Effect of Temperature on Rishitin and Steroid Glycoalkaloid Accumulation in Potato Tuber

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ABSTRACT

Browning and rishitin accumulation in tubers, either inoculated with an incompatible race of *Phytophthora infestans* or treated with sonicates of the fungus, were markedly reduced at 25 C or 30 C as compared to 19 C. Browning and rishitin accumulation were not evident at 14 or 37 C. Germination of zoospores and growth of the germ tubes were not affected by incubation at 25 C or 30 C for 12 hours. The accumulation of fungitoxic steroid glycoalkaloids, characteristic of the wound response, was increased by incubation at 25 C or 30 C and decreased markedly at 37 C as compared to 19 C. It appears that incubation at 25 C and 30 C accentuates the wound response, but inhibits browning and rishitin accumulation which are associated with the hypersensitive response to incompatible races of *P. infestans*.

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Potato tuber (*Solanum tuberosum* L.) cells invaded by an incompatible race of *Phytophthora infestans* (Mont.) d. By. die within 1-3 hours. The dying tissue rapidly browns and growth of the fungus is contained. In the compatible interaction the invaded host cells survive for at least 2 days. The death of host cells in the incompatible interaction appears associated with an alteration in host metabolism which produces an environment inhibitory to further development of the fungus.

The steroid glycoalkaloids, α-solanine and α-chaconine accumulate around sites of injury in tubers (1, 2, 8) and accumulation is due to de novo synthesis (11). Accumulation is suppressed by inoculation of the wounded surface with *P. infestans* and suppression is greatest with incompatible races (10, 11, 15). Sonicates of compatible and incompatible races of the fungus are equally effective in suppressing accumulation (10, 11). At least 16 terpenoids accumulate in tubers treated with sonicates of compatible or incompatible races of *P. infestans* or inoculated with incompatible races (7, 19). Two of the terpenoids, rishitin and phytuberin, are fungitoxic and appear associated with the "R" gene resistance mechanism (16, 17, 18, 19). Inoculation of tuber slices with a compatible race suppresses necrosis and accumulation of rishitin and phytuberin in response to subsequent inoculation with an incompatible race or treatment with sonicate (17, 18).

Growth and sporulation of *P. infestans* in potato leaves and tubers is maximal at 20-21 C. Tomiyama (13) reported that brief treatment of host tissues at 45-53.5 C reduced resistance to incompatible races of *P. infestans*. More recently Tomiyama (14) indicated that the time required for death of host cells after penetration by the fungus was reduced by inoculation at 25 C or 30 C as compared to that at 20 C. Our objective was to determine the effect of temperature on the accumulation of rishitin and steroid glycoalkaloids in response to an incompatible or compatible race of *P. infestans* or a sonicate of the fungus. This information would help ascertain whether resistance of tubers at temperatures other than the optimum for growth and sporulation of the fungus could be associated with increased browning, increased rishitin or steroid glycoalkaloid accumulation or a direct effect of temperature on growth of the pathogen.

MATERIALS AND METHODS.—All experiments were repeated twice and at least three determinations of steroid glycoalkaloids or rishitin were made per treatment per experiment.

Inoculation with P. infestans.—Wisconsin certified seed tubers, cultivar Kennebec (R_1), stored at 4 C were held for 24 hours at room temperature, washed with detergent and water, soaked in 70% ethanol for 3 minutes, cut into approximately 1-cm thick slices, and placed in 150 × 20-mm petri dishes lined with moistened filter paper. *P. infestans* race 4 (incompatible) and race 1.3.4 (compatible) were maintained on lima bean agar slants. Every 2 weeks, suspensions of sporangia were prepared from the slants and used to inoculate the above medium in petri dishes. After 2 weeks, 20 ml of water was added per petri dish and the spores were released by rubbing the surface of the medium with a glass rod (approximately 4×10^5 sporangia/ml). Zoospores were liberated by holding the sporangial suspensions at 12-15 C for 2 hours. The top surface of the tuber slices were sprayed with the chilled spore suspensions under aseptic conditions.

