Host-Pathogen Interactions XXXIV.
A Heat-Labile Activity Secreted by a Fungal Phytopathogen Releases Fragments of Plant Cell Walls that Kill Plant Cells

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Magnaporthe grisea (anamorphs: *Pyricularia oryzae* Cav., *P. grisea*), the ascomycetous causal agent of rice blast, was grown in liquid shake culture either on a minimal salts medium with a selected carbon source or on a complete (yeast extract-casein hydrolysate-sucrose) medium. When grown on pectin or isolated plant cell walls as the carbon source, the fungus secreted, into the culture medium, a heat-labile activity capable of killing plant cells. Plant cell death was monitored by the ability of suspension-cultured maize cells to incorporate [^{14}C]leucine into acid-precipitable material. The heat-labile killing activity was neither secreted when the fungus was grown on sucrose or xylan nor produced when the fungus was grown on a complete medium. Heat-stable wall fragments, capable of killing plant cells, were released from isolated maize suspension-cultured cell walls treated with the heat-labile killing activity.

Plant cell death is the initial physiological observation associated with the hypersensitive resistance response. The hypersensitive response is defined as a rapid, localized necrosis of one or a few cells at the site of attempted invasion by a pathogen leading to an incompatible interaction, that is, a resistant response by the host (Tomiyama 1982; Fritig et al. 1987; Tomiyama et al. 1979). The hypersensitive response occurs in a plant when challenged by a microbe unable to infect the plant, even if the microbe is a pathogen on other cultivars of that plant.

The hypersensitive response is a frequently reported phenomenon in plant pathology (see references in Hahn et al. 1989; Tomiyama 1982; Fritig et al. 1987; Tomiyama et al. 1979), but the biochemical mechanism by which the response is triggered has not been determined. Previously suggested inducers of the hypersensitive response in various plant systems include cell wall preparations from *Phytophthora infestans* (Mont.) de Bary (Doke and Tomiyama 1980; Currier 1981), lipids from *P. infestans* (Kurantz and Zacharius 1981; Preisig and Kuć 1988), a small peptide from intercellular fluids of fungal-infected tomato (De Wit et al. 1985), and the glycan moiety of a glycoprotein from germ tube walls of the uredospores of the fungal phytopathogen *Puccinia graminis* Pers. f. sp. *tritici* (Moerschbacher et al. 1986).

Other researchers have presented evidence that the hypersensitive response is associated with an activation of the plant plasmalemma K^+/H^+ exchange (Atkinson et al. 1985), with consumption of ATP (Tomiyama 1982), with increased protein synthesis (Tomiyama 1982), with increased lipoxygenase activity (Ocampo et al. 1986), with production of superoxide anion (Doke 1983), with increased cell wall hydroxyproline content (Hammerschmidt et al. 1984), with increased lignin deposition (Hammerschmidt et al. 1984; Beardmore et al. 1983), and with a decrease in the plasma membrane potential which was attributed to direct contact between the plant cell membrane and fungal hyphae (Tomiyama 1982). However, whether any of the above phenomena are the cause or the result of hypersensitive cell death or "injury" has not been established. We conclude that the trigger for hypersensitive cell death remains to be identified.

Treatment of plant cells or tissues with cell wall-degrading enzymes that are secreted by bacteria or fungi leads, in at least some instances, to death of the plant cells (for a review, see Hahn et al. 1989). It was suggested that these enzymes kill plant cells by weakening the cell wall to the point that the cell ruptures (Bateman and Basham 1976). However, a pectate lyase from *Erwinia* has been described that, at pH 5-6, has low macerating (that is, low wall-degrading) activity on potato tissue and yet is highly toxic to potato cells (Quantick et al. 1983). Furthermore, the purified polygalacturonases from *Aspergillus niger* van Tieghem and *Fusarium moniliforme* Sheldon macerate and kill potato tissue, but the addition of an excess of the polygalacturonase-inhibiting protein negates the macerating activity without apparently decreasing the killing activity (Cervone et al., in press). Thus, the killing activity of these enzymes may, in some instances, be dissociated from large-scale degradation of the cell wall.

Our laboratory has shown that acid-solubilized fragments of isolated sycamore cell walls are able to kill plant cells (Yamazaki et al. 1983). Several assays for cell death were utilized, including the ability of the cell wall

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fragments to inhibit incorporation of [^{14}C]leucine into acid-precipitable proteins. The related observations that cell wall fragments and wall-degrading enzymes can kill plant cells led us to investigate the possibility that the trigger for hypersensitive cell death is the release, by pathogen-secreted enzymes, of death-inducing plant cell wall fragments, oligosaccharins, from covalent attachment within the cell wall. The pathogen we selected for these studies was *Magnaporthe grisea* (Hebert) Barr (anamorphs: *Pyricularia oryzae* Cav., *P. grisea* (Cooke) Saccardo), the causal agent of rice blast (Ou 1980). We selected this fungus for our studies because it is readily manipulable by both classical (Valent et al. 1986) and molecular (Parsons et al. 1987) genetic techniques, making it an excellent organism to determine whether cell death induced by cell wall fragments is the biochemical mechanism by which hypersensitive cell death is triggered.

