Defense Responses Induced by Soluble Silicon in Cucumber Roots Infected by *Pythium* spp.

M. Chérif, A. Asselin, and R. R. Bélanger

Département de phytologie, Faculté des sciences de l'agriculture et de l'alimentation, Université Laval, Québec, Canada G1K 7P4.
Supported in part by a grant from the National Science and Engineering Research Council of Canada and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche.
Paper CRH-116 from Le Centre de Recherche en Horticulture.
We thank C. Labbé, J. Grenier, and S. Elouakfaoui for their help and technical assistance throughout this project.
Corresponding author: R. R. Bélanger.
Accepted for publication 26 October 1993.

ABSTRACT

Chérif, M., Asselin, A., and Bélanger, R. R. 1994. Defense responses induced by soluble silicon in cucumber roots infected by *Pythium* spp. Phytopathology 84:236-242.

The role of silicon (Si) in plant growth was investigated on the basis of induction of defense mechanisms in response to fungal attacks. Amendment of cucumber plants with soluble Si (Si+) resulted in a marked stimulation of chitinase activity and in a more intense and rapid activation of peroxidases and polyphenoloxidases after infection with *Pythium* spp. Additionally, glycosidically bound phenolics extracted from Si+ plants and subjected to acid or β-glucosidase hydrolysis (Gly extracts) displayed a strong fungistatic activity against *P. ultimum, P. aphanidermatum,* and *Cladosporium cucumerinum.* Extracts obtained in the same way from control plants were not inhibitory to fungal growth, regardless of Si treatment. Three zones with antifungal activity were revealed after separation of Gly extracts from infected Si+ plants on thin-layer chromatography plates. Increased β-glucosidase activity was detected in protein extracts of infected Si+ plants, which correlated with the presence of fungitoxic aglycones found in the roots of these plants and the plants' efficacy to suppress *Pythium* spp. These results associate Si with specific plant defense reactions and would explain the reported prophylactic properties of Si against fungal attacks. These reactions appear to be multicomponent, and resistance is contingent on the activation of a cascade of associated biochemical changes.

Silicon (Si) is the second most abundant element on the surface of the earth, yet its role in plant growth is apparently minimal and poorly understood (22,40). It is considered an essential element only in the cases of a few wetland and grassland species, in which it regulates the flowering stage (6). In other plant species, Si accumulates in varying degrees based on availability and the ability of plants to absorb it. However, attempts to associate silicon with metabolic or physiologic activities have been inconclusive (22). In addition, there has been accumulating evidence linking the presence of Si with reduction of the severity of fungal attacks (20,21). This phenomenon at first appeared to be specifically directed against foliar pathogens, until it was recently demonstrated that diseases caused by root-infecting fungi were equally suppressed (7). The mechanisms by which Si provides protection against pathogens are still elusive. It was suggested that silicon would accumulate at sites of fungal penetration, but a recent study using scanning electron microscopy and X-ray microanalysis failed to corroborate this hypothesis (11). At the same time, Chérif et al (10) reported that the restricted development of the pathogen appeared to be related to the intensification of physiologic barriers and accumulation of phenolic compounds and ligninlike material.

In this study, we present the first evidence that Si fertilization alters the timing and intensity with which several defense-associated mechanisms are expressed in response to fungal infection in the cucumber-*Pythium* interaction.

MATERIALS AND METHODS

Plant material and pathogen inoculations. Cucumber plants (*Cucumis sativus* L. 'Corona') were grown in a greenhouse with a hydroponic system as previously described (7). Seeds were sown in LC-1 Horticubes (Smithes-Oasis, Kent, OH) and fertilized daily with a nutrient solution of N-P-K (7-11-27) for 2–3 wk under greenhouse conditions. After transplanting, plants were grown in a nutrient solution that contained 13.0 mM NO3, 1.5 mM H_2PO_4, 7.0 mM K, 3.5 mM C, 1.0 mM Mg, 1.0-1.5 mM SO4, 18.8 μM Fe, 5.5 μM Mn, 0.9 μM Zn, 0.2 μM Cu, 18.1 μM B, and 1.0 μM Mo. Soluble silicon was fed to the plants by amending the nutrient solution with 1.7 mM (100 ppm) Si in the form of potassium silicate (Kasil no. 6, 23.6% SiO_2, PQ Corporation, Toronto) for the duration of the experiment. Adjustments were made to the nutrient solutions to compensate for the additional input of K. The experiment included six treatments: *P. ultimum* Trow inoculation with (Si+Pu+) or without Si amendments (Si−Pu+), *P. aphanidermatum* (Edson) Fitzp. inoculation with (Si+Pa+) or without Si amendments (Si−Pa+), and two controls with (Si+P−) and without Si amendments (Si−P−). Each treatment was replicated twice. The experimental design was reported in previous studies (7).

Inoculum was prepared from one strain of *P. ultimum* (Barr 447, Centre for Land and Biological Resources Research, Ottawa, Ontario, Canada) previously reported to be virulent on cucumber (8) and one isolate of *P. aphanidermatum* isolated from greenhouse-grown cucumber and provided by J. G. Menzies, Agassiz Research Station, Canada. The fungi were grown on potato-dextrose agar (PDA) in 9-cm petri dishes for 5 days. For each 100 L of nutrient solution to be inoculated, the equivalent of one petri-dish culture was blended in 100 ml of distilled water and added directly into the appropriate tank 2 wk after transplantation. Sterile PDA was used for the controls.

Preparation of enzyme extracts. Root samples were collected every other day for 12 days after inoculation and immediately frozen at −80 C. The samples were extracted with 0.1 M sodium phosphate buffer (pH 6.5) (1:1, w/v) by grinding at 4 C with a chilled mortar and pestle. Homogenates were centrifuged at 10, 000 *g* for 10 min at 4 C and were kept at −20 C. The supernatants were used as crude enzyme extracts, and protein concentration was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA).

Peroxidase and polyphenoloxidase assays. Peroxidase activity was determined by following the appearance of the brown coloration resulting from guaiacol oxidation in the presence of hydrogen peroxide (16). The reaction mixture contained 2.0 ml of 0.2 M phosphate buffer at pH 5.8, 0.05 ml of 0.02 M guaiacol, 0.5 ml of 0.38 M H_2O_2, and plant extracts containing 250 μg of protein. The reaction was carried out at room temperature. Absorbency increase was recorded with a Milton Roy spectrophotometer model Spectronic 1001 Plus (Fisher Scientific, Montréal) at 470 nm, 15 and 120 s after the addition of extracts.

Polyphenoloxidase activity was determined according to the intensity of dark-colored polymeric compounds formed by oxidation of catechol. The reaction mixture contained 5 ml of 0.5% catechol solution and plant extracts containing 250 μg of protein. The catechol solution was brought to 30 C before enzyme addition. Absorbency increase was recorded at 400 nm, 15 and 60 s after the addition of extracts.

For both assays, extracts were obtained from root samples collected from treatments Si−Pu−, Si+Pu−, Si−Pu+, and Si+Pu+. For each time period, treatment, and replicate experiment, two root samples from each of six plants were collected for a total of 24 measurements per treatment per time period. Enzyme activity was expressed as percentage of control (Pu−).

