# Effects of Controlled Atmospheres on Production of Sesquiterpenoid Stress Metabolites by White Potato Tuber

## POSSIBLE INVOLVEMENT OF CYANIDE-RESISTANT RESPIRATION[1]

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### ABSTRACT

**Levels of katahdinone (solavetivone), lubimin, rishitin, and phytuberin, sesquiterpenoid stress metabolites of white potato (*Solanum tuberosum*), were monitored in tuber slices which were challenged with an extract of *Phytophthora infestans* and incubated under controlled atmospheres. A mixture of ethylene in air enhanced stress metabolite production. This enhancement was amplified by higher partial pressures of oxygen. Stress metabolite production was inhibited by salicylhydroxamic acid. These results suggest the involvement of cyanide-resistant respiration in the production of potato stress metabolites, compounds which may serve as phytoalexins.**

The production of sesquiterpenoid stress metabolites (SSMs)[4] by *Solanum tuberosum* tubers after infection by an incompatible race of the late-blight fungus *Phytophthora infestans* is characteristic of the hypersensitive interaction (5, 6, 11, 19, 30, 32). Four of the major SSMs of *S. tuberosum,* katahdinone (solavetivone), lubimin, rishitin, and phytuberin, possess fungistatic and/or fungitoxic properties (2, 9, 12, 18, 33). The inhibition of fungal growth in hypersensitive interactions has been attributed to the presence of these SSMs in host tuber tissue (31). For this reason they are sometimes termed phytoalexins.

Infection of *S. tuberosum* by a compatible race of *P. infestans* results in fungal growth and the absence of SSMs, events characteristic of the susceptible interaction (35). Susceptibility and hypersensitivity are functions of the genetic make-up of the potato cultivar and the fungal race involved in the interaction, resulting in a cultivar-race specificity (22). However, if a cell-free extract of any race of *P. infestans* is used to challenge any cultivar of *S. tuberosum,* only the hypersensitive interaction is observed (34). Thus, all potato tubers have the capacity to produce SSMs although that capacity may not be expressed following infection by the intact fungus.

Published reports indicate an increase in over-all potato tuber respiration, as measured by $CO_2$ evolution, after exposure to ethylene (20, 23), and an amplification of the ethylene-induced respiratory surge by higher partial pressures of $O_2$ (3, 4). Such conditions activate a cyanide-resistant respiratory pathway in potato (28, 29).

The intent of our investigation was to determine whether the conditions leading to increased respiratory activity and to cyanide insensitivity in potato also influence the specific physiological events which result in SSM production and alter SSM levels. Such a result would implicate the physiology and biochemistry of SSM production as manifestations of cyanide-resistant respiration.

## MATERIALS AND METHODS

**Biological Material.** Tubers of white potato (*S. tuberosum* L. cv. Katahdin) were obtained from the University of Maine Aroostock Farms, Presque Isle. Tubers were stored at 10 C until treated. Tubers aged for 6 to 9 months postharvest proved to be most responsive and were utilized in this study. *P. infestans* (Mont.) De Bary, race 0, obtained from H. Van Etten (Cornell University), was cultured in a rye-steep medium (26) in 2-liter flasks at 20 C in a PsycroTherm Incubator Shaker with gyrotory platform (New Brunswick Scientific)[5] at 150 rpm for 7 to 14 days.

**Fungal Extract.** Preparation of the *P. infestans* extract, a modification of the method of Currier (7), is shown in Figure 1. Homogenization was performed for 10 min at maximum speed in a VirTis homogenizer, sonication for 10 min at maximum wattage with a Branson Sonifier. During both steps, the disrupted hyphal mat was cooled in an ice bath. Both centrifugations were carried out at 0 C for 1 hr.

**Treatment of Tubers.** Tuber treatment is shown in Figure 2. Preincubation and incubation took place in a modified pressure cooker at atmospheric pressure. All gases were humidified. Flow rate during both steps was 10 liters/hr. Compositions of gas mixtures were: E/A: 10 $\mu$l ethylene/liter air; E/$O_2$: 10 $\mu$l ethylene/liter (1% (v/v) $N_2$; balance $O_2$). Mixtures were obtained from Air Products and Chemicals and included a certified standard.

