEM) by critical point drying in a Balzers CPD 10, gold sputter coated with an Emscope SC 500, and were examined with a JEOL 35CF SEM. Samples prepared for transmission electron microscopy (TEM) were embedded in ERL epoxy resin (Spurrs, 1969), after the ethanol dehydration, and sectioned on a Reichert Ultracut E ultramicrotome. Silver-gold sections (.90nm) were stained with saturated uranyl acetate in water followed by lead citrate (Reynolds, 1963), and examined using a Philips 201 TEM. During the course of this study 299 appressoria were examined and photographed.

Most comparisons were made at 48 hrs after infection, since at 24 hours no examples of penetration of the epidermal cell wall were seen, and at 72 hours, primary hyphae had ramified well into host cells. Penetration was found to occur at roughly 48 hours. 283 appressoria were examined from samples of cucumber leaves at 48 hours after infection by C. lagenarium.

$KMnO_4$ Staining:

Ultrathin sections were collected on nickel grids, placed into an 1.5 ml Eppendorf microcentrifuge tube filled with 1% $KMnO_4$ for 30 minutes, and washed in distilled water (Hayat, 1970). $KMnO_4$ was used because it has been found to localize

lignin (Hayat, 1970; Hayat, 1986). Sections stained with $KMnO_4$ were examined for the presence and localization of manganese with a JEOL 100CX TEM equipped with a scanning attachment and a Link Systems AN 10000 energy dispersive X-ray analyzer.

Bromine staining procedure:

At 48 hours after inoculation, 1 mm square samples of the third leaf of induced and control plants and 1 mm long portions of inoculated and uninoculated pumpkin seedlings were fixed in 2% paraformaldehyde in 0.05M phosphate buffer, pH 6.8 (Hayat, 1972). Pumpkin seedlings served as an example of constitutive resistance that were compared with the induced resistance of cucumber plants. Samples were washed in buffer (4x) and brominated by the technique of Saka et al. (1978), where specimens dehydrated in a graded ethanol series were transferred to chloroform through intermediate ethanol/chloroform steps, placed into a solution of 0.3 ml bromine in 20 ml chloroform for 3 hours and washed in chloroform (4x) to remove bromine from the solution. Samples were left in chloroform for 10 days with the chloroform being changed daily to extract out unbound bromine. Samples were taken through a chloroform/ethanol transition into ethanol and through an ethanol/ERL epoxy resin transition before being placed into 100% ERL resin (Spurr, 1969). Samples were then embedded and sectioned as described in the section on transmission electron

microscopy. Sections were not stained with uranyl acetate or lead citrate and were examined for Br and Si using a JEOL 100 CX TEM equipped with a scanning attachment and a Link Systems AN 10000 energy dispersive X-ray microanalyzer. In this procedure 21 appressoria from seedling pumpkin and 16 appressoria from mature cucumber leaves were examined.

Energy dispersive X-ray microanalysis

X-ray spectra from 0.840 ev to 20.220 ev were collected for each sample examined.

Energy dispersive X-ray microanalysis (EDS) dot maps of $KMnO_4$ stained sections and brominated material were constructed by X-ray collection of a specified ion over a 1 hour time period. Black pixels in the computer image indicated the presence of the ion.

Diaminobenzidine staining

C. lagenarium infected leaves of control susceptible and induced resistant cucumber were fixed in glutaraldehyde, as above, followed by treatment with a solution of 0.2 mg diaminobenzidine (DAB) in 1.0 ml of 0.1 M Tris-HCl, pH 7.6, with 0.2% H_2O_2 for one hour (Goff, 1975). Peroxidase catalyzes the oxidation of DAB, producing an osmiophilic precipitant. Samples were then washed twice with buffer, postfixed (stained) with OsO_4 and prepared for TEM as above. Samples of control and induced cucumber were not treated with H_2O_2 to act as a check on treated samples. Ultrathin sections were not stained with uranyl acetate or lead

citrate. The presence of peroxidase was indicated by the formation of osmiophilic deposits (Goff, 1975).

RESULTS

Inoculation of plants

Cucumber plants which were injected with Pseudomonas syringae pv syringae developed necrotic lesions at the sites of injection (Fig. 1). The second true leaf, which developed after the injection, frequently developed a type of veinal chlorosis (Fig. 2). This was especially true if the first true leaf was injected when the second true leaf was less than 1.0 cm long. If the first true leaf was injected after the second true leaf was over 30% of its full size then the vascular chlorosis did not appear. Chlorotic leaves appeared to possess less laminar than injected plants without chlorosis and, in general, were smaller in size. Also in general, injected cucumber plants were smaller than the uninjected control plants (Fig. 3).

Ultrastructure of appressoria

At 24 hours after inoculation, Colletotrichum lagenarium conidia had germinated and produced a germ tube (Fig. 4). The germ tube was variable in length. Appressoria formed from the end of the germ tube (Fig. 4). No ultrastructural differences were seen in the appearance of the fungus or timing of germination on induced or uninduced tissue (Figs. 5 and 6). An extracellular matrix produced by appressoria

Fig. 1. First true leaf of Cucumis sativa cv SMR 58 plant induced for resistance one week earlier by infection into the abaxial surface with Pseudomonas syringae pv syringae. Necrotic lesions (N) are the result of bacteria injection.

Fig. 2. Second leaf of injected cucumber plant, such as in Fig. 1, where the injection has resulted in veinal chlorosis.

Fig. 3. Injected SMR 58 cucumber plants are located on the left of rear bench next to uninjected controls on the right of rear bench. The controls (arrowhead) are larger in size than the injected plants.

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2

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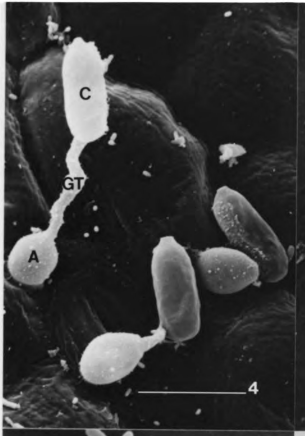
Fig. 4. Scanning electron micrograph of germinated conidia (C) of Colletotrichum lagenarium with germination tube (GT) of variable length and appressoria (A) 24 hrs. after inoculation upon control leaves. Bar = 10 μm.

Fig. 5. Scanning electron micrograph of conidium (C) with appressorium (A) on control leaf 24 hours after inoculation with C. lagenarium showing matrix layer (ML) around appressorium. Bar = 1 μm.

Fig. 6. Scanning electron micrograph of germinated spore on induced leaf 24 hrs. after inoculation with C. lagenarium, showing matrix layer (ML) (arrows) around appressorium (A). Conidium (C) and Bacteria (B). Bar = 1 μm.

Fig. 7. Transmission electron micrograph of ultrathin section of appressorium (A), portion of conidium (C), and plant cell wall (CW) from control plant 48 hrs. after inoculation with C. lagenarium. Section was uranyl acetate and lead citrate stained. Conidium wall appears less electron-dense than appressorium wall. The plant cell wall under the appressorium is indented (arrow). Internal flange (F), extracellular matrix layer (ML), and bacterium (B). Bar = 1 μm.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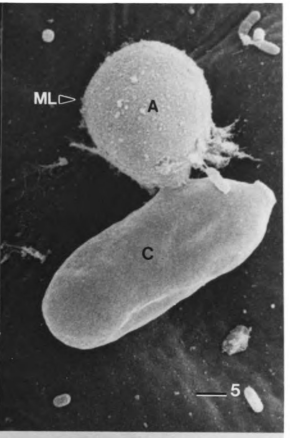
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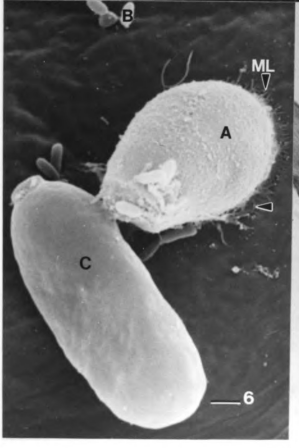
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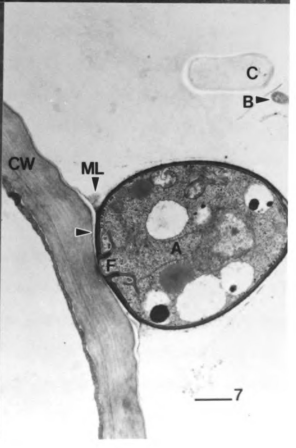
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was located at the area of contact between appressoria and host plant and appeared to bind appressoria to the plant surface (Figs. 5, 6 and 7). By 48 hours after inoculation, penetration pegs formed in appressoria on control and induced cucumbers and had broken through the plant epidermal outer cell walls (Fig. 8). Appressorial walls appeared more electron-dense than spore walls due to melanization (Fig. 7). The appressorial wall also possessed two wall layers: the electron-dense, thick outer and an inner, more electron-lucent layer (Fig. 9). The inner layer of the appressorial wall turned inwards into the cytoplasm of the appressorium (Figs. 7 and 9) and, based on examination of serial sections, formed a funnel-like structure (Fig. 9). Cone-like structures arose from the ends the ends of the internal flange and were apparently continuous with the innermost layer of the appressoral internal flange (Fig. 9). The innermost layer of the internal flange appeared to be continuous with the wall of the penetration peg (Fig. 8). Sections prepared without post-staining with uranyl acetate or lead citrate showed that the cone-like structures stained poorly with OsO_4 compared with the internal flange itself (Figs. 9 and 10). The cone-like structures, towards the top, often branched into different layers (Fig. 6). Portions of the internal flange and the cone-like structures were seen in most appressorial sections and appeared to occupy much of the basal portion of the appressorium (Table 1).

TABLE 1

Occurrence and appearance of internal flanges observed in ultrathin sections of appressoria

	Whole cross sections of internal flange	Partial cross section of internal flange	No internal flange
Control	11	10	8
Induced	18	27	22

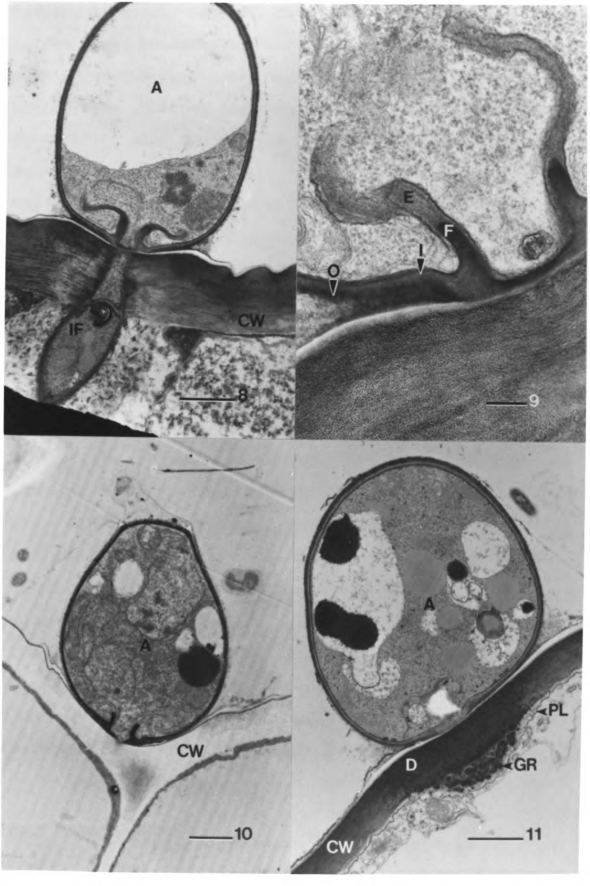
Numbers are taken from the inspection of micrographs of samples, 48 hours after inoculation with *Colletotrichum lagenarium*, prepared by glutaraldehyde/osmium tetroxide fixation.

