Elicitors of Terpenoid Accumulation in Potato Tuber Slices

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ABSTRACT

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Autoclaved sonicates of the Oomycetes, *Phytophthora infestans*, *Phytophthora parasitica*, *Pythium aphanidermatum*, *Achlya flagellata*, and *Aphanomyces euteiches* elicited the accumulation of rishitin and lubimin in potato tuber slices. Slices inoculated with a zoospore suspension of *P. infestans* or mycelial fragments of *P. aphanidermatum*, *A. flagellata*, and *A. euteiches* also accumulated the phytoalexins. Spore suspensions of *Helminthosporium carbonum*, *H. victoriae*, and *Colletotrichum lagenarium*, but not autoclaved sonicates of spores and mycelia, elicited accumulation of the terpenoids. The following elicited little or no accumulation of the terpenoids: all other fungi or their sonicates; three bacteria, either live or after being heated at 65 C for 20 min.; tissue of tomato, potato, cucumber, and bean; 15 carbohydrates; nine lipids; two saponins, and poly-L-lysine. Among the lipids, L-α-phosphatidyl choline, but not L-α-lysophosphatidyl choline, elicited extensive browning of tuber slices. Glucans from three *Phytophthora* spp. elicited the accumulation of low levels of the terpenoids.

Additional key words: phytoalexins, elicitors.

Rishitin, lubimin, and phytuberin are fungitoxic terpenoids that accumulate in potato slices inoculated with incompatible races of *Phytophthora infestans* (8, 9, 13, 15), fungi nonpathogenic on potato (13, 14), or bacteria (3, 8). Cell-free sonicates of compatible and incompatible races of *P. infestans* also elicit accumulation of the terpenoids (14). Phytuberol is one of several relatively nonfungitoxic terpenoids which also accumulate in infected potatoes (4). Varns et al. (14) reported that several chemicals or injury that browned potato slices did not elicit the accumulation of rishitin and phytuberin.

The purpose of this work was (i) to determine if a relationship exists between groups of fungi with different major cell wall components (2) and their ability to elicit the accumulation of terpenoids; (ii) to check the elicitor activity of various metabolites and homogenized plant tissues.

MATERIALS AND METHODS

General.—In all experiments, 0.3 ml of the material being tested was applied to the surface of Kennebec potato tuber slices, which then were incubated in the dark at 19 C for 84 hr (15). Homogenized fungi, heat-killed fungi and bacteria, plant tissues, and chemicals were applied at 10 mg dry weight/ml. Fungi, bacteria, and all chemicals and homogenates tested were applied to the surface of Kennebec potato tuber slices as described previously (15). Unless stated otherwise, all cultures were from stocks maintained in our laboratory.

Inoculation with living fungi and bacteria.—*Phytophthora infestans* (Mont.) de By. race 4 was grown on lima bean agar medium at 19 C for 10 days. Suspensions of sporangia (10^5/ml) and zoospores were prepared and applied to Kennebec potato tuber slices.

Helminthosporium carbonum Ulstrup race 1, *H. victoriae* Meehan & Murphy isolate HW-1, *Colletotrichum lagenarium* (Pass.) Ell. & Halst and *Neurospora crassa* Shear & Dodge were grown on potato-dextrose agar (Difco). *Zygorhynchus* sp., *Mucor rouxi* (Calmette) Wehmer [American Type Culture Collection (ATCC) 4855], *Sporobolomyces roseus* Kluijver & V. Neil (ATCC 24257), *Rhodotorula lactosa* Hasegawa (ATCC 9536), *Candida utilis* (Henneberg) Lodder & Kreger-V.Rij (ATCC 9226), and *Saccharomyces cerevisiae* Hansen were grown on yeast-peptone glucose (YPG) agar. The fungi were grown for 7-10 days at 24 C and suspensions of spores or cells were prepared by adding sterile distilled water to the cultures and scraping the surface with a bent glass rod. The suspensions were filtered through a double layer of cheesecloth, and adjusted by dilution with water to 10^6 conidia or cells/ml. *Pythium aphanidermatum* (Edson) Fitz., *Achlya flagellata* Coker, *Aphanomyces euteiches* Drechs. and *Phytophthora parasitica* Dast. were grown on YPG liquid medium at 24 C for 7-10 days. Mycelial mats were separated from the medium, washed with sterile water, weighed, and homogenized for 2 min in a Waring Blendor under aseptic conditions. The suspension was applied to potato slices.