β-glucosidase activity after polyacrylamide gel electrophoresis (PAGE). Samples from cucumber-root extracts (crude enzyme extracts) or *Pythium* liquid media were subjected to native PAGE, using the Davis system in 15% (w/v) polyacrylamide gels. After electrophoresis, each gel was incubated for 5 min in 200 mM sodium phosphate buffer (pH 7.0), transferred to a glass plate, and overlayed with a 3-MM Whatman paper (Canlab, Baxter Corporation, Pointe-Claire, Qc) in 200 mM sodium phosphate buffer (pH 7.0) containing 1 mM 4-methyl-umbelliferyl-β-D-glucoside. After 1 h at 37 C in a closed container, the paper was removed and β-glucosidase activity was visualized by UV transillumination (302 nm)

Chitinase and β-1,3-glucanase activities after PAGE. Sample preparation and staining for chitinase activity after PAGE under native conditions for acidic or neutral proteins (Davis system) were performed according to Trudel and Asselin (34). Gels were stained with Calcofluor White M2R (American Cyanamid Co., Wayne, NJ), and chitinase activities were detected as dark bands against the fluorescent background of intact glycol chitin excited by UV (365 nm) (35).

Detection of β-1,3-glucanase activity was performed by native PAGE as previously described (13), using 15% (w/v) polyacrylamide gels containing laminarin at a final concentration of 2 mg/ml. After electrophoresis, gels were incubated at 37 C for 45 min in 50 mM sodium acetate buffer (pH 5.0) and then immersed in aniline blue fluorochrome (sirofluor at 35 mg/ml in 100 mM glycine-NaOH, pH 11.5) (13) for at least 15 min. Lytic zones were visualized without a destaining step with a long-wave UV transilluminator (365 nm) (C-62, UV Products, San Gabriel, CA).

Extraction and fractionation of phenolic compounds. Root tissues frozen at −80 C were lyophilized and ground to a fine powder. Samples (0.2 g) were mixed with 20 ml of 80% acidified methanol (pH 2.0), protected from oxidation by replacing oxygen with nitrogen and eliminating light, and extracted overnight on a rotary shaker. After extraction, the homogenate was vacuum-filtered and washed with 20 ml of 80% acidified methanol. Total phenolic compounds in crude extracts were quantified by the Folin procedure (33), and gallic acid was used as a reference.

For fractionation of phenolics, crude extracts from root tissues (125 g of fresh-tissue equivalents) collected 6 days after inoculation with *Pythium* spp. were prepared as described. The different extracts were rotor-evaporated at 40 C. The aqueous residue was diluted with water two times and partitioned three times against anhydrous ethyl ether. The ethyl ether fraction was evaporated, and the residue, designated as "the free phenolic constituents (FPC)," was dissolved in 5 ml of methanol. The aqueous residue from the ethyl ether extraction still contained the phenolic glycosides. This fraction was divided into two flasks for acid and enzymatic hydrolyses. Acid hydrolysis was performed in a water bath at 100 C. One of the aqueous fractions was diluted with an equal volume of 4 N HCl and hydrolyzed for 1 h. After cooling, the hydrolysate was partitioned against anhydrous ethyl ether as described, and the residue, designated as "the aglycones of glycosidically bound phenolics (Gly)," was dissolved in 2.5 ml of methanol.

The other aqueous fraction was subjected to hydrolysis with β-glucosidase. This fraction was concentrated to near dryness, and the dried material was dissolved in 100 ml of 0.2 M citric-phosphate buffer solution (pH 5). Aliquots (30 ml each) from the buffered mixture were hydrolyzed with almond β-glucosidase (50 mg, 5.5 units/mg) at 37 C for 72 h. The liberated aglycones were extracted three times with 50 ml of ethyl ether. The extract was dried and dissolved in 0.5 ml of methanol.

Antifungal activity of phenolic and enzyme extracts. *P. ultimum* and *P. aphanidermatum* were grown on PDA (three replicates) in 9-cm petri dishes. Five-millimeter sterile filter-paper disks were saturated with 30 or 60 μl of each test substance, and the solvent was evaporated with a nitrogen stream. The dry disks were arranged in a circle on the agar near the margin of the growing fungal colony. Antifungal activity of crude cucumber enzyme extracts was evaluated as described above, except that filter-paper disks were not dried before deposition on PDA. Plates were incubated at 25 C and observed 12 to 24 h after treatment.

Chromatogram inhibition assay. The different phenolic fractions were evaluated for fungitoxicity directly on chromatograms. Varying amounts were spotted on silica gel thin-layer chromatography (TLC) plates (Silica Gel 60 F-254, Sigma Chemical Co., St. Louis, MO) and developed with cyclohexane:ethyl acetate (1:1, v/v) or with chloroform:methanol (9:1, v/v). After drying for 2–3 h, the plates were sprayed with a conidial suspension of *Cladosporium cucumerinum* Ellis & Arth. according to Bailey and Burden (3). The plates were incubated in a humid chamber for 72 h at room temperature, and zones of inhibition appeared as white spots on a grey background composed of *C. cucumerinum* spores and mycelium.

Effect of Gly extracts on oospore germination and radial growth of *Pythium* spp. To test the inhibition of hyphal growth and germination of oospores, flasks containing 200 ml of PDA were prepared, and root extracts were added at different concentrations. Because the root Gly extracts were not readily soluble in water and methanol inhibited *Pythium* growth, 2 ml of the determined extracts were evaporated, dissolved in a minimal volume of ethyl acetate, and 200 μl of polyethylene glycol (PEG) 200 was added. The ethyl acetate was evaporated with a nitrogen stream. Sterile distilled water (2 ml) was added, and the sample was sonicated for 5 min to provide an aqueous dispersion. The dispersion was diluted to different concentrations, and aliquots were added to the flasks containing sterile molten PDA to provide the concentrations of extracts. Extracts were tested at concentrations of 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 μl/ml. Fifteen-milliliter aliquots of suspensions were immediately dispersed into 9-cm petri dishes.

Inhibition of radial growth of *Pythium* spp. was tested by seeding plates with 6-mm agar plugs taken from the margin of 4-day-old *P. ultimum* or *P. aphanidermatum* cultures. Four replicates of three plates were used for each fungus at each extract concentration, and the plates were incubated in the dark at 25 C. Growth measurements were determined 24 h after incubation. The test was repeated twice, and the results are presented as the percentage of radial growth relative to the control (PDA containing a comparable quantity of PEG, but no extracts).

Germination of *P. aphanidermatum* oospores was tested on PDA plates prepared as described. Oospores of *P. aphanidermatum* were prepared according to Pieczarka and Abawi (29). The oospores were suspended in distilled water at 3,000–4,000 oospores per milliliter. Aliquots (100 μl) were pipetted onto PDA plates containing 0.0, 0.2, 0.4, 0.8, 1.2, 1.6, or 2.0 μl of Gly extracts per milliliter of PDA. Four replicates (three plates per replicate) were used for each concentration of plant extracts. Inoculated plates were incubated at 25 C for 12 h. Germination of 100–120 oospores per plate was determined with an inversion microscope. An oospore was considered to have germinated if the length of

the germ tube exceeded the length of the oospore. The test was repeated twice, and results are presented as the percentage of inhibition of germination.