**SSM Extraction.** Extraction protocol is shown in Figure 3. All solvents were reagent grade.

**Chromatography.** Methanolic solutions containing SSMs were spotted on Uniplate 250-$\mu$ Silica Gel G TLC plates (Analtech) which were developed to a height of 155 mm in an ascending solvent system of ethyl acetate-cyclohexane (1:1, v/v). Plates were stained by spraying with chloroform saturated with antimony

[1] A brief account of this research was presented at the Annual Meeting of the American Society of Plant Physiologists at the University of Wisconsin, Madison, on August 17, 1977 (1).

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[4] Abbreviations: SSM: sesquiterpenoid stress metabolite; E/A: ethylene in air; E/$O_2$: ethylene in oxygen; SHAM: salicylhydroxamic acid.

[5] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

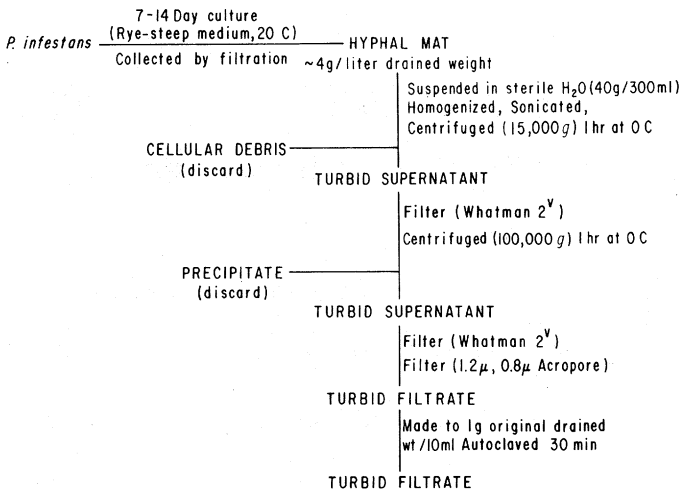


FIG. 1. Preparation of *P. infestans* cell-free extract.

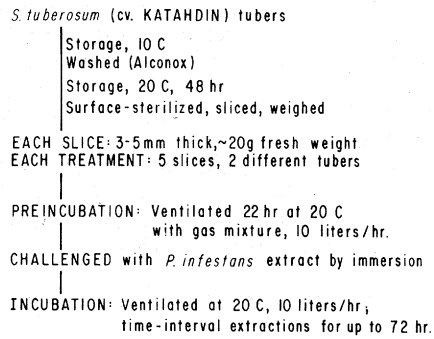


FIG. 2. Experimental treatment of *S. tuberosum* tubers.

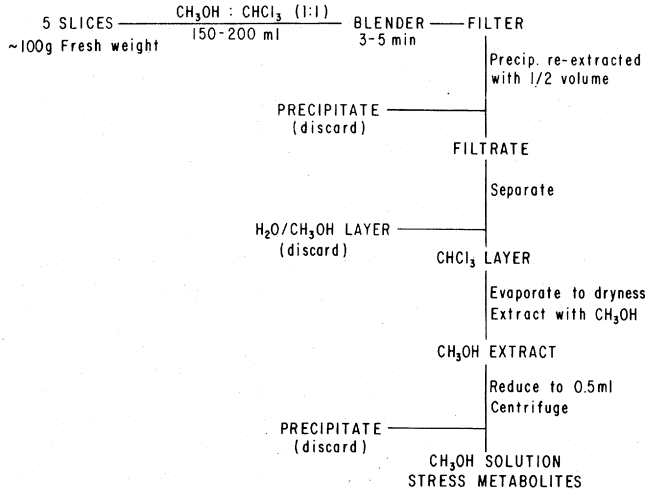


FIG. 3. Preparation of *S. tuberosum* extracts containing SSMs.

trichloride and were heated at 110 C for 5 min. $R_F$ values and staining properties, after heating, of major SSMs were: rishitin, 0.39, violet; lubimin, 0.5, violet; phytuberin, 0.83, orange; and katahdinone, 0.83, yellow.

Separation and quantification of SSMs by GLC were performed by the method of Heisler *et al.* (10). Relative standard deviations (%) for the SSMs were: katahdinone, 2.2; lubimin, 3.0; rishitin, 2.4; and phytuberin, 3.1. SSM concentration of 1 μg/g (dry weight) tuber tissue was used as the threshold value to determine the activation of SSM production.