Xanthomonas pruni (Smith) Dowson, *Pseudomonas pisi* Sackett, and *Bacillus subtilis* Cohn were grown in nutrient-dextrose broth on a rotary shaker at 22 C for 48-72 hr. The bacteria then were sedimented at 3,000 *g* and

washed with 0.1 M phosphate buffer pH 7.0 three times by centrifugation. The bacterial suspensions were adjusted to 10^9 cells/ml and applied to potato slices.

Inoculation with heat-killed fungi and bacteria.—*Alternaria* sp. was grown on YPG liquid medium for 7-10 days at 24 C. All the other fungi were grown on the media as described except that agar was omitted. The mycelial mats were separated from the media and washed three times with tap water followed by two washings with distilled water. The mats were stored at −20 C. Before being applied to slices, the frozen mats were added to water and homogenized in a Waring Blendor for 5 min. The homogenate was sonicated three times, 4 min each time, at 4 C in a Sonifier Cell Disruptor Model W 185 (Heat Systems-Ultrasonics, Inc., Plainview, L. 1,; NY 11803) at 60 watts output. Examination of the sonicates under a light microscope showed that more than 75% of the cells were disrupted. The sonicates were autoclaved at 121 C for 5 min, cooled, and applied to potato slices. The bacteria were grown, washed, and sedimented as described earlier. The sedimented bacteria were resuspended in water, heated at 65 C for 20 min, and applied to potato slices.

Treatment with plant tissues.—Tomato (*Lycopersicon esculentum* Mill. 'Bonny Best'), potato (*Solanum tuberosum* L. 'Kennebec'), cucumber (*Cucumis sativus* L. 'Wisconsin SMR 58'), and bean (*Phaseolus vulgaris* L. 'Top Crop') were grown in a greenhouse at 24-30 C until the first two true leaves were completely developed. The soil was removed by washing them once with tap water and twice with distilled water. The roots, stems, and leaves of the plants were separated, weighed, and frozen at −20 C. Fruits and seeds of the same cultivars were obtained commercially and frozen. Tissue was homogenized in a Waring Blendor with 0.1 M phosphate buffer pH 7.0. Streptomycin (25 μg/ml) was added to a portion of the homogenate, and the remainder was autoclaved at 121 C for 5 min.

Treatment of tuber slices with chemicals.—The polysaccharides tested were: laminarin (Sigma Chem. Corp., St. Louis, MO 63198 and ICN Pharmaceuticals, Inc., Cleveland, OH 44128), soluble starch (ICN), cellulose (Sigmacell type 100), α-cellulose (Sigma), glycogen (type III, Sigma), dextran (MW 15,000-20,000, ICN), pustulan (Calbiochem, La Jalla, CA 92307), citrus pectin (ICN), xylan (Calbiochem), hyaluronic acid (grade I, Sigma), chitin (Sigma), N-acetylglucosamine (Sigma), mannan (Sigma), and galactan (Calbiochem). The lipids were: L-α-phosphatidyl ethanolamine (type II, Sigma), L-α-phosphatidyl choline (type III, Sigma), L-α-lysophosphatidyl choline (Sigma) refined lecithin soy, (ICN), sphingomyelin (ICN), cholesterol (ICN), farnesol (ICN), phytol (General Biochemicals, Chagrin Falls, OH 44022), and squalene (ICN). The saponins were from Calbiochem and ICN and poly L-lysine (type VI-A) was from Sigma. Each of these chemicals was dissolved or suspended in 50 mM phosphate buffer pH 7.0.

Three glucans isolated from *Phytophthora* spp. were tested. The insoluble hyphal cell wall glucan of *Phytophthora cinnamomi* (17) was a gift from S. Bartnicki-Garcia; a glucan isolated from *Phytophthora megasperma* var. *sojae* was a gift from P. Albersheim; a glucan isolated from *P. infestans* race 4 was isolated by the method of Hodgson et al. (6) as modified by Wood et al. (16).

