Ultrastructural and Cytochemical Aspects of the Biological Control of *Botrytis cinerea* by *Candida saitoana* in Apple Fruit

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Accepted for publication 8 January 1998.

ABSTRACT

El-Ghaouth, A., Wilson, C. L., and Wisniewski, M. 1998. Ultrastructural and cytochemical aspects of the biological control of *Botrytis cinerea* by *Candida saitoana* in apple fruit. Phytopathology 88:282-291.

Biocontrol activity of *Candida saitoana* and its interaction with *Botrytis cinerea* in apple wounds were investigated. When cultured together, yeast attached to *Botrytis* sp. hyphal walls. In wounded apple tissue, *C. saitoana* restricted the proliferation of *B. cinerea*, multiplied, and suppressed disease caused by either *B. cinerea* or *Penicillium expansum*. In inoculated apple tissue without the yeast, fungal colonization caused an extensive degradation of host walls and altered cellulose labeling patterns. Hyphae in close proximity to the antagonistic yeast exhibited severe cytological injury, such as cell wall swelling and protoplasm degeneration. Colonization of the wound site by *C. saitoana* did not cause degradation of host cell walls. Host cell walls in close contact with *C. saitoana* cells and *B. cinerea* hyphae were well preserved and displayed an intense and regular cellulose labeling pattern. In addition to restricting fungal colonization, *C. saitoana* induced the formation of structural defense responses in apple tissue. The ability of *C. saitoana* to prevent the necrotrophic growth of the pathogen and stimulate structural defense responses may be the basis of its biocontrol activity.

Additional keywords: epiphytic yeasts, gray mold, Penicillium rot, postharvest decay.

Currently, synthetic fungicides are the primary means of controlling postharvest diseases. However, concern over the presence of chemical residues in the food chain, the development of fungicide-resistant strains of postharvest pathogens, and the deregistration of some of the more effective fungicides have generated interest in the development of alternatives that are both effective and economically feasible (10,17,19). Among the alternatives that are being explored, the use of microbial biocontrol agents has shown significant potential (18,20). In this regard, several antagonistic yeasts have been isolated and shown to protect a variety of fruit against postharvest pathogens (4,9,14,16,18). Currently, two antagonistic microorganisms, a yeast, *Candida oleophila* Montrocher, and a bacterium, *Pseudomonas syringae*, are commercially available under the trade names Aspire and Biosave-110, respectively.

Although the use of antagonistic yeasts to control postharvest disease has been demonstrated with several commodities, their commercialization will depend on whether they are capable of effectively controlling decay of fruit from different locations with variable inoculum loads, types of infection, and levels of mechanical injury. In addition, microbial biocontrol agents will be expected to display curative activity comparable to that observed with synthetic fungicides. Currently available antagonistic microorganisms do not appear to be able to control previously established infections and are most effective when applied prior to infection by the pathogen (17,18).

The mechanisms by which yeast exert their biocontrol activity have not been fully elucidated. Biological activity of antagonistic yeasts may involve nutrient competition (4,6,7), site exclusion (6, 21), direct parasitism, and perhaps induced resistance (17,21,22). The antagonistic yeast *Pichia guilliermondii* Wickerham and *Debaryomyces hansenii* Lodder & Krejer-Van Rij have been shown to attach directly to the cell walls of postharvest pathogens (22), and their biocontrol activity can be diminished by the addition of nutrients to the wound site (7), thus indicating that attachment and nutrient competition may be implicated in their antagonism to higher fungi. As we learn more about the mechanism by which antagonistic yeasts suppress disease, more effective methods of formulating, applying, and selecting antagonists will ultimately emerge.

The current study was undertaken to determine the biocontrol activity of *C. saitoana* Nakase & Suzuki against major postharvest pathogens and to delineate some of the cytological events that occur during the interaction between the antagonistic yeast *C. saitoana* and *Botrytis cinerea* Pers.:Fr. in apple fruit.

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Publication no. P-1998-0211-01R
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MATERIALS AND METHODS

Yeast and pathogens. *C. saitoana* strain 240 was isolated from the surface of orange fruit following procedures described previously (19). *C. saitoana* was grown at 24°C for 48 h in shake-flask cultures of nutrient yeast broth. Yeast cells were pelleted by centrifugation with a Sorval RC-58 centrifuge (DuPont Instruments, Wilmington, DE) at 3,000 × *g* for 20 min, resuspended in sterile distilled water, and centrifuged again. The resulting pellets were dispersed in sterile distilled water, and the concentration of the yeast was adjusted to 10^8 CFU ml^{-1} using a hemacytometer.

Isolates of *Penicillium expansum* Link and *B. cinerea* were isolated from infected apple fruit and maintained on potato dextrose agar (PDA). A spore suspension was obtained by flooding 2-week-old cultures of *B. cinerea* and *P. expansum* with sterile distilled water containing 0.1% (vol/vol) Tween 80. Spore counts were determined with a hemacytometer, and spore suspensions from the pathogens were adjusted with sterile water to obtain 10^6 conidia per ml for *B. cinerea* and 10^4 conidia per ml for *P. expansum.*

Effect of *C. saitoana* on postharvest decay. Ripe apples (cv. Red Delicious) were harvested at the Appalachian Fruit Research

Station, Kearneysville, WV, and sorted to remove fruit with injuries or infections. Apples were stored at 4°C for 24 h prior to their use for biocontrol studies. In initial screening tests, we observed that biocontrol activity increased with increasing dosage of *C. saitoana* and decreasing dosage of the pathogen. *C. saitoana* at a concentration of 10^6 CFU ml^{-1} was not effective in controlling decay caused by *B. cinerea* and *P. expansum* applied at doses $\geq 10^3$ conidia per ml. *C. saitoana* was most effective in controlling decay caused by *B. cinerea* and *P. expansum* when applied to apple wounds at 10^8 CFU ml^{-1}.

Apples were wounded (3 mm by 5 mm deep) as previously described (13). Wounds were treated with 50 µl of cell suspension of *C. saitoana* at 10^8 CFU ml^{-1} or sterile distilled water, and challenge-inoculated with 30 µl of conidial suspension of *B. cinerea* or *P. expansum*. Noninoculated wounds were treated with sterile distilled water. Fruit were stored at 24°C under high (95%) relative humidity (RH) in enclosed plastic trays. Each treatment was applied to four replicates of 20 fruit each within each experiment. The entire experiment was repeated three times. Fruit were evaluated for symptoms daily over a period of 14 days, and diseased fruit were discarded at each inspection. An arcsine transformation was applied to the data prior to analyses of variance. Data from the three experiments were combined, because statistical analysis determined homogeneity of variances. Treatment means were separated by Duncan's multiple range test.

Wound colonization by *C. saitoana*. Apples were wounded, treated with 50 µl of yeast suspension, challenge-inoculated with a conidial suspension of *B. cinerea* or treated with sterile distilled water, and stored at 24°C as described above. For each treatment, 40 fruit in four replicates of 10 fruit each were arranged in a randomized complete block design. The entire experiment was performed twice, with similar results that were combined for statistical analysis.

Tissue samples from the different treatments were collected at various time intervals (0, 6, 24, 48, 72, 96, and 168 h) after treatment. At each sampling time, tissue samples containing the wounds were removed with a number 7 cork borer from four apples randomly selected from each treatment. Tissue samples were homogenized in 5 ml of sterile water, vortexed, dilution-plated in triplicate on yeast maltose agar medium, and the plates incubated at 24°C. Colonies were counted after 48 h, and the results are expressed as the mean number of CFU per wound.