Reagents. Chemicals used for electrophoresis and protein molecular mass markers were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Calcofluor White M2R, almond β-glucosidase, 4-methyl-umbelliferyl-β-D-glucoside, salicin, coniferyl alcohol, and chlorogenic acid were obtained from Sigma Chemical Co. (St. Louis, MO). TLC plates were obtained from BDH Inc. (Ville St.-Laurent, Qc). All other reagents were analytical grade.

Data analysis. Data were analyzed with Super ANOVA (Abacus Concepts, Berkeley, CA) software.

RESULTS

Effect of Si amendments on peroxidase and polyphenoloxidase activities. Peroxidase and polyphenoloxidase (PPO) activities in

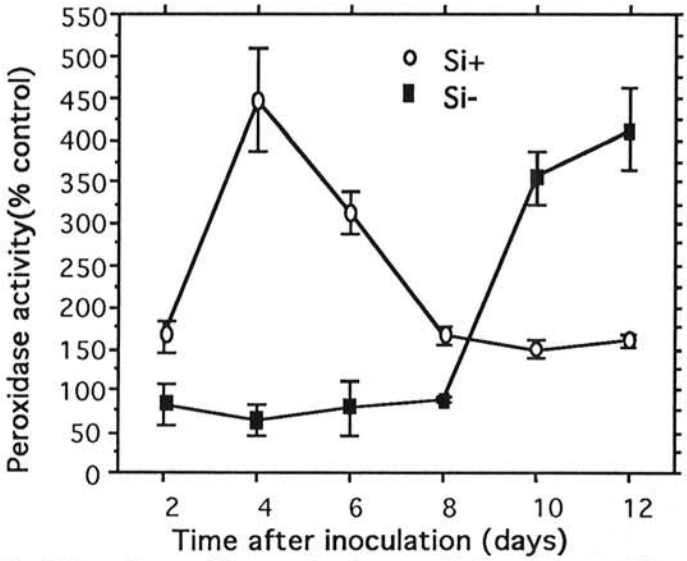

Fig. 1. Peroxidase activity over time in roots of silicon-amended (Si+) and unamended (Si−) cucumber plants infected by *Pythium ultimum*. Peroxidase activity is expressed as a percentage of the control. Vertical bars represent the SD from the mean. Each value is the mean SD based on 24 measurements in duplicate experiments.

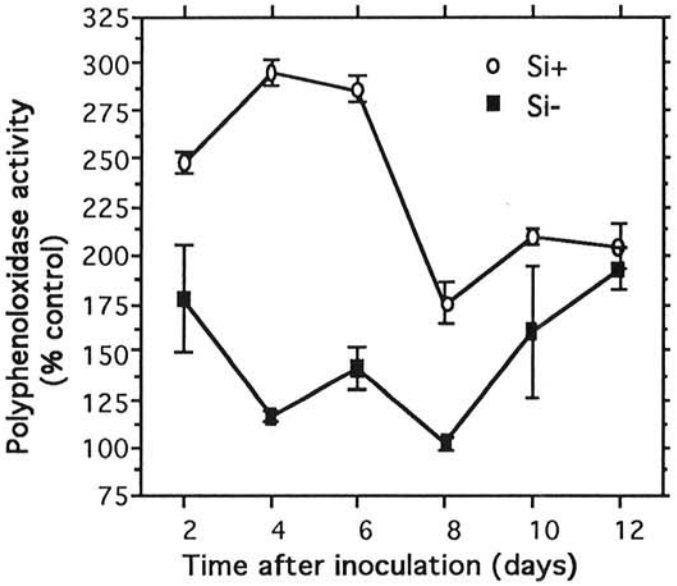

Fig. 2. Polyphenoloxidase activity over time in roots of Si-amended (Si+) and unamended (Si−) cucumber plants infected by *Pythium ultimum*. Polyphenoloxidase activity is expressed as a percentage of the control. Vertical bars represent the SD from the mean. Each value is the mean ±SD based on 24 measurements in duplicate experiments.

