

## A Self-Inhibitor in Uredospores of the Coffee Rust Fungus

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### ABSTRACT

Uredospores of the coffee rust fungus (*Hemileia vastatrix*) were shown to contain a potent self-inhibitor. The compound was extracted from the spores with water, partitioned into ether, and chromatographed on thin-layer plates of silica gel. The compound had an  $R_f=0.3$  in a benzene:ether solvent (80:20, v/v) system, and the

extract from 2 mg of spores was required to obtain the ED<sub>50</sub>. Neither of the known self-inhibitors from spores of two other species of rust fungi (*Puccinia* spp. and *Uromyces* spp.) effectively inhibited germination of *H. vastatrix* uredospores.

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*Additional key words:* germination, coffee rust.

Known self-inhibitors isolated from species of rust fungi are methyl esters of cinnamic acid derivatives (9, 10, 11). Two esters, methyl ferulate and methyl 3,4-dimethoxycinnamate were shown to be responsible for the self-inhibition phenomenon in species of *Puccinia* (9) and *Uromyces* (10).

Although many studies have been published on the germination of coffee rust uredospores, remarkably few studies have been made of their physiology (6, 8, 13, 14). This may be due to the fact that the spores are borne on uredophores which penetrate through the stomata rather than in a compact sorus which erupts through the epidermis as in *Puccinia graminis*. The effect of this is to create a dispersed pustule which ages from the center outwards; the massive accumulation of spores which occurs within a uredinium is not achieved. In addition, the heavy rains typical of the tropics make it difficult to collect the gram quantities of spores usually required for studies on uredospores.

The present report is concerned with the detection of the coffee rust self-inhibitor and a brief description of some of its properties. For this purpose, self-inhibitors are defined as substances in extracts of fungal spores which inhibit their own germination (1). Extracts from uredospores were shown previously to be inhibitory to germination (12).

**MATERIALS AND METHODS.**—*Collection of spores.*—Uredospores of the coffee rust fungus (*Hemileia vastatrix*, race 2) were harvested from naturally infected coffee trees (*Coffea arabica* L.) by suction with a cyclone separator (5, 15) or by gentle scraping using the edge of a celluloid vial. Microscopic inspection showed that the spores were clean and free of contaminating fungi. A special effort was made to eliminate leaves bearing rust pustules infected with *Verticillium hemileiae* Bour., a fungal parasite of the rust fungus. Spores were stored in the refrigerator at 4 C.

*Germination.*—The spores are sticky and clump easily but the procedure finally adopted largely eliminated this problem. Routinely, 10 mg of spores were dispersed by shaking vigorously in 10 ml of a

1.0% water solution of Tween 20 (20% aqueous polyoxyethylene sorbitan monolaurate, Sigma Chemical Co., St. Louis, Missouri, U S A). One-tenth ml of the spore suspension was then pipetted into the cavity of a hanging-drop slide, the slide placed in a petri dish containing water, and incubated at 23 C in the dark. Usually spores used for germination tests had been stored at 4 C for less than one wk. Long-term storage was achieved by freezing the spores at -20 C or in dry ice, but heating at 40 C for 2 min was required to revive the spores. Coffee rust spores germinate by emission of one to six germ tubes (4), so we scored germination if one germ tube was evident.

Toxicity of chemical fractions was determined by probit analysis (7). Water extracts were tested by first mixing with the spore suspension before plating for incubation. Ether extracts were tested by pipetting the desired volume into the cavity of the slide, evaporation of the ether, and addition of 0.1 ml of the spore suspension to the slide. Routinely, spores were counted after 6 h of incubation, but results were

