Germination Self-Inhibitor from Cronartium comandrae Aeciospores

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ABSTRACT

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An unidentified compound associated with the self-inhibitory action of germinating aeciospores of *Cronartium comandrae* was extracted with 0.02 M ammonium bicarbonate. The inhibitor was partially purified by partition into chloroform and further purified by thin-layer chromatography. When added to suspensions of inhibitor-free germinating aeciospores, the purified inhibitor prevented germ tube emergence, but had no apparent effect when added immediately after visible germ tube emergence. Some of its physical and chemical properties are presented.

There are at least two known self-inhibitors of rust spore germination. Methyl 3,4-dimethoxy-*cis*-cinnamate is the inhibitor found in most uredospores (1, 3, 7). Methyl 3-methoxy 4-hydroxy-*cis*-cinnamate (methyl-*cis*-ferulate) has been identified as an inhibitor in *Puccinia graminis tritici* (2, 6, 8). Most research has been concerned with self-inhibitors of uredospore germination in the Pucciniaceae. Little is known of self-inhibitors of other spore forms in this family or in the related Melampsoraceae and Coleosporiaceae.

We extracted a germination self-inhibitor from aeciospores of *Cronartium comandrae* Pk. (Melampsoraceae) which was superficially similar in its mode of action to the cinnamic acid derivatives (2, 5) but which apparently was dissimilar chemically.

MATERIALS AND METHODS

Galls on shortleaf pine (*Pinus echinata* Mill.) bearing mature aecia of *C. comandrae* were collected in mid-April, 1974 and 1975, from areas of natural infection in Madison and Newton counties in Arkansas. The galls were brought into the laboratory, air-dried overnight, and spores were loosened and removed by giving each aecia-bearing gall a sharp rap against a counter top. Spores were passed through a 246-μm (60-mesh) sieve to remove debris and then air-dried for 2 days. Spores not used immediately were stored in liquid nitrogen in flame-sealed glass tubes. Immediately before use, spores were placed at 100% relative humidity for 24 hours at 20 C in the dark. To test for the presence of a self-inhibitor, spores at varying concentrations were germinated in the dark in 0.1% water agar (pH 6.9) in shake culture at 20 C. At appropriate time intervals, aliquants of the germinating spores were fixed in acid fuchsin-lactophenol stain (10) and examined under the light microscope to determine percentage germination.

To extract the germination self-inhibitor, 16-g batches of hydrated aeciospores were shaken for varying time intervals in 2 liters of 0.02 M NH_4HCO_3 (pH 7.8) at 20 C in the dark. The resulting pale orange extract was filtered through an 8-μm Millipore filter to remove the spores. The filtrate was concentrated at 25 C in a rotary evaporator to a final volume of 70 ml. Spectranalyzed acetone was added to give a final concentration of 90% (v/v), and the preparation was kept at 4 C for 24 hours. The white and brown precipitates that formed were removed by filtration through glass fiber papers. Most of the acetone was evaporated from the filtrate under a stream of nitrogen, and the remaining water layer then was placed in a vacuum desiccator overnight and taken almost to dryness to insure removal of most of the remaining acetone. The water layer then was extracted with portions of spectranalyzed chloroform until no more orange-colored material was extracted into the chloroform layer. The combined chloroform extracts were concentrated under a stream of nitrogen before chromatography.

Thin-layer chromatography (TLC) was performed on aluminum-backed silica gel 60/kieselguhr F-254 sheets (Brinkmann Instruments, Inc., 110 River Road, Des Plaines, IL 60016). The sheets were prechromatographed immediately before use in chloroform:acetone (1:1, v/v). The chloroform extract then was chromatographed in a saturated atmosphere of acetone:benzene:petroleum ether (b.p. 30-60 C) (1:2:3, v/v/v). The extract from 4 g of spores was chromatographed on each TLC sheet. The chromatograms were examined under visible and ultraviolet light, and then cut into approximately 1 cm^2 sections. The sections were eluted with 4 ml chloroform:acetone (1:1, v/v) by shaking for 30 minutes, and aliquants were dried and tested for inhibitory activity at a concentration equivalent to 40 mg original spores/ml. Spots containing inhibitory activity were

further purified by rechromatography, using the system described above.