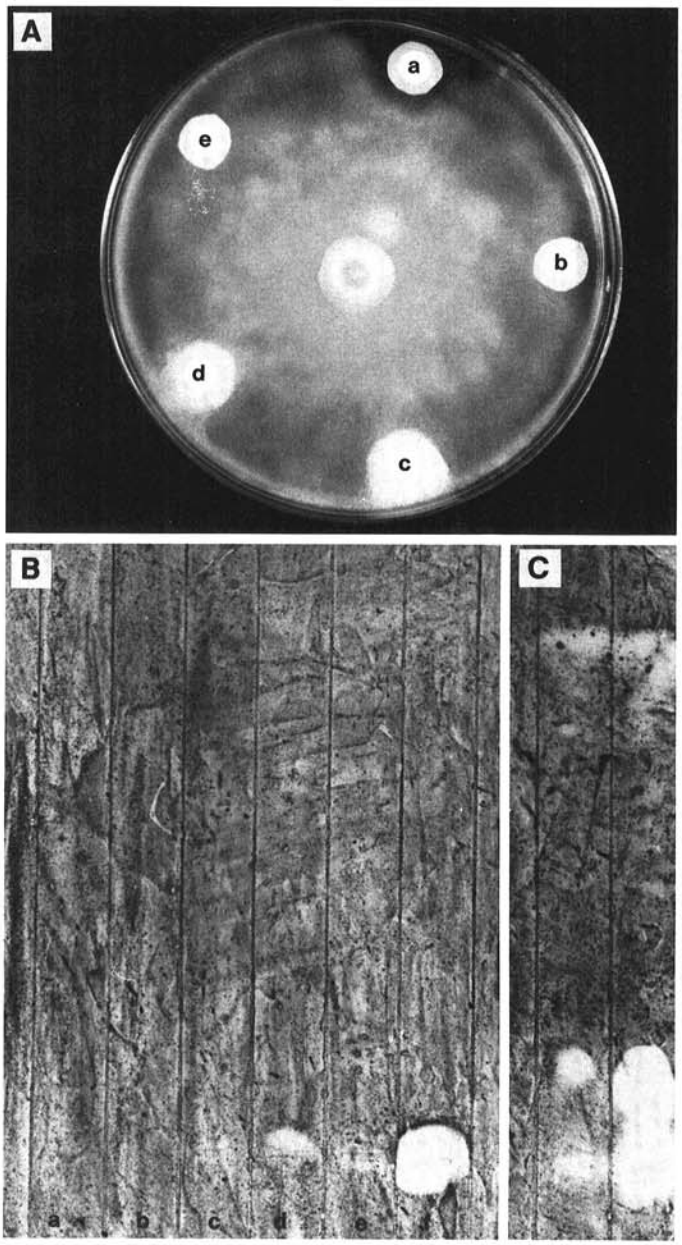

Fig. 3. A, Inhibition of *Pythium ultimum* growth by cucumber phenolic extracts. Potato-dextrose agar plates were inoculated with a mycelial disk of *P. ultimum* and incubated at 25 C for 24 h. Subsequently, 5-mm-diameter sterile filter-paper disks were saturated with 30 μl of the following test substances and, after solvent evaporation, were placed on agar near the margin of the growing fungal colony: 1) glycosidically bound phenolics after hydrolysis with HCl (Gly), extracted from cucumber roots amended with Si and inoculated with *P. ultimum* (Si+P+); 2) Gly extracts from cucumber roots unamended with Si and inoculated with *P. ultimum* (Si−Pu+); 3) free phenolic constituents (FPC) from Si+Pu+ plants; 4) FPC from Si−Pu+ plants; 5) Gly extracts from Si+Pu−; **B,** fungitoxicity of root phenolic fractions on thin-layer chromatograms (TLC). Aliquots (60 μl) from FPC (a, c, and e) and HCl-hydrolyzed Gly (b, d, and f), extracted from Si+Pu− plants (a and b), Si−Pu+ plants (c and d), and Si+Pu+ plants (e and f), were spotted on a silica gel TLC plate and developed with cyclohexane:ethyl acetate (1:1, v/v). After drying, the plates were sprayed with a conidial suspension of *Cladosporium cucumerinum* and incubated for 72 h at room temperature; **C,** TLC of Gly extracted from Si+Pu+ plants after HCl hydrolysis. Aliquots of 30 μl (left lane) and 60 μl (right lane) were spotted on a silica gel TLC plate and developed with chloroform:methanol (9:1, v/v). Three zones of inhibition with Rf values of approximately 0.0, 0.2, and 0.85 are revealed after incubation in the presence of *C. cucumerinum*.

Si+ and Si− plant roots infected by *P. ultimum* are shown in Figures 1 and 2, respectively. Their activities in inoculated plants were calculated as a percentage of activities in control plants. Si+ and Si− control plants showed similar peroxidase and PPO activities. In *Pythium*-infected plants, peroxidase activity was significantly higher in Si+ than in Si− plants 2 days after inoculation and reached a maximum level 4 days after inoculation (Fig. 1). In infected Si− plants, a similar increase in peroxidase activity was found only after 10 days, the time at which most Si− plants had reached an advanced stage of senescence. A rapid initial increase in PPO activity, followed by very high activity during a period of 4 days was observed in Si+ plants (Fig. 2). This increase in PPO activity after infection by *Pythium* was not observed in Si− plants. However, a substantial increase in PPO activity was found in Si− plants 14 to 16 days after inoculation, when the majority of plants were either dead or had reached an advanced stage of root decay (*data not shown*).

Effect of Si treatment on total phenolic compounds. There were no major differences in total phenolic levels between infected Si+ and Si− plants, but differences were observed between inoculated and uninoculated plants. At 6 days after inoculation, the content of phenolic compounds in *Pythium*-inoculated plants was more than double that of the uninoculated controls (*data not shown*).

Antifungal activity of phenolic and enzyme extracts. Phenolic compounds were fractionated into FPC and the aglycones of Gly. These phenolic fractions were tested for their ability to inhibit the growth of *P. ultimum* and *P. aphanidermatum* on PDA. FPC extracts from all treatment combinations had no inhibitory effect on growth of *Pythium* spp. (Fig. 3A, c and d). However, Gly fractions extracted from infected Si+ plants caused a distinct zone of inhibition (Fig. 3A, a), whereas a fainter zone of inhibition with Gly extracts of infected Si− roots was apparent (Fig. 3A, b). The Gly fractions extracted from Si+ (Fig. 3A, e) and Si− uninoculated controls had no inhibitory effect on fungal growth. The crude enzyme extracts also were tested, but no fungal inhibition was observed, even when the extracts were subjected to lyophilization and concentration before testing for lytic activity (*data not shown*).

Chromatogram inhibition assay. The two phenolic fractions (FPC and Gly) were evaluated for fungitoxicity on chromatograms using the solvent system ethyl acetate:cyclohexane (1:1, v/v) (Fig. 3B). Although the FPC fractions showed no toxicity against *C. cucumerinum* in the different treatments (Fig. 3B, a, c, and e), the Gly fractions induced a zone of inhibition that was particularly apparent for extracts from Si+ plants inoculated with *P. ultimum* (Fig. 3B, d and f). No inhibition zones were observed in the case of Gly fractions extracted from Si+ or Si− plants that were not inoculated with *Pythium* (Fig. 3B, b).

When varying amounts from the fungitoxic extracts (Fig. 3B, d and f) were tested, results showed that as little as 5 μl of the Gly fraction from infectd Si+ plants gave a clearly visible zone of inhibition (Fig. 4B), whereas more than 30 μl of Gly fraction from infected Si− plants was needed to obtain a similar result (Fig. 4A). The compound(s) responsible for fungal inhibition in Gly extracts did not migrate after development in ethyl acetate:cyclohexane (1:1, v/v) (Fig. 3B, f). However, after development of the plates in chloroform:methanol (9:1, v/v), two additional zones of inhibition with Rf values of approximately 0.2 and 0.85 were observed (Fig. 3C). Exposure of the chromatogram