Interaction of *C. saitoana* and *B. cinerea* on the surface of apple. The interaction between *C. saitoana* and *B. cinerea* was determined initially on the surface of apple wounds. Apples were wounded (12 mm diameter) using a razor blade, and each wound was overlaid with a sterile cellophane disk. A 20-ml spore suspension of *B. cinerea* (10^6 conidia per ml) was pipetted onto the cellophane disks, and then 40 µl of yeast suspension of *C. saitoana* was added to the *Botrytis* sp. spores. Alternatively, yeast cells were placed at the margin of hyphal growth after an incubation period of 16 h. Fruits were incubated at 24°C under high humidity in enclosed plastic trays for an additional 24 h. For each treatment, eight fruit were used, and from each replicate fruit, two small sections of the cellophane disk showing the *B. cinerea* and yeast interaction were processed for light microscopy and electron microscopy studies for a total of $n = 16$.

For light microscopy, sections of cellophane with *Botrytis* sp. hyphae and yeast cells were photographed with a Zeiss Axiophot light microscope (Carl Zeiss, Inc., Thornwood, NY) before and after rinsing with a stream of distilled water containing 1.0% (vol/vol) Tween 20. From each treatment, at least 8 separate cellophane disks from the 16 total samples were examined. The interaction between *C. saitoana* and *P. expansum* was also conducted using the same procedure as described above for *B. cinerea.*

For low-temperature scanning electron microscopy, sections of cellophane with *Botrytis* sp. hyphae and yeast cells were rinsed with distilled water containing 1.0% (vol/vol) Tween 20, sectioned, and processed. Cryopreparation of the samples was carried out with an Oxford CT-1500 cryopreparation system (Oxford Instruments Limited, Oxford, England) and cold stage-mounted on a Cambridge S-120 scanning electron microscope (SEM) (Cambridge Instruments, Cambridge, England). Sections were mounted on aluminum stubs using silver paint as an adhesive, quenched in liquid N_2-slush, transferred to the cold stage, and etched at –80°C for 5 min. Subsequently, samples were removed from the specimen, chamber-coated with gold-palladium at –160°C, and then transferred to the cold stage and held at –165 to –175°C. Samples were examined with a Cambridge S-120 SEM at 5 to 10 kV. At least 6 separate cellophane disks were examined from the 16 total samples collected for each treatment.

Interaction of *C. saitoana* and *B. cinerea* in apple wounds. To further characterize the biocontrol activity of *C. saitoana*, a cytological study of the pathogen-yeast interaction in apple wounds was conducted. Apples were wounded, treated with cell suspensions of *C. saitoana* or sterile water, challenge-inoculated with spore suspensions of *B. cinerea,* and stored at 24°C as described above. Each treatment was applied to four replicates of 20 fruit each. Treatments were arranged in a randomized complete block design, and the experiment was repeated twice. The experiment was maintained for 1 week, and tissue samples were collected 72 h after pathogen inoculation, when macroscopic lesions were visible among inoculated controls.

Tissue samples from five fruit per treatment were removed from the center of infected areas using a number 7 cork borer, incubated for 2 h at 4°C in 3% (vol/vol) glutaraldehyde in 25 mM sodium phosphate buffer (pH 6.8), and rinsed with the same buffer. Samples were dehydrated in a graded ethanol series, and then embedded in Spurr's epoxy resin (Polysciences, Warrington, PA). Half of the tissue samples were postfixed for 1 h at 4°C in 1.0% (wt/vol) osmium tetroxide in 25 mM sodium phosphate buffer (pH 6.8) prior to dehydration. For each treatment, six to eight blocks from the five total samples were sectioned, and at least 10 sections from each block were examined.

For light microscopy, tissue blocks were sectioned with glass knives. Cross sections of tissue were mounted on glass slides, stained with 1.0% toluidine blue-O for 10 min, and rinsed with deionized water. Sections were examined using a Zeiss Axiophot light microscope equipped with a camera using bright field and Nomarski differential interference contrast optics. The number of wall appositions were determined on thick cross-sections of apple tissue with a total area of 3.0 cm^2. For each treatment, approximately four thick sections were examined and the data were analyzed by analysis of variance procedures.

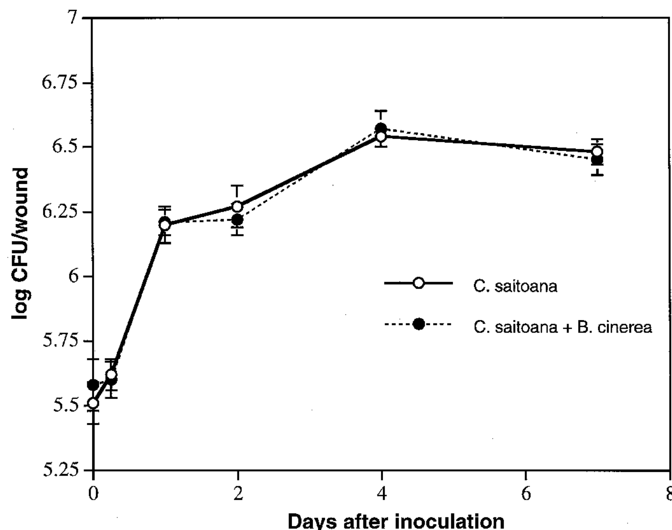

Fig. 1. Population dynamics of *Candida saitoana* in apple wounds stored at 24°C. Bars represent standard deviations.

For transmission electron microscopy, ultrathin sections fixed either with glutaraldehyde only or glutaraldehyde and osmium tetroxide were collected on nickel grids (200 mesh). Sections were stained with uranyl acetate and lead citrate, or processed for cytochemical labeling before examination with a Hitachi H-600 transmission electron microscope (Nissei Sangyo American, Mountain View, CA) at 75 kV.

Preparation of gold-conjugated probe and cytochemical labeling. Cellulase isolated from cultures of *Trichoderma reesei* was conjugated to colloidal gold following the procedure described by Berg et al. (2). Briefly, the cellulase-gold complex was prepared by adding 100 µg of cellulase with stirring to 10 ml of colloidal gold, pH 4.5, for 5 min at room temperature. The solution was further stabilized with 0.5 ml of 1.0% (wt/vol) polyethylene glycol 20,000 (PEG), centrifuged for 1 h at 4°C and 14,000 × *g*, and the pellet was suspended in 5 ml of phosphate-buffered saline (PBS), pH 5.0, containing 0.01% Tween 20. The enzyme-gold complex was centrifuged again under the same conditions and the pellet collected in 0.5 ml of the same buffer. The cellulase-gold complex was stored at 4°C. Cellulase isolated from cultures of *T. reesei* and all other reagents were purchased from Sigma Chemical Co. (St. Louis). The colloidal gold suspension was obtained from Electron Microscopy Sciences (Washington, PA).

Ultrathin sections from the glutaraldehyde-OsO_4-fixed samples were first floated for 5 min on a drop of 10 mM PBS (pH 7.3) containing Tween 20. Thereafter, the sections were transferred to a drop of the gold-complexed cellulase diluted 1:10 in PBS-Tween, pH 5.0, for 30 min at room temperature in a moist chamber. After rinsing thoroughly with PBS (pH 7.3) followed by double-distilled water, grids were contrasted with uranyl acetate and lead citrate prior to examination with a Hitachi H-600 transmission electron microscope at 75 kV.