Sonicates.—Two-week-old cultures of *P. infestans* race 4 grown in petri dishes were frozen with dry ice. The fluffy mycelial mat was scraped off and held at −20 C. Five to 10 g of the frozen fungus was suspended in 20 ml of 50 mM borate buffer (pH 8.8, 1 mM EDTA and 2 ppm streptomycin) and homogenized for 2 minutes at maximum speed in a Virtis Model 45 homogenizer. The solution was then sonicated for 1 minute with a Branson 10 Kc sonifier at approximately 60 watts output. Sonication was repeated four times and the solution was cooled in an ice bath during homogenization and sonication. The sonicate was diluted with buffer to give a final concentration of about 10 ml/g frozen fungus. Upper surfaces of tuber slices were treated with 0.5 ml diluted sonicate/slice.

Incubation.—Treated and untreated slices were incubated at 14, 19, 25, 30, and 37 C. Temperature variation was approximately ± 1 C. At intervals the slices were removed, peeled, and the top 1.0 mm extracted or frozen at −20 C.

Extraction.—Fresh or frozen tissue was blended in chloroform, acetic acid, methanol (50:5:45, v/v) (CAM). The homogenate was allowed to stand overnight, filtered under reduced pressure through Whatman No. 54 filter paper and the residue was washed twice with CAM. The combined CAM extracts were evaporated to dryness and resuspended in 2% acetic acid. The dilute acetic acid solution was extracted twice with equal volumes of chloroform.

The combined chloroform extracts were evaporated to dryness in a rotary evaporator. The residue was resuspended in sufficient chloroform to give 20 g fresh tissue/ml and 10 μliters of a 4,400 μg/g solution of methyl myristate was added as internal standard. These extracts contained rishitin and phytuberin. Samples were spotted on precoated silica gel G plates (Analtech 20 × 20 cm) and developed in cyclohexane:ethyl acetate (1:1, v/v). Rishitin was detected with a saturated chloroform solution of antimony trichloride (Carr-Price reagent) and appeared brilliant red without heating.

The dilute acetic acid extract was reduced to approximately 1/20 its original volume in a rotary evaporator. The concentrated extract, containing steroid glycoalkaloids, was centrifuged at 10,000 *g* for 10 minutes to remove insoluble material. Concentrated ammonium hydroxide was added to the supernatant to raise the pH to at least 10, and the mixture was heated in an 80 C water bath for 30 minutes then cooled in a refrigerator for at least 4 hours. The precipitated steroid glycoalkaloids were collected by centrifugation at 10,000 *g* for 10 minutes and dried in a vacuum desiccator. The dried pellet was dissolved in 2 ml of 5% methanolic acetic acid.

Quantitation.—Rishitin was quantitated by gas-liquid chromatography on a 91.4 cm × 3.2 mm stainless steel column of 3% QF-1 on Chromosorb Q [246/177 μm (60/80 mesh)] at 180 C. The peak height relative to that of the internal standard was used to calculate the amount of rishitin in the sample. A standard curve was prepared using purified rishitin.

Steroid glycoalkaloids were quantitated by a colorimetric method based on the Alberti reaction (9, 10). Portions of the dilute acetic acid extracts were dried under reduced pressure. Three ml of 50% ethanol:sulfuric acid (1:2, v/v) were slowly added to the residue held in an ice bath and 1 ml of 1% formaldehyde was then slowly added to the cold solution. The reddish-purple color which developed after 90 minutes at room temperature was measured at 562 nm. A standard curve was prepared using commercial α-solanine.

