Terpenoid Accumulation and Browning in Potato Sprouts Inoculated with Phytophthora infestans

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ABSTRACT

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Zoospores of races 4 and 1,2,3,4 of *Phytophthora infestans* elicited browning and the accumulation of the terpenoids rishitin, lubimin, phytuberin and phytuberol in sprouts of Kennebec (R_1) Russet Burbank (r) and Red Pontiac (r) potatoes. There was little or no difference in the quantities of the terpenoids which accumulated in sprouts from potatoes inoculated with a compatible or incompatible race of *P. infestans*. Autoclaved, cell-free sonicates of *P. infestans, Pythium aphanidermatum, Achlya flagellata, Phytophthora parasitica,* and *Aphanomyces euteiches* also elicited browning and the accumulation of terpenoids. As with zoo spores, there was little or no difference in the extent of browning or quantities of terpenoids that accumulated in sprouts from tubers of a cultivar which lacked R genes for resistance and those from a cultivar with R_1. Kennebec potato sprouts treated with sonicates of *P. infestans* browned more intensely and accumulated more of the terpenoids at 19 C than at 14 or 25 C, but neither browning nor terpenoid accumulation were detected at 30 C and 36 C. Sprouts inoculated with zoospores of *P. infestans* browned first closest to the growing point and then over the length of the inoculated area. There was no difference in the appearance of browning on sprouts treated with cell-free sonicates of the fungi. Growth and sporulation of *P. infestans* were not apparent on inoculated sprouts. The steriod glycoalkaloid content in sprout sections 5 cm from the growing tip was threefold that in the 5 cm at the base.

Additional key words: Elicitors, resistance.

Terpenoid phytoalexins in potatoes may be part of a defence mechanism against invading pathogenic microorganisms (8, 10, 11, 17). They accumulate sooner and reach higher concentrations in incompatible interactions of potato tubers with *P. infestans* than in compatible interactions, and this phenomenon has been correlated with R gene resistance (8, 11, 17). Rishitin (17) lubimin (11), phytuberin and its derivative phytuberol (2, 18) have been studied most extensively. Varns et al. (18) reported that less rishitin accumulated in inoculated sprouts of resistant than in susceptible cultivars, and several authors (5, 16) used this report as an argument against the involvement of the terpenoids in the resistance mechanism.

Steroid glycoalkaloids are present in all parts of the potato plant, and sprouts contain the highest concentration (13). It has been demonstrated that steroid glycoalkaloids inhibit the growth of *P. infestans* and other fungi (1).

The purpose of this work was to assess browning and quantitatively determine the accumulation of terpenoids in potato sprouts inoculated with *P. infestans,* and to relate these processes to the resistance of sprouts and tubers to this fungus.

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MATERIALS AND METHODS

General.—In all experiments, drops of a zoospore suspension or sonicate, 15-20 μl/drop, were applied in three rows along the length of cut sprouts lying in a glass tray lined with moistened filter paper. Unless otherwise stated, the sprouts were incubated in the dark at 19 C for 84-96 hr after inoculation.

Potato sprouts were obtained by placing tubers of cultivars Kennebec (R_1), Russet Burbank (r) and Red Pontiac (r) into plastic pots containing an equal mixture of moist sand and peat moss. The pots were kept in a dark room until the etiolated sprouts reached a height of 20-25 cm. The sprouts then were cut above the surface of the potting mixture and immediately inoculated.

Races 4 and 1,2,3,4 of *Phytophthora infestans* (Mont.) de By were grown on lima bean agar medium (9) for 8-10 days at 19 C. Suspensions of sporangia (10^5/ml) and zoospore release were obtained as previously described (19). Race 4 of *P. infestans* is incompatible and race 1,2,3,4 is compatible on Kennebec tubers and foliage. Both races are compatible on Russet Burbank and Red Pontiac.

For the preparation of sonicates, *P. infestans* was grown in lima bean broth and incubated as described above. *Pythium aphanidermatum* (Edson) Fitz., *Achlya flagellata* Coker, *Aphanomyces euteiches* Drechs., and *Phytophthora parasitica* Dast. were grown in yeast-

peptone-glucose broth (9) at 24 C for 7-10 days. Before application to the sprouts, the mycelial mats were separated from the media, washed, frozen at −20 C, sonicated, and autoclaved as described previously (9). The sonicates were applied to the sprouts at a concentration equivalent to 10 mg dry weight of fungus per milliliter.

Extraction and quantitation of the sesqui and norsesquiterpenoids.—The top 1-2 mm of inoculated sprout tissue was homogenized in methanol with a Waring Blendor. The mixture was filtered through Whatman No. 2 filter paper in a Büchner funnel and the residue was washed twice with methanol. The filtrate and washings were combined, evaporated almost to dryness in a rotary evaporator, dissolved in a mixture of chloroform, water, and 7.5% acetic acid (200:200:1, v/v) and the chloroform phase was dried in a rotary evaporator (9). The dry residue was suspended in methanol, centrifuged at 600 *g*, and the clear yellow supernatant was chromatographed on TLC plates with cyclohexane:ethyl acetate (1:1, v/v). Terpenoids were detected by spraying plates either with sulfuric acid or vanillin-sulfuric acid reagent (9). To test the diffusibility of terpenoids from inoculated sprouts into aqueous solutions, sprouts were cut into 5-cm sections and gently stirred for 10 min in either five volumes of water or 0.1 M phosphate buffer, pH 7.0. The solutions were decanted and the procedure was repeated twice. The aqueous solutions were extracted with chloroform, and the terpenoids were analyzed as described.

