Effect on Host Hypersensitivity of Suppressors Released During the Germination of *Phytophthora infestans* Cystospores

N. Doke, N. A. Garas, and J. Kuć

The first author: Plant Pathology Laboratory, Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya, Japan 464. The second and third authors: Department of Plant Pathology, University of Kentucky, Lexington 40546

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ABSTRACT

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Germinating cystospores of *Phytophthora infestans* released substances into the germination fluid which suppressed the hypersensitive reaction of potato tuber tissue to an incompatible race of the fungus. The suppressors were partially characterized as water-soluble glucans containing $\beta(1\rightarrow3)$ linkages. A nonanionic glucan isolated from germination fluid of compatible *P. infestans* race 1,2,3,4 suppressed hypersensitive cell death and the accumulation of rishitin in potato tuber tissue (Kennebec, R1-resistance gene) infected with incompatible race 4. The nonanionic glucan from race 4 was a less active suppressor. The amount of nonanionic and anionic glucans in the germination fluids from both races increased equally during the germination of the cystospores. Possible roles for the glucans as determinants of specificity in the potato-*P. infestans* interaction are discussed.

Additional key words: host-parasite specificity, potato late blight.

Specificity in plant-pathogen interactions has been well documented (3). Little information is available, however, to explain specificity except for interactions between hosts and fungi that produce host-specific toxins (19,21–23). Elicitors and suppressors of the hypersensitive reaction of potato to *Phytophthora infestans* have been reported (4–6,8,10,14,18,25). Elicitors appear to be high molecular weight wall components of the fungus which nonspecifically elicit the hypersensitive reaction of potato tubers with or without R gene resistance (10,16,18,25,26). On the other hand, suppressors were characterized as relatively low molecular weight water-soluble glucans that suppressed the hypersensitive reaction of potato tuber infected with an incompatible race of the fungus or treated with elicitors (5,6,13,14,25). It also was reported that suppressors were found in the germination fluids of *P. infestans* spores, races 1 and 0 (8). In the present report, suppressors released by germinating cystospores of *P. infestans*, races 4 and 1,2,3,4, were isolated, partially characterized, and compared with the suppressors isolated from mycelia and zoospores.

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MATERIALS AND METHODS

Plants and fungi. Kennebec (R1) potato tubers were used in all assays. The tubers were stored at 4C and kept at room temperature for 1 day before use. Two races of *P. infestans*, race 1,2,3,4 (compatible) and race 4 (incompatible) were used for the isolation of suppressors from zoospores, mycelia, and germination fluids. Zoospores of race 4 were used to elicit the hypersensitive response in tuber tissue disks of cultivar Kennebec.

Microbiological procedure. The methods described earlier were used routinely for the maintenance of *P. infestans* (6,14) and preparation of zoospore suspensions (7). Zoospore suspensions were used to inoculate potato tuber disks ($5–8 \times 10^5$ spores per milliliter) and prepare germination fluids (4×10^6 spores per milliliter). Zoospores were encysted and germinated by shaking reciprocally at 100 strokes per minute in distilled water containing 10^{-4}M $CaCl_2$ at 18 C (8). Potato disks, (18 mm in diameter, 3 mm thick) were inoculated on the upper surface with the suspension of zoospores (50 μl, spread over the surface of each disk).

Extraction and purification of the hypersensitivity inhibition factor. Liquid containing germinated cystospores was filtered through a 0.4-μm membrane filter. The filtrate (300 ml) was shaken with an equal volume of 80% phenol in a separatory funnel

for 15 min, and the aqueous phase was shaken for 15 min, first with an equal volume of a chloroform-methanol mixture (1:1) and then chloroform. After evaporation of residual chloroform under reduced pressure, the aqueous solution was dialyzed overnight against distilled water at 4 C (dialysis tubing with mol wt cutoff 3,500) and the nondialyzable fraction was freeze-dried. The freeze-dried material was dissolved in 0.01 M tris-HCl buffer, pH 7.4, and applied to a column (1.8 × 20 cm) of DEAE-cellulose (Whatman Inc., Clifton, NJ 07014) that had been washed and equilibrated with the buffer. The column was eluted with 100 ml of the buffer followed by the buffer containing 0.1 M NaCl at a flow rate of 3.5 ml/10 min. Fractions (4-ml) were collected and their carbohydrate content and ultraviolet light absorbance were determined. Major carbohydrate-containing fractions were dialyzed against 0.01 M tris-HCl buffer and concentrated by ultrafiltration with an Amicon UM2 filter. Solution containing carbohydrate (6 ml) was applied to a Sephadex G75 column (1.4 × 50 cm, void volume 40 ml) equilibrated with 0.01 M tris-HCl buffer, pH 7.4. The column was eluted with the buffer and 2-ml fractions were collected. The carbohydrate content and ultraviolet absorbance were determined. Suppressors isolated from zoospores and mycelia as described earlier (6,14) also were subjected to DEAE-cellulose and Sephadex G75 column chromatography.