Germination and growth of P. infestans.—A zoospore suspension of *P. infestans* race 4 was used to inoculate water agar and lima bean agar plates. The plates were incubated at 14, 19, 25, 30, and 37 C. At various time

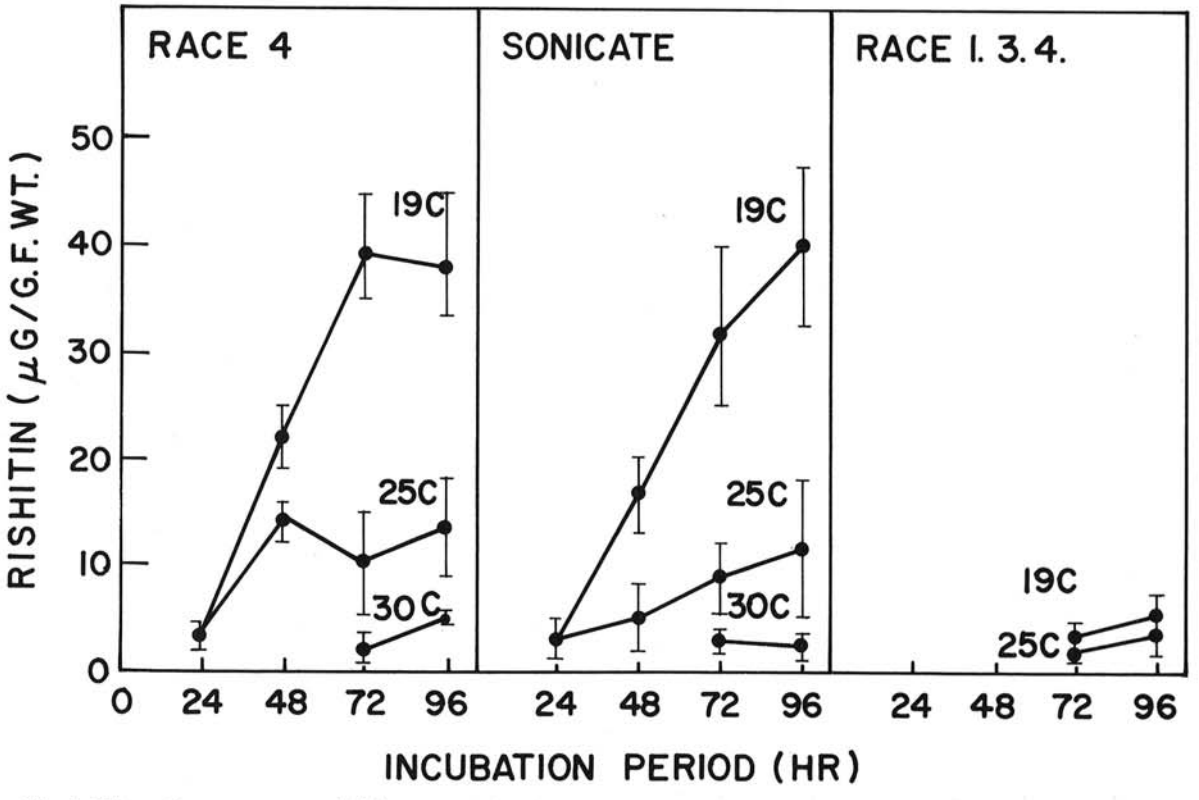

Fig. 1. Effect of temperature on rishitin accumulation in the top 1.0 mm of potato tuber slices inoculated with compatible race 1.3.4, incompatible race 4, or treated with a cell-free sonicate of race 4 of *Phytophthora infestans*.

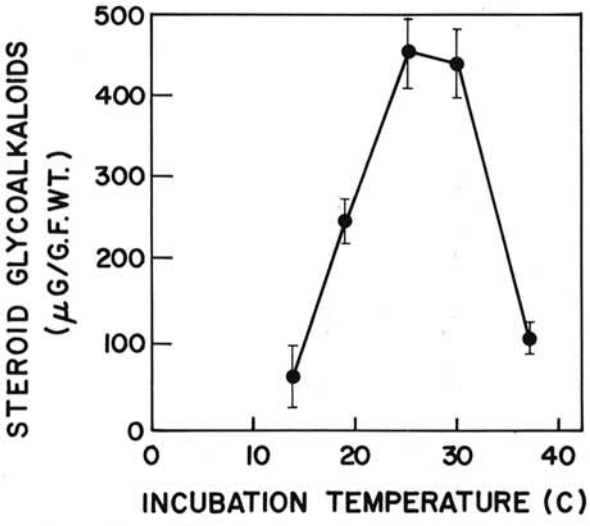

Fig. 2. Effect of temperature on steroid glycoalkaloid accumulation in cut potato tuber slices held for 72 hours. Steroid glycoalkaloids are expressed as α-solanine.