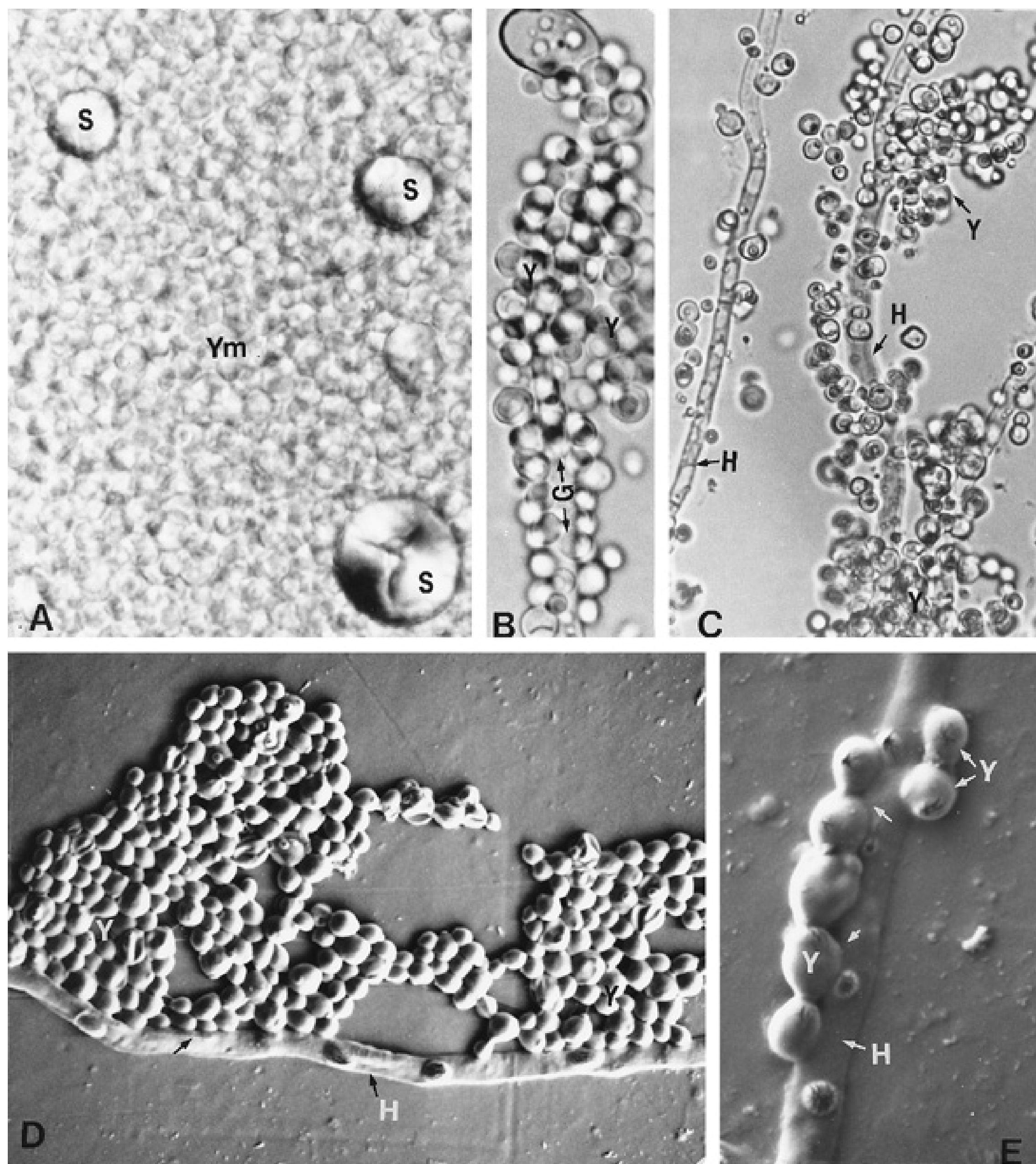

Fig. 2. Light and scanning electron micrographs of *Candida saitoana* and *Botrytis cinerea* growing on cellophane on the surface of apple. **A,** *Botrytis* sp. spores entrapped in a matrix of yeast cells (×1,700). **B,** Yeast cells encircling *Botrytis* sp. hyphae (×1,750). **C,** *C. saitoana* cells attached to *Botrytis* sp. hyphal wall despite extensive rinsing of samples with distilled water containing 1.0% (vol/vol) Tween 20 (×1,400). **D,** Yeast cells attached to hyphal wall of *B. cinerea* (×1,400). **E,** A higher magnification of **D** yeast cells that appear embedded in the hyphal cell wall of *B. cinerea* (×3,500). H = hypha, G = *Botrytis* sp. germling, S = spores, Ym = yeast matrix, and Y = yeast cells.

Specificity of the different labelings was verified using the following control tests: (i) incubation of sections with cellulase-gold complex to which one of the following polysaccharides was previously added at 1.0 μg ml^{-1}: β-1,4-glucan from barley; polygalacturonic acid; laminarin; glycolchitin; or glycolchitosan; (ii) incubation of sections with uncomplexed cellulase followed by the gold-complexed cellulase; (iii) incubation of sections with bovine serum albumin (BSA)-gold complex; and (iv) incubation with stabilized gold suspension.

RESULTS

Effect of *C. saitoana* on postharvest decay. Application of *C. saitoana* to apple wounds significantly reduced the incidence of decay caused by either *B. cinerea* or *P. expansum.* Fruit treated with *C. saitoana* and then inoculated with either pathogen showed no symptoms of infection for up to 7 days of storage at 24°C; in control fruit, lesions were visible by the third day of storage. By the seventh day, all of the control fruit had lesions ranging in size from 50 to 63 mm in diameter. In fruit treated with *C. saitoana*, lesion development and incidence of decay proceeded at a much slower rate than among the water controls. After 14 days of storage, 9 and 14% of the apples treated with *C. saitoana* and inoculated with *B. cinerea* or *P. expansum*, respectively, were diseased. Infected fruit had lesions approximately 12 mm in diameter. All of the control apples inoculated with *B. cinerea* or *P. expansum* alone were diseased. *C. saitoana* multiplied identically in apple wounds in the presence or absence of *B. cinerea.* An initial increase in the population of *C. saitoana* occurred after 6 h at 24°C. Within 24 h, *C. saitoana* population size increased nearly 10-fold and stabilized thereafter (Fig. 1).

Attachment of *C. saitoana* to hyphal walls. Light microscopy of the cellophane disks inoculated with *C. saitoana* and *B. cinerea* indicated that, by 24 h after inoculation, the yeast completely colonized the cellophane disks and formed a matrix of cells that appear to entrap the spores of *B. cinerea* and suppress germination (less than 30%) (Fig. 2A). Hyphae from germinating *Botrytis* sp. spores often were completely encircled by yeast cells (Fig. 2B). When the *Botrytis* sp. was allowed to grow for 16 h before adding *C. saitoana*, the yeast cells attached tightly to *Botrytis* sp. hyphae despite extensive rinsing of samples using distilled water with 1.0% (vol/vol) Tween 20 (Fig. 2C). Similar attachment of *C. saitoana* to hyphal walls also was observed when *P. expansum* and *C. saitoana* were cultured together (data not shown). In the absence of *C. saitoana*, all the spores of *B. cinerea* and *P. expansum* germinated and formed dense mycelia having hyphae with streaming cytoplasm (data not shown). Closer examination by scanning electron microscopy indicated that the yeast cells attached to each other (Fig. 2D) and became embedded in the hyphal walls (Fig. 2E).