Terpenoids were quantitated with a Varian Gas Chromatograph series 1400 equipped with a 180 cm × 0.64 cm OD, 2 mm ID, Pyrex glass column packed with 3% OV-225 on 80/100 Supelcoport (Supelco Inc., Bellefonte, PA 16823) and a flame ionization detector. Nitrogen at the rate of 40 ml/min was used as a carrier gas. Temperatures of injection port, oven, and detector were 230, 180, and 225 C, respectively. The amounts of the terpenoids were calculated based on standard curves with methyl arachidate (Sigma Chemical Co., St. Louis, MO 63178) as an internal standard.

Extraction and quantitation of the steroid glycoalkaloids.—Two 5-cm sections of uninoculated sprouts were cut from sprouts 20-25 cm long: a section 5 cm from the growing point and one 5 cm from the cut base of the sprout. The extraction was essentially as described by Shih et al., (13, 15). Sprouts were blended with chloroform:acetic acid:methanol (50:5:45, v/v), 10 ml/g fresh weight, filtered through Whatman No. 2 filter paper, and the residue again was blended and filtered. The combined filtrates were evaporated almost to dryness in a rotary evaporator at <45 C. The residue was shaken with 100 ml of 0.2 M acetic acid and the steroid glycoalkaloids were separated from other terpenoids by adding 100 ml of chloroform. The acetic acid phase was washed three times with chloroform and freeze-dried. Ten ml of distilled water then was added to the dried residue, and the steroid glycoalkaloids were precipitated by adding 2 ml concentrated ammonium hydroxide and holding the solution at 80 C for 30 min. The preparation was kept for 4 hr at 4 C. The precipitate was collected by centrifugation at 10,000 *g* for 30 min and dried under reduced pressure. The residue was dissolved in 2 ml of 5% acetic acid in methanol and used for qualitative estimation on silica gel G thin-layer chromatography (TLC) plates and colorimetric quantitation. The TLC plates were developed in chloroform: acetic acid: methanol (50:5:45, v/v) and sprayed with a saturated solution of antimony trichloride in chloroform. Steroid glycoalkaloids were quantitated spectrophotometrically as described previously (15).

RESULTS

Sprouts of susceptible and resistant tubers, inoculated with zoospores or treated with sonicates of *P. infestans*, browned intensely and accumulated approximately equal concentrations of rishitin, lubimin, phytuberin, and phytuberol (Table 1). Growth and sporulation of the fungus were not evident on the sprouts. Autoclaved sonicates of *P. aphanidermatum*, *A. flagellata*, *A. euteiches*, and *P. parasitica* also caused browning and terpenoid accumulation (Table 1). Browning and terpenoid accumulation of sprouts were not related to the resistance or susceptibility of the tubers to *P. infestans*. Sprouts inoculated with zoospores of *P. infestans* turned brown first (24-36 hr) in the region of the growing point of the sprouts and browning gradually extended (36-60 hr) throughout the length of the sprout below the sites where inoculum was applied. No differences in the appearance of browning was evident when the sprouts were treated with the autoclaved sonicates of fungi. Young leaves on sprouts from resistant and susceptible cultivars browned intensely when inoculated with zoospores or treated with

TABLE 1. Accumulation of terpenoids in Kennebec (K), Russet Burbank (RB) and Red Pontiac (RP) potato sprouts inoculated with zoospores (Z) or treated with autoclaved sonicates(s) of fungi

	Quantity of terpenoids (μg/g fresh weight)											
	Phytuberin			Phytuberol			Rishitin			Lubimin		
Fungi	K	RB	RP	K	RB	RP	K	RB	RP	K	RB	RP
(Z) *Phytophthora infestans* race 4	4	7	Tr[a]	5	4	Tr	71	64	89	26	21	42
(Z) *Phytophthora infestans* race 1,2,3,4	5	6	Tr	9	5	Tr	86	75	68	31	34	27
(S) *Phytophthora infestans* race 4	8	5	Tr	7	6	Tr	58	63	74	28	22	30
(S) *Phytophthora parasitica*	Tr	Tr		Tr	Tr		45	51		19	13	
(S) *Pythium aphanidermatum*	8	4		4	6		68	81		22	16	
(S) *Achlya flagellata*	0	0		0	0		37	28		11	19	
(S) *Aphanomyces euteiches*	0	0		0	0		29	11		8	5	

[a]Tr = Trace ≤ 3 μg/g fresh weight.

sonicates of fungi.