intervals, a plate at each temperature was removed and the percent spore germination (25-100 spores) determined. The length of the germ tube was measured for 25 germinated spores with an ocular micrometer.

RESULTS.—Browning was apparent on slices treated with sonicate or inoculated with an incompatible race and held at 19 C. Browning was markedly reduced on slices treated with sonicate or inoculated and held at 25 C or 30 C. This reduction was most pronounced at 30 C. Browning was not detected on slices held at 14 or 37 C.

Little or no rishitin accumulated in uninoculated slices, slices inoculated with the compatible race at all temperatures studied, and slices inoculated with the incompatible race or treated with the sonicate at 14 or 37 C. Rishitin accumulated in slices inoculated with the incompatible race or treated with sonicate at 19, 25, or 30 C with maximum accumulation occurring at 19 C (Fig. 1).

Spore germination and growth of the germ tubes for 12 hours was not affected by incubation at 14, 19, 25, or 30 C. The respective average growth rates over 12 hours were 0.26, 0.30, 0.29, and 0.24 μm/minute. Spores germinated at 37 C but growth of the fungus was stopped. The germinated spores survived 24 hours at 37 C and started growing again when held at 19 C. Incubation of the tubers at 25 or 30 C markedly reduced the growth and sporulation of the compatible fungus as seen after 4-5 days.

The optimal temperature for steroid glycoalkaloid accumulation was 25-30 C (Fig. 2). Suppression of steroid glycoalkaloid accumulation by an incompatible interaction or sonicate was evident at 19, 25, and 30 C (Fig. 3).

DISCUSSION.—Lauritzen (5, 6) demonstrated that increased temperature accelerated the wound response in sweet potatoes and gladiolus corms and enhanced resistance to fungi. The results reported here with steroid glycoalkaloids are consistent with his observations.