Fig. 8. Appressorium on control plant, transmission electron micrograph of ultrathin section, 48 hrs. after inoculation with C. lagenarium, the infection peg (IF) has broken through plant epidermal cell wall (CW). Section stained with uranyl acetate and lead citrate. Cell wall next to infection peg is less electron-dense than surrounding wall. Wall under appressorium (A) appears indented towards the plant cell cytoplasm but rest of cell wall layers do not appear to be disturbed. Bar = 1 μm.

Fig. 9. Appressorium from control plant. Transmission electron micrograph of the appressorial cell wall shows outer (O), and inner (I) layers. The internal flange (F) and the extension (E) differ in electron density from each other. Bar = 0.2 μm.

Fig. 10. Transmission electron micrograph of ultrathin section of appressorium (A) and plant epidermal cell wall (CW) from control plant 48 hrs. after inoculation with C. lagenarium. Section was not stained with uranyl acetate and lead citrate. Bar = 1 μm.

Fig. 11. Transmission electron micrograph of ultrathin section, stained with uranyl acetate and lead citrate, of appressorium (A) on protected tissue at 48 hrs after infection with C. lagenarium. Cell wall (CW) of plant under appressorium is electron-dense (D) with electron-dense granules (GR) between host cell wall and plasmalemma (PL). Bar = 1 μm.


A
IF
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8
E
F
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A
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10
A
PL
GR
D
CW
11

Effects of fungus on plant cell wall

The areas beneath the appressoria, the cuticle and upper portion of the plant cell wall of both control and induced plants were indented, suggesting that penetration by penetration pegs through the cuticle was achieved by mechanical force (Fig. 7). Control and induced plant cell walls immediately next to successful penetrations were less electron-dense than the wall areas 1μm further away from the point of penetration (Fig. 8). The cell wall layers nearer the cytoplasm were cleanly cut and not compressed as was the cuticle and outer layers of the cell wall (Fig. 8). This could indicate the breakdown of cell walls by enzymatic degradation (Fig. 8).

Certain plant cells in contact with appressoria, in both control and induced plants, had cell walls which were more electron-dense than surrounding plant cells (Fig. 11). This was noted in 4 out of 34 appressoria in the control treatment at 48 hours and 8 out 85 appressoria in the induced plants at 48 hours (Table 2). No cases were seen in which these electron-dense cell areas were penetrated by the fungi.

In appearance the electron-dense plant cell areas were not much wider than the appressorium actually in contact with this area (Fig. 11). In addition, a number of electron-dense granules occurred between the electron-dense plant cell wall and the plasmalemma (Fig. 11).

When $KMnO_4$ was used to stain sections in which the

TABLE 2

The presence of electron-dense cell walls at the sites of infection caused by *Colletotrichum lagenarium*

	48 hours after inoculation	72 hours after inoculation
Control	4/34	0/4
Induced	8/85	1/6

Samples were prepared from the third leaf of cucumber plants which were induced for resistance by injections of *Pseudomonas syringae* pv *syringae* into the first and second true leaves. *Colletotrichum lagenarium* was inoculated onto the third leaf and the sites of inoculation were fixed 48 hours and 72 hours later with glutaraldehyde and osmium tetroxide. The numerator expresses the number of appressoria in contact with electron-dense plant cell walls. The denominator is the total number of appressoria.

electron-dense epidermal cell areas occurred under appressoria, the electron-density of these sites did not appear to increase (Figs. 12 and 13). EDS indicated that Mn from the $KMnO_4$ stain was localized in this portion of the host cell (Fig. 14). The staining of this area by $KMnO_4$ indicated the presence of lignin. In addition, silicon was found to be localized in the electron-dense plant cell areas (Fig. 15).

To determine if silicon was actually present in the electron-dense plant cell area before the tissue was prepared, to confirm the localization of lignin that was obtained by $KMnO_4$ staining, and to find whether the electron-dense plant cell areas were electron-dense without OsO_4 post-fixation, control and induced cucumber leaves were inoculated with C. lagenarium and sampled 48 hours later and then prepared by the bromine technique.

The brominination and further preparation of mature cucumber leaves, inoculated 48 hours previously with C.lagenarium, extracted much of the cytoplasmic content of the cells (Fig. 16). Electron-dense plant cell areas were also found proximal to appressoria in this treatment (Fig. 16). This occurred in induced plants, in 1 out of 4 cases and in uninduced plants in 2 out of 12 cases all of which were analyzed for bromine. In appearance, the electron-dense areas appeared to be very similar to those seen in glutaraldehyde/ OsO_4 fixed cucumber leaves.

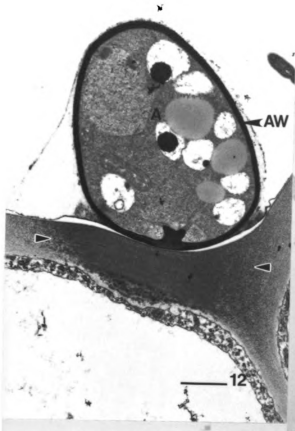
All of brominated cucumber leaf samples with electron-

Fig. 12. Transmission electron microscopy ultrathin section, stained with $KMnO_4$, of appressorium (A) on induced plant inoculated 48 hrs previously with C. lagenarium. Appressorial walls (AW) are heavily stained by $KMnO_4$ due to the presence of melanin. The region of the plant cell wall between the arrowheads and proximal to the appressorium is electron-dense. Bar = 1 μm.

Fig. 13. Unstained serial section of specimen in Fig. 12. Region of plant cell wall between the arrowheads corresponds with that of Fig. 12. Appressorium (A). Bar = 1 μm.

Fig. 14. EDS dot map from a serial section of specimen in Fig. 12 indicating the localization of manganese. The electron-dense cell wall (marked by arrowheads in Fig. 12) of the plant under the appressorium is more heavily stained than surrounding areas of the plant cell wall. Appressorium (A).

Fig. 15. EDS dot map of Si in Fig. 13. The arrowheads correspond in position to those found in Figs. 12, 13, and 14.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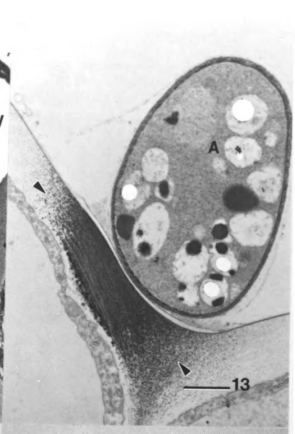
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A
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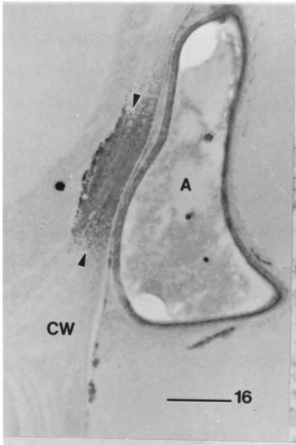
Fig. 16. Transmission electron microscopy ultrathin section of brominated mature cucumber leaf that was fixed 48 hours after inoculation with C. lagenarium. Plant cell wall (CW) proximal to appressorium (A) is electron-dense (marked by arrowheads). Bar = 1 μm.

Fig. 17. Br EDS dot map of Fig. 16. arrowheads below appressorium (A) correspond to the plant cell wall (CW) similarly marked in Fig. 16.

Fig. 18. Si EDS dot map of Fig. 16 with arrowheads in the same position as those in the thin section.

Fig. 19. Transmission electron micrograph of ultrathin section of tissue, from protected plant at 48 hrs after inoculation with C. lagenarium. Section was stained with $KMnO_4$. Plant cell wall under appressorium (A) is more electron-dense than surrounding wall. Appressorium is misshapened and appears necrotic. Bar = 1 μm.



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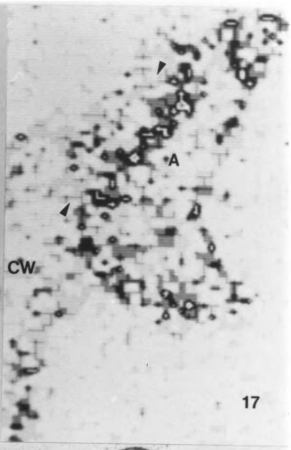

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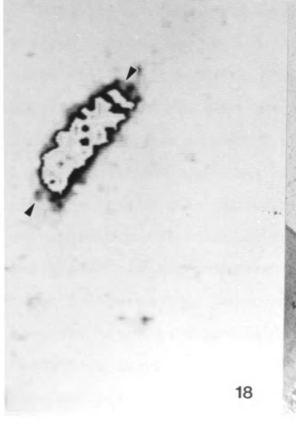

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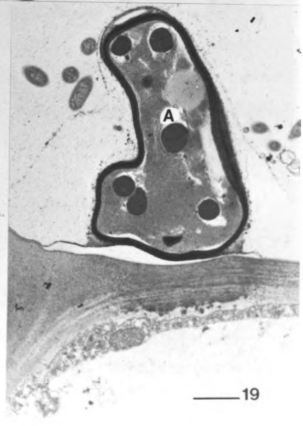

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dense cell areas were then analyzed for bromine and silicon. It was found that in brominated samples, no examples of bromine concentration in the electron-dense areas could be found by EDS (Fig. 17), although silicon was readily found (Fig. 18).

Necrotic appressoria

Induced and control plants inoculated with C. lagenarium showed many of the same features including, necrotic appearing appressoria (Fig. 19). In many cases necrotic appressoria were associated with deposition of cell wall material (Fig. 19). Numbers of necrotic appressorium were often found together on both induced and control plants (Fig. 20).

Papillae

Papillae usually formed where penetration hyphae had pierced through the cell wall (Figs. 21 and 22). The cytoplasm around papillae had large numbers of mitochondria, golgi, abundant vesicles and rough endoplasmic reticulum. In both induced and control plants, papillae consisted of a layer of granular material (Fig. 21). The plant cell membrane around this layer was highly invaginated. The extraplasmalemmal material continued to surround hyphae as the hyphae elongated through cells (Fig. 23).

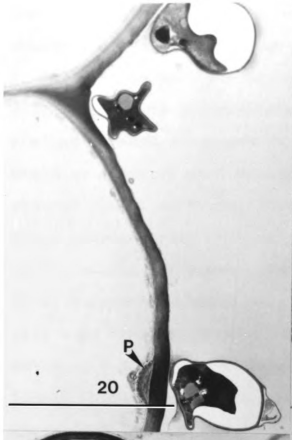
At 72 hours, this layering of papillae and cytoplasm was observed often to continue as primary hyphae ramified throughout the plant cells (Fig. 22). Both the papillae and the host plant cytoplasm surrounding the hyphae were thin.

Fig. 20. Transmission electron micrograph of ultrathin section of necrotic appressoria on induced tissue 48 hrs. after inoculation with C. lagenarium. Pockets of unsuccessful penetrations, as well as successful ones, often occurred. Papilla (P). Bar = 10 μm.

Fig. 21. Transmission electron micrograph of ultrathin section, stained with uranyl acetate and lead citrate, of papilla of protected plant at 48 hrs after inoculation with C. lagenarium. Papilla (P) with granular material. Plasmalemma of plant cell (arrowhead) surrounds papilla. Bar = 1 μm.

Fig. 22. Transmission electron micrograph of ultrathin section of primary hypha (PH), 72 hrs., showing successful penetration of C. lagenarium into protected plant. Section was stained with $KMnO_4$. Material between hypha and plasmalemma appears to have smaller particles than at 48 hrs. (Fig. 21). The material surrounding the hypha, adjacent to the plant cell wall (arrow), is stained with $KMnO_4$ indicating the presence of lignin. Bar = 1 μm.

Fig. 23. Transmission electron micrograph at 48 hours after inoculation of induced leaf with C. lagenarium. Section was stained with $KMnO_4$. Primary hypha (PH) has elongated into plant cell and is surrounded by papilla (P). Bar = 1 μm.