Extraction and quantitation of the terpenoids.—The upper 1.0-mm layer of tuber slices was collected and frozen at −20 C. The slices then were blended with

TABLE 1. Effect of heat-killed fungi and bacteria on the accumulation of terpenoids in potato tuber slices

Microorganism	Major cell wall[a] components	Quantity of terpenoids[b]			
		Phytuberin	Phytuberol	Rishitin	Lubimin
Phytophthora infestans	Glucan-Cellulose	6	4	59	113
Phytophthora parasitica		5	6	42	71
Pythium aphanidermatum		9	7	90	47
Achlya flagellata		Trace	Trace	25	32
Aphanomyces euteiches		Trace	Trace	18	29
Helminthosporium carbonum	Glucan-Chitin	0	0	Trace	Trace
Helminthosporium victoriae		0	0	Trace	Trace
Neurospora crassa		0	0	0	Trace
Alternaria sp.		0	0	0	0
Mucor rouxi	Chitin-Chitosan	0	0	Trace	Trace
Zygorhynchus sp.		0	0	0	0
Sporobolomyces roseus	Chitin-Mannan	0	0	0	0
Rhodotorula lactosa		0	0	0	0
Candida utilis	Glucan-Mannan	0	0	0	0
Saccharomyces cerevisiae		0	0	0	0
Xanthomonas pruni	Gram −	0	0	0	0
Pseudomonas pisi	Gram −	0	0	0	0
Bacillus subtilis	Gram +	0	0	0	0

[a]Based on the classification of Bartnicki-Garcia (2).

[b]Expressed as micrograms per gram fresh weight of the top 1.0-mm of potato slices. Trace ≤3 μg per gram fresh weight of the top 1.0-mm of potato slices.

methanol (5 ml/g fresh weight tissue) for 4 min, the suspensions filtered under reduced pressure through Whatman #2 filter paper, and the residue blended again with methanol and filtered. The combined filtrates were evaporated in a rotary evaporator at 40-45 C. The almost-dry material was suspended in a mixture of chloroform, water, and 7.5% acetic acid (200:200:1, v/v) in a separatory funnel. The chloroform phase was dried in a rotary evaporator at 40-45 C and the dried material was suspended in methanol (1.0 g fresh weight of potato tissue per 2 ml of methanol). This preparation was centrifuged at 600 *g* and the clear yellowish supernatant liquid was chromatographed on precoated silica gel G TLC plates (Analtech) to estimate terpenoid accumulation. The TLC plates were developed in cyclohexane: ethyl acetate (1:1, v/v), dried, and sprayed with either concentrated sulfuric acid, vanillin-sulfuric acid or antimony trichloride in perchloric acid.

The methanol solution of the chloroform soluble fraction was used for gas-liquid chromatography. Quantitation of the terpenoids was performed in a Series 1400 Varian Aerograph Gas Chromatograph fitted with a 200.0 cm × 0.64 cm OD, 2-mm pore, Pyrex glass column packed with 3% OV-225 on 80/100 Supelcoport (Supelco, Inc. Bellefonte, PA 16823) at 180 C with nitrogen as the carrier gas at a flow rate of 40 ml/min. The amounts of the terpenoids were calculated based on standard curves using methyl arachidate (Sigma) as the internal standard.

All experiments described in this report were repeated three times and average values are reported. The quantities of terpenoids which accumulated varied between experiments by as much as 30%, but the pattern of accumulation remained unchanged.

RESULTS

Autoclaved sonicates of Oomycetes, which have glucan-cellulose as major cell-wall components (2), elicited the accumulation of terpenoids and browning of tuber slices (Table 1). Autoclaved sonicates of other fungi and heat-treated bacteria, with different major cell-wall components, failed to elicit browning or accumulation of more than a trace of terpenoids.

Living fungi with glucan-cellulose or glucan-chitin as major cell-wall components elicited terpenoid accumulation and browning (Table 2). Fungi and bacteria which did not elicit browning did not elicit the accumulation of terpenoids. Homogenates of roots, stems, leaves, fruits, seeds, or tubers of all plants tested did not elicit terpenoid accumulation or browning. Homogenates of potato sprouts elicited the accumulation of 8-11 μg of lubimin per gram fresh weight of potato tissue. The noninfected sprouts did not contain measurable amounts of this compound.