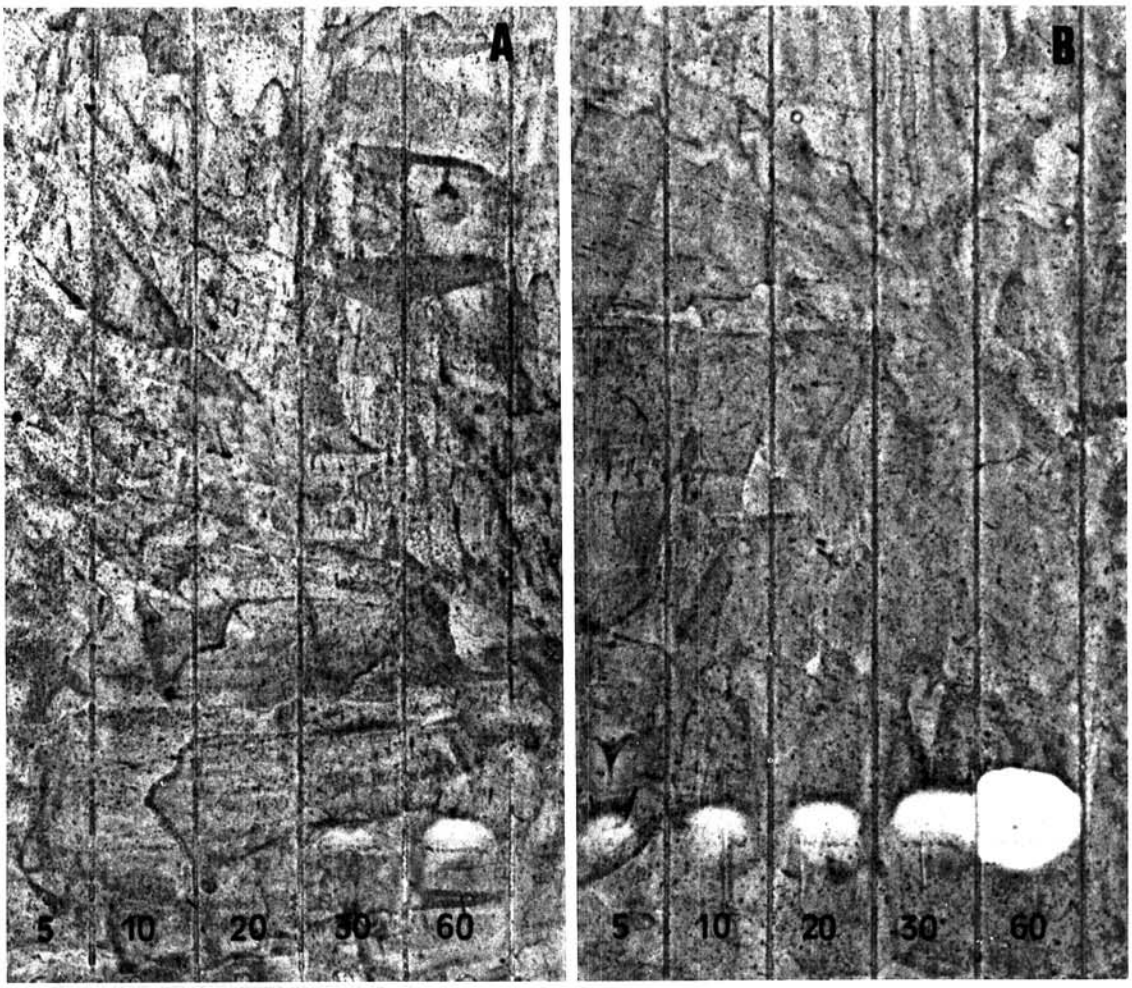

Fig. 4. Thin-layer chromatograms of varying amounts (5–60 μl) of glycosidically bound phenolics extracted from roots of cucumber plants infected with *Pythium ultimum* and grown in **A,** a Si-free or **B,** a Si-amended solution after hydrolysis with HCl. Plates were developed with cyclohexane:ethyl acetate (1:1, v/v) and after drying were sprayed with a conidial suspension of *Cladosporium cucumerinum* and incubated for 72 h at room temperature.

to ultraviolet light (254 nm) revealed a dark-blue spot corresponding to the zone of Rf 0.0, but the areas of Rf 0.2 and 0.85 were not detected under ultraviolet light.

Effect of Gly extracts on oospore germination and radial growth of *Pythium* species. Oospore germination of *P. aphanidermatum* was markedly reduced when PDA plates were amended with Gly extracts from infected Si+ plants (Table 1). At the highest concentration (2 μl/ml), Gly extracts inhibited oospore germination by more than 90%. Inhibition of germination also was observed with Gly extracts from infected Si− plants (Table 1), but concentrations four to eight times higher were necessary to cause the same effect.

Gly extracts from infected Si+ plants inhibited the radial growth of *P. ultimum* at all concentrations, with a marked effect at the highest concentration (2 μl/ml) (Table 2). These extracts were less inhibitory to *P. aphanidermatum*, which was sensitive only to concentrations greater than 0.2 μl/ml. Gly extracts from infected Si− plants were significantly less toxic to *P. ultimum* and showed no effects on growth of *P. aphanidermatum* at all concentrations tested (Table 2).

Detection of β-glucosidase activity in root crude enzyme extracts. Incubation of the Gly fraction in the presence of almond β-glucosidase yielded fungitoxic aglycones similar to those obtained after acid hydrolysis (*data not shown*). It was of interest, therefore, to determine whether β-glucosidase activity, which could hydrolyze the phenolic fractions into toxic products, could be detected in root protein extracts and the activity correlated with the induced resistance observed in Si+ plants after inoculation with *Pythium* spp. Protein extracts from cucumber roots inoculated or not with *P. ultimum* were analyzed for β-glucosidase activity. As shown in Figure 5A and B, one major β-glucosidase band was detected in Si− and Si+ plants 2 days after inoculation with *P. ultimum*. The intensity of fluorescent bands found in Si+ root extracts was higher than those of Si− root extracts, indicating a higher β-glucosidase activity in the former extracts. These differences were more noticeable at days 4, 6, and 8 after inoculation (Fig. 5B). Low-intensity β-glucosidase activity was detected in the uninoculated controls 8 days later (Fig. 5C). No differences were observed between uninoculated Si+ and Si− plants (*data not shown*).

Effect of Si amendments on chitinase and β-1,3-glucanase activity. Acidic chitinase enzymes in control and *Pythium*-infected roots of cucumber amended or not with Si were investigated at various times after inoculation, using 15% native PAGE gels. Five major chitinase bands, designated a–e, were detected in the root extracts (Fig. 6A and B). Among the five bands, four of them, a, b, d, and e, were present at equal intensity in both the uninoculated Si+ and Si− plants (Fig. 6A and B, 0). Two days

TABLE 1. Effect of Gly extracts[a] from roots of cucumber plants grown in nutrient solutions amended (Si+) or not (Si−) with soluble silicon on oospore germination of *Pythium aphanidermatum*

Concentration (μl/ml)	Percent inhibition[b]	
	Si−	Si+
0.2	15 (±3)	47 (±5)
0.4	27 (±4)	64 (±3)
0.8	48 (±7)	75 (±8)
1.2	50 (±5)	77 (±8)
1.6	47 (±3)	87 (±7)
2.0	56 (±4)	92 (±3)

[a] Aglycones of glycosidically bound phenolics (Gly) obtained, after acid hydrolysis, from root tissues collected 6 days after inoculation with *P. aphanidermatum*.
[b] Germination of 100–120 oospores per plate after 12 h at 25 C. Each value is the mean (±SD) based on 24 measurements in duplicate experiments.

TABLE 2. Effect of Gly extracts[a] from roots of cucumber plants grown in nutrient solutions amended (Si+) or not (Si−) with soluble silicon on the radial growth of *Pythium ultimum* and *P. aphanidermatum*

Concentration (μl/ml)	Radial growth (%)[b]			
	P. ultimum		*P. aphanidermatum*	
	Si−	Si+	Si−	Si+
0.2	107 (±3)	86 (±10)	122 (±9)	106 (±8)
0.4	89 (±5)	79 (±6)	116 (±7)	109 (±3)
0.8	84 (±2)	74 (±4)	109 (±3)	91 (±5)
1.2	79 (±5)	49 (±8)	104 (±4)	87 (±3)
1.6	77 (±3)	47 (±5)	103 (±3)	80 (±2)
2.0	67 (±1)	0	102 (±2)	69 (±4)

[a] Aglycones of glycosidically bound phenolics obtained, after acid hydrolysis, from root tissues collected 6 days after inoculation with *Pythium* spp.
[b] Measurement of radial growth was performed 24 h after incubation at 25 C. Each value is the mean (±SD) based on 24 measurements in duplicate experiments.