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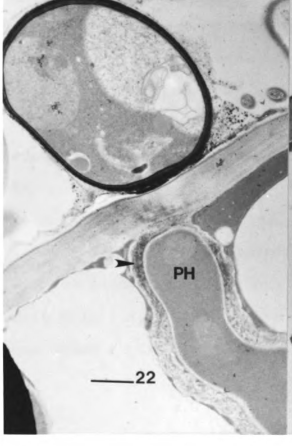

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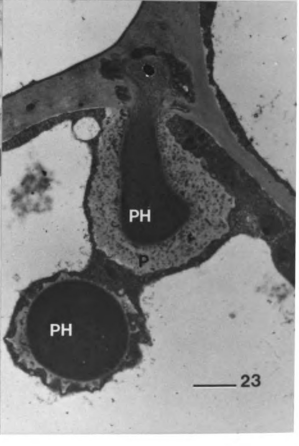

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The plant cell cytoplasm around the papillae possessed few organelles at 72 hours after inoculation.

Papillae in sections of induced and uninduced tissue prepared by glutaraldehyde/osmium tetroxide were often stained by $KMnO_4$ treatment (Figs. 24 and 25). The amount of staining could be seen by comparing unstained and $KMnO_4$ stained serial sections. Transmission EM energy-dispersive X-ray microanalysis (EDS) of $KMnO_4$ stained sections confirmed the localized accumulation of manganese from the $KMnO_4$ treatment in papillae (Fig. 26). Appressorial walls were also heavily stained. This was due to the presence of melanin, a polyphenolic compound similar in structure to lignin which also reacted with $KMnO_4$ (Figs. 12 and 14).

Peroxidase localization

Staining for peroxidase in infected control and induced cucumbers using DAB revealed a layer of material around the outside of appressoria (Figs. 27 and 28). This layer was only faintly stained with uranyl acetate and lead citrate staining (Fig. 21) and somewhat more densely stained with $KMnO_4$ (Fig. 12).

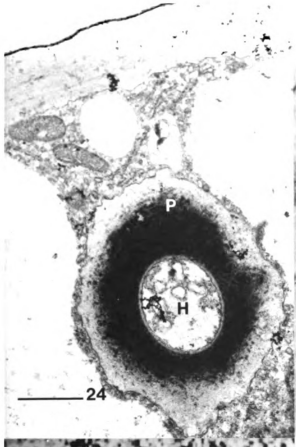
In addition, DAB-OsO_4 stained electron-dense plant cell walls that were under appressoria in both control and induced plants (Figs. 27 and 28). In some cases the plant cell wall directly under the appressoria did not stain while the areas surrounding this did stain with DAB (Fig. 29).

Fig. 24. Transmission electron micrograph of ultrathin section of hypha (H) with papilla (P), on a protected plant inoculated 48 hrs. previously with C. lagenarium. Section was stained with $KMnO_4$. Material in papilla around hypha is electron-dense, indicating the presence of lignin. Bar = 1 μm.

Fig. 25. Unstained transmission electron microscopy ultrathin serial section of same material as Fig. 24. Papilla (P), hypha (H). Bar = 1 μm.

Fig. 26. Scanning transmission electron microscopy X-ray microanalysis dot map demonstrating the presence of manganese (from $KMnO_4$) in a serial section of hypha shown in Fig. 24. Papilla (P), hypha (H).

Fig. 27. Transmission electron microscopy ultrathin section of diaminobenzidine-OsO_4 stained protected tissue at 48 hrs. after inoculation with C. lagenarium showing increased staining (arrowhead) around outside of appressorium and also staining between electron-dense plant cell area and plant plasmalemma, demonstrating the localization of peroxidase. Bar = 1 μm.



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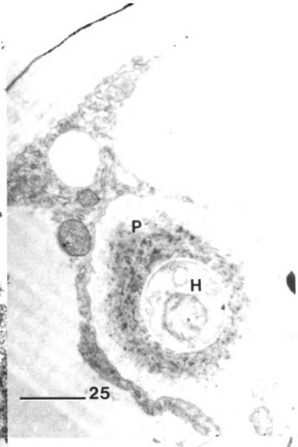

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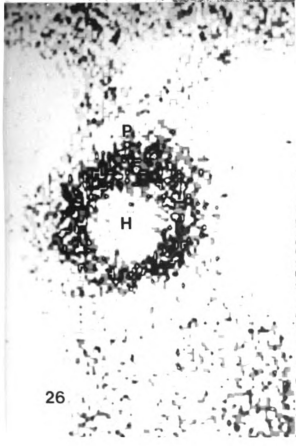

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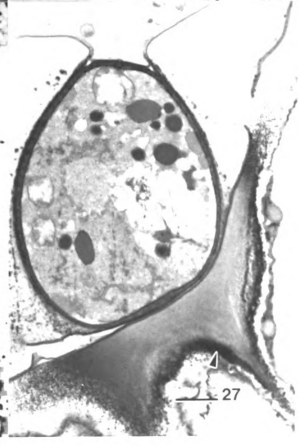

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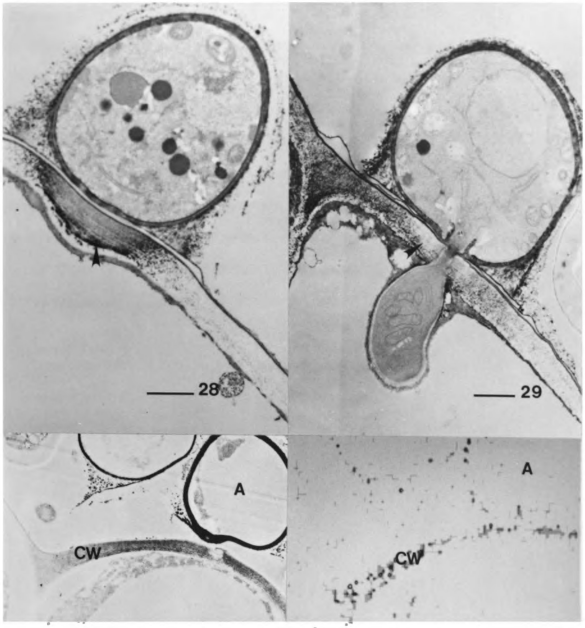
Fig. 28. Transmission electron micrograph of section of control plant, inoculated 48 hours before fixation with C. lagenarium, and stained for peroxidase using diaminobenzidine-OsO_4 (DAB). The interface between the electron-dense area of the plant cell wall and the plasmalemma as well as the outer surface of the appressorium are stained by the DAB. Bar = 1 μm.

Fig. 29. Transmission electron micrograph of material from induced plant which had been inoculated with C. lagenarium 48 hours before and stained for peroxidase using DAB. The plant cell wall around area of fungal penetration lacks staining (arrowhead). Bar = 1μm.

Fig. 30. Transmission electron micrograph of the epidermis of the hypocotyl of seedling of the Spookie cultivar of pumpkin (resistant) which had been inoculated 48 hours earlier with C. lagenarium and fixed with paraformaldehyde and incubated in bromine/chloroform solution. Appressorium (A) and plant cell wall (CW). Bar = 1 μm.

Fig. 31. Scanning transmission electron microscopy X-ray microanalysis dot map from a serial section of Fig. 30 indicating the localization of bromine. The electron-dense cell wall of the plant under the appressoria (A) is more heavily stained than surrounding areas of the plant cell wall (CW) indicating the presence of lignin. Cell walls of adjacent cells are not stained.



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Bromination of pumpkin hypocotyls

Pumpkin hypocotyls were as heavily extracted by bromine treatment as were mature leaves (Fig. 30). The EDS for bromine in seedling pumpkin cells showed that areas of greater bromine concentration were found in the cell walls of the cells proximal to appressoria and not in neighboring cells (Fig. 31). The portion of the pumpkin cell wall that contained bromine was greater relative to the size of the cell than the electron-dense area of the cucumber leaf (Fig. 12 and 13).

DISCUSSION

Appressoria ultrastructure

Appressorial walls were found to be made up of two layers, an electron opaque outer wall and an electron lucent inner wall. Xuei et al. (1988) also described appressoria on control leaves as having an electron-opaque inner wall and electron-lucent outer wall. In addition a loose fibrillar material surrounded the outer appressorial wall. It was poorly seen in uranyl acetate/lead citrate stained glutaraldehyde/osmium tetroxide fixed sections but $KMnO_4$ and especially, DAB-OsO_4 staining increased its visibility. A similar layer was also found in C. lindemuthianum (Wolkow et al., 1983).

The internal flange existed in the appressoria as an inturning of the cell wall surrounding the penetration peg. Xuei et al. (1988) also noted the presence of these elaborations of the wall structure in C. lagenarium appressoria, an identical structure in C. lindemuthianum was termed a internal flange (Mercer et al., 1975). Prominent continuations to the internal flange were seen in this study and were also reported to occur in C. lindemuthianum where the structure was called the associated cone-like structure (Mercer et al., 1975).

In this study, appressorial walls of C. lagenarium were

stained by $KMnO_4$. Melanin is known to a constituent of the walls of appressoria in C. lindemuthianum (Wolkow et al., 1983) as well as other fungi (Howard and Ferrari, 1989). $KMnO_4$ is known to stain appressorial walls (Hayat, 1986) and melanin, with a polyphenolic structure like that of lignin would therefore account for the observed appressorial wall staining by $KMnO_4$.

Matrix layer

In the area of contact between appressoria and plant surface there occurred an extracellular matrix between appressoria and plant cell walls. The matrix appeared in both SEM and TEM. There is evidence to believe that the threadlike appearance of the matrix seen in Fig. 2 is an artifact since SEM of frozen hydrated specimens do not show this feature (Ralph Nicholson, personal communication). The matrix might be related in some way to the fibrillar material that was found to surround appressoria. This matrix appeared to bind appressoria to the plant surface and, perhaps, allowed for the application of pressure by appressoria against the plant surface, thereby causing the plant cell wall indentations observed under appressoria.

Effects of fungus on plant cell wall

In the successful penetrations through the epidermis of control and induced plants by appressoria, evidence was seen in micrographs for the compression of the cuticle and outermost layers of the host cell wall beneath the appressorium. The inner wall layers of the epidermal cell

did not appear to be compressed by the penetration pegs but the cell wall next to the penetration peg did not stain with uranyl acetate and diaminobenzidine-OsO_4 as intensely as the surrounding cell wall. C. lagenarium was found to form an indentation on the epidermal outer cell wall and penetrated through plant cell walls without causing further disruption of the wall layering. Xuei et al. though, reported little or no compression. Wolkow et al. (1983) found that the base of the appressorium of C. lindemuthianum also often fit into a indentation in the plant cell wall and only the upper part of the plant cell wall was affected, the rest appeared undisturbed. In contrast, C. graminicola (Politis and Wheeler, 1973), was found to break through plant cell walls without compressing the walls.

An inhibitor of the melanin pathway was used to eliminate the electron-dense portion of the appressorial wall in C. lindemuthianum (Wolkow et al., 1983). This decreased the ability of appressoria to penetrate through normal Bryophyllum epidermis, but not dewaxed epidermis (Wolkow et al., 1983). This suggested that the pressure of the rigid melanized appressorial walls against the plant surface accounted for the ability of appressoria to penetrate through plant cuticles. Wolkow et al. (1983) also determined that a fungal cutinase inhibitor delayed, but did not stop, penetration and that melanization was greatest on the thickest film of formvar plastic upon which the conidiospores were germinated. Bonnen and Hammerschmidt

(1989a) found that, while C. lagenarium did produce cutinase, cutinase was not necessary for the penetration of plant hosts by appressoria (Bonnen and Hammerschmidt, 1989b).

These studies are in accord with the observation that only the outer layers of the cucumber plant cell appeared to be pushed by the appressoria of C. lagenarium. These findings indicated that mechanical pressure of appressoria allowed for the further penetration by means of enzymatic degradation of plant cells by the fungus.