Light microscope observations of yeast-pathogen interaction in apple wounds. Light microscopic examinations of thick sections from inoculated apple wounds collected 72 h after inoculation showed a massive colonization of the apple wounds by *B. cinerea.* Pathogen proliferation in wounded tissue resulted in the collapse of cell walls and internal disorganization. Invading hyphae that ramified throughout the parenchyma tissue beneath the wound

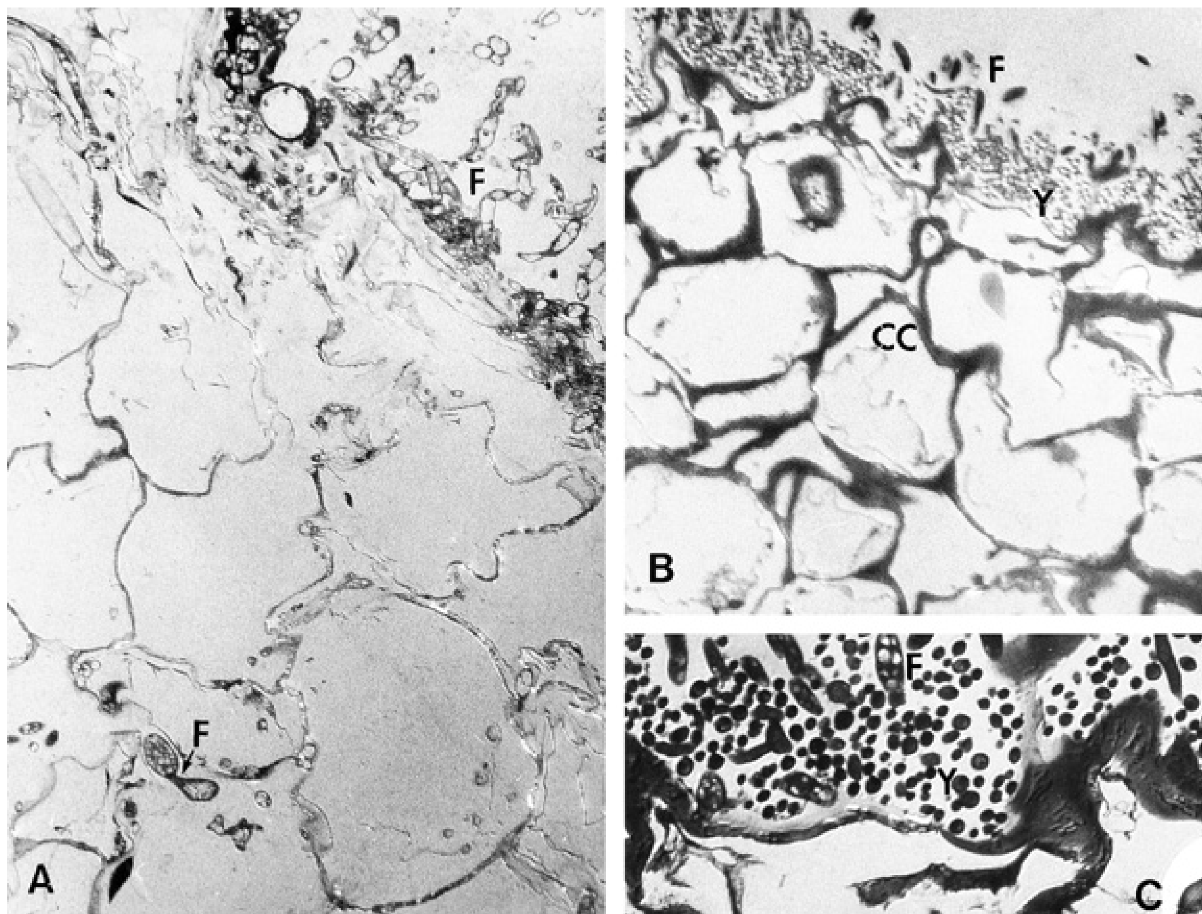

Fig. 3. Light micrographs of nontreated and yeast-treated apple tissue 72 h after inoculation with *Botrytis cinerea*. **A,** Inoculated, water-treated control showing the intense colonization of the apple tissue. *B. cinerea* proliferated intercellularly, intracellularly, and intramurally. Arrow designates wall penetration site (×400). **B,** Tissue treated with yeast cells followed by inoculation with *B. cinerea.* Note the colonization of the wound cavities by *Candida saitoana* cells. *B. cinerea* cells were restricted to wound site and were often surrounded by antagonistic yeast cells (×400). **C,** Higher magnification (×800) of **B**. F = fungal cells, CC = cortical cell, and Y = yeast cells.

site caused extensive cellular degradation (Fig. 3A). Cell walls of invaded host cells and those adjacent to them often appeared severely damaged, indicated by their marked discoloration. In the cell layer immediately beneath the wound site, *B. cinerea* cells grew inter- and intracellularly, as well as intramurally (Fig. 3A).

In apple tissue treated with *C. saitoana* and then inoculated with *B. cinerea*, *C. saitoana* prevented fungal colonization of the apple wounds (Fig. 3B and C). Examination of at least 36 thick sections from five different fruit showed a threefold decrease in the number of invading hyphae in infected tissue. Few fungal hyphae were detected in the wounded tissue layer treated with *C. saitoana*, and the invading hyphae were usually surrounded by antagonistic yeast cells. *C. saitoana* multiplied in the wound site and formed a matrix of yeast cells that covered the cell layers ruptured during wounding (Fig. 3B and C). Cells ruptured in the process of wounding, and the parenchyma tissue beneath the wound site appeared well preserved. The ground tissue consisted of highly vacuolated, thin-walled parenchyma cells. Wounded tissue treated only with *C. saitoana* showed no sign of cellular alterations and was similar to noninoculated, wounded tissues (data not shown).

Ultrastructural observations. Examination of sections from noninoculated apple wounds showed that the ground tissue beneath the wounded cells appeared normal and was not altered. Cell walls generally were well preserved and lined with a thin layer of vacuolated cytoplasm (Fig. 4A). In the pathogen-inoculated tissue sampled 72 h after inoculation, fungal cells grew extensively in the wound sites. *B. cinerea* cells proliferated both inter- and intracellularly and, in some cases, intramurally, causing extensive cell collapse in the ground tissue. Host wall alterations such as swelling (Fig. 4B) and complete wall breakdown (Fig. 4C and D) often were observed. In many cases, host walls in contact with invading hyphae were completely altered (Fig. 4C and D, arrow). Host wall swelling and shredding also were observed following intramural growth of the fungus (Fig. 4D). In infected tissues, *B. cinerea* hyphae appeared normal and were metabolically active (Fig. 4B to D). Invading hyphae had a dense, polysome-rich cytoplasm with numerous organelles and were delimited by a thin, electron-opaque wall. Ingress of the pathogen into the ground tissue did not trigger the formation of defense barriers such as wall appositions or occlusion of intercellular space.