## RESULTS AND DISCUSSION

Rishitin production in ethylene in air (E/A) was 25% greater than in air alone at 72 hr (Fig. 4). The slopes of the two curves were similar after 24 hr, indicating that the primary effect of ethylene was exerted at an earlier time (Fig. 4, inset). Rishitin was first detected in air at 8 hr, in E/A at 1 hr. At 12 hr, the rishitin level was 500% higher in E/A than in air. The slopes of the two curves were divergent up to 12 hr. Thus, ethylene exerted a dual effect, causing rishitin to appear sooner and to accumulate more rapidly. Ethylene did not cause SSM production in unchallenged tuber slices.

To determine the effect of ethylene exposure time on SSM production, slices were preincubated in E/A for various lengths of time prior to challenge and E/A incubation. Twelve-hr preincubation in E/A resulted in a higher level of rishitin than did 22-hr preincubation (Fig. 5). Similar results were observed with phytuberin and lubimin (unpublished data).

To determine whether the ethylene-induced elevation of SSM

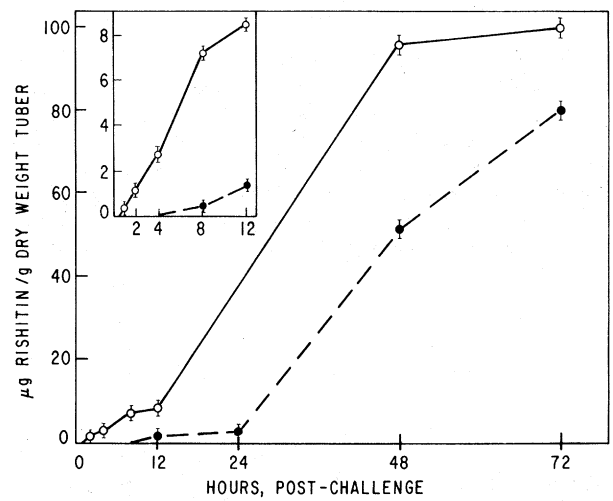


FIG. 4. Effect of E/A on rishitin accumulation in *S. tuberosum* tuber slices challenged with *P. infestans* extract. Inset: effect of E/A on rishitin accumulation at early times. (○): 10-hr preincubation in air, 12 hr in E/A (total: 22 hr), incubation in E/A; (●): 22-hr preincubation in air, incubation in air. Vertical bars on each point indicate 1 SD.

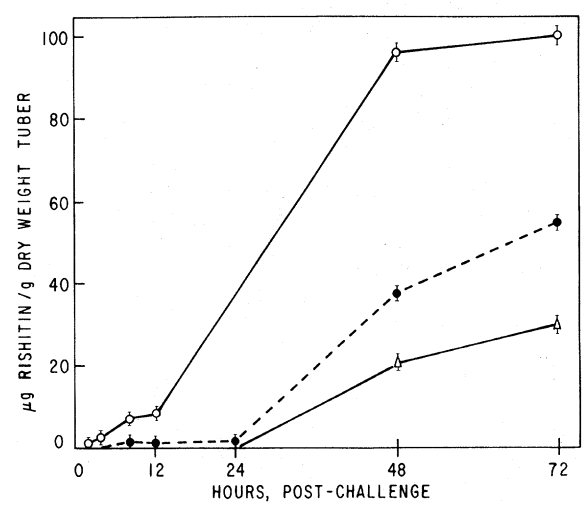


FIG. 5. Effect of duration of E/A preincubation on rishitin accumulation in *S. tuberosum* tuber slices challenged with *P. infestans* extract. (○): 10-hr preincubation in air, 12 hr in E/A (total: 22 hr), incubation in E/A; (●): 22-hr preincubation in E/A, incubation in E/A; (△): 22-hr preincubation in air, incubation in E/A. Vertical bars on each point indicate 1 SD.

levels could be amplified by higher partial-pressures of $O_2$, a mixture of ethylene in oxygen ($E/O_2$) was employed. The rishitin level was 55% higher at 48 hr and 120% higher at 72 hr in $E/O_2$ than in E/A (Fig. 6). The phytuberin level was 220% higher at 48 hr and 490% higher at 72 hr in $E/O_2$ than in E/A (Fig. 6). Furthermore, at 72 hr the E/A curves appeared to plateau while the $E/O_2$ curves were still rising. The SSM levels in challenged $O_2$-treated tuber slices and in challenged air-treated slices were nearly identical, indicating that the large increase of rishitin and phytuberin in challenged $E/O_2$-treated slices was not due to high $O_2$ tensions alone.