Assay of suppressor activity. The activities of suppressors were determined by measuring their effect on cell death and terpenoid accumulation in potato disks infected with race 4 of *P. infestans* as described earlier (4,6,9,14).

Chemical and enzymatic methods. Nonanionic polysaccharide was treated with laminarinase (Calbiochem, Los Angeles, CA 90063 USA), amylase (Sigma Chemical Co., St. Louis, MO 63178 USA), and cellulase (Calbiochem) and hydrolyzed with 1 N HCl as described earlier (6). The products were separated by thin-layer chromatography (TLC) on silica gel G with chloroform:methanol (6:4, v/v), and chloroform:methanol:17% ammonium hydroxide (40:40:20, v/v) as developing solvents (20). Sugars were detected after spraying with diphenylamine-aniline (2) and *P*-anisidine HCl (15). Aminosugars and amino acids were detected with ninhydrin reagent (1). Total sugar content was determined by the phenol-sulfuric acid colorimetric assay with D-glucose as a standard (12).

RESULTS

Synchronized germination. Shaking suspensions in distilled water containing 10^{-4} M $CaCl_2$ at 20 C accelerated encystment and germination of zoospores of races 1,2,3,4 and 4 as previously reported for race 1 and 0 (8). More than 80% of the zoospores had encysted 1 hr after shaking, and ~40, 85, and 100% of the cystospores of both races had germinated after 1.5, 3.0, and 4.5 hr, respectively (Fig. 1). Germination fluids were collected after shaking for 3 and 6 hr, respectively.

Evidence for the presence of a suppressor in germination fluid. The germination fluid from race 4 and 1,2,3,4 cystospores suppressed cell death and the accumulation of rishitin in Kennebec tuber disks infected with incompatible race 4 (Table 1). The germination fluid from race 1,2,3,4 was more active than that from race 4. The fluids did not elicit cell death or the accumulation of rishitin (Table 1). These results are consistent with those obtained with zoospores of races 0 and 1 on the potato cultivar Rishiri (R1) (8).

Purification of suppressors. The glucans in germination fluids have similar elution patterns on DEAE-cellulose as do the glucans

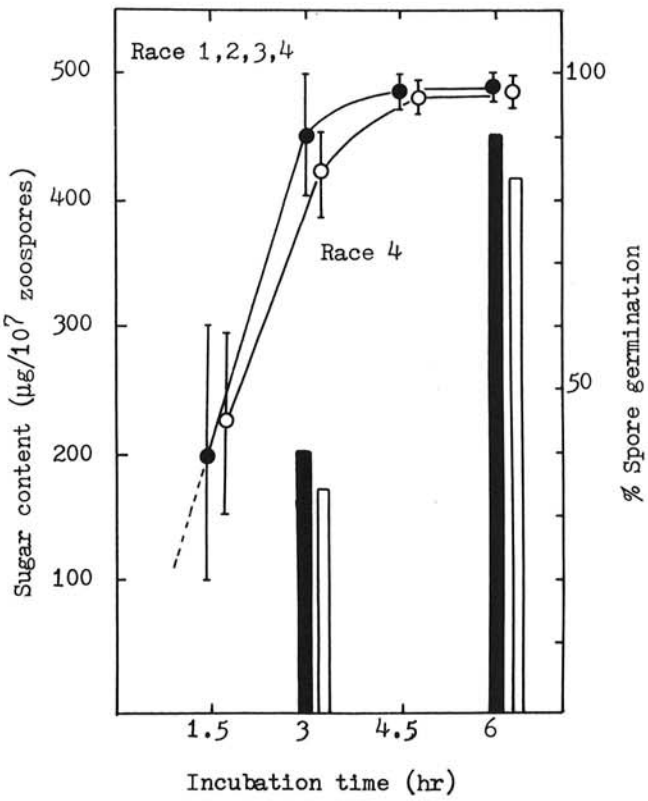

Fig. 1. Time course changes in the germination of *Phytophthora infestans* (●———●, race 1,2,3,4; o———o, race 4), and content of anionic and nonanionic glucans in the germination fluid (solid bars, race 1,2,3,4; open bars, race 4). Zoospore suspensions (4×10^6/ml) containing 10^{-4} M $CaCl_2$ were shaken at 100 strokes per minute at 20 C. Glucan content was calculated from the total sugar content of each fraction eluted from Sephadex G-75 gel. Values reported are the averages of those from two experiments.