MATERIALS AND METHODS

Plant cell suspension cultures. Suspension-cultured rice cells (*Oryza sativa* L. cv. Yamabiko) derived from callus tissue of immature embryos were the kind gift of Horst Lörz (Max-Planck-Institut für Züchtungsforschung, Köln, BRD [West Germany]) and have been maintained in this laboratory since 1985. The suspension-cultured rice cells were subcultured at a 1:10 dilution every 14 days into N6 medium (Chu et al. 1975) supplemented with 1 μg/L of kinetin.

Suspension-cultured maize cells, line BM-7, (*Zea mays* L. cv. Black Mexican Sweet) were derived from callus induced on stem tissue from 10–14-day-old seedlings and were the kind gift of Christopher M. Donovan (University of Minnesota, St. Paul). The maize cells, which have been maintained in this laboratory since 1983, were subcultured at a 1:10 dilution every 7 days into a medium containing Murashige and Skoog salts (Murashige and Skoog 1962), 20 g/L sucrose, 132 mg/L L-asparagine, 2 mg/L glycine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, 0.1 mg/L thiamine·HCl, and 0.5 mg/L 2,4-dichloro-phenoxyacetic acid.

Suspension-cultured sycamore (*Acer pseudoplatanus* L.) cells, which were derived from a culture originally started by Lamport (1964), have been maintained by this laboratory since 1960. The suspension-cultured sycamore cells were subcultured every 7 days at a 1:10 dilution into the medium described by York et al. (1985) containing 20 g/L of sucrose rather than 1 g/L.

The three species of suspension-cultured cells were maintained in the dark at 24–25° C on 25.4-mm-diameter orbit gyratory shakers (Model G-10, New Brunswick Scientific, Edison, NJ) rotating at 100 revolutions per minute (rpm).

Isolation of plant cell walls. Cell walls were isolated from the suspension-cultured maize, rice, and sycamore cells using the procedure described by York et al. (1985), except that, following the organic solvent washes, the α-amylase treatment was omitted.

Preparation of acid-released cell wall fragments. Fragments were solubilized from isolated cell walls by partial acid hydrolysis as described by Yamazaki et al.

(1983), except that the fragments were desalted not by dialysis but by chromatography in deionized water on a Bio-Gel P-2 column (3 × 80 cm) (for fragments prepared from isolated sycamore or maize cell walls) or on a Sephadex G-10 column (2.5 × 67 cm) (for fragments prepared from isolated rice cell walls). The fragments from sycamore and maize cell walls were lyophilized and redissolved at 5 mg/ml in deionized water for use in the [^{14}C]leucine incorporation assay. The Sephadex G-10 fractions of the rice cell wall fragments were tested directly in the [^{14}C]leucine incorporation assay.

Fungus culture. *M. grisea* strain Ken 60-19 was a gift of Barbara Valent (E. I. du Pont de Nemours & Co., Wilmington, DE). The fungus was stored in sterile glassine envelopes at −20° C in the form of desiccated hyphae-impregnated cellulose filters (Valent et al. 1986). Working stocks of the fungus were maintained under a fluorescent light on oatmeal agar (prepared from 100 g of rolled oats steeped in 1 L of deionized water at 70° C for 1 hr, filtered through cheesecloth, and made up to 2 L of medium). New oatmeal agar petri dishes were inoculated using plugs (approximately 2 mm) of older plates for a maximum of three transfers before the fungus was started fresh from the desiccated hyphae (Valent et al. 1986). The minimal salts medium used for liquid shake cultures was that of Vogel (1964) supplemented with 1 mg/L of thiamine·HCl and a carbon source, which was variously (per liter) 10 g sucrose, 5 g citrus pectin (No. 3444, Sunkist Growers, Corona, CA), 5 g β-1,4-D-xylan (Pfaltz & Bauer, Inc., Flushing, NY), or 2.5 to 5 g plant cell walls. The complete medium contained (per liter) 10 g sucrose, 3 g yeast extract, and 3 g casein hydrolysate.

Seed cultures were started by washing conidiospores from an oatmeal agar petri dish using approximately 2 ml of sterile water and a glass spreader and inoculating 100 ml of 0.6% yeast extract containing 1% sucrose with 1 × 10^6 conidiospores (number determined by counting on a hemacytometer). The seed cultures were incubated overnight on a shaker at 100 rpm, and 10 ml aliquots of these cultures were used to inoculate each liter of liquid medium. Liquid shake cultures were harvested 9 to 14 days after inoculation, because trial cultures on pectin had been found to contain the highest levels of heat-labile killing activity at this age.

Protein determination. Protein was determined by the Bradford method (Bradford 1976) using reagents from Bio-Rad, Richmond, CA. Bovine serum albumin (Sigma, St. Louis, MO) was used as the standard.