Plant cell wall reactions to fungus

Certain plant cell walls in contact with appressoria were more electron-dense than adjacent areas of the plant cell wall. These electron-dense cell areas were found in both control and induced plants. Electron-dense cell areas were found in samples prepared by the bromine technique and by glutaraldehyde/ osmium tetroxide fixation. In no case was it seen that penetration pegs broke through electron-dense areas. For this reason electron-dense cell areas appeared to be an effective defense against infection. Electron-dense areas of plant cells were intensely stained without uranyl acetate/lead citrate or $KMnO_4$. The electron-density of the staining did not seem to increase greatly when $KMnO_4$ was used to stain the sections. EDS localized Mn from the $KMnO_4$ staining, which indicated that lignin occurred there. Bromine stained material did not have Br concentrated in the electron-dense cell walls.

Why $KMnO_4$ staining indicated lignin in mature leaves but Br_2 did not is not known, though it might have to do with the chemistry of lignin in the cell walls. It was found earlier that the composition of lignin deposited as result of infection and wounding is different from lignin that is normally deposited (Hammerschmidt et al., 1985; Hammerschmidt, 1984). Saka et al. (1978) found that bromine reacts more with syringyl units than with the guaiacyl units.

Silicon was also found in the electron-dense cell walls. It is possible that the Si was introduced during tissue preparation but the fact that Si was also found in electron-dense cell areas in the tissues prepared by bromination shows this is unlikely since brominated mature leaves were heavily extracted during preparation and the only solutions used in common for the two treatment were phosphate buffer, ethanol, and Spurr's embedding media.

Silicon was likely bound into the plant cell wall. For this reason it was relatively immobile despite the processing procedures used. Because of this bound nature, the EDS dot maps for silicon most likely reflect the situation in the living plant.

Sakai and Thom (1979) reported that silicon was preferentially found in the middle lamella of stomatal cells of sugar cane and suggested that silicon interacted with lignin and other phenolics of the middle lamella as well as other cell wall constituents. Raven (1983) postulated that

energetically it is more favorable for plants to deposit silicon rather than deposit lignin.

Lignin and silicon in the electron-dense areas of the cucumber cell wall could act as a barrier to further penetration by the fungus and this would then account for the fact that no penetration was seen through electron-dense areas. Penetration through lignifications of the epidermis of cucumber petiole has been reported (Hammerschmidt and Kuć, 1982). Whether petiole epidermal cells are siliconized is not known.

Pumpkin seedlings were inoculated to serve as an example of constitutive resistance, as opposed to the induced resistance of cucumbers. No electron-dense deposits were found in seedling pumpkin hypocotyls. Bromine staining gave evidence that lignification occurred in the epidermal cells of the hypocotyl of seedling pumpkin. The total area of lignification in the pumpkin hypocotyl epidermal cell wall as seen in EDS bromine dot maps was greater than the electron-dense areas of cucumber epidermal cells. Staining with phloroglucinol/HCl showed that epidermal cells of the petiole of cucumber plants did lignify when infected with C. lagenarium (Hammerschmidt et al., 1982). The outer epidermal wall of the infected, as well the anticlinal wall between the infected and as adjacent cells, were lignified as seen by phloroglucinol staining and light microscopy. Lignifications of the epidermal cells of the leaf blade, when viewed by light microscopy, were confined to just

around the area pierced by the penetration peg and did not extend to the anticlinal walls (personal communication, R. Hammerschmidt). This corresponds in size to the electron-dense plant cell walls seen in electron microscopy and indicates that, in cucumber leaf epidermal cells, the electron-dense cell walls and phloroglucinol-HCl stained lignifications are the same. The pumpkin hypocotyl lignifications were more similar to those seen in cucumber petiole in terms of the size and the lack of silicon.

Silicon was not found in seedling pumpkin. Kunoh and Ishizaki (1975) also did not report silicon in cucumber cotyledons infected by C. lagenarium. Although the localization of silicon may be artifactual, the fact that silicon was found in tissues prepared in such divergent ways as the bromine and the glutaraldehyde/OsO_4 techniques would argue that silicon was actually present in the electron-dense regions of the infected plant cell wall. It would, therefore, seem likely that petiolar epidermis of cucumbers and hypocotyl of seedling lignify upon infection with C. lagenarium, but that mature leaves of cucumber plants accumulate silicon. In addition, silicon has been shown to be important in the defense of cucumber plants against infection by Fusarium wilt and by powdery mildew, Sphaerotheca fuliginea (Miyake and Takahashi, 1983; Adatia and Besford, 1986).

Papillae

Electron-dense deposits not only occurred in the cell wall but also as granules between the plant cell wall and the plant plasmalemma. These granules were previously described as papillae (Xuei et al., 1988). In this research, the location of the granules between the plasmalemma and cell wall is also the same as described in papillae (Xuei et al., 1988). In addition, granules were more electron-dense and larger than the material that composed papillae. Granulation in the area of the infection site has been reported to precede papillae formation in C. graminicola on susceptible maize (Cadena-Gomez and Nicholson, 1987). The study, though, was done with light microscopy so the appearance of granulation in maize and its similarities to granules in cucumbers can not be determined.

We also noted that papillae possessed a layer of granular material, which was between the plasmalemma and the outside of the hyphae, surrounding the penetration hyphae 48 hours after inoculation. At 72 hours, as the primary hypha elongated, this layer of material was still present between the hypha and plant host plasmalemma, although the particles of the material in the papillae at 72 hours were smaller than at 48 hours. This layer stained with $KMnO_4$ indicating the presence of lignin. A layer that was similarly granular was also reported in papillae of C. lindemuthianum infecting beans (O'Connell et al., 1985). Hammerschmidt and Kuć (1982) found that mycelia of C. lagenarium, incubated with

coniferyl alcohol, hydrogen peroxide, and a crude cucumber peroxidase preparation, had a lignin-like substance deposited on it. Lignification was also seen in papillae around C. lagenarium and Helminthosporium carbonum in squash and pumpkin (Hammerschmidt et al., 1985).

Numbers of necrotic appearing appressorium were seen on control and induced plants, often in association with papillae. Whether the papillae blocked penetration by the appressoria and caused the cells to die or whether the papillae formed after the appressoria became necrotic is not known.

Peroxidase

DAB-OsO_4 staining occurred in both induced and uninduced plants at 48 hours after inoculation with C. lagenarium, demonstrating that increased amounts of peroxidase were present in the electron-dense plant cell walls. Peroxidase is thought to cause the polymerization of lignin (Negrel and Lherminier, 1987).

Cell wall barriers to infection

Barriers to infection in the form of lignification of cell walls and papillae, as well as the development of electron-dense deposits between plasmalemma and cell wall of the host plant, occurred in both control and induced cucumbers. These mechanisms of defense are, therefore, common both to induced and control cucumbers, and the differences in extent of symptom development are probably related to the number of successful vs. blocked infection

attempts.

CHAPTER III

EFFECTS OF HEATSHOCK UPON THE CELL WALL THICKNESSES AND APPEARANCES OF HYPOCOTYL OF CUCUMBER SEEDLINGS

ABSTRACT

There were distinct differences between the ultrastructure of heat shocked and control seedlings, most notably the dilation of the endoplasmic reticulum and the increased electron-density of nucleoli of heat shocked seedlings. Morphometry of the thickness of epidermal cell walls of the hypocotyls of heat shocked cucumber seedlings gave some evidence that heat shock caused an increase in thickness.

INTRODUCTION

Resistance to infection by pathogens can be induced by the prior localized infection of the host plant by fungal, bacterial or viral pathogens (Jenns and Kuć, 1980). This type of induced resistance can last for several weeks (Hammerschmidt et al., 1976).

If the deposition of new cell wall material is responsible for resistance, it should be possible with morphometric analysis to show a difference in cell wall thickness between cells of induced plants and controls. The aim, therefore, of this portion of the research was to: 1) determine the differences between the epidermal cell wall thicknesses of heatshocked and control seedling cucumbers and: 2) determine if any difference in thickness could be correlated with disease resistance.

METHODS AND TECHNIQUES

Experiment 1

Seeds of cucumbers, Cucumis sativa cv Marketer were placed in 1% sodium hypochlorite for 10 minutes and then washed in dH_2O. Seeds were then placed on moist paper toweling. After 6 days, 10 seedlings were placed in 50° Water for 40 seconds and fixed in 2.7% glutaraldehyde in

0.1M HEPES, pH 7.2. Ten control seedlings were also fixed. Additional control and heat shocked seedlings were placed on germination paper and rolled into 4 tubes of 10 seedlings each which were put into 250 ml beakers which had 50 ml of dH_2O added into them to keep the tubes moist. Beakers and tubes were wrapped in aluminum foil so as to prevent the exposure of seedlings to light. At both 24 and 48 hours 10 seedlings each of control and heat treated were fixed in 2.7% glutaraldehyde. Samples were washed in buffer (4x) 24 hours after fixation, postfixed in 1% OsO_4 in 0.1 M HEPES pH 7.2 for 90 minutes, washed in buffer (2x), dehydrated in a graded ethanol series, infiltrated and embedded in ERL epoxy resin (Spurr, 1969). Silver-gold sections were also taken on a Sorvall Porter-Blum MT 2B, placed on copper grids and viewed with a Philips 201 transmission electron microscope (TEM).

Experiment 2

Seedlings germinated as in Exp. 1 were placed into 22.5_oC, 35°C, 40°C, 45°, 50° and along with a control which was not placed into water were fixed in 2.7% glutaraldehyde in 0.1M HEPES, pH 7.2. Control and 50°C heatshock seedlings were also fixed at 24 and 48 hours. Samples were prepared for TEM as in Exp. 1.

Morphometry

1 μm sections of specimen blocks were placed onto glass slides and stained with toluidine blue. The sections were covered with permount and coverslips.

Slides were analyzed by a Zeiss Photometric Digitizer, courtesy of Dr. Ann Cornell-Bell of the Eye Research Institution on Staniford St., Boston, Massachusetts. A total of sixty measurements were taken from each hypocotyl cross section, the measurements being made in three places of twenty cells. Since the cross section of the cucumber hypocotyl resembles the shape of a clover leaf the twenty cells counted from each section were picked from each of the four corners, five cells at a time.

The mean, variance, F-test for variance ratio and T-test to compare means of a sample were determined for the samples.

RESULTS

Morphometry

The epidermal cells of the corners of the hypocotyl were measured (Fig. 1). The epidermal outer cell wall of the heat treated samples were significantly thicker than the controls at 0, 24 and 48 hours (Table 1). When this experiment was repeated and additional temperature steps were run it was found that the results did not replicate those of the first experiment (Table 2) and cell wall thicknesses decreased, as the temperature increased (Table 3).

TABLE 1

Comparison of epidermal outer cell wall thickness of control and heat shocked hypocotyl 0, 24 and 48 hours after experimental hypocotyls were treated.

	0 Hour	24 Hour	48 Hour
Control	1.72±0.55 N=600 A	1.52±0.39 N=600 C	1.66±0.42 N=600 E
Heat shock	2.06±0.58 N=600 B	1.80±0.46 N=600 D	1.84±0.52 N=600 F

The means of the control and heat shocked cell wall thicknesses at 0, 24 and 48 hours were compared using Student's t-test done at a significance level of 0.05.

TABLE 2

The second comparison of epidermal outer cell wall thickness of control and heat shocked hypocotyl 0, 24 and 48 hours after experimental hypocotyls were treated.

	0 Hour	24 Hour	48 Hour
Control	1.76±0.47 N=300 A	1.54±0.35 N=300 C	1.60±0.34 N=300 D
Heat shock	1.57±0.37 N=300 B	1.54±0.31 N=300 C	1.57±0.41 N=300 D

The means of the control and heat shocked cell wall thicknesses at 0, 24 and 48 hours were compared using Student's t-test done at a significance level of 0.05.