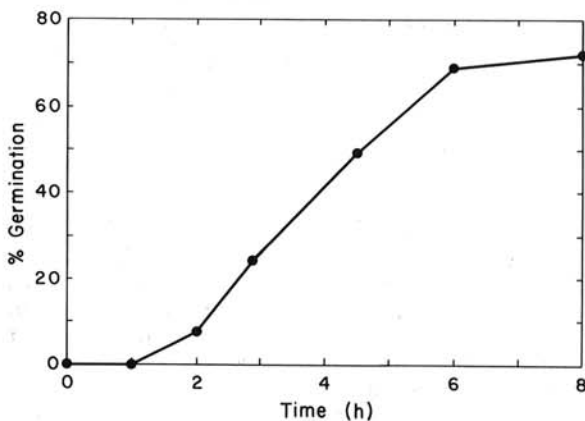


Fig. 1. Germination of *Hemileia vastatrix* uredospores in 1% Tween 20 (20% aqueous polyoxyethylene sorbitan monolaurate) in the dark at 23 C.

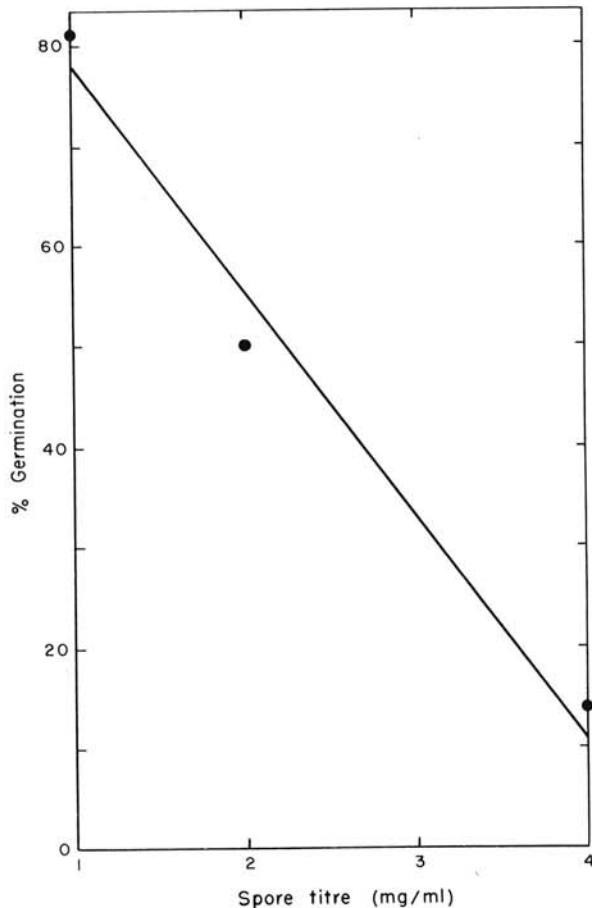


Fig. 2. The effect of crowding on germination of *Hemileia vastatrix* uredospores.

unchanged if germination was allowed to proceed overnight for convenience.

When buffered solutions were required, a 0.002 M phosphate buffer ( $\text{KH}_2\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$ ), pH 6.8, was used in place of water. Germination in the buffer was equivalent to that in water.

**Extraction of self-inhibitor.**—A weighed quantity (1.0 g) of spores was stirred in 100 ml distilled water for 1 h. The spore suspension was filtered through sheets of Whatman No. 1 filter paper, and the water extracted three times with 100 ml of diethyl ether. The ether was then reduced to a small volume in a flash evaporator under vacuum.

**Partial purification of self-inhibitor.**—The ether extract was chromatographed on thin-layer (0.25 mm) plates of silica gel (Merck 60 F254, Germany). After development in a solvent system of benzene:diethyl ether (80:20, v/v), the plates were dried and the inhibitor located by assaying 10-mm square zones of silica scraped from the plate. The silica removed from the plates was extracted with ether and the ether pipetted onto the slides for the germination tests. The remainder of the inhibitor was then extracted from the proper zone of the plate by

elution of the zone with ether. The inhibitor was not successfully sublimed using a short-path distillation apparatus (9, 10, 11).