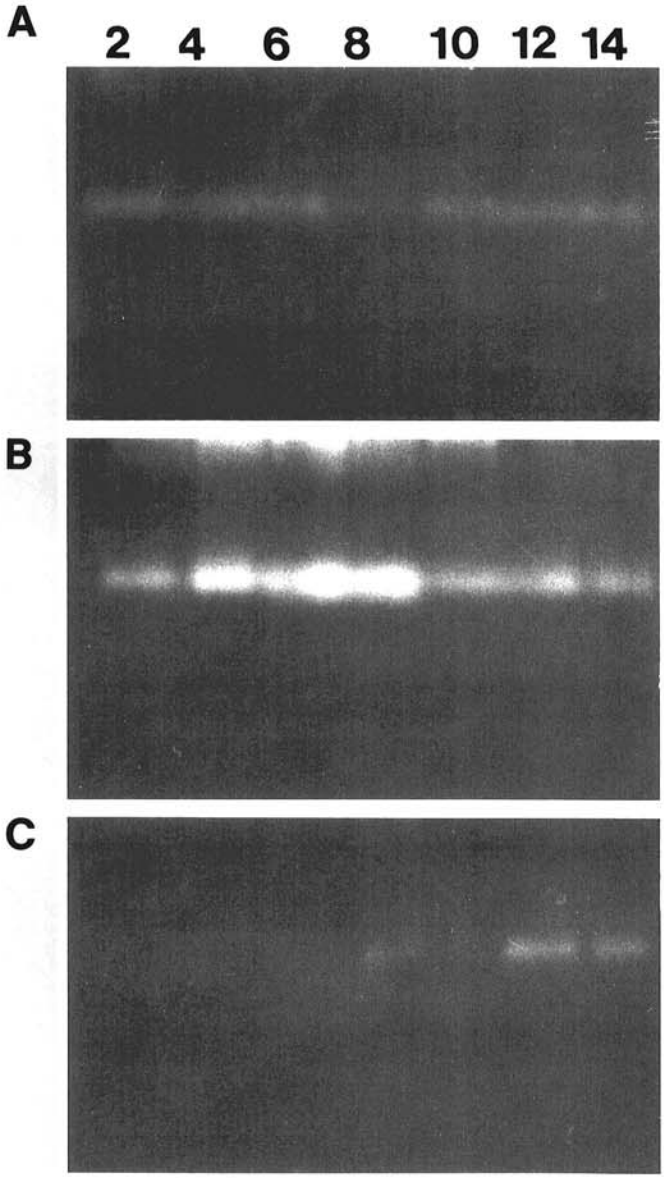

Fig. 5. β-glucosidase activity after electrophoresis in 15% (w/v) native polyacrylamide gels (Davis system for acidic proteins). After electrophoresis, each gel was incubated in 200 mM sodium phosphate buffer, pH 7.0, transferred to a glass plate, and overlayed with a 3-MM Whatman paper in sodium phosphate buffer containing 1 mM 4-methyl-umbelliferyl-β-D-glucoside. β-glucosidase activity was visualized after 1 h at 37 C by UV transillumination. Proteins in root enzyme crude extracts subjected to polyacrylamide gel electrophoresis: **A,** extracts from roots of Si-unamended cucumber plants inoculated with *Pythium ultimum*; **B,** extracts from roots of Si-amended cucumber plants inoculated with *P. ultimum*; and **C,** extracts from roots of Si-amended control plants. In all cases, samples were collected 2–14 days after inoculation with the pathogen.

after inoculation with *P. aphanidermatum* the activity of "b" disappeared and remained undetectable until the end of the experiment, regardless of the Si treatment, whereas a new band, "c," appeared (Fig. 6A and B). The activity of "c" in infected Si+ plants increased with time and remained very high 12 days after inoculation, although this band was no longer detectable 8 days after inoculation in Si− plants. Another major difference observed between infected Si+ and Si− plants was the fact that "a" activity was no longer detected in Si− protein extracts by 6 days after inoculation (Fig. 6A, a), whereas this activity was always present in Si+ extracts and remained very high at day 12 (Fig. 6B, a). The activity of bands "d" and "e" were found in all root extracts, with increasing intensity in infected Si+ roots (Fig. 6B) compared to Si− roots (Fig. 6A). Similar differences in chitinase patterns were observed for plants inoculated with *P. ultimum* (*data not shown*).

Extracts from control and *Pythium*-infected roots were analyzed for the detection of β-1,3-glucanase activity after electrophoresis in the Davis system, but no qualitative or quantitative differences were found between Si+ and Si− extracts (*data not shown*).

DISCUSSION

The relationship between the uptake of Si and plant growth has been investigated for more than 100 yr, but a physiological role for Si in plant growth has not been determined (22). Because of observations that Si may protect plants against fungal pathogens (1), several attempts have been made to determine the possible mechanisms by which this element enhances disease resistance and interferes with fungal development (20,21). Recently, it was convincingly shown that soluble Si reduces mortality and disease symptoms attributed to *P. ultimum*, a root pathogen, and that the restricted protection appears to be related to the intensification of plant cytological responses (7,10). Evidence that major metabolic changes that might enhance disease resistance occur in Si+ plants in response to fungal infection and that these changes are not observed in Si+ plants before inoculation has been presented in the present study.

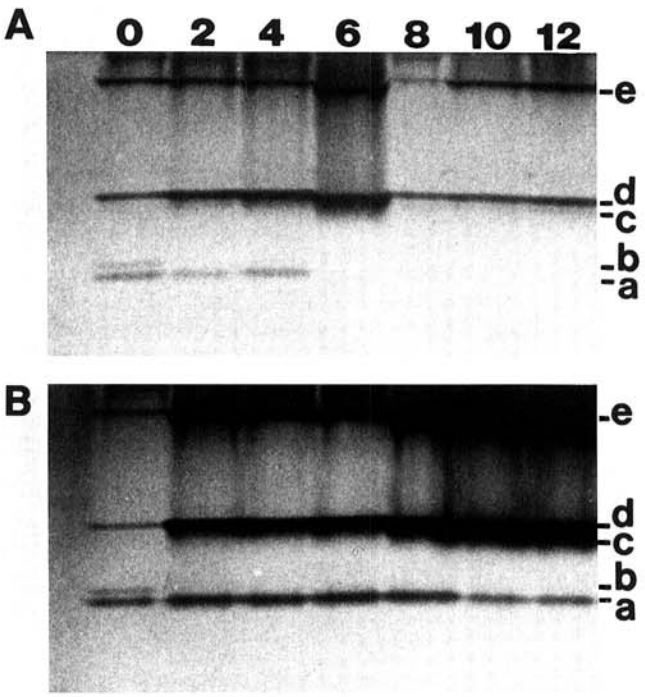

Fig. 6. Chitinase activity after electrophoresis in 15% (w/v) native polyacrylamide gels (Davis system for acidic proteins). Proteins in root extracts (20 μl), collected at various time intervals (0–12 days) after inoculation with *Pythium aphanidermatum*, from cucumber plants grown in nutrient solutions **A,** unamended or **B,** amended with 1.7 mM soluble Si were separated in gels containing 0.01% (w/v) glycol chitin as substrate for chitinase activity. After electrophoresis, gels were incubated in 50 mM acetate buffer at pH 5.0 for 45 min at 37 C and then stained with Calcofluor White M2R. Bars on the right indicate the various chitinase bands (a–e).