Assay for [^{14}C]leucine incorporation. A modification of the assay described by Yamazaki et al. (1983) was used. Maize cells, 4 to 7 days after transfer, were collected on a piece of muslin in a beaker and agitated to obtain the smaller cell clumps that passed through the fabric. These cells were collected by mild vacuum filtration onto a double layer of 4.25-cm GF/A glass-fiber filters (Whatman International, Clifton, NJ) in a Büchner funnel; the cells were washed with and suspended in fresh medium at approximately 1.5% packed cell volume. This cell suspension (250 μl), 50 mM sodium succinate, pH 6.0 (100 μl), and deionized water (150 μl minus the volume of sample to be tested for activity) were added to 19 ×

48 mm glass vials. The vials were placed on a 19-mm-diameter orbit gyratory shaker (Model G-2, New Brunswick Scientific) at 100 rpm. After a 30-min incubation period, a sample containing a possible killing factor or control substance (1 to 150 μl) was added to each vial, bringing the final assay volume to 500 μl. After an additional 1 hr incubation, 0.05 of μCi of L-[U-^{14}C]leucine (specific activity 270–330 mCi/mmol, ICN, Irvine, CA) in 20 μl of water was added to each vial. Two hours after the addition of the radiolabeled leucine, 5 ml of 10% (w/v) trichloroacetic acid (TCA) was added to each vial, and the vials were stored at 4° C for at least 1 hr to precipitate proteins.

The contents of each vial were agitated and then immediately passed through a 2.4-cm 934-AH glass-fiber filter using a 12-sample filtering manifold (Model 1225, Millipore, Bedford, MA). The vials were rinsed with approximately 5 ml of cold 10% TCA, and this was poured through the filter. Each filter was then washed two more times with approximately 5 ml of cold 10% TCA to wash out unincorporated radiolabeled leucine. The filters were placed in scintillation vials, 2 ml of cocktail (Scinti Verse I, Fisher Scientific, Atlanta, GA) was added, and the vials were counted for 1 min in a Beckman LS 1801 scintillation counter to obtain the counts per minute of [^{14}C]leucine incorporated.

Each sample was assayed in triplicate; the results are presented as the average counts per minute of [^{14}C]leucine incorporated into the triplicates plus or minus the standard deviation. Each experiment included the negative control of no added killing factor (that is, 150 μl of water) and the positive control of the addition of 100 μl of 0.1 mg/ml cycloheximide. The water control in each experiment was defined as 0% inhibition (percent inhibition = 100 [1-experimental value/control value], where experimental value is defined as cpm [^{14}C]leucine incorporated into maize cells treated with a killing factor, and control value is defined as cpm [^{14}C]leucine incorporated into untreated maize cells); the cycloheximide control typically inhibited the [^{14}C]leucine incorporation by 75 to 80%. The data establishing the sensitivity of the maize cells to the heat-labile killing activity were converted to percent inhibition and fitted, by means of computer-assisted linear regression analysis, to logarithmic curves illustrating percent inhibition rather than counts per minute of [^{14}C]leucine incorporation. One killing unit (KU) was defined as that amount of activity that resulted in 50% inhibition (from the best-fitted dose-response curves) of [^{14}C]leucine incorporation.

Acid-solubilized fragments from sycamore and maize cell walls were also tested in a [^{14}C]leucine incorporation assay identical to the one described above, except that suspension-cultured sycamore cells rather than maize cells were used.

Comparison of killing activity production during the growth of *M. grisea* on the isolated cell walls of rice, maize, and sycamore cells. Two hundred-milliliter cultures of *M. grisea* were started with 2 ml of seed culture and grown on minimal salts media containing 0.5% rice, maize, or sycamore cell walls. Two-milliliter aliquots were removed at 2-day intervals until the culture had been grown for 22 days. The individual aliquots were passed through GF/

A glass-fiber filters (Whatman), and sodium azide was added (0.02% w/v). The aliquots were stored at 4° C until all of the aliquots had been collected. The aliquots were then dialyzed at 4° C for approximately 24 hr against three 8 L changes of 50 mM sodium succinate, pH 6.0. Each aliquot was analyzed at several concentrations for heat-labile killing activity in the [^{14}C]leucine bioassay. Each dose-response curve thus obtained for the individual aliquots was fitted to the best logarithmic curve, and the volume of culture filtrate required to cause 50% inhibition of [^{14}C]leucine incorporation (that is, one KU) was calculated from these curves.