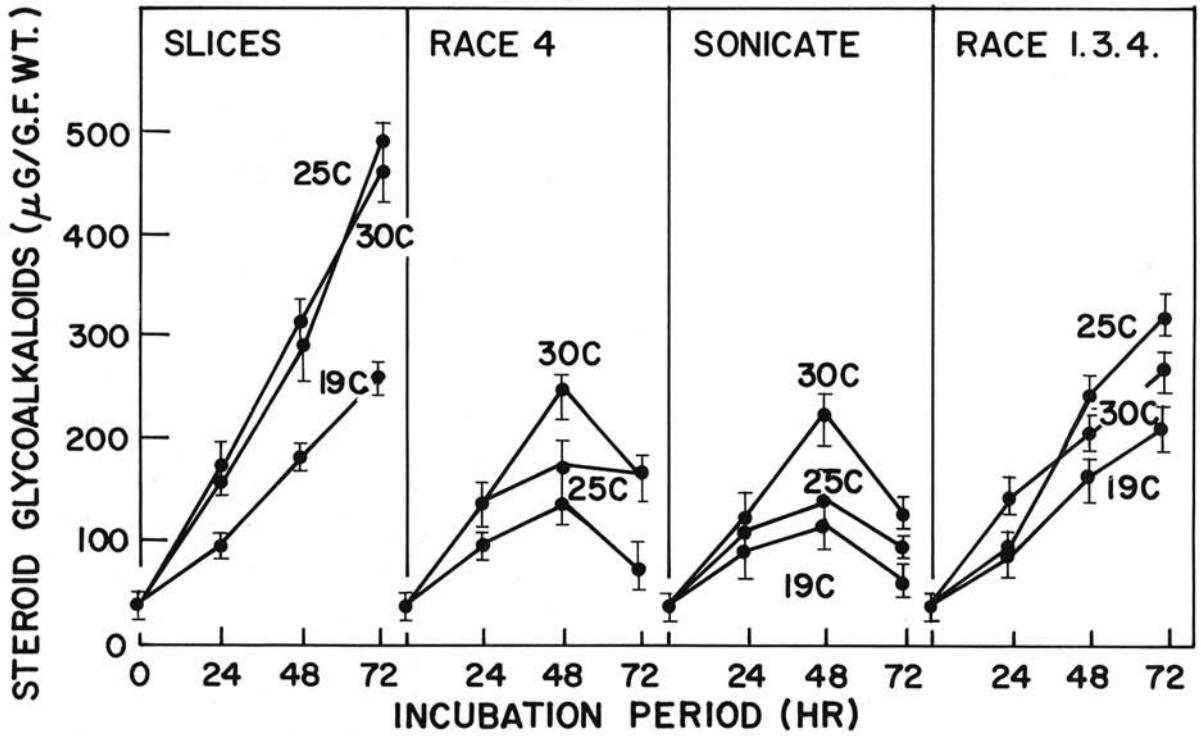

Fig. 3. Effect of temperature on steroid glycoalkaloid accumulation in potato tuber slices and slices inoculated with compatible race 1.3.4, incompatible race 4, or treated with a cell-free sonicate of race 4 of *Phytophthora infestans*. Steroid glycoalkaloids are expressed as α-solanine.

The suppression of steroid glycoalkaloid accumulation caused by inoculation with *P. infestans* or treatment with sonicate, evident at 19 C, occurred at higher temperatures (Fig. 3) even though rishitin accumulation was markedly reduced at these temperatures (Fig. 1). Suppression of steroid glycoalkaloid accumulation has previously been associated with rishitin accumulation (10). However, the results presented here suggest that there is not always an association between these phenomena; i.e., suppression of steroid glycoalkaloids does not always result in the accumulation of rishitin.

Tomiyama's studies (14) with incompatible races of *P. infestans* suggested accelerated death of host cells at elevated temperatures. Our data indicate browning and rishitin accumulation were markedly decreased at 25 or 30 C. This temperature effect was directly on the tuber tissues rather than on the fungus since the effect was evident with both the incompatible fungus and its sonicate. Since treatment with the sonicate at 25 or 30 C caused less rishitin accumulation than at 19 C (Fig. 1), death or containment of the fungus in infected tubers is not the only metabolic control for rishitin accumulation, and rapid death of host cells (14) does not always result in a high level of rishitin accumulation.

If the hypersensitive response of the tuber, as determined by browning and rishitin accumulation, is decreased by incubation at 25 or 30 C while the germination and initial growth of the fungus is unaffected, what contains the growth of the fungus? We might expect all tubers to become more susceptible at temperatures above 19 C because rishitin accumulation is reduced at higher temperatures. The fungus, however, does not grow well after 12-24 hours at 25 or 30 C in nutrient media or in vivo. Thus, the mechanism for restricting growth in potato at higher temperatures appears unrelated to the accumulation of rishitin.

At the optimum temperature for the growth of *P. infestans,* in culture or in the host, the incompatible fungus and its sonicate induce maximum browning and rishitin accumulation. Most fungi (pathogens and nonpathogens of potato) have optimum temperatures for growth above 19 C and probably closer to 24 C. The accumulation of steroid glycoalkaloids may contribute to protection at these elevated temperatures, and once accumulated, high levels of steroid glycoalkaloids may also protect potato against *P. infestans.*

Even at optimal temperatures for rishitin accumulation, the rate of periderm formation (4) and the role of compounds accumulating in the compatible interaction (3, 12) should be considered to elucidate the nature of the host-parasite interaction.