TABLE 1. Effect of germination fluids or nonanionic glucans obtained from germination fluids of *Phytophthora infestans* cystospores on hypersensitive cell death and rishitin accumulation in potato tuber disks infected with an incompatible race of *P. infestans*

Treatment[a]	Dead cells[b] (%)	Rishitin (μg/g fresh wt)[c]
Distilled water + race 4 zoospores	96	85
Germination fluid of race 1,2,3,4 + water	3	ND[d]
Germination fluid of race 1,2,3,4 + race 4 zoospores	38	25
Germination fluid of race 4 + water	4	ND
Germination fluid of race 4 + race 4 zoospores	85	55
Glucan of race 1,2,3,4 + water	3	ND
Glucan of race 1,2,3,4 + race 4 zoospores	28	21
Glucan of race 4 + water	3	ND
Glucan of race 4 + race 4 zoospores	72	63

[a] Tubers were cut into disks 18 mm in diameter and 3-mm thick. The disks were aged for 6 hr, and the upper surface of each disk was then covered with 100 μl of water, germination fluid from 1.8×10^6 cystospores of race 4 or 1,2,3,4, or nonanionic glucan (80 μg/ml) from race 4 or 1,2,3,4. After 12 hr the disks were covered with water or 40 μl of a zoospore suspension of race 4 or 1,2,3,4 ($5–8 \times 10^5$ spores per milliliter).
[b] Observed 18 hr after inoculation with race 4. Approximately 300 infected or control cells were counted per treatment.
[c] Measured 72 hr after inoculation with race 4.
[d] ND = None detected.

from zoospores and mycelia (Fig. 2). Fractionation of the glucans from germination fluids of both races revealed anionic and nonanionic fractions which resembled those obtained from glucans isolated from zoospores and mycelia. The elution patterns of the anionic and nonanionic glucans on Sephadex G-75 gel also were similar to the patterns obtained from the fractionation of glucans from zoospores and mycelia (Fig. 3). None of the fractions containing sugar from the anion exchange and Sephadex G-75 columns, except the anionic fraction of DEAE-cellulose, had a UV absorption maximum. The elution patterns of the glucans in germination fluid from compatible and incompatible races appeared quantitatively and qualitatively similar.

Some properties of the glucans. Complete hydrolysis of the anionic and nonanionic fractions with acid followed by separation of the products by TLC revealed glucose to be the only monosaccharide. Ninhydrin-positive components were not detected. The nonanionic fractions were treated with laminarinase, α-amylase, and cellulase and the reaction mixtures were separated by TLC. The fractions treated with laminarinase revealed a major spot corresponding to glucose in addition to several minor spots having lower R_f values. The fractions treated with cellulase or α-amylase did not have these components, but did have a carbohydrate component which remained at the origin. Following treatment with laminarinase, more than 85% of the carbohydrate in the nonanionic fraction was recovered as glucose. These results indicate that the nonanionic fractions consist predominantly of $\beta(1\rightarrow3)$-linked glucosyl residues. Insufficient quantities of the anionic glucan were isolated from germination fluids for biological assays or chemical and enzymatic hydrolysis.