TABLE 3

A comparison of the epidermal outer cell wall thickness of hypocotyls of the cucumber cultivar Marketer which had been placed into water at different temperatures

Undunked	1.76±0.47
22°C	1.84±0.38
35°C	1.67±0.38
40°C	1.62±0.39
45°C	1.40±0.46
50°C	1.57±0.37

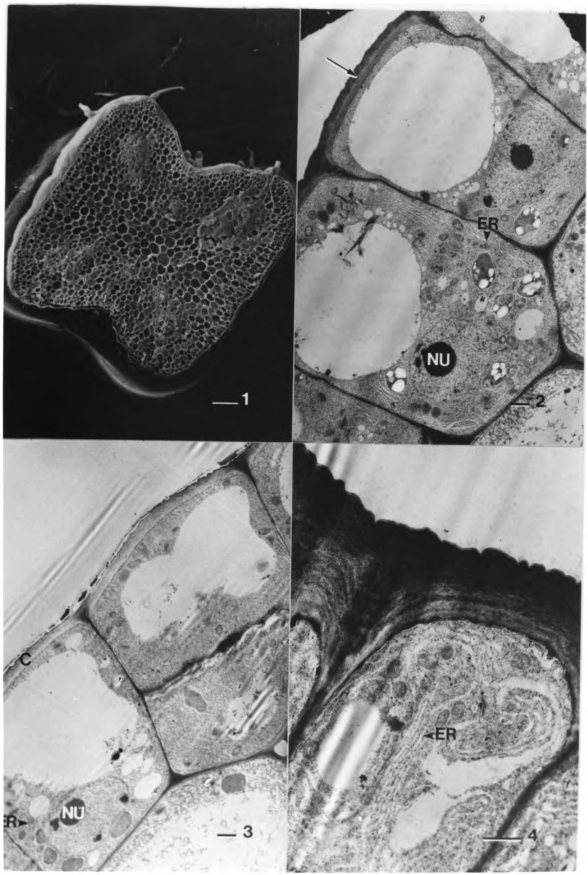
The means and standard deviations are from 300 measurements.

Fig. 1. Scanning electron micrograph of cucumber hypocotyl cross section. Bar = 100 μm.

Fig. 2. Transmission electron micrograph of heat shocked cucumber seedling hypocotyl epidermal cells. The endoplasmic reticulum (ER) are swollen, the nucleoli (NU) are electron-dense and the epidermal outer cell walls are layered (arrow). Bar = 1 μm.

Fig. 3. TEM micrograph of control cucumber hypocotyl epidermal cells. Endoplasmic reticulum (ER), nucleoli (NU) and epidermal outer cell wall (C). Bar = 1 μm.

Fig. 4. TEM of epidermal cells of cucumber hypocotyl from seedling which had been placed into 35°C water. Endoplasmic reticulum (ER). Bar = 1 μm.


1
ER
NU
2
C
NU
3
ER
4

Electron microscopy

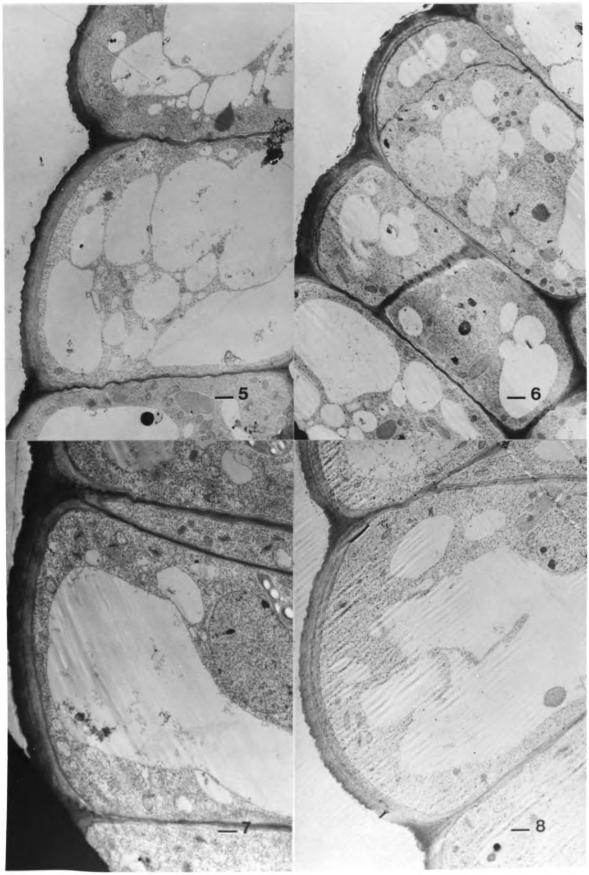
The epidermal cells of the heat shocked seedlings differed in a number of features from the control seedlings. Immediately after heatshock the endoplasmic reticulum was dilated, compared to controls (Figs. 2 and 3). Dilation of the endoplasmic reticulum also occurred at 35°C (Fig. 4). The nucleoli of heat shocked cells were more electron-dense than controls (Figs. 2 and 3). The outer epidermal cell wall became demarcated into layers in heat shocked cells but remained a single layer in controls (Figs. 2 and 3). At 24 hours the epidermal cells no longer exhibited these differences (Figs. 5 and 6). This was also true at 48 hours (Figs. 7 and 8).

Fig. 5. TEM of epidermal cells of cucumber hypocotyl 24 hours after heat shock. Bar = 1 μm.

Fig. 6. TEM of epidermal cells of control cucumber hypocotyl 24 hours after experimental seedlings were heat shocked. Bar = 1 μm.

Fig. 7. TEM of epidermal cells of cucumber hypocotyl 48 hours after seedlings were heat shocked. Bar = 1 μm.

Fig. 8. TEM of epidermal cells of control cucumber hypocotyl 48 hours after experimental seedlings were heat shocked. Bar = 1 μm.


5
6
7
8

DISCUSSION

The hypothesis that the basis for induced resistance in heat shocked cucumber seedlings was due to increased deposition of cell wall material was tested by comparing heatshocked with control seedlings. Previously it was found that the concentration of cell wall associated materials extensin and peroxidase increased in induced seedlings (Stermer and Hammerschmidt, 1984). In the first experiment the cell wall thicknesses of control and heat shocked cucumber seedlings were compared at 0, 24 and 48 hours. At 24 hours the plants became disease resistant as a result of the heat shock (Stermer and Hammerschmidt, 1984). The first experiment seems to support the hypothesis by showing that the epidermal outer cell walls increased in thickness as a result of heatshock at 24 and 48 hours. A statistical significant increase in cell wall thickness was also seen at 0 hour when it would be thought that deposition of new cell wall material would not have time to occur.

To determine whether the increase in cell wall thickness at 0 hour in heat shocked seedlings was a result of water imbibition seedlings, in the second experiment, were treated in water at 22.5°C, 35°C, 40°, 45°C, and 50°C. If the cell wall thickness increased as a result of the heat shock the increases would be most evident at the greater temperatures.

If seedlings were imbibing water the increases would start at 22.5°C and increase gradually as the temperatures went up to 50°C. The measurements of cell wall thickness followed neither course and actually diminished at the higher temperatures. Included in the samples were control and heat shocked seedlings at 24 and 48 hours. No difference was found in the cell wall thickness of the control and heat shocked seedlings at either 24 or 48 hours.

Some differences were seen in the fine structure of the cells of heat treated plants. The cells from heat shocked plants at 0 hour had dilated endoplasmic reticulum, darkly staining nucleoli, and an outer cell wall demarcated into two layers.

The present evidence gives no indications of what the mechanism of disease resistance in heat shocked cucumber seedlings is. Observation of the infection process of heat shocked seedling cucumbers by Colletotrichum lagenarium using histochemistry, such as was done in Chapter II might provide more information. Morphometric analysis proved to be unsuitable for a study of this type, perhaps because of the large error in the measurement of such small structures or to other factors involving random differences between different batches of seedlings which resulted in the apparent differences in the first experiment between the control and heat shocked seedlings.

CHAPTER IV

EFFECTS OF INDUCED RESISTANCE ON PEROXIDASE, PHENOLICS AND EXTENSIN CONCENTRATIONS IN CUCUMBER LEAVES

ABSTRACT

Lignin, extensin and peroxidase are involved in disease resistance in cucumber plants. Both lignin and extensin are structural components of the cell wall. Peroxidase is thought to polymerize the precursors of lignin and extensin. Carbazole staining showed that cucumber leaf peroxidase occurred in greater concentration in induced cucumber leaves compared to control leaves. The staining did not seem to be localized to any particular cell type. The concentrations of phenolics, peroxidase and extensin were determined for epidermal peels of petioles and for the whole petiole. The difference between the epidermal layer of the petiole and the entire petiole and between induced and control tissues could therefore be determined. Assays for phenolics in cell walls showed no difference in the concentration of phenolics in control and induced epidermis and petiole. Peroxidase isozymes were more concentrated in protected than control epidermis and petiole and in induced epidermis than in petiole. Hydroxyproline levels were higher in the epidermis of the petiole compared to the entire petiole.

INTRODUCTION

Peroxidase occurs at an increased concentration in cucumber plants induced to become resistant by prior infection (Hammerschmidt et al., 1982). Lignin occurs at a higher concentration in induced plants, compared to control plants, after induced and control plants were inoculated with C. lagenarium (Hammerschmidt and Kuć, 1982). Extensin was shown to be involved in resistance (Hammerschmidt et al., 1984). Peroxidase is part of the pathway in which polyphenolic precursors are polymerized to form lignins (Negrel and Lherminier, 1987). Peroxidase, lignin and extensin have not been localized in induced plants so that the particular tissues in which these materials are enhanced is not known.

Lignins and extensins are thought to be responsible for resistance in cucumber plants by the stiffening of cell walls, thereby inhibiting penetration of fungi through the epidermis of plants (Hammerschmidt and Kuć, 1982; Hammerschmidt et al., 1984). Induced resistance was eliminated once the epidermis was removed, but this was not true for genetic resistance (Richmond et al., 1979). For these reasons, most of the enhancement of peroxidase in induced resistant cucumbers should occur in the epidermis.

To determine the location of peroxidase, lignin and

extensin enhancement and to confirm that enhancement does indeed occur, cucumber plants were induced to be resistant. The amounts of peroxidase, lignin and extensin were compared in controls versus induced plants by measuring the amounts in epidermis peeled from petioles. Amounts in entire petioles were also determined so that the degree of enhancement in the epidermis could be determined. Since peroxidase can be stained using histochemical techniques, it was used to examine the leaves of control and induced plants for sites of enhancement.

METHODS AND TECHNIQUES

Preparation of plant samples

Cucumber seeds were placed into 1% sodium hypochlorite for 10 minutes, rinsed in dH_2O, placed onto moist paper toweling in a pan covered by aluminum foil, and kept at room temperature for 6 days. When seedlings were about 1 inch high, they were planted into pots and placed in a greenhouse. Once the first leaves of plants were approximately 2/3 full size, *Pseudomonas syringae* pv *syringae* (see below) was injected into leaves through abaxial stomata using a 5 ml syringe without a needle. One week later, similar injections were made into second leaves. Leaves were harvested one week after the second injections. Epidermal peels from petioles and whole petioles were frozen. The procedures for preparation of epidermal peels

and petioles are described in the section on acetone powders below. Samples of three leaves of each treatment were fixed for histochemistry.

Bacteria culture

Pseudomonas syringae pv *syringae* were inoculated into nutrient broth media (NB media) and placed on rotary shader for 24 hours. 5 ml of culture was diluted with 100 ml of dH_2O to ca. 10^8 cfu/ml and used for injections.