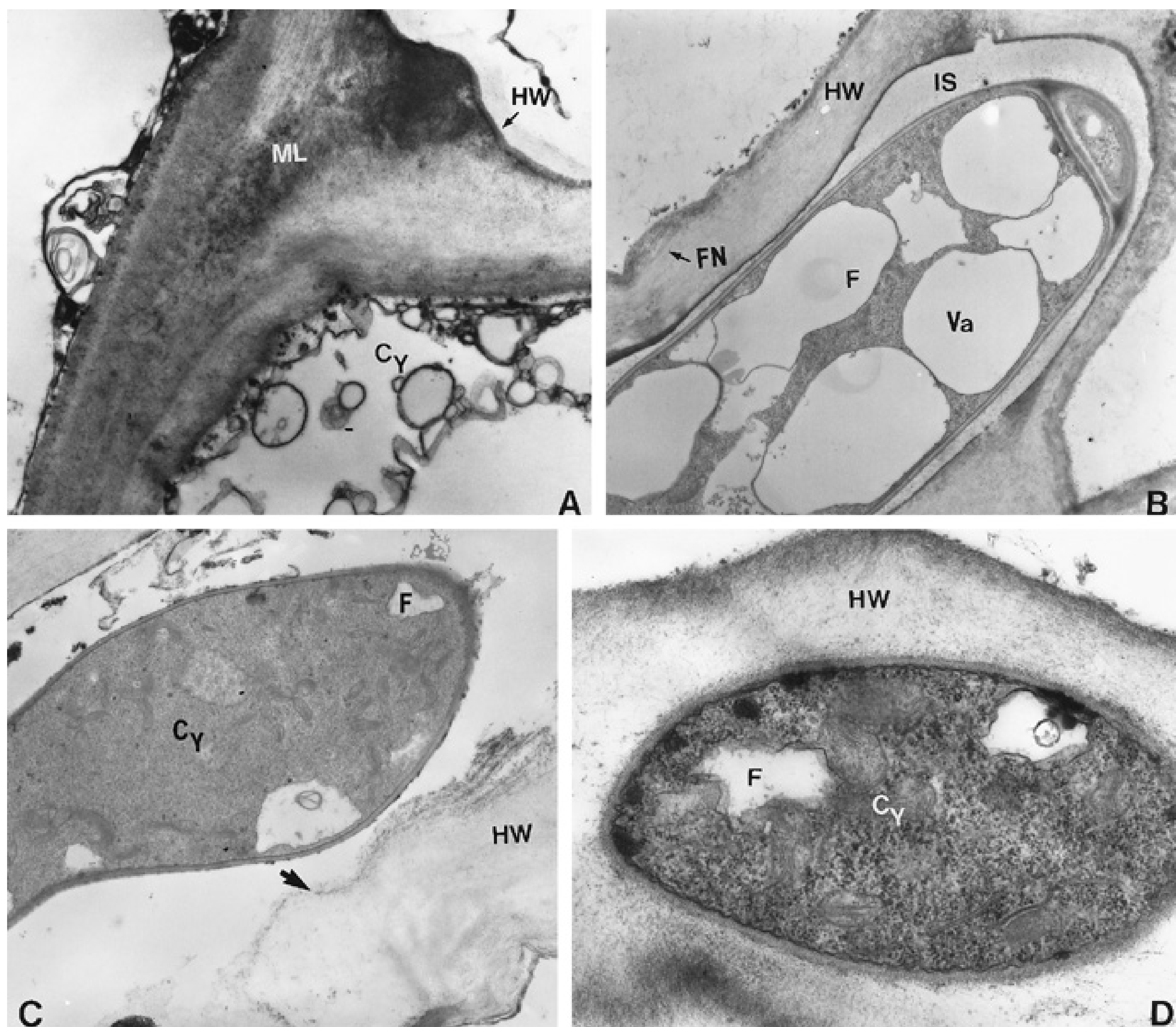

Fig. 4. Transmission electron micrographs of noninoculated and inoculated control apple tissue 72 h after inoculation with *Botrytis cinerea.* **A,** Noninoculated control, host wall appears well preserved (×10,600). **B to D,** Inoculated control. **B,** Intercellular growth of the pathogen (×5,450). **C,** *B. cinerea* hyphae growing in an intercellular space along host cell wall. Note the extensive degradation of the host cell wall facing invading hyphae (×6,600). **D,** Pathogen growth within host wall was followed by pronounced alteration and swelling of the cell wall (×24,000). Cy = cytoplasm, F= fungal cell, FN = fibrillar network, HW = host wall, IS = intercellular space, ML = middle lamella, and Va = vacuole.

In noninoculated apple tissue treated with *C. saitoana*, yeast cells were restricted mainly to the ruptured parenchyma cells (Fig. 5A). Colonization of the wounds by the antagonistic yeast did not induce degradation of the host walls. The interaction between the yeast and host cell walls did not result in any apparent alteration of wall structure (Fig. 5A to C). In the ruptured cell layer, deposition of fibrillar material around the yeast cells (Fig. 5A) and along host cell walls often was seen (Fig. 5B and C, arrow). In some instances, yeast cells appeared to be embedded in the fibrillar material deposited along the host cell wall (Fig. 5C).

In apple tissue treated with *C. saitoana* and then inoculated with *B. cinerea*, *C. saitoana* prevented the proliferation of *B. cinerea.* Few *Botrytis* sp. cells were detected at the wound site and in the ground tissue beneath the wound site (Fig. 3C). While most of the invading hyphae in contact with antagonistic yeast displayed indications of cellular disorganization (Fig. 6), few fungal cells appeared normal (Fig. 7). Examination of at least eight ultrathin sections per block showed that 68% of the *Botrytis* sp. hyphae surrounded by *C. saitoana* cells were severely damaged (Figs. 5D and 6A to D). Affected hyphae in contact with the antagonistic yeast displayed severe cellular alterations that included cell wall swelling, extensive vacuolation, and cytoplasm degeneration (Fig. 6A to D). Similar cellular alterations also were noted in *Botrytis* sp. cells cocultured with *C. saitoana* in vitro (data not shown). In extreme cases, *B. cinerea* cells with convoluted walls either were depleted of their cellular content (Fig. 6C) or their cytoplasm as-