**RESULTS AND DISCUSSION.**—*Germination.*—Using our procedures, coffee rust uredospores were found to begin to germinate in 2 h (Fig. 1). Complete germination at 23 C was achieved in 6 h in a nearly linear fashion, so germination was counted at 6 h for convenience. Collections of these field-harvested spores varied somewhat in overall germination from 70-85% especially if the spores were stored longer than one wk. This decline in germination could be reversed by heating the spores at 40 C for 10 min, provided that the spores had not been stored longer than 2 mo. Routinely, this corrective heat treatment was omitted, since the spores were not stored longer than one wk. Germination of coffee rust uredospores is not as abrupt nor as well synchronized as spores of the bean or wheat rust fungi.

*Crowding effect.*—Increasing the concentration of spores used for germination from 1 mg/ml to 4 mg/ml almost completely suppressed germination (Fig. 2). This typical crowding effect, long reported for other uredospores (1, 2, 3, 16), strongly suggested that coffee rust uredospores contained a self-inhibitor, and that the suspected inhibitor could be released by washing the spores with water. Enough inhibitor was obtained by washing 25 mg of spores in one ml of water to inhibit germination 50% as determined by probit analysis ( $\text{ED}_{50} = 25$  mg spores/ml).

Partitioning of the water extract with ether, followed by germination tests of the ether extract, however, showed by probit analysis that enough inhibitor was actually obtained from 2 mg of spores to inhibit germination 50% ( $\text{ED}_{50} = 2$  mg spores). This suggested that the water extract may have contained a germination stimulator as well as the inhibitor and that this stimulator was eliminated during the partitioning process.

*Partial purification.*—Partial purification of the unidentified self-inhibitor was carried out by chromatography on thin-layer plates of silica gel developed in a solvent of benzene-ethyl ether (80:20, v/v). The  $R_f$  value found for the inhibitor was 0.3 as determined by germination assays of ether extracts of silica gel scraped from the plates. Insufficient compound was present to permit observation by ultraviolet light. The  $R_f$  values for methyl 3,4-dimethoxycinnamate and methyl ferulate determined simultaneously were 0.8 and 0.6, respectively. The unknown coffee rust inhibitor did not sublime in the short-path distillation apparatus used so successfully with the ester inhibitors of bean rust and wheat rust.

*Tests of known self-inhibitors.*—Equimolar mixtures of the *cis* and *trans* isomers of the inhibitors of bean rust and wheat stem rust, methyl 3,4-dimethoxycinnamate and methyl ferulate, respectively, were tested for capacity to inhibit germination of coffee rust uredospores. Neither compound was active. However, in a test of a wide range of organic acids and their methyl esters,

cinnamic acid (CA), and 3,4-dimethoxycinnamic acid (DM) were found to be slightly effective. CA had an ED<sub>50</sub> of 7 µg/ml, whereas DM had an ED<sub>50</sub> of 15 µg/ml. The significance of these values is not known because the content of the *cis* isomer in the acids is uncertain. Only the *cis*-isomers of the known self-inhibitors are active (11). However, since methylation of the acids rendered the compounds completely inactive, it is clear that these spores were responding to the acids and not their methyl esters.

The effect of pH on germination was tested using 0.002 M solutions of potassium phosphate buffer in the range from pH 6.3 to 7.9, and pH 6.8 provided the best germination. Solutions of cinnamic acid at pH 6.8 were then used, and the same inhibition was obtained as was found using cinnamic acid in water solution. The *R<sub>f</sub>* values of CA and DM on silica gel plates using the benzene-ether solvent were 0.3 and 0.2, respectively. These values suggest again that the unknown compound might be a free organic acid rather than a methyl ester as was encountered previously (11), but insufficient compound was present to permit use of common indicator sprays for organic acids.

Coffee rust uredospores clearly contain a self-inhibitor. It is extracted from the spores by washing with water using typical procedures (1, 2, 3, 9, 10, 11, 16), and is soluble in diethyl ether. In the single chromatographic system employed, the compound migrates with the free organic acids and not with the methyl esters.

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