Preparation of the heat-labile killing activity. The 1-L liquid shake cultures of *M. grisea*, grown in minimal salts on sucrose, xylan, pectin, or rice cell walls, were passed through 12.5-cm 934-AH glass-fiber filters in a Büchner funnel using vacuum filtration. Sodium azide (0.01% w/v) was added to each filtrate, and the filtrates were poured into 75-mm-diameter dialysis tubing (M_r 12,000–14,000 cutoff, Spectrum Medical Industries, Los Angeles, CA). The dialysis sacks were placed in a bucket, and polyethylene glycol compound (M_r 15,000–20,000, Sigma) was poured over them. After approximately 1 wk at 4° C, the contents of the tubing were collected by rinsing the tubing twice with approximately 40 ml of cold 50 mM sodium succinate, pH 4.5. This material was placed in 45-mm-diameter dialysis tubing and dialyzed in the cold for approximately 10 hr against two 18-L changes of 50 mM sodium succinate, pH 4.5. The dialyzed samples were recovered and the tubing rinsed with buffer so that the final volume of each concentrated, dialyzed filtrate was 150 ml.

An aliquot (10 ml) of each sample was assayed for cell killing activity in the [^{14}C]leucine incorporation assay. The remaining dialyzed filtrate was applied at 4° C to a 1 × 2 cm CM-Sephadex C-50 (Pharmacia, Piscataway, NJ) column equilibrated in 50 mM sodium succinate, pH 4.5. The columns were washed with approximately 5 ml of buffer, and then the bound material was eluted with 4 ml of 2 M NaCl in buffer. The eluates were immediately desalted at 4° C on a 1.5 × 28 cm Sephadex G-15 (40–120 μm) column using the succinate buffer. The void fractions of each desalting run were pooled and made up to 10 ml with succinate buffer. Aliquots (200 μl) of these desalted CM-Sephadex eluates were analyzed for killing activity using the [^{14}C]leucine incorporation assay.

The pH of the sodium succinate buffer used for the ion exchange chromatography was adjusted to 4.5 because this pH had been determined to give optimal binding of the heat-labile killing activity to the cation exchange column. Succinate buffer, at the concentrations used, had no effect on the ability of the cells to incorporate [^{14}C]leucine into protein.
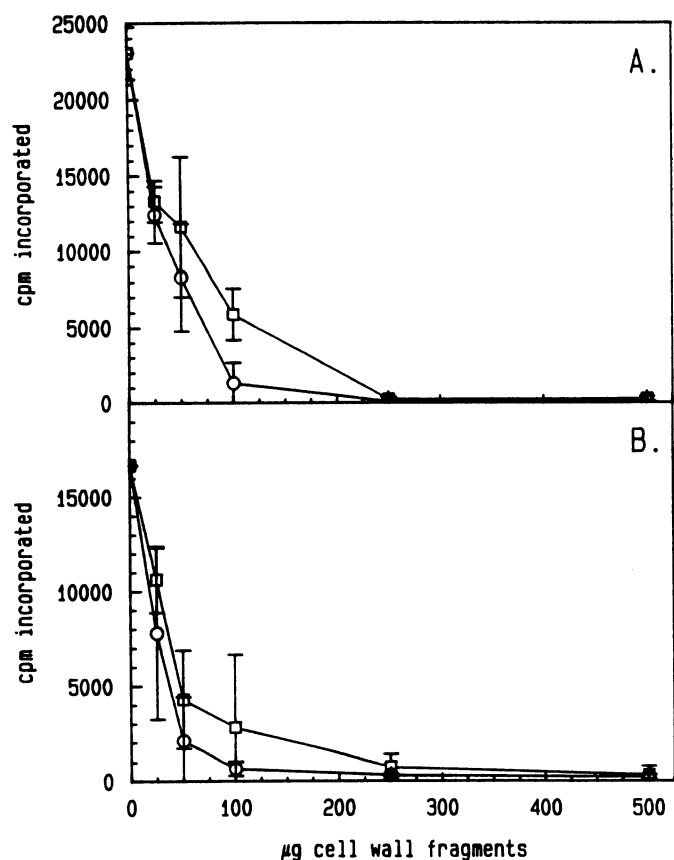
Treatment of cell walls with the heat-labile killing activity. One gram of isolated maize cell walls was suspended in 50 ml of 10 mM sodium succinate containing 10 mM CaCl$_2$, pH 6.0, in a 50-ml polypropylene screw-cap tube (Sarstedt Inc., Princeton, NJ) with 1 ml toluene added to inhibit microbial growth. The cell walls were incubated for 1 to 2 days at room temperature with occasional shaking and were then collected on a 40- to 60-μm sintered-glass funnel (60-ml capacity). The cell walls were washed

on the funnel with fresh buffer and then resuspended in 50 ml of fresh buffer. A second 1- to 2-day incubation period was conducted, and the washing was repeated. The twice-suspended and twice-washed cell walls were re-suspended in 50 ml of fresh buffer with 1 ml of toluene.