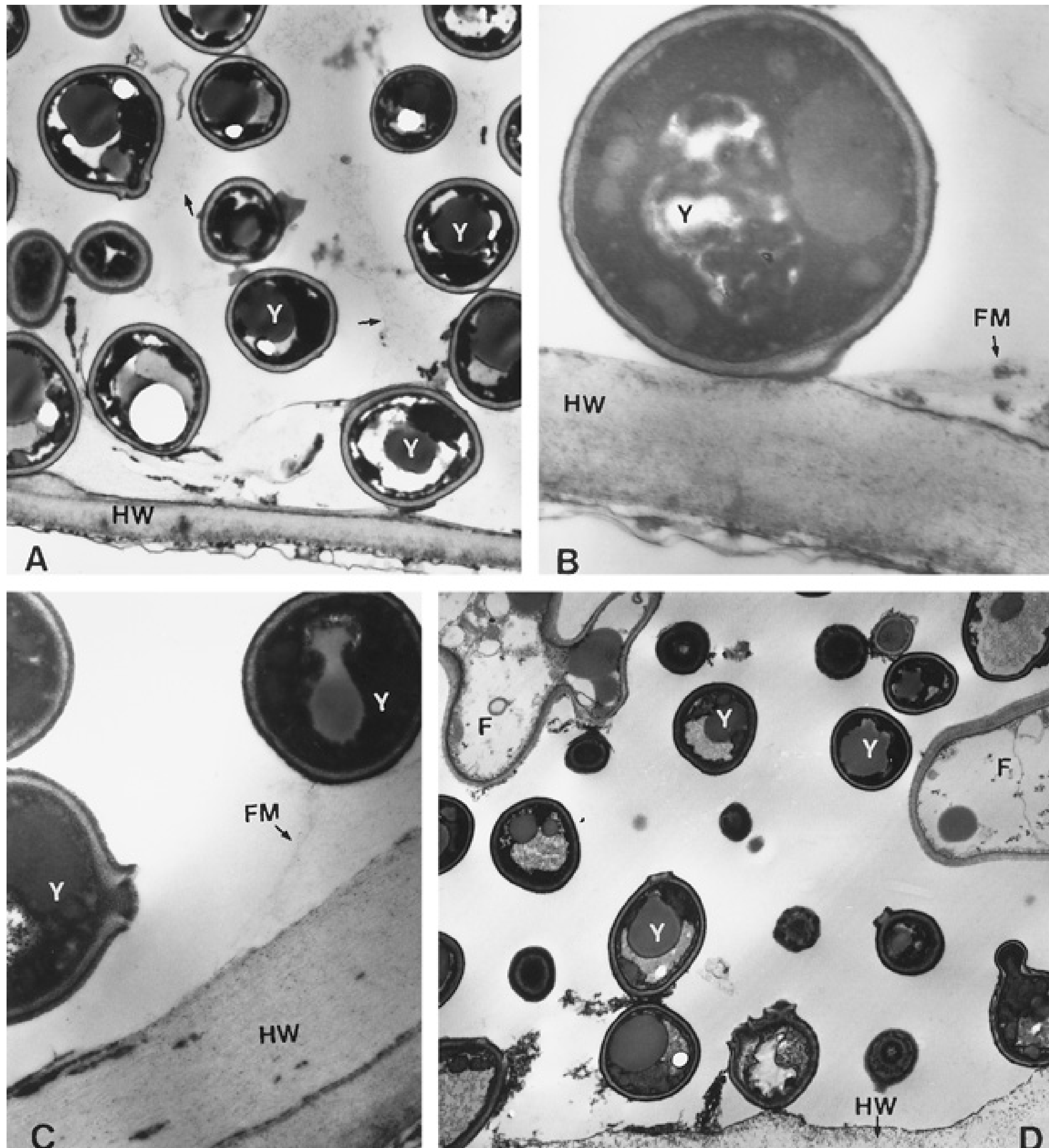

Fig. 5. Transmission electron micrographs of yeast-treated apple tissue 72 h after inoculation with *Botrytis cinerea.* **A to C,** Tissue treated with antagonistic yeasts alone. **A,** Colonization of the wound cavities by *Candida saitoana* cells. Yeast cells appear to be surrounded by extracellular material (×6,700). **B,** Yeast cell in intimate contact with host cell wall. Host wall in contact with yeast cells showed no apparent sign of alteration. Note the deposition of fibrillar material along the host cell wall (×20,000). **C,** Yeast cell embedded in an extracellular material (×14,500). **D,** Tissue treated first with antagonistic yeasts and then inoculated with *B. cinerea.* Note the cellular alteration displayed by invading hyphae and the healthy appearance of antagonistic yeast cells (×5,500). F= fungal cell, FM = fibrillar material, HW = host wall, and Y= yeast cell.

sumed an electron-dense appearance with no recognizable organelles (Fig. 6D). The hyphal-yeast interaction often resulted in the convolution and swelling of the hyphal cell wall (Fig. 6C and D), and in many cases, a complete breakdown of wall structure was observed (Fig. 6C). In contrast, yeast cells appeared normal and their ultrastructure was similar in every aspects to that previously described (5,21,22). Yeast cells were surrounded by compact cell walls and the only discernible structures in their dense cytoplasm were lipid bodies (Fig. 6). No sign of plasmolysis or protoplasm extraction as a result of fixation technique was detected.

Localization of cellulose subunits. Treatment of noninoculated apple tissue with the cellulase-gold complex resulted in specific deposition of gold particles over cell walls. Host cell walls of noninoculated tissue were thick, layered, and had an intense and regular labeling pattern (Fig. 7A). Examination of several sections from control tissue inoculated with *B. cinerea* showed that the proliferation of the pathogen in the ground tissue disrupted the structural layering of cellulose (Fig. 7B and C). Host wall penetration by invading hyphae caused the disintegration of the wall into a network of labeled fibrils (Fig. 7B and C). The intensity of gold

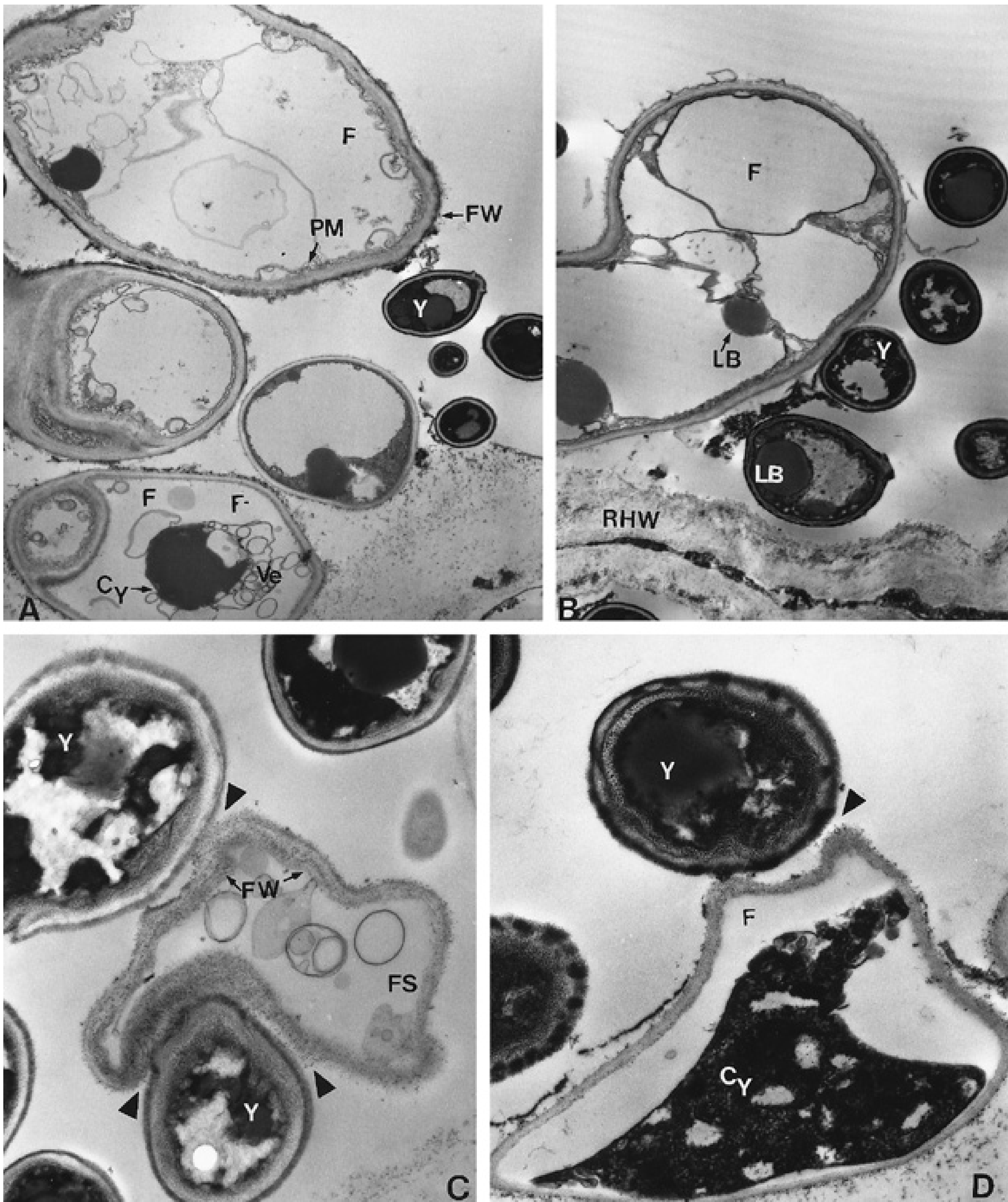

Fig. 6. Transmission electron micrographs of yeast-treated apple tissue 72 h after inoculation with *Botrytis cinerea.* **A to D,** Interaction between yeast and fungal cells in apple wound. Fungal cells in close contact with yeast cells show various degrees of alterations that include cytoplasmic degeneration (**A, C, and D**), intense vacuolation (Fig. 5B), and cell wall swelling and convolution (**C and D**). **A,** ×5,500; **B,** ×7,000; **C,** ×14,700; and **D,** ×16,000. Cy = cytoplasm, F = fungal cell, FS = fungal shell, FW = fungal wall, LB = lipid body, PM = plasma membrane, RHW = ruptured host cell, and Y= yeast cell.

labeling over penetrated host cell walls was markedly reduced, and some areas of the walls were nearly free of labeling (Fig. 7B, arrowhead). Reduction in labeling intensity was also observed over host walls in close contact with invading hyphae (data not shown).