Histochemistry and microscopy

Samples from three of the third true leaves from each treatment were fixed overnight in 0.25% glutaraldehyde, 1% paraformaldehyde in 0.05M, pH 6.8, phosphate buffer. Samples were washed in buffer with 0.5M sucrose, then buffer with 1.15M sucrose. Samples were placed into Tissue Tek and sectioned in a cryostat at -20°. Cryostat sections were placed into wells of a spot plate. Peroxidase staining solution (20 μl of 40 mg/ml of 3-amino-9-ethyl carbazole in n,n dimethyl formamide was added to 0.01% H_2O_2 in 0.05M sodium acetate buffer, pH 5.6.) was added to sections for one minute. Sections were then taken out of wells and placed into 0.05M sodium acetate, pH 5.6 and finally into drops of sodium acetate on slides. Cover slips were placed on slides and sealed with nail polish.

Acetone powders

Wet weight for cucumber plant epidermal peels and whole petioles were determined. The samples were then placed into mortars to which LN_2 was added. Samples were ground into

fine powder. 10 ml of acetone at -20°C was twice added to sample. Slurry was filtered and resultant powder stored at -20°C.

Assay for cell wall bound phenolics

100 mg of acetone powders from uninjected control and Pseudomonas syringae pv syringae injected, induced plants were placed into 5 ml of 1.0N NaOH for 20 hours at room temperature. Samples were centrifuged in a Precision Vari-Hi-Speed Centricone at a setting of 70. The supernatant was pipetted off from the cell walls pellet. The pellet was washed with 3 ml of dH_2O, 3 times and the washes were combined with the supernatant. The pellet was discarded. The combined supernatant and dH_2O wash was acidified to pH 2.5 with concentrated HCl. 10 ml of ethyl acetate was added to the acidified solution (3x), vortexed, and pipetted off. The ethyl acetate extract was dried in a Rotavapor at 35°C and redissolved with 0.25 ml methanol. The methanol extract was spotted onto a TLC plate and run with $CHCl_3$: Acetic Acid: H_2O (4:1:1) which was mixed together and the lower phase drawn off and used. A total of three TLC plates were run.

Assay for peroxidase

20 mg of acetone powders were extracted with 1 ml of 0.5M sucrose in 0.01M phosphate buffer pH 6.0. 100 µl of extract was mixed with 10 µl of 50% dextrose and layered onto PAGE. The voltage was set at 100V until the bromophenol blue tracking dye reached the stacking gel at which point current

was turned up to 200V. Gels were stained for peroxidase for 1 hour in a solution of 3-amino-9-ethyl carbazole added to 0.01% H_2O_2 in 199 ml of 0.05M sodium acetate, pH 5.6. 8 gels were run, in total.

Assay for hydroxyproline

Acetone powders (0.01g of bulked cell wall material from the assay for peroxidase) were prepared for hydroxyproline assay by the method of Hammerschmidt et al. (1984) by being placed into 5 ml of 0.015 M Sörensen's phosphate buffer, pH 7.2 (Hayat, 1972). The material was centrifuged and the pellet was washed in 10 ml of buffer (5x). The material was washed in dH_2O (4x), centrifuged, and the pellet was frozen at -20°C. The frozen material was lyophilized at 60 millitorr and -20°C. 0.005g of material was hydrolyzed with 400 µl of 5.5 N HCl at 110°C overnight. Hydrolyzed material was blown dry in a stream of air. The dried material was resuspended in 400 µl of 0.001 N HCl. 200 µl of the solution was assayed for hydroxyproline (Lamport, personal communication; Lamport and Miller, 1971).

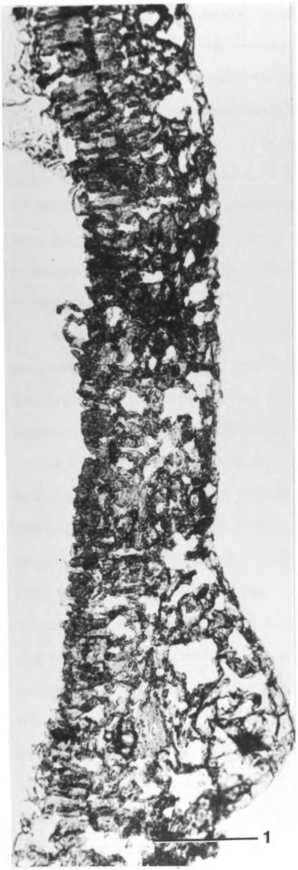
RESULTS

Microscopy

Carbazole treated induced cucumber leaf sections were deeply stained by the treatment (Fig. 1). Control leaf sections were green, though some carbazole staining occurred on the epidermis (Fig. 2). The induced leaves appeared to

Fig. 1. Light micrograph of cucumber leaf, induced by injection with Pseudomonas syringae pv syringae, sectioned with a cryostat and stained with carbazole for peroxidase. Bar = 100μm.

Fig. 2. Light micrograph of control cucumber leaf, not injected, sectioned with a cryostat and stained with carbazole for peroxidase. Bar = 100 μm.


1


2

be stained more intensely in the palisade mesophyll. During the staining of the sections, the spot plate wells containing induced leaf sections could readily be differentiated from the control since the staining solution in the well turned red.

Cell wall bound phenolics

No differences in intensity or compound occurred between the comparisons of cell wall bound phenolics in control and induced epidermis and petiole.

Peroxidase

The isozymes that were most noticeable in the polyacrylamide agarose gel electrophoresis of the anionic isozymes of peroxidase in unprotected and protected epidermis and whole petioles of cucumber were the 3 bands which were the most anodic. The bands of isozymes from protected petiole were darker than those of unprotected petiole 6 times out of 8 whereas unprotected petiole was darker 2 out of 8 times. Protected epidermis was darker than unprotected epidermis 7 out of 8 times while both appeared the same 1 out 8 times. Protected epidermis appeared darker than protected petiole 6 of 8 times, protected petiole was darker than protected epidermis 1 of 8 times, and both appeared the same 1 of 8 times. The ranking of the comparisons between the different tissues on the intensity of the three most anodic peroxidase bands was Protected epidermis < Protected petiole < Unprotected petiole ≤ Unprotected epidermis.

Hydroxyproline

The concentration of hydroxyproline in cucumber petiole did not appear to be affected by the use of Pseudomonas syringae pv syringae as an inducing agent (Table 1). The concentration of hydroxyproline in the epidermis of petioles was higher than the concentration in the entire petiole (Table 1).

TABLE 1

Measurements of the concentrations of hydroxyproline in the tissues of bulked samples from the cucumber epidermises and petioles used in the PAGE assay for peroxidase.

	µg 0.005 g Dry weight	µg 1.0 g Fresh weight
Unprotected petiole	1.90 µg	9.45 µg
Unprotected epidermis	1.22 µg	18.89 µg
Protected petiole	2.40 µg	9.90 µg
Protected epidermis	0.90 µg	15.83 µg

Cucumber plants were induced by injection of Pseudomonas syringae pv syringae into the first and second leaves. The petiole of the third leaf was used for hydroxyproline determinations. Petioles were either stripped of epidermis, for the measurement of hydroxyproline in epidermis or used whole for determination of hydroxyproline in the bulk petiole.

DISCUSSION

The finding of increased peroxidase in induced leaf tissue corroborates the previous findings of Hammerschmidt et al. (1982) of increased peroxidase levels in induced leaves. Reaction product appeared somewhat more concentrated in the palisade mesophyll than in the epidermis, spongy mesophyll or vascular tissue. Comparisons of induced and control petiole and epidermis using PAGE indicated that soluble peroxidases were more concentrated in induced petioles than control petioles and that induced petiole epidermis contained a higher concentration of peroxidase than the bulk petiole. This indicates that in the petiole of cucumber, induced plants have a higher concentration of peroxidase than the uninduced control plants and that there is a higher concentration of peroxidase in the epidermis than in the bulk petiole tissue. This supports the work of Richmond et al. (1979) which indicated that the epidermis is the site at which induced resistance operates. The peroxidases were also found as a set of three bands concentrated to the aniodic end of the gel as was reported by Smith and Hammerschmidt (1988).

The lack of a difference in the concentration of phenolics between epidermal strips and bulk petiole and protected and unprotected plants agrees with the observation

that lignification followed challenge inoculation by a pathogen in induced plants (Hammerschmidt and Kuć, 1982).

The lack of difference in hydroxyproline concentration between the induced and control petioles indicates that extensin levels do not change after cucumber plants are induced for resistance.

CHAPTER V

TECHNIQUES FOR THE ULTRASTRUCTURAL LOCALIZATION OF LIGNIN USING POTASSIUM PERMANGANATE AND BROMINE STAINING AND EDS

ABSTRACT

Bromine and potassium permanganate were used to localize lignin in a herbaceous plant using energy dispersive X-ray microanalysis (EDS) and transmission electron microscopy (TEM). Bromine was used as part of the preparation procedure, and in separate experiments, $KMnO_4$ was used as either a fixative or a post-section stain. Lignin deposition was associated with sites of infection caused by the fungus, Colletotrichum lagenarium in pumpkin seedlings. The procedure for using Br_2 as an ultrastructural stain for lignin in herbaceous plants included a modification of the procedure originally developed for woody plant by adding a paraformaldehyde prefixation step. These stains provided data on the ultrastructural localization of lignin which contributed to the elucidation of its role in the interactions between pathogenic fungi in resistant plant hosts.

INTRODUCTION

Of the techniques available for the ultrastructural localization of lignin, it was necessary to decide which was the most useful for the cucumber model system. To make this choice, it was important to compare the different techniques in a similar situation to determine the relative merits of each technique. For this reason the $KMnO_4$ and Br_2 procedures were modified for use with herbaceous plants and compared ultrastructurally. Energy dispersive X-ray microanalysis was used to localize Mn or Br_2 in the treated samples.

MATERIALS AND METHODS

The upper 1 cm of the hypocotyl of the Spookie cultivar of pumpkin, 6 days old, grown at 23°C in the dark was inoculated with $3x10^6$ conidia/ml of Colletotrichum lagenarium which was grown on PDA for 10 days. Hypocotyl segments were fixed 48 hours after inoculation by one of the following protocols:

(1) 2% paraformaldehyde in 0.05 M sodium phosphate buffer, pH 6.8 (Hayat, 1972) for 4 hours, 3 buffer washes, dehydrated in a graded ethanol series, placed into 1:1 ethanol:chloroform, 100% ethanol, then a solution of 0.5%

bromine in chloroform for 3 hours with agitation (Saka et al., 1978), placed in $CHCl_3$ for 10 days during which the $CHCl_3$ was replaced 8 times, followed by 1:1 propylene oxide:$CHCl_3$, 100% propylene oxide, and four changes of ERL epoxy resin (Spurr, 1969), and then embedded (Spurr's resin) and polymerized at 65°C for 8 hr.

(2) 1% $KMnO_4$ at 23°C for 15 minutes, four buffer washes.

(3) 1% $KMnO_4$ at 23_oC for 15 minutes, four buffer washes, 2.67% glutaraldehyde in buffer, overnight at 4°, followed by four buffer washes.

(4) 2.67% glutaraldehyde in buffer for overnight at 4°C, four buffer washes, 1% $KMnO_4$ at 23°C for 15 minutes, and again four buffer washes.

(5) 2.67% glutaraldehyde in buffer for overnight at 4°C, and four buffer washes.

(6) 2.67% glutaraldehyde in buffer for overnight at 4_oC, four buffer washes, 1% OsO_4 at 4°C for 90 minutes, and two buffer washes.