Aliquots of desalted CM-Sephadex eluates were added to the washed cell wall suspension and mixed; control samples were included that contained only the cell wall suspension or the cell wall suspension with an equivalent amount of heat-treated (120° C for 10 min in a screw-cap tube) CM-Sephadex eluate. Following the addition of CM-Sephadex eluates, 3-ml aliquots of the cell wall suspensions were removed at various times using an inverted, disposable 5-ml pipet and filtered through a 10- to 15-μm sintered-glass funnel (15-ml capacity) into Teflon-lined screw-capped glass tubes (13 × 100 mm). The capped tubes were placed in a heating block for 5 min at 120° C. The samples were then frozen at −20° C and lyophilized. The samples were redissolved in 0.6 ml of deionized H_2O and analyzed for killing activity in the [^{14}C]leucine assay.

RESULTS

Effects of acid-solubilized cell wall fragments on [^{14}C]leucine incorporation into maize and sycamore cells. The acid-solubilized, desalted sycamore and maize cell wall fragments both severely inhibited the abilities of sycamore
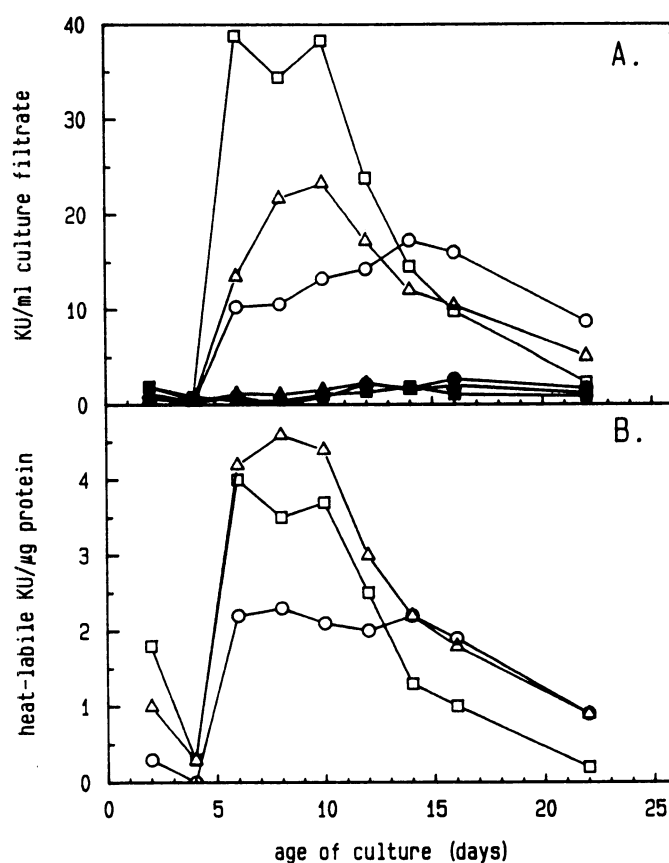
(Fig. 1A) and maize (Fig. 1B) cells to incorporate [^{14}C]leucine. The sycamore wall fragments had a slightly greater effect on both cell types. The Sephadex G-10 fractions of acid-solubilized rice cell wall fragments inhibited the ability of maize cells to incorporate [^{14}C]leucine (data not shown); the inhibitory material extended from the void volume of the column well into the partially included volume.

Comparison of the production of the killing activity during the growth of *M. grisea* on the isolated cell walls of rice, maize, and sycamore cells. The results are plotted as KUs per milliliter vs culture age in days (Fig. 2A). Essentially all of the killing activity in the culture medium was heat-labile. While the highest activity per volume of culture filtrate was produced when the fungus was grown on rice cell walls, the specific activity was at least as high when sycamore cell walls were used as the carbon source (Fig. 2B).

Comparison of the production by *M. grisea* of heat-labile killing activity when grown on four carbon sources. The killing activities of the concentrated, dialyzed culture filtrates from 10-day-old, 1-liter cultures of *M. grisea* grown on sucrose, xylan, pectin, and rice cell walls (0.25% w/v) are compared in Figure 3. Heat-labile killing activity

Fig. 1. Ability of acid-solubilized fragments from isolated sycamore (O) and maize (□) cell walls to inhibit [^{14}C]leucine incorporation into proteins of suspension-cultured sycamore (A) and maize (B) cells.

Fig. 2. Production of heat-labile killing activity by *Magnaporthe grisea* grown on isolated plant cell walls as the carbon source. Maize cell walls (O), rice cell walls (□), and sycamore cell walls (△); closed symbols represent the corresponding heat-treated samples. (A), Killing units (KUs) per milliliter of culture filtrate. (B), Heat-labile KUs per microgram of protein (relative to bovine serum albumin).

was produced when *M. grisea* was grown on pectin or on rice cell walls as a carbon source; the level of killing activity was higher when the carbon source was rice cell walls rather than when the carbon source was pectin. The low level of killing activity produced when the fungus was grown on sucrose or xylan was all heat-stable (Fig. 3). No detectable heat-labile killing activity was produced when the fungus was grown on a complete (yeast extract-casein hydrolysate-sucrose) medium because the ability of the culture filtrate to inhibit [^{14}C]leucine incorporation was unaffected by heat treatment (data not shown).