Sections from apple tissue treated with *C. saitoana* and then inoculated with *B. cinerea* revealed an intense and regular labeling of host walls. However, the intensity of labeling appeared lower than that observed in noninoculated controls (Fig. 7A versus 7D and E). The rapid colonization of ruptured tissue by *C. saitoana* was not followed by a marked alteration in the intensity and distribution of cellulose labeling similar to that seen in inoculated controls (Fig. 7B and C versus 7D and E). A heavy deposition of gold particles was observed over host cell walls in close contact with antagonistic yeast and healthy fungal cells (Fig. 7E). Labeling was also intense and regular over wall portions closely appressed against the antagonistic yeast (Fig. 7D). Treatment of sections with stabilized gold alone or cellulase-gold complex to which 1.0 µg of β-1,4-glucan from barley per ml was added resulted in no labeling (data not shown).

Induction of structural defense responses. Several sections of apple tissue treated with either *C. saitoana* and then inoculated with *B. cinerea*, or with *C. saitoana* alone, showed that the yeast

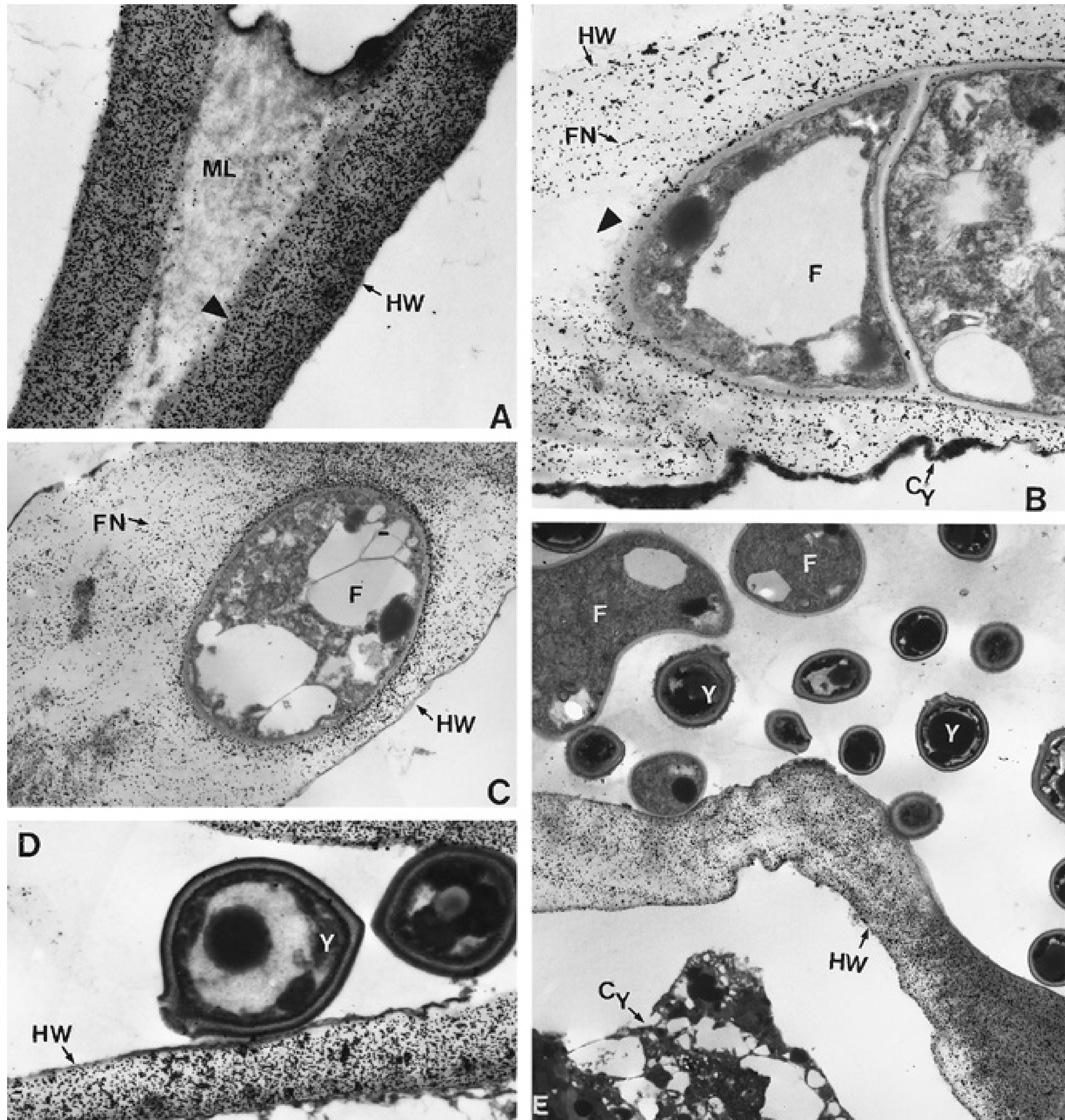

Fig. 7. Transmission electron micrographs of apple tissue 72 h after inoculation with *Botrytis cinerea* and labeled with the gold-complexed exoglucanase for localization of cellulosic β-1,4-glucan. **A,** Noninoculated control. Note the intense and regular deposition of gold particles over host cell wall (×12,700). **B and C,** Inoculated control. **B,** Pathogen growth in host wall is followed by complete disruption of the cellulose labeling. Small portion of the wall appears to be free from labeling (×12,000, arrowhead). **C,** Intramural growth of the *Botrytis* sp. hyphae caused alteration in the labeling distribution and intensity (×6,000). **D and E,** Tissue treated with antagonistic yeasts and then inoculated with *B. cinerea.* **D,** Intense and regular labeling was often observed over host walls in close contact with yeast cells (×13,000). **E,** Reduction in labeling intensity was observed over host walls in close contact with yeast cells and fungal cells (×6,300). Cy = cytoplasm, F = fungal cell, FN = fibrillar network, HW = host wall, ML = middle lamella, and Y = yeast cell.

induced the formation of structural defense responses in apple tissue. In apple tissue treated with *C. saitoana* prior to inoculation with *B. cinerea*, formation of host structural barriers was detected mainly in the first tissue layers underlying the ruptured cells and was absent in the inner ground tissue. The most common defense reactions observed were the formation of papillae (Fig. 8A and B) and hemispherical protuberances along host cell walls (Fig. 8C and D). In many cases, the papilla (wall apposition deposited on the inside of the cell walls) appeared impregnated with electron-opaque material (Fig. 8B). The hemispherical protuberances are deposited outside the cell and appeared to interfere with pathogen ingress (Fig. 8D). Similar defensive reactions were also observed in apple tissue treated with *C. saitoana* alone. The magnitude of the induction of these structural reactions did not appear to increase upon challenge with *B. cinerea*, as indicated by the estimated number of wall appositions per cm^2 of tissue. In apple tissue treated with *C. saitoana* alone or with *C. saitoana* prior to inoculation with *B. cinerea*, the number of wall appositions was estimated at 5.3 and 6.3 per cm^2, respectively. In contrast, in noninoculated and inoculated controls, the number of wall appositions was estimated at 0.7 and 1.0 per cm^2, respectively.