Amendment of nutrient solutions with soluble Si resulted in an increase in peroxidase and PPO activities in Si+ roots within 2 days after infection with *Pythium*, whereas a comparable activity was not found in Si− roots until 10 days after inoculation, at a time when plants were moribund. The relationship between peroxidase and PPO activities and the Si-stimulated accumulation of polymerized phenolics (10) may be of major importance. These oxidases are reported to have an important function in plant disease resistance, by polymerizing phenols to rigid barriers and forming lignins after polymerization of hydroxy and methoxycinnamic alcohols (18,38). The occurrence of necrotic and highly damaged fungal hyphae within electron-dense phenolic polymers observed in a previous study (10) is chronologically connected with peroxidase and PPO activities observed in infected Si+ plants in the present study. Several reports have demonstrated that oxidative products of phenolics are highly toxic to plant pathogens (25,30,36,37). Whereas marked differences in peroxidase and PPO activities were found between cucumber roots inoculated with Si+ and Si−, significant differences in total phenolics content were not found during the first 10 days after inoculation. These results may indicate that the participation of the oxidative metabolism of phenolic compounds is more important than the total phenolic constituents of the roots in cucumber resistance against *Pythium*, as reported by Wang and Pinckard (39) in cotton bolls.

Gly fractions, extracted from infected Si+ and Si− cucumber roots, yielded aglycones effective in inhibiting the growth of *Pythium*. Because more than one inhibitory band seems to be involved in plant response, measurements of one factor alone, after purification of the different bands, would not be directly related to *Pythium* survival in host tissues and would not be informative about Si effect. Data presented here indicate a strong relationship between Si treatment, resistance of cucumber plants to *Pythium* attack, and levels of precursors of inhibitory aglycones. These levels were much higher in infected Si+ roots. Similar inhibitory aglycones were not found in hydrolyzed control extracts, suggesting that the presence of the pathogen was necessary under our experimental conditions to induce the accumulation of specific glycoside(s) from which inhibitory aglycones can be released. These glycosides showed no or very low toxicity to fungal growth in vitro before acid or enzymatic hydrolysis. The enzymatic release of aglycones from Gly compounds in plant tissues invaded by pathogens may result from the activity of the naturally occurring β-glucosidase in plant tissues after injury to cells by metabolites elaborated by invading pathogens (14,31) and/or from the activity of β-glucosidase released by the invading pathogen (26,31). In this study, we have shown that a high β-glucosidase activity capable of splitting 4-methyl-umbelliferyl-β-D-glucoside was detected in the crude enzyme extracts of infected Si+ plants. The high β-glucosidase activity detected in protein extracts of Si+ plants, in conjunction with the high amount of glycosidic phenolics found in phenolic extracts, correlates with the plant's efficacy in suppressing the pathogen. The faint stimulation of β-glucosidase found in protein extracts of the controls could well be related to a plant response.

Because inhibitory aglycones may constitute a part of the plant defense reactions, and their concentrations increased after infection with the pathogens, it is very possible that they act in the plant like phytoalexins. Hammerschmidt and Kuć (19) suggested that coniferyl alcohol may act as a phytoalexin in cucumber plants in the systemically induced protection against fungal pathogens. However, these authors could not find detectable amounts of coniferyl alcohol in infected and control plant extracts. Preliminary studies conducted in our laboratory (M. Chérif and R. R. Bélanger, *unpublished data*) showed that no one of the three inhibitory aglycones detected by the chromatogram assay was identical to commercial coniferyl alcohol. However, these preliminary studies demonstrated that the phenolic aglycone corresponding to Rf 0.85 cochromatographed with chlorogenic acid in different solvents and responded similarly under UV illumina-

tion. Chlorogenic acid produced an inhibitory zone when as little as 0.5 μg was bioassayed against *C. cucumerinum*. The resistance of many crop plants to different fungal diseases has been positively correlated with the concentration of chlorogenic acid in root tissue (5,12,15,17,28). However, further biochemical studies are needed to purify, identify, and characterize the inhibitory substances and to determine their mechanism of action.

Our data indicated a stimulation of chitinase activities in *Pythium*-infected, Si+ plants. Glucanohydrolases, such as chitinases and β-1,3-glucanases, play an important role in plant defense mechanisms against pathogens (4,23,27). However, considering that chitin, although reported to occur in the walls of *Pythium* hyphae (9), is a very minor wall component compared to cellulose and other glucans, induction of chitinases may be considered as one of the general metabolic changes that may participate in resistance against other plant pathogenic fungi (27).

In recent years, there has been an increasing interest in the use of biotic and abiotic elicitors in agriculture, and several functions have been identified (32). Different elicitors induced plant defense mechanisms and suppressed the growth of plant pathogenic fungi. Nevertheless, as pointed out by Sequeira (32), induction of resistance by means of elicitors, prior to fungal infection, diverts energy from normal processes and may result in important losses in yield. Based on our results, Si fertilization appears to offer the advantage of inducing plant defense only in response to infection with the pathogen.

In conclusion, our results support the hypothesis that Si is able to induce plant defense mechanisms that are expressed in response to fungal infection. This response is multicomponent and appears to be contingent on the activation of a cascade of associated biochemical changes leading to rapid and extensive defense reactions. The multicomponent nature of this response may explain the nonspecificity of Si-induced resistance to a wide spectrum of unrelated pathogens (1,2,7,24).