LITERATURE CITED

1. ALLEN, E. H. 1965. Steroid glycoalkaloids in the disease resistance of white potato tubers. Ph.D. Thesis, Purdue Univ., Lafayette, Indiana, 83 p.
2. ALLEN, E. H., and J. KUĆ. 1964. Steroid alkaloids in the disease resistance of white potato tubers. Phytopathology 54:886 (Abstr.).
3. HUGHES, J., and T. SWAIN. 1960. Scopolin production in potato tubers infected with Phytophthora infestans. Phytopathology 50:398-400.
4. ISHIZAKA, N., and K. TOMIYAMA. 1970. Effect of Phytophthora infestans infection on wound periderm formation in cut potato tuber tissue. Ann. Phytopathol. Soc. Jap. 36:243-249.
5. LAURITZEN, J. I. 1935. Factors affecting infection and decay of sweet potatoes by certain storage rot fungi. J. Agric. Res. 50:285-329.
6. LAURITZEN, J. I., and R. C. WRIGHT. 1934. Factors affecting Gladiolus in storage. J. Agric. Res. 48:265-282.
7. LOCCI, R., and J. KUĆ. 1967. Steroid alkaloids as compounds produced by potato tubers under stress. Phytopathology 57:1272-1273.
8. MC KEE, R. K. 1959. Factors affecting the toxicity of solanine and related alkaloids to Fusarium caeruleum. J. Gen. Microbiol. 20:686-696.
9. PASESHNISCHENKO, V. A., and A. R. GUSEVA. 1956. Quantitative determination of potato glycoalkaloids and their preparative separation. Biochemistry (USSR) 21:606-611.
10. SHIH, M., J. KUĆ, and E. B. WILLIAMS. 1973. Suppression of steroid glycoalkaloids accumulation as related to rishitin accumulation in potato tubers. Phytopathology 63:821-826.
11. SHIH, M., and J. KUĆ. 1973. Incorporation of ^{14}C from acetate and mevalonate into rishitin and steroid glycoalkaloids by potato tuber slices inoculated with Phytophthora infestans. Phytopathology 63:826-829.
12. SUBRAMANIAN, S., J. L. VARNS, and J. KUĆ. 1970. Biochemical response in the compatible potato tuber–Phytophthora infestans interaction. Proc. Indiana Acad. Sci. 80:367 (Abstr.).
13. TOMIYAMA, K. 1957. The influence of a pre-infectional heat treatment on the resistance of potato plant to Phytophthora infestans. Ann. Phytopathol. Soc. Jap. 22:237-242.
14. TOMIYAMA, K. 1973. Effect of temperature on the process of hypersensitive death of a potato plant cell infected by an incompatible race of Phytophthora infestans. Ann. Phytopathol. Soc. Jap. 39:73-78.
15. TOMIYAMA, K., N. ISHIZAKA, N. SATO, T. MASAMUNE, and N. KATSUI. 1968. "Rishitin" A phytoalexin-like substance. Its role in the defense reaction of potato tubers to infection. Pages 287-292 *in* Biochemical regulation in diseased plants or injury. The Phytopathological Soc. Jap., Tokyo.
16. TOMIYAMA, K., T. SAKUMA, N. ISHIZAKA, N. SATO, N. KATSUI, T. MASAMUNE, and M. TAKASUGI. 1968. A new antifungal substance isolated from resistant potato tuber tissue infected by pathogens. Phytopathology 58:115-116.
17. VARNS, J., and J. KUĆ. 1971. Suppression of rishitin and phytuberin accumulation and hypersensitive response in potato by compatible races of Phytophthora infestans. Phytopathology 61:178-181.
18. VARNS, J., and J. KUĆ. 1972. Suppression of the resistance response as an active mechanism for the susceptibility in the potato–Phytophthora infestans interaction. Pages 465-468 *in* R. K. S. Wood and A. Graniti, eds. Phytotoxins in plant diseases. Academic Press, New York. 530 p.
19. VARNS, J. L., J. KUĆ, and E. B. WILLIAMS. 1971. Terpenoid accumulation as a biochemical response of the potato tuber to Phytophthora infestans. Phytopathology 61:174-177.