DISCUSSION

In recent years, there has been an interest in the development of nonantibiotic-producing antagonistic yeasts for the biological control of postharvest diseases (17,18). In the current study, we demonstrated that *C. saitoana* proliferated in apple wounds, attached to

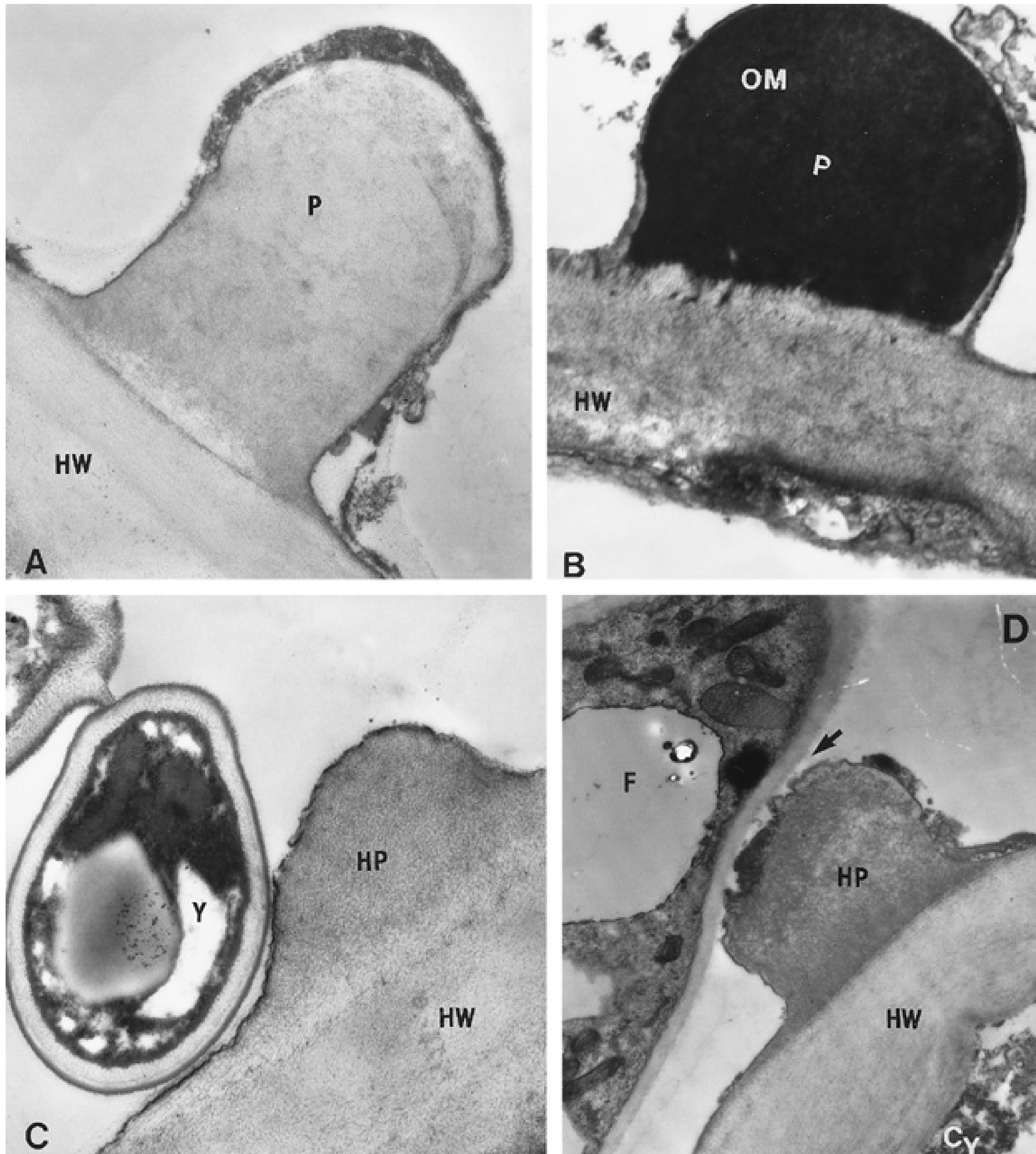

Fig. 8. Transmission electron micrographs of yeast-treated apple tissue 72 h after inoculation with *Botrytis cinerea.* **A and B,** Deposition of papilla along host cell wall (**A,** ×15,000). **B,** Complete impregnation of papilla with an electron-opaque material (×20,000). **C and D,** Formation of hemispherical protuberance outside the host cell (**C,** ×21,000). **D,** Wall apposition appears to interfere with fungal ingress (×13,000). Cy = cytoplasm, F = fungal cell, HP = hemispherical protuberance, HW = host wall, OM = opaque material, P = papilla, and Y = yeast cell.

Botrytis sp. cell walls, prevented the proliferation of *B. cinerea* in apple wounds, stimulated several host defense reactions, and controlled decay of apple fruit caused by *B. cinerea* and *P. expansum.*

This cytological study of the infection process of apple tissue by *B. cinerea* shows that the growth pattern of *B. cinerea* is mainly necrotrophic. The massive colonization of apple tissue by *B. cinerea* resulted in extensive host cell wall degradation and tissue collapse. Similar wall alterations have also been reported in other *Botrytis* sp.-host interactions and were attributed to a synergistic action between cell wall-degrading enzymes and organic acids, namely oxalic acid (11,12,15). Enzymatic degradation of the host cell wall by expanding *B. cinerea* hyphae was, in part, supported by the observed swelling of host cell walls and the alteration of cellulose labeling. Evaluation of the cellulose labeling patterns in infected tissue showed that growth of hyphae within the host cell wall caused a complete disruption of the structural layering of cellulose labeling.

The observed restriction of fungal ingress and preservation of host wall integrity strongly indicate that *C. saitoana* may have affected the ability of *B. cinerea* cells to degrade host tissue and establish a nutritional relationship. Indeed, in many cases, the interaction between *C. saitoana* and *B. cinerea* cells at the wound site caused severe cytological damage to *B. cinerea* cells. Hyphal cells in close contact with yeast cells showed various degrees of alteration that ranged from cell wall swelling to degeneration of the cytoplasm. The cellular damage evident in *Botrytis* sp. cells was similar in many respects to that reported in aged and nutrient-deprived fungal cells (3). It is quite possible that the observed cellular alterations of *B. cinerea* cells may be the result of nutritional starvation caused by rapidly multiplying *C. saitoana.* Nutrient competition is believed to play a major role in the biocontrol activity of various antagonistic yeasts (6,7). This has been supported by the fact that their biocontrol activity can be overcome by the addition of nutrients (7).

Examination of sections from both apple wounds and in vitro studies showed that *C. saitoana* cells were able to attach to each other and to the hyphal cell walls of *B. cinerea* and concomitantly cause swelling and, in extreme cases, the complete disruption of hyphal wall structure. A similar attachment pattern was also observed in vitro with other yeast species (5,21,22) including the antagonistic yeast *Pichia guilliermondii*, when cocultured with *B. cinerea*, and was attributed to a lectin-type recognition (22). The partial degradation of *B. cinerea* cell walls by *Pichia guilliermondii* was attributed to its tenacious attachment to hyphal walls in conjunction with its production of β-1,3 glucanase (22). Like *Pichia guilliermondii*, *C. saitoana* is also capable of producing fungal wall-degrading enzymes, namely chitinase and β-1,3 glucanase (data not shown); therefore, upon attachment, an intramural release of lytic enzymes by *C. saitoana* can be anticipated. This, in part, may explain the alterations observed in fungal wall areas in contact with *C. saitoana* cells. As to the role played by the attachment capability of antagonistic yeasts in the observed control of lesion development, it has been suggested that attachment may enhance nutrient competition as well as interfere with the ability of the pathogen to initiate infection. Analysis in planta of the effect of treatments that negate attachment may provide further insight regarding the extent of the role played by attachment in the biocontrol activity of antagonistic yeasts.

Treating apple wounds with *C. saitoana* cells also stimulated several structural defense reactions such as the formation of papillae and other protuberances along host cell walls in tissue layers underlying the wounded area. The observed wall appositions were often completely or partially impregnated with amorphous electron-opaque substances. Similar materials have been reported in other structural barriers (8) and were believed to contain phenolic-like compounds. The activation of structural defense processes by *C. saitoana* could help the tissue restrict the spread of invading pathogens. Mechanical defensive reactions have been shown to prevent pathogen invasion in several plant-pathogen systems (1).

In conclusion, this study demonstrates the effectiveness of the antagonistic yeast *C. saitoana* in controlling postharvest decay of apple caused by *B. cinerea* and *P. expansum.* The biocontrol activity of *C. saitoana* appears to stem from its ability to restrict fungal proliferation, directly interfere with infection hyphae, and stimulate structural defense responses.

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