The samples from treatments #2-5 were dehydrated in a graded ethanol series and embedded in ERL epoxy resin (Spurr, 1969). At least three blocks from all the treatments were sectioned with a Reichert Ultracut E microtome, although, in some of the treatments only one block was found to have instances of fungal infection. Sections from treatments #5 and #6 were placed on nickel grids and stained with 1% $KMnO_4$ for 30 minutes in a filled and sealed 1.5 ml Eppendorf microcentrifuge tube and then

washed well with dH_2O (Hayat, 1970). Sections were viewed with either a Philips 201 transmission electron microscope (TEM) or a JEOL 100CX II TEM. Energy dispersive X-ray microanalysis (EDS) spectra were collected and dot maps for bromine and manganese were made using the JEOL 100CX II in the scanning transmission mode (STEM) at 100 kV with a Link Systems AN 10000 energy dispersive X-ray analyzer. Each dot map was generated by 20 scans which took approximately one hour.

RESULTS

Conidia of Colletotrichum lagenarium germinated to produce appressoria by 24 hours after inoculation. By 48 hours, appressoria had formed penetration pegs that had broken through the outer walls of the plant epidermal cells. It was at this stage that reaction by the plant to infection by the fungus could be studied.

As expected the method for Br staining resulted in poor ultrastructural preservation of the plant cells. Even with the addition of a paraformaldehyde prefixation step, the treatment with chloroform extracted the cytoplasm. Cell walls were the primary visible elements of structure (Fig. 1). Cytoplasm was present, though organelles and other constituents of the cytoplasm could not be distinquished.

The walls of the cells proximal to appressoria were often more electron-dense than walls of those cells that were not

Fig. 1. Ultrathin section of bromine-incubated, paraformaldehyde-fixed material showed poor ultrastructural preservation. Cytoplasm (CY) was present, though organelles and other constituents of the cytoplasm could not be differentiated. Walls directly under appressorium (A) were more electron dense than walls of neighboring cell (arrow). Bar = 1 μm.

Fig. 2. Energy dispersive X-ray microanalysis (EDS) dot map showing localization of Br in Fig. 1. The wall of the cell under the appressorium (A) was more heavily stained than the neighboring cell (arrow).

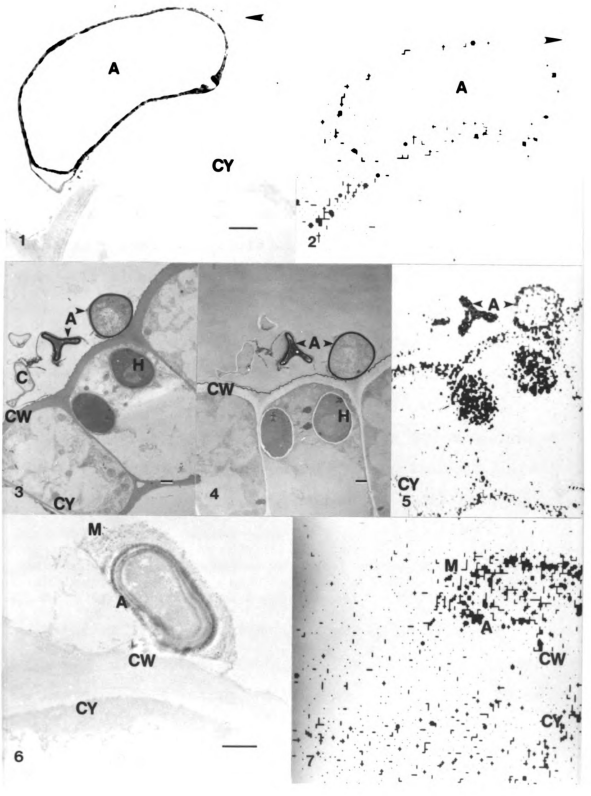
Fig. 3. Ultrathin section of material fixed with glutaraldehyde and OsO_4; sections from epoxy blocks were stained with $KMnO_4$. Preservation of sample was comparable to that seen in standard UA/ Pb citrate stained samples. Cell walls (CW) were heavily stained with $KMnO_4$. Cytoplasm (CY) was not as heavily stained. Fungal conidia (C), appressoria (A) and hyphae (H) are present. Bar = 1 μm.

Fig. 4. Photomicrograph of a serial section of Fig. 3 which was not stained with $KMnO_4$. The cell walls of plant (CW) and fungal hyphae (H) were electron-lucent, compared to the electron-dense walls of the stained section in Fig. 3. The wall of the appressoria (A) was electron-dense. Bar = 1 μm.

Fig. 5. EDS dot map for Mn in Fig. 3. The heaviest concentrations of Mn were found in hyphae and plant cell walls which correspond to the areas which were more electron-dense (Fig. 3). High concentration in cell walls of the appressoria (A) which were electron-dense in both Figs. 3 and 4. The cytoplasm (CY, same area as in Fig. 3) is only lightly stained.

Fig. 6. Ultrathin section of sample fixed with glutaraldeyde followed by $KMnO_4$. The plant cell wall (CW) was less electron-dense than the plant cytoplasm (CY), though there were layers in the cell wall which were more heavily stained by the $KMnO_4$. The outer matrix layer (M) and cytoplasm of the appressorium (A) were also well stained. The appressorium cell wall was very electron dense. Bar = 1 μm.

Fig. 7. EDS of Fig. 6. The greater concentration of Mn was found in the cytoplasm (CY) of the plant cell and in the appressorium (A) and its matrix layer (A). The plant cell wall (CW) had little Mn associated with it.
electron-density of the cell walls of infected cells, as compared to non-infected cells, was caused by increased amounts of bromine, as determined by EDS (Fig. 2). This indicated that lignin was present in the walls of infected cells.


A
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in contact with appressoria (Fig. 1). The increased Specimens fixed with glutaraldehyde and osmium tetroxide, followed by $KMnO_4$ staining of ultrathin sections showed preservation equal to that found in standard section staining treatments with uranyl acetate and lead citrate, though there was a greater number of staining artifacts from the $KMnO_4$ (Fig. 3). Comparison of stained and unstained serial sections indicated that increases in electron-density occurred in the plant cell wall and in the hyphae. Appressorial cell walls and certain portions of plant cells were electron-dense in the sections unstained by $KMnO_4$ (Figs. 3 and 4). In order to determine whether increases in electron-density were due to $KMnO_4$ or to another cause, such as OsO_4 post-fixation, EDS was used to determine the distribution of manganese (Fig. 5). The EDS Mn dot maps showed that Mn occurred in the areas where $KMnO_4$ staining resulted in increased electron-density, such as the plant cell wall and in fungal hyphae, but that Mn also was localized in the appressorial wall, which was electron-dense in both $KMnO_4$ stained and unstained sections.

Material fixed with $KMnO_4$, alone (method #2), as well as before or after glutaraldehyde (methods #3-4), were similar in that the cell walls were not heavily stained and the cytoplasm was poorly preserved, yet heavily stained (Fig. 6). The EDS of these samples showed that little of the manganese was localized in the cell walls of the host plant, but rather most of the Mn X-ray signal originated from

cytoplasm and appressoria (Fig. 7).

DISCUSSION

A lignin staining technique utilizing EDS to localize bromine, which had previously been used on wood cells (Saka et al., 1978), was applied here to an herbaceous specimen, to study the localization of lignin in pumpkin seedlings infected with the fungus, Colletotrichum lagenarium.

Lignin in infected epidermal cell walls in herbaceous plants could be determined by the localization of bromine with EDS (Table 1). The level of staining in both the cytoplasm and in the rest of the background of epoxy sections was very low. Cytoplasm, however, was heavily extracted by the treatment, leaving few recognizable cytoplasmic constituents. From the perspective of overall specimen image quality, the bromine method appears more suitable for specimens where the quality of the preservation of cytoplasm is unimportant.

Lignification in herbaceous plants has been previously studied by means of $KMnO_4$, which when used as a fixative, acts also well as a stain for lignin (Hepler et al., 1970). In this study, plant and fungal cell cytoplasm became heavily stained during $KMnO_4$ fixation, but plant cell walls were not (Table 1). Energy dispersive X-ray microanalysis (EDS) showed that there was little Mn in plant cell walls in samples from the $KMnO_4$ fixation treatments (alone or before

Table 1.
Comparison of techniques used in the fixation and staining of plant samples for the presence of lignin.

Treatment	Quality of Ultrastructural Preservation*	Ease of Localization of Lignin*
1. FA/Br	+/-	++++
2. $KMnO_4$	+	+/-
3. $KMnO_4$/Glut	+	+/-
4. Glut/$KMnO_4$	++	+/-
5. Glut $KMnO_4$ on section	+++	++
6. Glut/OsO_4 $KMnO_4$ on section	++++	+++

The order of the treatments is the same as that found in the Materials and Methods. In treatments 5 and 6 ultrathin sections were stained with $KMnO_4$.
Paraformaldehyde (FA), Glutaraldehyde (Glut).
*Quality of ultrastructural preservation refers to the integrity of membrane, cytoplasm, and organelles.
Ease of localization of lignin refers to the amount of signal compared to background in the dot maps.
The meaning of the symbols are: +/- poor, + fair, ++ good, +++ very good, ++++ excellent.

glutaraldehyde). Since the overall staining of the cytoplasm by $KMnO_4$ is very heavy, $KMnO_4$ fixation would not be useful in determining whether lignin is occurring in papillae. Doubtlessly the wide variety of materials present in cytoplasm include materials such as the polyphenolics which were found to be a primary material that reacts with $KMnO_4$ (Bland et al., 1971). Fixation with glutaraldehyde alone did not improve Mn localization in sections stained with $KMnO_4$.

As expected, glutaraldehyde/osmium tetroxide fixed material had better preservation of fine structure than did the other techniques (Table 1). Ultrathin sections from specimens fixed in this way and subsequently stained with $KMnO_4$ resulted in depositions of amorphous, electron-dense puddles. Fungal walls and cytoplasm, cell walls of the plant, and to a lesser degree plant cytoplasm, were stained by $KMnO_4$, as demonstrated by both EDS and bright field TEM. Staining of appressorial walls was due to the presence of melanin, a polyphenolic which also reacts with $KMnO_4$ (Hayat, 1986).

Cytoplasmic and general background staining of sections of glutaraldehyde/osmium tetroxide fixed material was higher than that of the bromine technique but much lower than that of the $KMnO_4$ fixation techniques. Cell walls of both plant and fungus from thin sections treated with $KMnO_4$ were heavily stained with permanganate but that differences in the cell wall structure were obscured by the high

concentration of Mn. As was done in this study, unstained serial sections should be considered as an important control for this procedure.

In summary, the bromine staining technique corroborated with EDS resulted in excellent localization of lignin and acceptable fixation of plant cell walls, but had the distinct disadvantage of extraction and poor preservation of cytoplasm. The $KMnO_4$ thin section staining technique resulted in good preservation of cytoplasm, but poorer elucidation of cell wall structure due to an increase of staining over the bromine technique. Both procedures allowed the localization of specific stain (Br or Mn) with EDS.

The $KMnO_4$ fixation procedure resulted in poor fixation and inferior localization of $KMnO_4$ stained lignin. One advantage of this technique, however, was that the substructure of plant cell walls could be distinguished under bright field TEM more readily, due to the lower levels of staining that occurred, compared to the $KMnO_4$ section staining method. For these reasons both the $KMnO_4$ staining technique and the bromine procedure were used for further study of lignification during resistance against infection.

CHAPTER VI

PRELIMINARY OBSERVATIONS ON THE IMMUNOCYTOCHEMICAL LOCALIZATION OF EXTENSIN AND PEROXIDASE IN THE LEAF OF CUCUMBERS INDUCED FOR RESISTANCE

ABSTRACT

In preliminary experiments an antibody to peroxidase bound to much of the cytoplasm and cell wall of the leaves of induced and control cucumber plants. An antibody to extensin did not exhibit any specificity.

INTRODUCTION

Both extensin and peroxidase are thought to be involved in disease resistance in cucumber plants (Hammerschmidt et al., 1984; Hammerschmidt et al., 1982). Extensin is a component of plant cell walls and presumably the nonspecificity of induced resistance in cucumbers is due to the increase in the impenetrability of cell walls as a result of either increased cell wall deposition or increased crosslinkage of cell walls or possibly both. Extensin has been found to increase after resistance was induced by both prior infection and heat shock (Stermer and Hammerschmidt, 1984).