Katahdinone accumulation was unaffected by $E/O_2$. Lubimin accumulation was affected only slightly, being 75% higher in $E/O_2$ than in air at 48 hr and 37% higher at 72 hr (Fig. 7). Unlike the $E/O_2$ curves for rishitin and phytuberin, the $E/O_2$ curves for katahdinone and lubimin began to plateau at 72 hr. The $O_2$ availability is critical in maintaining the rate of production and/or limiting the rate of turnover of rishitin and phytuberin, but not so for katahdinone and lubimin.

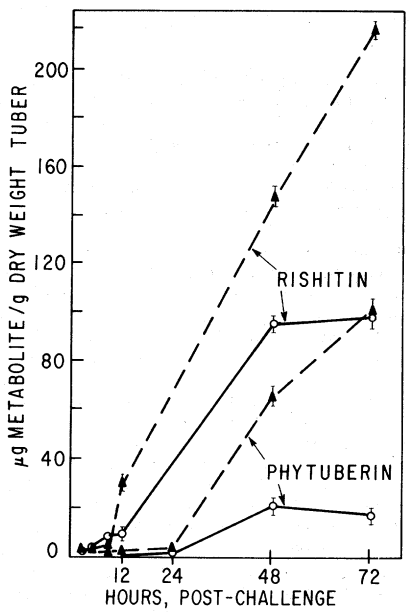


FIG. 6. Effect of $O_2$ tension on rishitin and phytuberin accumulation in ethylene-stimulated *S. tuberosum* tuber slices challenged with *P. infestans* extract. (▲): preincubation and incubation in $E/O_2$; (○): preincubation and incubation in E/A. Vertical bars on each point indicate 1 SD.

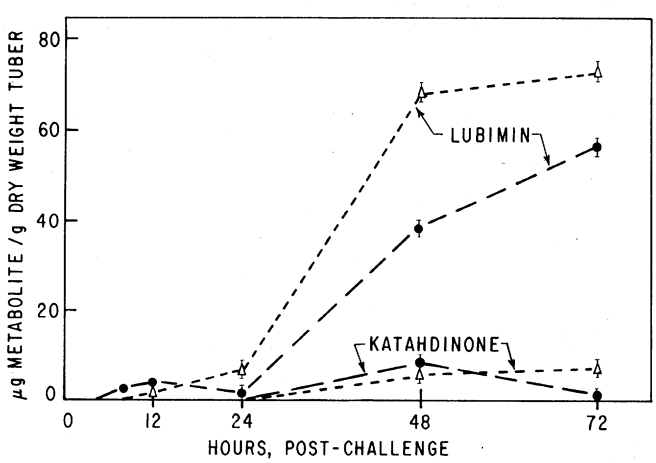


FIG. 7. Effect of $E/O_2$ on lubimin and katahdinone accumulation in *S. tuberosum* tuber slices challenged with *P. infestans* extract. (△): preincubation and incubation in $E/O_2$; (●): preincubation and incubation in air.

The E/A and $E/O_2$ treatments also caused an increase in levels of other stress metabolites which, to date, are unidentified. The color intensity of a series of compounds which stained blue after TLC, antimony trichloride, and heating increased significantly in E/A-treated tuber slices over air-treated slices; color intensity increased further with $E/O_2$. One such compound has a bicyclic fused-ring configuration with empirical formula $C_{17}H_{22}O_2$ and mol wt 258 (unpublished data). Its similarity in structure to rishitinol (31) suggests a common biosynthetic pathway. Also, a compound of $R_F$ 0.59 which stained similarly to phytuberin and which appeared in challenged slices prior to the appearance of phytuberin increased in TLC color intensity with E/A and $E/O_2$.

Observations of macroscopic symptomology correlated well with SSM production. In air, necrosis was observed 48 hr after challenge when the combined level of katahdinone, lubimin, rishitin, and phytuberin was 118 μg/g (dry weight) slice. In $E/O_2$, necrosis was first observed at 24 hr and was more pronounced at 48 hr than in air, and the combined level of SSMs at 48 hr was 286 μg/g (dry weight) slice.

Challenge experiments performed in atmospheres of 100% $N_2$ and 100% CO showed no necrosis and no SSMs after 72 hr. Thus, aerobiosis is a necessary condition for both events to occur.