Not more than 25-30% of the terpenoids were released when the inoculated sprouts were washed either with distilled water or 0.1 M phosphate buffer, pH 7.0.

Accumulation of terpenoids and browning both were influenced by the temperature of incubation (Table 2). Highest terpenoid accumulation and browning was obtained at 19 C; at 30 and 36 C neither browning nor terpenoid accumulation was detected.

The content of steroid glycoalkaloids in the top 5 cm of the sprout was higher than in the 5 cm at the base. Values of 9.8 and 3.2 mg/g fresh weight were obtained from top and bottom of Russet Burbank sprouts, respectively. In Kennebec, the values were 11.6 and 3.4 mg/g fresh weight for top and bottom, respectively. Thin-layer chromatography indicated that extracts of Russet Burbank and Red Pontiac contained both α-solanine and α-chaconine. Extracts of the top 5 cm of Kennebec, but not of Russet Burbank or Red Pontiac sprouts, had three additional steroid alkaloids. These appeared to be α-solamarine, β-solamarine, and tomatidenol on the basis of TLC with the solvents described by Shih and Kuć (14) to separate α-solamarine, β-solamarine, and tomatidenol from extracts of potato foliage and aged sliced tubers of Kennebec. Only α-solanine and α-chaconine were detected in the bottom 5 cm of Kennebec sprouts.

DISCUSSION

This work confirms the report by Varns et al. (18) that sprouts of cultivars susceptible to *P. infestans* browned and accumulated rishitin. In our work, however, little or no difference in browning and terpenoid accumulation was apparent in sprouts inoculated with zoospores of *P. infestans* which are compatible or incompatible to the tubers. Varns et al. (18) did not quantitate terpenoid accumulation but they estimated more browning and terpenoid accumulation in inoculated sprouts of compatible tubers. The age of tubers or differences in applying inoculum may be the reason for the discrepancy between data presented by Varns and those reported in this paper. In both studies, mycelial growth and sporulation were not apparent on sprouts. Tuber slices inoculated with compatible races of the fungus do not brown and extensive mycelial growth and sporulation are evident on inoculated surfaces 96-120 hr after inoculation. The lack of mycelial growth and sporulation of *P. infestans* on sprouts from tubers resistant or susceptible to late blight, as well as the ability of compatible and incompatible races of the fungus to elicit browning and terpenoid accumulation in sprouts, suggests that sprouts of potatoes are resistant. The ability of cell-free fungal sonicates of various oomycetes to elicit browning and terpenoid accumulation verifies the earlier work reported for tubers (9) and further supports the contention that resistance or susceptibility of potato to late blight is not determined by the presence or absence of genetic information coding for the synthesis of the terpenoids. If the terpenoids have a major role in restricting development of *P. infestans,* the speed and magnitude of their accumulation would appear to be critical factors affecting susceptibility and resistance. In contrast with the reports of Varns et al. (18) and our data, Dorozhkin et al. (4), reported the detection of higher amounts of terpenoids in sprouts of incompatible as compared to compatible tubers.

In our study, 70-75% of the total terpenoids remained in sprouts after washing. This does not agree with other reports of the diffusibility of terpenoids from tubers and leaves (7, 11). Dorozhkin et al. (4), however, reported higher amounts (2-3 ×) of terpenoids in extracts than in diffusates from sprouts.

The observation that terpenoid accumulation is greater at 19 C than at 14 C or 25 C verifies the work reported earlier for tubers (3, 6). The effect of temperature is on host rather than on fungus metabolism since cell-free sonicates of the fungus were used to elicit terpenoid accumulation (Table 2). Thus, a possible defense mechanism of the host is most active at a temperature which is optimal for development of the pathogen in the host.

The appearance of browning first at the tips of sprouts inoculated with zoospores of *P. infestans* may be the result of enchanced fungal death caused by the higher concentration of steroid glycoalkaloid at the tips. Sonicates of *P. infestans* rapidly elicit both necrosis and terpenoid accumulation. Though α-solamarine and β-solamarine were earlier reported in foliage and aged slices of Kennebec tubers (14), this work presents the first report of the steroid glycoalkaloids in Kennebec sprouts (14). Paseshnichenko (12) reported higher amounts of steroid glycoalkaloids in young as compared to old plants.

Differences in necrosis and terpenoid accumulation were demonstrated in different organs of the same potato plant having R-3 genes (20). Since sprouts of susceptible cultivars appear resistant (18, and the present work) it is not possible to generalize that resistance or susceptibility are expressed in the entire plant or that the same mechanism or factor controls the disease reaction in all plant tissues.

TABLE 2. The effect of temperature on the accumulation of terpenoids in Kennebec potato sprouts treated with autoclaved sonicates of *Phytophthora infestans* race 4

Temp	Quantity of terpenoids (μg/g fresh weight)			
(C)	Phytuberin	Phytuberol	Rishitin	Lubimin
14	4	Tr	22	13
19	8	11	68	29
25	Tr[a]	Tr	31	18
30	0	0	0	0
36	0	0	0	0

[a]TR = Trace ≤ 3 μg/g fresh weight.

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