LITERATURE CITED

1. Akai, S. 1953. Studies on *Helminthosporium* blight of rice plants. VII. On the relation of silicic acid supply to the outbreak of *Helminthosporium* blight or blast disease of rice plants. Ann. Phytopathol. Soc. Jpn. 17:109-112.
2. Aleshin, N. E., Avakyan, E. R., Dyakun Chak, S. A., Aleshin, E. P., Baryshok, V. P., and Vorokov, M. G. 1986. Role of silicon in resistance of rice to blast. Dokl. Akad. Nauk SSSR 291:499-502.
3. Bailey, J. A., and Burden, R. S. 1973. Biochemical changes and phytoalexin accumulation in *Phaseolus vulgaris* following cellular browning caused by tobacco necrosis virus. Physiol. Plant Pathol. 3:171-177.
4. Boller, T. 1988. Ethylene and the regulation of antifungal hydrolases in plants. Oxf. Surv. Plant Mol. Cell Biol. 5:145-174.
5. Bryde, R. J. W., Fielding, A. H., and William, A. H. 1960. The role of oxidized phenols in the varietal resistance of apples to brown root rot. Pages 95-99 in: Phenolics in Plant Health and Disease. J. B. Pridham, ed. Pergamon Press, Inc., Oxford. 131 pp.
6. Chen, C. H., and Lewin, J. 1969. Silicon as a nutrient element for *Equisetum arvense*. Can. J. Bot. 47:125-131.
7. Chérif, M., and Bélanger, R. R. 1992. Use of potassium silicate amendments in recirculating nutrient solutions to suppress *Pythium ultimum* on long English cucumber. Plant Dis. 76:1008-1011.
8. Chérif, M., Benhamou, N., and Bélanger, R. R. 1991. Ultrastructural and cytochemical studies of fungal development and host reactions in cucumber plants infected by *Pythium ultimum*. Physiol. Mol. Plant Pathol. 39:353-375.
9. Chérif, M., Benhamou, N., and Bélanger, R. R. 1993. Occurrence of cellulose and chitin in the hyphal walls of *Pythium ultimum*: A comparative study with other plant pathogenic fungi. Can. J. Microbiol. 39:213-222.
10. Chérif, M., Benhamou, N., Menzies, J. G., and Bélanger, R. R. 1992. Silicon-induced resistance in cucumber plants against *Pythium ultimum*. Physiol. Mol. Plant Pathol. 41:411-425.
11. Chérif, M., Menzies, J. G., Benhamou, N., and Bélanger, R. R. 1992. Studies of silicon distribution in wounded and *Pythium ultimum*-infected cucumber plants. Physiol Mol. Plant Pathol. 41:371-385.
12. Chiranjivi Rao, K., Krishna Murthy, T. N., Lalitha, E., and Rajalakshmi, V. K. 1968. Phenols in relation to resistance of sugarcane varieties to red rot disease. Curr. Sci. (Bangalore) 37:532-534.
13. Côté, F., El Ouakfaoui, S., and Asselin, A. 1991. Detection of β-glucosidase activity on various β-1,3 and β-1,4-glucans after native and denaturing polyacrylamide gel electrophoresis. Electrophoresis 12:69-74.
14. Davis, D., Waggoner, P. E., and Dimond, A. E. 1953. Conjugated phenols in the *Fusarium* wilt syndrome. Nature (Lond.) 172:959-961.
15. Echandi, E., and Fernández, C. E. 1962. Relation between chlorogenic acid content and resistance to coffee canker incited by *Ceratocystis fimbriata*. Phytopathology 52:544-546.
16. Fehrmann, H., and Dimond, A. E. 1967. Peroxidase activity and *Phytophthora* resistance in different organs of the potato plant. Phytopathology 57:69-72.
17. Gayed, S. K., and Rosa, N. 1975. Levels of chlorogenic acid in tobacco cultivars, healthy and infected with *Thielaviopsis basicola*. Phytopathology 65:1049-1053.
18. Grisebach, H. 1981. Lignin. Pages 451-478 in: The Biochemistry of Plants, vol. 7. E. E. Conn, ed. Academic Press, New York.
19. Hammerschmidt, R., and Kuć, J. 1982. Lignification as a mechanism for induced systemic resistance in cucumber. Physiol. Plant Pathol. 20:61-71.
20. Kunoh, H., and Ishizaki, H. 1975. Silicon levels near penetration sites of fungi on wheat, barley, cucumber and morning glory leaves. Physiol. Plant Pathol. 5:283-287.
21. Leusch, H.-J., and Buchenauer, H. 1989. Effect of soil treatments with silica-rich lime fertilizers and sodium trisilicate on the infection of wheat by *Erysiphe graminis* and *Septoria nodorum* depending on the form of N-fertilizer. J. Plant Dis. Prot. 96:154-172.
22. Lewin, J., and Reimann, B. E. F. 1969. Silicon and plant growth. Annu. Rev. Plant Physiol. 20:289-304.
23. Mauch, F., Hadwigar, L. A., and Boller, T. 1988. Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combination of chitinase and β-1,3-glucanase. Plant Physiol. 88:936-942.
24. Menzies, J. G., Ehret, D. L., Glass, A. D. M., Helmer, T., Koch, C., and Seywerd, F. 1991. Effects of soluble silicon on the parasitic fitness of *Sphaerotheca fuliginea* on *Cucumis sativus*. Phytopathology 81:84-88.
25. Noveroske, R. L., Kuć, J., and Williams, E. B. 1964. Oxidation of phloridzin and phloretin related to resistance of *Malus* to *Venturia ineaqualis*. Phytopathology 54:92-97.
26. Oku, H. 1959. Biochemical studies on *Cochliobolus miyabeanus*: V. β-glucosidase activity of the fungus and the effect of phenolglucoside on the mycelial growth. Annu. Rep. Takamine Lab. 11:190-192.
27. Pan, S. Q., Ye, X. S., and Kuć, J. 1992. Induction of chitinases in tobacco plants systemically protected against blue mold by *Perenospora tabacina* or tobacco mosaic virus. Phytopathology 82:119-123.
28. Patil, S. S., Powelson, R. L., and Roy, A. Y. 1964. Relation of chlorogenic acid and free phenols in potato roots to infection by *Verticillium albo-atrum*. Phytopathology 54:98-103.
29. Pieczarka, D. J., and Abawi, G. S. 1978. Populations and biology of *Pythium* species associated with snap bean roots and soils in New York. Phytopathology 68:409-416.
30. Retig, N. 1974. Changes in peroxidase and polyphenoloxidase associated with natural and induced resistance of tomato to Fusarium wilt. Physiol. Plant Pathol. 4:145-150.
31. Schroth, M. N., and Hildebrand, D. C. 1965. β-glucosidase in *Erwinia amylovora* and *Pseudomonas syringae*. Phytopathology 55:31-33.
32. Sequeira, L. 1990. Induced resistance: Physiology and biochemistry. Pages 663-678 in: New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases. P. Dunn and R. Baker, eds. Alan R. Liss, Inc., New York.
33. Swain, T., and Hillis, W. E. 1969. The phenolic constituents of *Prunus domestica*. J. Sci. Food Agric. 10:63-68.
34. Trudel, J., and Asselin, A. 1989. Detection of chitinase activity after polyacrylamide gel electrophoresis. Ann. Biochem. 178:362-366.
35. Trudel, J., Audy, P., and Asselin, A. 1989. Electrophoretic forms of chitinase activity in Xanthi-nc tobacco, healthy and infected with tobacco mosaic virus. Mol. Plant-Microbe Interact. 2:315-324.
36. Urs, N. V. R., and Dunleavy, J. M. 1975. Enhancement of the bactericidal activity of a peroxidase system by phenolic compounds. Phytopathology 65:686-690.
37. Vidhyasekaran, P. 1988. Physiology of Disease Resistance in Plants, vol. 1. Pages 49-81. CRC Press, Inc., Boca Raton, FL. 160 pp.
38. Vidhyasekaran, P. 1988. Physiology of Disease Resistance in Plants, vol. 1. Pages 121-135. CRC Press, Inc., Boca Raton, FL. 160 pp.
39. Wang, S. C., and Pinckard, S. A. 1973. Peroxidase activity in the developing cotton boll and its relation to decay by *Diplodia gossypina*. Phytopathology 63:1095-1099.
40. Werner, D., and Roth, R. 1983. Silica metabolism. Pages 682-694 in: Encyclopedia of Plant Physiology, New Series. Vol. 15B. A. Lächli and R. L. Bicleski, eds. Springer-Verlag, New York.