CM-Sephadex chromatography of the culture filtrates. The abilities of the desalted eluates from CM-Sephadex columns of the filtrates of *M. grisea* grown on four carbon sources to inhibit [^{14}C]leucine incorporation are summarized in Figure 4. There was little difference between the heat-stable and heat-labile killing activities when the fungus was grown on xylan or sucrose; approximately 85% of the killing activity of the fungus grown on pectin and all of the activity of the fungus grown on rice cell walls were heat-labile (activities of heat-treated CM-Sephadex eluates not shown). The percents of the heat-labile killing activities of the fungus grown on pectin and rice cell walls

recovered from the CM-Sephadex column are summarized in Table 1. Assays of the culture filtrates and CM-Sephadex eluates after storage for 6 days on ice suggested that the activities of the CM-Sephadex eluates were more stable than the culture filtrates (Table 1). The loss of activity was greater in the filtrate of *M. grisea* grown on rice cell walls than in the filtrate of *M. grisea* grown on pectin.

Release of heat-stable killing activity from maize cell walls. Treatment of isolated maize cell walls with desalted CM-Sephadex eluate of the *M. grisea* culture filtrate of the fungus grown on rice cell walls released heat-stable killing activity (Fig. 5A). The CM-Sephadex eluate released, over a 2-hr period, a steadily increasing amount of heat-stable killing fragments from the cell walls. Only low levels of heat-stable killing fragments were eluted from the cell walls in the presence of heat-treated CM-Sephadex eluate or in the absence of the eluate.

Maize cell walls were treated with the CM-Sephadex eluate of the culture filtrate obtained from *M. grisea* grown on pectin. More heat-stable killing activity was released from the walls by this treatment than when the cell walls received no treatment or when the cell walls were treated
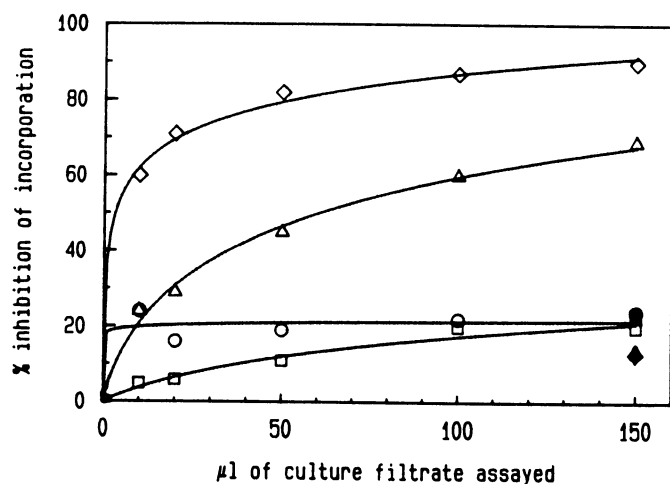
Fig. 3. Heat-labile killing activity of the culture filtrate of *Magnaporthe grisea* grown on various carbon sources. *M. grisea* grown on minimal salts containing sucrose (O), xylan (□), pectin (△), and rice cell walls (◇). Closed symbols represent the levels of inhibition of [^{14}C]leucine incorporation caused by 150 μl of heat-treated filtrate. Data points are plotted as percent inhibition with the best-fitted logarithmic curves (as determined by linear regression analysis) drawn through the points.
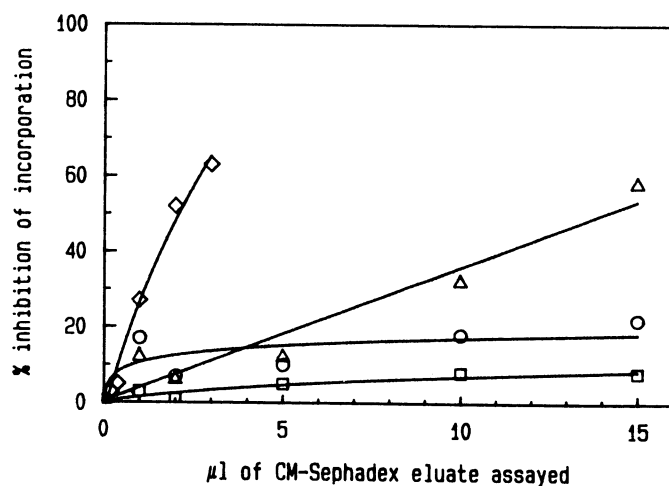
Fig. 4. Heat-labile killing activity of the desalted CM-Sephadex eluates. *Magnaporthe grisea* was grown on sucrose (O), xylan (□), pectin (△), or rice cell walls (◇). Data points are plotted as percent inhibition with the best-fitted logarithmic curves drawn through the points. Inhibition caused by heat-treated eluates (data not plotted) was the same as that caused by the untreated eluates for the sucrose- and xylan-derived material; heat-treated, pectin-derived CM-Sephadex eluate and rice cell wall-derived CM-Sephadex eluate caused 0 and 16% inhibition of incorporation, respectively.