The increase in rishitin and phytuberin levels caused by ethylene agrees with the findings of Lisker *et al.* (17) who reported an Ethrel-stimulated increase in these two SSMs in challenged tuber slices. The marginal effect of ethylene on katahdinone and lubimin levels is not surprising since these compounds are precursors to rishitin (13–15). The increase in rishitin in ethylene-treated slices necessitates accelerated rates of both synthesis and turnover for katahdinone and lubimin, leaving over-all accumulated levels unaffected. Kinetic studies with radioactive katahdinone and lubimin are planned to determine the stoichiometry of their conversion to rishitin as affected by ethylene.

The detection of rishitin within 1 hr after challenge of ethylene-treated slices is the earliest reported appearance of a stress metabolite to date. Shih (25) used isotope incorporation of labeled acetate and mevalonate to demonstrate *de novo* synthesis of rishitin after *P. infestans* inoculation. Utilizing blasticidin S, Doke *et al.* (8) reported that the *de novo* protein synthesis which occurs during the first 3 hr after *P. infestans* infection is essential to rishitin synthesis. Under the conditions employed, rishitin was not detected until 7 to 24 hr following infection. Kurantz and Kalan (16) reported that tuber slices preincubated in chloramphenicol prior to challenge with *P. infestans* extract produce the usual spectrum of SSMs; and that slices preincubated in chloramphenicol, cycloheximide, or actinomycin D metabolize exogenously applied katahdinone to other SSMs in the same manner as slices not treated with these antibiotics. The appearance of rishitin in ethylene-treated slices within 1 hr after challenge seems too rapid to be accounted for by challenge-induced *de novo* protein synthesis of enzymes necessary for early rishitin production. Among the plausible explanations is one that suggests that challenge causes activation of preexisting enzymes which synthesize rishitin.

Solomos and Laties (29) reported that the capacity for cyanide-resistant respiration must be present in order for ethylene to stimulate respiration and that both cyanide and ethylene either activate or affect the link between the conventional electron transport chain and the cyanide-resistant pathway (28). Rich *et al.* (21) showed the alternate pathway to generate $H_2O_2$ by the incomplete reduction of $O_2$. Chin and Frenkel (3, 4) have reported that the ethylene-induced respiratory surge and peroxide generation can be amplified by higher partial pressures of $O_2$.

Although E/A and $E/O_2$ have been reported to stimulate respiration and to lead to cyanide insensitivity in whole potato tubers, we are unaware of any reports indicating such effects on freshly cut tuber slices. The act of slicing, itself, initiates the onset of these events in tuber tissue (27), and it is not known whether they are hastened by E/A or $E/O_2$. To test the effects on SSM

production of conditions known to stimulate respiration and to lead to cyanide insensitivity, whole tubers were preincubated in $E/O_2$ for 12 hr, sliced, weighed, challenged with *P. infestans* extract, incubated in air, and extracted for SSMs at specified time intervals. SSM levels were enhanced by this treatment over levels observed in tubers preincubated in air as judged by visual inspection of TLC chromatograms.

Our work showed that the amplification of the ethylene-induced increase in SSM levels by higher partial pressures of $O_2$ is similar to the effect of $O_2$ on the ethylene-induced respiratory surge. This similarity led us to hypothesize that cyanide-resistant respiration may be involved in SSM production. Preliminary experiments were conducted to test this hypothesis. Russet Burbank and Katahdin tuber slices which were preincubated in air for 10 hr and in $E/O_2$ for 12 hr; treated with $10^{-2}$ M SHAM, an inhibitor of cyanide-resistant respiration (24); challenged; and incubated in $E/O_2$ resulted in complete inhibition of SSM production. When preincubation and incubation of slices were carried out entirely in air with all other conditions unchanged, SSM production was also completely inhibited by SHAM. When whole tubers were preincubated in air for 10 hr and in $E/O_2$ for 12 hr, sliced, treated with SHAM, challenged, and incubated in $E/O_2$, complete inhibition of SSM production was also observed.

Our results indicate that the regulation of cyanide-resistant respiration, by promotion with ethylene and by inhibition with SHAM, leads to corresponding effects on SSM production. The possible involvement of cyanide-resistant respiration in stress metabolite production and in plant disease resistance is presently under study in our laboratory. Confirmation of our hypothesis would assign a significant function to this pathway whose biological role is currently a source of speculation (27).

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