Polysaccharides, saponins, and poly-L-lysine did not elicit terpenoid accumulation or browning. Among the lipids, L-α-phosphatidyl ethanolamine elicited the accumulation of traces of rishitin and 12-14 μg of lubimin per gram fresh weight of potato tissue. Soy lecithin or L-α-phosphatidyl choline, but not L-α-lysophosphatidyl choline, elicited browning but did not elicit the accumulation of terpenoids. The glucans extracted from *P. cinnamomi* (17) and *P. infestans* (6, 16) at 10 mg/ml induced 6 and 5 μg rishitin and 16 and 18 μg lubimin per gram fresh weight of potato tissue, respectively. The glucan from *P. megasperma* var. *sojae* (1 or 10 μg per slice) elicited the accumulation of little or no rishitin and lubimin. At a concentration of 20 μg/slice, which is considerably higher than the concentration reported to

TABLE 2. Accumulation of terpenoids in potato tuber slices inoculated with fungi and bacteria

Fungi and Bacteria	Quantities of terpenoids[a]			
	Phytuberin	Phytuberol	Rishitin	Lubimin
Phytophthora infestans	9	7	51	97
Phytophthora parasitica[b]	Trace	4	31	59
Pythium aphanidermatum[b]	6	5	54	62
Achlya flagellata[b]	Trace	Trace	16	25
Helminthosporium carbonum	14	20	60	87
Helminthosporium victoriae	10	16	73	102
Colletotrichum lagenarium	4	Trace	34	42
Neurospora crassa	Trace	Trace	22	29
Mucor rouxi	0	0	Trace	Trace
Zygorhynchus sp.	0	0	Trace	Trace
Sprorobolomyces roseus	0	0	Trace	Trace
Rhodotorula lactosa	0	0	Trace	Trace
Candida utilis	0	0	Trace	Trace
Saccharomyces cerevisiae	0	0	Trace	Trace
Pseudomonas pisi	0	0	6	6
Xanthomonas pruni	0	0	0	0
Bacillus subtilis	0	0	0	0

[a]Expressed as micrograms per gram fresh weight of the top 1.0-mm of potato slices. Trace ≤3 μg per gram fresh weight of the top 1-mm of potato slices.

[b]Potato slices were inoculated with a suspension of mycelium fragments.

elicit glyceollin in soybean (1), the glucan elicited the accumulation of 3 μg rishitin and 5 μg lubimin per gram fresh weight of the upper 1 mm of potato slice.

DISCUSSION

The accumulation of phytoalexins in many plants is elicited by a broad spectrum of compounds which include inorganic salts, antibiotics, pesticides, polysaccharides, peptides, and lipids (7). It appears the elicitation of terpenoid accumulation in potato tubers is more specific. Lecithin caused the browning of potato slices, but it did not elicit terpenoid accumulation. A lack of elicitor activity also has been reported for other chemicals or injury which caused browning (14). The mechanism by which lecithin enhances the browning of potato slices is unknown; however, the toxicity of lecithin to apple fruit has been reported (10, 11). Slices of carrot roots, sweet potato roots, pepper fruit, or cucumber fruit did not brown when treated with soy lecithin or L-α-phosphatidyl choline (authors' *unpublished data*).

Fungi with glucan-cellulose or glucan-chitin as major components of their cell walls elicit the accumulation of terpenoids. Autoclaved sonicates of the former, but not of the latter, group of fungi also elicit accumulation. Possible explanations for this observation include: the presence of different elicitors with different thermolabilities in the two groups of fungi; differences in the association of elicitors with a non-elicitor core, and, therefore, differences in their thermolabilities; and the need for living fungus in the glucan-chitin group to release elicitors.

Not all fungi that do not cause disease on potato tubers elicit browning and terpenoid accumulation. It appears unlikely, therefore, that elicitors of phytoalexin accumulation are the sole determinants of resistance, even though in interactions where elicitors have been reported they are active at extremely low concentrations (1). It is also unlikely that classical phytoalexins are the sole determinants of resistance. Many nonpathogens of potato elicited little or no accumulation of terpenoid phytoalexins but did not cause disease or grow on tuber slices (Table 2). It is entirely possible that several coordinated but metabolically distinct mechanisms contribute to the specificity of interactions between plants and microorganisms.

Saponins may be part of the elicitor complex (4); however, the saponins tested showed little or no elicitor activity. Though poly L-lysine and bean tissue have been reported to be elicitors of pisatin (5, 12), they did not elicit the accumulation of terpenoids in potato tubers.

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