Table 1. Recovery of heat-labile killing activity from CM-Sephadex initially (0 days) and after 6 days on ice (6 days)

Carbon source	Step	50% dose (μl)[a]		KU/ml[b]		Volume (ml)[c]	Total KU		Percent recovery	
		0 days	6 days	0 days	6 days		0 days	6 days	0 days	6 days
Pectin	Filtrate	59.3	67.4	16.9	14.8	140	2,370	2,072	-	-
	CM-Sephadex	14.0	13.3	71.4	75.2	10	714	752	30	36
Rice cell wall	Filtrate	3.27	8.64	306	116	140	42,800	16,240	-	-
	CM-Sephadex	2.10	2.93	476	341	10	4,760	3,410	11	21

[a]Volume that gives 50% inhibition of [^{14}C]leucine incorporation, or 1 killing unit.
[b]KU = killing unit.
[c]Volumes were the same at 0 and 6 days.

with CM-Sephadex eluate that had been heat-killed (Fig. 5B).

No heat-stable killing activity was released from maize cell walls treated with the CM-Sephadex eluate of the culture filtrate obtained after the fungus had been grown on xylan (Fig. 5C). The walls were treated with a volume of the xylan-derived CM-Sephadex eluate equivalent to the volume of pectin-derived CM-Sephadex eluate that would have contained 100 KUs. No heat-stable killing
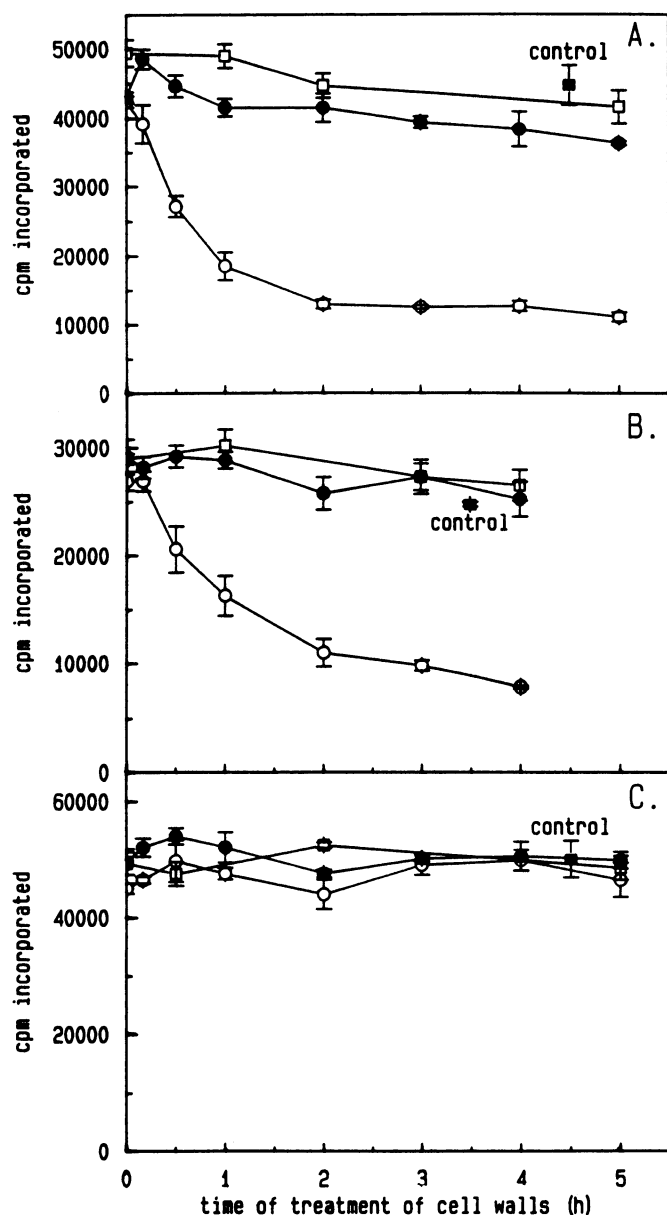

Fig. 5. Release of heat-stable killing fragments from isolated maize cell walls by treatment with CM-Sephadex eluates of the culture filtrate of *Magnaporthe grisea* grown on three carbon sources. Cell walls (1 g in 50 ml of buffer) treated with CM-Sephadex eluate (O); cell walls treated with an equivalent amount of heat-treated eluate (●); and cell walls receiving no treatment (□). The "control" level of [^{14}C]leucine incorporation was by maize cells that received no treatment. **A**, *M. grisea* grown on rice cell walls (100 killing units [210 μl] of CM-Sephadex eluate used). **B**, *M. grisea* grown on pectin (50 killing units [0.7 ml] of CM-Sephadex eluate used). **C**, *M. grisea* grown on xylan (1.4 ml of CM-Sephadex eluate used).

activity was obtained when cell walls were untreated or when the walls were treated with heat-killed CM-Sephadex eluate of the culture filtrate of *M. grisea* grown on xylan.