Peroxidase is thought to cause the polymerization of both lignin and extensin in plant cell walls (Negrel and Lherminier, 1987). The concentration of peroxidase, in plants, increases after localized infection of mature plants and heatshock of seedlings (Hammerschmidt et al., 1982; Stermer and Hammerschmidt, 1984).

The localization of extensin and peroxidase to the epidermal cells, the site of induced resistance in cucumbers (Richardson et al., 1979) would corroborate the ideas that both extensin and peroxidase are involved in the response by cucumbers to infection.

METHODS AND TECHNIQUES

Cucumber seedlings

6 day old cucumber seedlings (Cucumis sativa cv Marketer) were heat shocked by plunging into 50°C water for 40 seconds. Control and heat treated seedlings were sampled at 0, 24 and 48 hours after treatment. In addition, both control and heat treated seedlings were infected with Cladosporium cucumerinum at 24 hours after the heat treatment and were sampled at 48 hours after the heat treatment. The uppermost 1 mm of the seedlings from the different treatments were fixed in 0.25% glutaraldehyde, 2% paraformaldehyde in 0.1 M HEPES, pH 7.2 for 3 hours. Samples were dehydrated in 25% ethanol at 0°C and 50%, 75%, 95%, and 100% at -30°C, then placed in Lowicryl and polymerized with U.V light at -30°C for 3 days. Samples were also fixed in 2.7% glutaraldehyde in 0.1 M HEPES, pH 7.2 for 24 hours, post-fixed in 1% OSO_4 in buffer for 2 hours, dehydrated in ethanol and embedded in ERL media (Spurr, 1969).

Cucumber plants

Cucumber plants (Cucumis sativa cv Marketer) were injected, with Pseudomonas syringae pv syringae, into the first true leaf when that leaf was at 2/3 of its mature size. 10 injections were made using a syringe without a needle into the abaxial surface. Portions of the second

leaf were fixed for 12 hours in 2.7% glutaraldehyde in 0.1 M HEPES, pH 7.2, 6 days after injections. Samples were post-fixed in 1% OsO_4 in buffer for 2 hours, dehydrated in ethanol and embedded in ERL media (Spurr, 1969).

Immunocytochemistry

Sections were cut with a diamond knife, placed on 300 mesh nickel grids and stored in a vacuum desiccator.

The methods of Bendayan (1984) were used to stain sections:

1). Grids with Spurr's media embedded sections were etched with saturated sodium metaperiodate, tissue side down, for one hour.

2). Spurr's media and Lowicryl embedded sections were rinsed by placing section side down on surface of dH_2O for 2-3 minutes in each spot (4x), grids were shaken off between each wash.

3). Grids were placed on drops of PBS with 1% ovalbumin for 5 minutes.

4). Transfer without shaking or rinsing to 1:100 antibody (anti-extensin, Ray Hammerschmidt, or anti-peroxidase, Jennifer Smith) for 1 hour.

5). Rinsed on PBS in spot plates for 5 minutes (4x).

6). Transfered to drops of PBS with 1% ovalbumin for 5 minutes.

7). Incubated on colloidal gold/Staphylococcus Protein A (Bendayan, 1983) diluted 5 to 10 fold with PBS for 30 minutes.

8). Thoroughly jet washed with PBS and dH_2O.

9). Dried and stained with 2% aqueous uranyl acetate for 10 minutes.

PBS formula

pH 7.4 PBS is composed of 8g NaCl, 1.44g $Na_2HPO_4x2H_2O$, 0.2g KH_2PO_4, 0.2g KCl, and brought up to 1 liter with dH_2O.

RESULTS AND DISCUSSION

Spurr's media embedded mature cucumber leaves, when stained with anti-peroxidase and 12 μn colloidal gold had labeling on most of the cell wall (Fig. 1) and cytoplasm in both control and induced sections (Fig. 2 and 3). Hypocotyl sections were also labeled on both cell wall and cytoplasm (Fig. 4).

In all these cases, there was varying amounts of background, due to nonspecific binding, which can be seen where colloidal gold particles occurred in extracellular areas, such as outside the epidermal outer cell wall (Fig. 1) and in the gaps created by the shrinkage of starch granules in chloroplasts (Fig. 2).

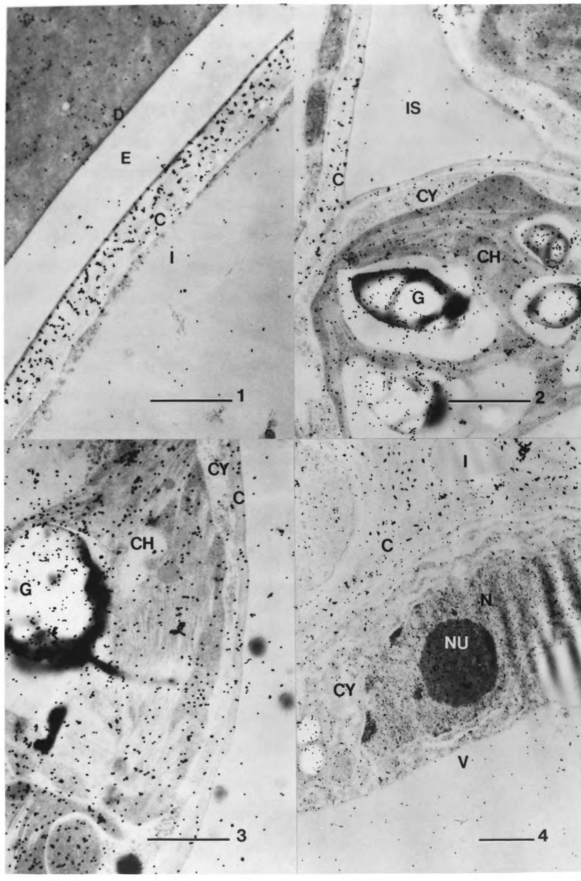
The lack of specificity of the anti-peroxidase can be seen by the large amounts of staining occurring in areas where peroxidase would not be expected, such as the starch grains of chloroplasts (Figs. 2, 3 and 4). The nonspecific binding of the antibody to such locations may be due to the use of Freund's adjuvant which has been found to cause cross

Fig. 1. TEM of ultrathin section of epidermal outer cell wall of SMR 58 cucumber plants. Tissues were embedded in Spurr's epoxy. Sections were incubated with anti-peroxidase and Protein A/ colloidal gold. Cell wall (C) has a heavy concentration of gold particles but extra- (E) and intracellular (I) spaces have only a scattering of particles. A greater number of particles were found in a layer which covered much of the epidermis (D). Bar = 1 μm.

Fig. 2. TEM of mesophyll cell from control, uninjected SMR 58 cucumber plant, the samples of which were embedded in Spurr's epoxy. Sections were incubated in anti-peroxidase and Protein A/ colloidal gold. Little binding of particles occurs in the intercellular space (IS) but there was binding over much of the cytoplasm (CY), chloroplasts (CH), starch granules (G) and cell wall (C). Bar = 1 μm.

Fig. 3. TEM of induced resistant SMR 58 cucumber plant. Thin sections were incubated with anti-peroxidase and Protein A/ colloidal gold. Most of binding occurred on chloroplasts (CH), starch grains (G), ground cytoplasm (CY) and cell wall (C). Bar = 1 μm.

Fig. 4. TEM of the hypocotyl of 48 hour heat shocked and 24 hours after inoculation of Cladosporium cucumericum onto seedlings. Sections were incubated with anti-peroxidase and colloidal gold. Particles were found on the nucleus (N), nucleolus (NU), ground cytoplasm (CY) and cell wall (C) but not as heavily on the intercellular space (I) and vacuole (V). Bar = 1 μm.


D
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reactivity to components of plants due to the similarities of the cell walls of bacteria which make up the adjuvant and the cell walls and other polysaccharides of plants (G. de Zoeten, personnel communication).

Lowicryl embedded material, besides being more difficult to section and having poorer contrast preservation of fine detail than epoxy embedded material, did not yield a greater specificity of anti-peroxidase localization.

Anti-extensin, obtained from R. Hammerschmidt, did not bind to sections to any greater degree than normal rabbit serum.

To continue this research new antibodies to extensin and peroxidase need to be produced. One method to gain greater specificity of antibody to antigen would be exposing the antigens to the fixative used in sample preparation. This would cause the antibody to be produced to the conformation of the antigen as it would occur in the fixed samples. As mentioned above Freund's adjuvant could not be used.

Both extensin and peroxidase are glycosylated. Antibodies to the glycosylated portion of the protein would probably cross react with other glycosylated molecules resulting in non-specific binding. Deglycosylation of the proteins would yield antibodies which would likely be blocked from the appropriate epitope by the glycosylation. One solution might be to make antibodies to the fixed deglycosylated form of the molecules and affinity purify against the fixed glycosylated forms which should result in

antibodies which would bind to the antigen in fixed samples. The problem is whether fixation of the deglycosylated form of the protein would cause the protein to take up a different conformation than the fixed glycosylated form.

The possibility of the production of an antibody to lignin should be examined. The precursors of lignin are comparatively low weight molecules but antibodies have been produced to other low weight molecules in plants such as abscisic acid (Sossountzov et al., 1986). Localization of lignins would corroborate the histochemistry of lignin in Chapter II. Since lignin is well bound into the cell walls normal preparation procedures should suffice. If possible, antibodies to the precursors of lignin as well as the polymer should be made to see if the relative abundances of the precursors in the cytoplasm of induced and infected cucumbers could be determined and compared to the amounts of lignin polymerized in the cell wall.

SUMMARY

Cucumber plants exhibit a modification of epidermal cells where the outer wall, that is in contact with fungal appressoria of Colletotrichum lagenarium, becomes electron-dense. Electron-dense cell walls were never seen without appressoria. Appressoria were never seen to penetrate through these walls. Energy dispersive X-ray microanalysis (EDS) showed that one component of these walls, and probably the one that accounted for the electron-density, was silicon. Samples prepared by two different techniques still were found to have silicon in the electron-dense cell walls so the silicon is not likely an artifact of sample preparation. Electron-dense cell walls were histochemically stained for lignin using potassium permanganate and bromine. EDS showed that Mn was bound into the electron-dense cell wall, indicating lignin, but that Br was not. The reason for the failure of this histochemical test to indicated lignin while the potassium permanganate test did is not known but might be caused by the special properties of lignin deposited as a response to infection.

Papillae developed around the sites of fungal penetration. Staining with potassium permanganate indicated that lignin localized into papillae. The localization of Mn

form the stain to papillae was corroborated by EDS.

The ultrastructural reactions to infection were common to both induced and control plants infected by C. lagenarium. The differences were likely quantitative rather than qualitative.

Heat shock of cucumber seedlings was shown to cause induced resistance. To determine whether an increase in epidermal cell wall thickness caused induced resistance, measurements were made of heat shock and control seedlings. Heat shock of seedlings caused several ultrastructural effects, most notably an increase in the distance between the layers of the endoplastic reticulum and an increase in the electron-density of nuclei. Measurements of the thickness of the outer wall of epidermal cells indicated that heat shock caused the cell walls to increase in thickness but this increase did not occur consistently.

Measurements of peroxidase in the epidermis of the petiole and the bulk petiole of induced and control mature plants showed that the induced tissue had a greater concentration of peroxidase than control and that induced petiolar epidermis a greater concentration than the bulk petiole. This is in accord with the hypothesis that the epidermis is responsible for the effect of induced resistance since peroxidase is thought to be responsible for the polymerization of lignin and extensin.

Measurements of the concentration of phenolics in induced and control petiolar epidermis and bulk petiole did not

demonstrate any differences but the concentrations of hydroxyprolines, a constituent of extensin, were found to be higher in epidermal cells but no difference was seen between control and induced tissue.

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