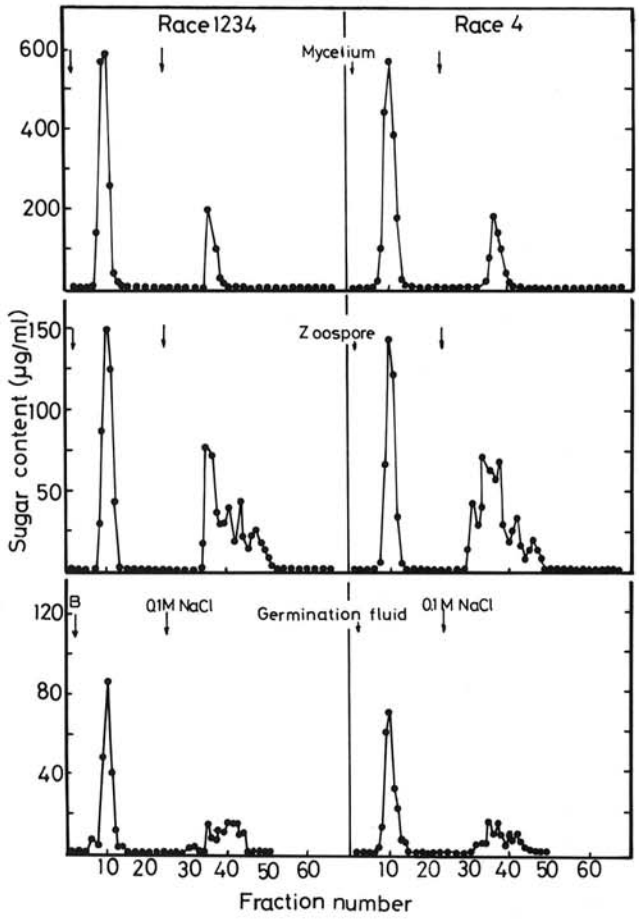

Fig. 2. Chromatographic fractionation (4-ml fractions) on DEAE-cellulose of glucans from mycelia, zoospores, and germination fluids of *Phytophthora infestans*. Germination fluid was collected after shaking for 6 hr. The column was eluted with 0.01 M tris-HCl buffer, pH 7.4; followed by the buffer containing 0.1 M NaCl.

Suppressor activity of glucans. One hundred microliters of a solution of the nonanionic glucan (80 μg/ml) purified from the germination fluid of race 1,2,3,4 and 4 was applied to the surface of potato tuber disks 6 hr after cutting. After 12 hr, the disks were inoculated on the treated surface with race 4 and incubated in a moist petri dish at 18 C in the dark. Death of infected cells was markedly suppressed by the glucans and the glucan from race 1,2,3,4 was more active than the glucan from race 4 (Table 1). No significant difference in the infection rate (number of infected cells per square millimeter) between treated and nontreated tissue was observed. Most of infection hyphae grew through the first infected cell to adjacent cells in both treated and nontreated tissues 18 hr after inoculation. Although the length of infection hyphae in the cells was not measured, it appeared that there was no effect of treatment with glucan on the growth of infection hyphae, at least while the fungi grew in the first cells that were penetrated. Eighteen hours after inoculation, control tissue inoculated with the fungus was slightly browned, but tissue treated with glucan before inoculation remained white. Forty-eight hours after inoculation, control tissue was much more darkly browned than tissue treated with the glucans. There also was some difference, however, in the intensity of browning between tissues treated with glucan from races 1,2,3,4 and 4. Treatment with glucan from race 1,2,3,4 suppressed the browning more strongly than that from race 4. The accumulation of rishitin in the tissue treated with both glucans was suppressed compared with that in control tissue (Table 1). The tissue treated with glucan from race 1,2,3,4 germination fluid accumulated less rishitin than that treated with glucan from race 4. Treatment of slices with glucans from both races, not followed by inoculation, did not elicit the accumulation of rishitin 72 hr after treatment. Glucose or the products of hydrolysis after treatment of glucans with laminarinase did not have suppressor activity.

Release of glucans during germination. The content of anionic and nonanionic glucans in the germination fluid was determined after shaking zoospore suspensions for 3 or 6 hr. The glucan content was calculated from the total sugar content of each fraction