DISCUSSION

The [^{14}C]leucine incorporation assay provided a reproducible means of testing samples for the ability of enzymes or cell wall fragments to kill plant cells. Assays involving incorporation of labeled precursors into macromolecules by plant cells or protoplasts have been shown to reliably measure cell death (Yamazaki *et al.* 1983; Breiman and Galun 1981; Zilkah and Gressel 1978). Work in this laboratory has shown that the assay of heat-labile killing activity with the [^{14}C]leucine incorporation assay correlated well with other assays of cell death, for example by using the vital dyes fluorescein diacetate and triphenyltetrazolium chloride (Bucheli *et al.*, in press). Furthermore, cells treated with heat-labile killing activity did not grow on agar petri dishes, whereas cells treated with heat-killed, heat-labile killing activity develop callus tissue in a manner identical to untreated cells. In addition, necrotic lesions formed quickly in tobacco leaf tissue injected with heat-labile killing activity (our unpublished results). Thus, we concluded that the [^{14}C]leucine incorporation assay provided a reliable measure of the death of cultured maize cells.

The work described in this paper demonstrated that killing fragments could be solubilized, by partial acid hydrolysis, not only from sycamore cell walls (as described by Yamazaki *et al.* 1983) but also from maize and rice cell walls. Furthermore, both the sycamore and maize fragments killed sycamore and maize cells, and rice cell wall fragments killed maize cells; the rice wall fragments were not tested on sycamore and rice cells. Thus, the killing fragments capable of being solubilized by acid appear to be present in the cell walls of an evolutionarily broad range of plants, and the fragments derived from the cell walls of a certain species of plant will kill the cells of that plant as well as the cells of the other species tested. The apparent ubiquity of these fragments supports the hypothesis that they play a major role in plant defense responses.

The results presented in this paper have established that *M. grisea* secretes into culture fluid heat-labile (presumably enzymatic) components capable of killing maize cells. The killing factors were secreted when *M. grisea* was grown on pectin or on isolated plant cell walls as the carbon source, but the fungus did not secrete the killing factors when grown on sucrose, xylan, or on a complete (yeast extract-casein hydrolysate-sucrose) medium.

The heat-labile killing factors secreted by *M. grisea* were able to release, from isolated maize cell walls, heat-stable fragments that kill maize cells. The heat-labile factors solubilized maize cell wall fragments that kill maize cells regardless of whether the fungus was grown on cell walls or on citrus pectin as the carbon source; no heat-stable killing fragments were solubilized when maize cell walls were treated with culture filtrate that did not contain demonstrable heat-labile killing activity, that is, the culture filtrate of *M. grisea* grown on xylan. The fragments released from cell walls retained their ability to kill maize cells, even when incubated with heat-labile killing factors for

up to 50 hr (data not presented).

Heat-stable cell wall components possessing the ability to kill maize cells were slowly solubilized from the cell walls in buffer in the absence of enzymes. The tendency of components capable of killing plant cells to leach out of cell walls in the absence of added enzymes can be seen in the results summarized in Figures 5A and 5B. Therefore, it was necessary to thoroughly prewash the isolated walls with buffer (as described previously) before using them for the experiments described in this paper.

Our results extend those of Hahne and Lörz (1988), who demonstrated that a mixture of commercially available enzymes killed suspension-cultured wheat and tobacco cells and that heat-stable components generated by treating the wheat and tobacco cells with the enzymes were themselves capable of killing the wheat and tobacco cells (assayed by vital staining).

Our demonstration that cell wall fragments are able to kill plant cells contrasts with the results of Basham and Bateman (1975), who demonstrated that a purified pectate lyase from *Erwinia chrysanthemi* Burkholder *et al.* could macerate and kill the cells of potato tuber, cucumber fruit, and tobacco pith tissue (killing assayed by vital staining), but treatment of isolated tobacco pith tissue cell walls with the pectate lyase did not release detectable products capable of killing plant cells. Similarly, Ishii (1988) showed that pectin lyase and xylanase, but not cellulase, killed rice cells, as determined by dry weight. Treatment with xylanase or pectin lyase caused a 99 and 71% decrease in the dry weight, respectively, measured 5 days after treating the cells. No heat-stable material that decreased cell viability was detected in filtrates of rice cells treated with the xylanase.

The observation that pathogen-secreted enzymes which kill plant cells are able to solubilize plant cell wall fragments which kill plant cells may be an important clue to the mechanism by which hypersensitive cell death is triggered. This possibility is supported by the facts that pathogens produce enzymes capable of degrading cell walls when attempting to invade plant tissues (Cooper 1987), and that cell wall fragments have been shown to be capable of eliciting a variety of biological functions, including plant defense responses (for reviews, see Darvill *et al.* 1985; Hahn *et al.* 1989). Attempts are being made to purify and characterize the active component(s) of heat-labile killing factors and the heat-stable plant cell wall fragments that are able to kill plant cells.

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