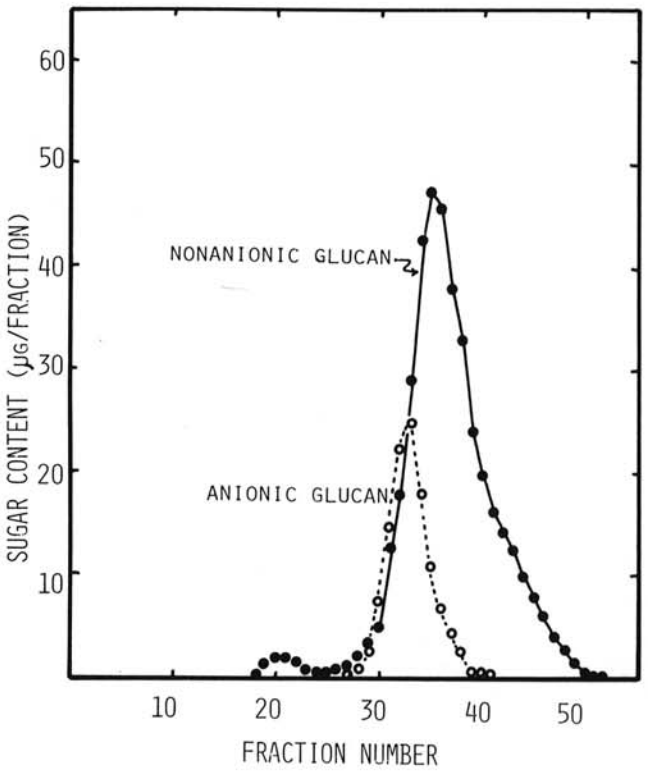

Fig. 3. Chromatographic fractionation (2-ml fractions, two different runs) of anionic and nonanionic glucans, separated by DEAE-cellulose column chromatography, on Sephadex G-75 gel. Sample was isolated from the germination fluid of cystospores of race 1,2,3,4 of *Phytophthora infestans*.

eluted from Sephadex G-75 gel. The content of glucan in the germination fluid of both races apparently increased 3–6 hr after shaking. There was no significant difference in the amount of glucan released during the germination of cystospores of compatible and incompatible races (Fig. 1).

DISCUSSION

Suppressors of the hypersensitive reaction were demonstrated in the germination fluids of race 1,2,3,4 and 4 of *P. infestans;* this is consistent with the work reported for zoospores of race 1 and 0(8). The suppressors in germination fluids were purified and found to consist principally of glucosyl residues linked $\beta(1\rightarrow3)$. Anionic and nonanionic glucans were isolated from germination fluids. The glucans appeared similar to suppressors which were purified from zoospores and mycelia and partially characterized by means of the identical chromatographic, chemical, and enzymatic procedures (5,6). The suppressors are similar to the mycolaminaran glucans isolated from *Phytophthora palmivora* (5,6,27,28). Anionic and neutral glucans were isolated from zoospores and neutral glucans were isolated from mycelium of *P. palmivora*, whereas anionic and neutral glucans were isolated from zoospores, spore germination fluids, and mycelium of *P. infestans*. Since the glucans in the germination fluids also appeared to be contained in the zoospores and mycelia, it is possible that the glucans were released by the rupture of zoospores and mycelia during incubation and the filtration process rather than released from germ tubes. Cystospores and germinated cystospores, however, are enveloped by a cell wall and are resistant to breakdown. Three hours after incubation, most zoospores had encysted and began to germinate (Fig. 1). The increase in the amount of glucan in germination fluid 3 to 6 hr after incubation, when cystospores were actively germinating, suggests that suppressors are released during germination.

There was no significant difference between compatible and incompatible races in the amount of glucans in the germination fluid. Thus, the differences in suppressor activity is not due to differences in the amounts of glucans released during germination of the cystospores. Qualitative differences in suppressors may be important in determining the differences in activity. Glucans isolated from zoospores and mycelia of a compatible race also suppressed the hypersensitive reaction more strongly than those from an incompatible race (5,6). These experimental results, together with those reported for other cultivars and races, suggest a possible explanation for host-parasite specificity in the potato-*P. infestans* interaction at the level of the (R) genes (5,6,8,13,14,25). Race-cultivar specificity in the disease may be due to a specific suppression of the hypersensitive response of host cells by fungal components rather than specific elicitation of the hypersensitive response of host cells (17). This is supported by the observation that an elicitor has not been detected in the germination fluid (4,9) and that the elicitors from *P. infestans* are nonspecific (18,26). Earlier reports that preinfection with a compatible race of *P. infestans* suppressed hypersensitive cell death (24) and the accumulation of terpenoid phytoalexin (25) in potato tissue infected with an incompatible race, are consistent with this report.

Cytological observations of the infection process of *P. infestans* demonstrated that attachment of the infection peg of incompatible races to cell membrane is associated with physiological change at the site of contact. These changes may result in cell death. Attachment of infection pegs of compatible races caused no changes detectable under a light microscope and did not cause rapid cell death (16). It is possible that cytological changes at the site of the initial interaction between the surface of the incompatible infection peg and the host cell membrane are caused by the interaction between the elicitor on the surface of the infection peg and the receptor on host cell membrane. Glucans released from germination tubes of compatible races may inhibit the effective interaction between elicitor and the receptor. This theory is supported by the following experimental observations: the elicitor binds to some components in the microsomal fraction of potato tuber tissue, the bound elicitor does not elicit the accumulation of rishitin when added to potato slices, and glucans from compatible, but not incompatible, races prevent inactivation (binding) of the elicitor (6,11).

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