To estimate time of action of the germination self-inhibitor, the inhibitor purified by TLC (spot A) was added at 10- to 15-minute intervals to suspensions of germinating aeciospores. Percent germination was determined immediately before addition of the inhibitor. Percent inhibition was determined after 4 hours' total germination time.

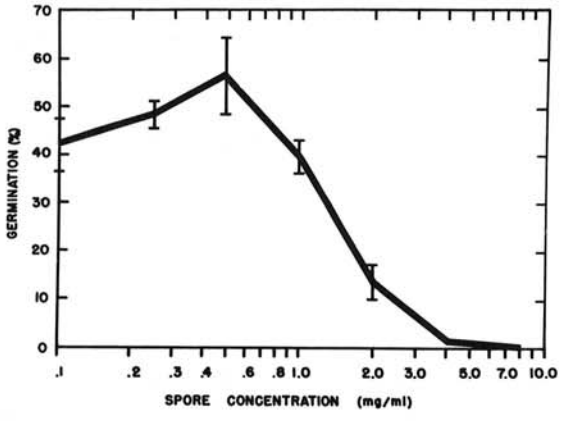

Fig. 1. Germination of *Cronartium comandrae* aeciospores as a function of spore concentration. Vertical bars indicate one standard deviation.

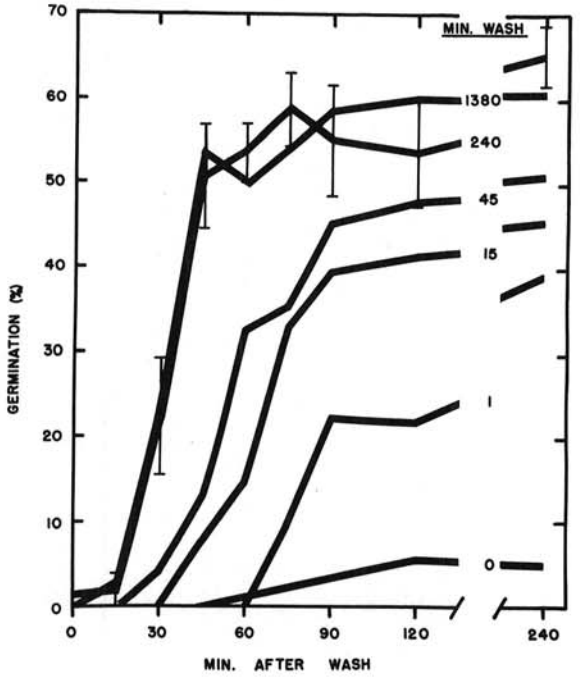

Fig. 2. Germination of *Cronartium comandrae* aeciospores following varying extraction times to remove the self-inhibitor. Vertical bars indicate one standard deviation. Comparable deviations were obtained for the remaining lines but were omitted for sake of clarity.

Gas chromatography was performed with a Varian Aerograph, Series 2800, gas chromatograph. A 3.66 m × 3.16 mm (12′ × 1/8″) column was packed with 8% DC-200 + 4% DEGS (Varian) and used at 120 C with a flow rate of 8 ml/minute. Samples were not derivatized.

Infrared (IR) spectra were determined with a Perkin-Elmer Model 337 grating infrared spectrometer. The spectra were taken of neat samples, using sodium chloride windows.

RESULTS

Germination of nonextracted *C. comandrae* aeciospores was achieved in liquid shake culture only after dilution of the germination self-inhibitor (Fig. 1). A spore concentration of 8 mg/ml was sufficient to inhibit germination completely. As the concentration was decreased, however, germination steadily increased until a dilution of 0.5 mg/ml was reached. Alternatively, germination was achieved by extracting the self-inhibitor and replacing it with fresh culture medium (Fig. 2). Extraction periods of 0-1,380 minutes were tested. A 240-minute extraction period was sufficient to achieve maximal germination. An extraction period of only 1 minute, however, was sufficient to give a significant increase in germination.

Thin-layer chromatography of the chloroform extract resulted in one main spot (spot A, yellow-colored, R_f 0.59) which contained most of the inhibitory activity, allowing only 3% germination. However, the spot that chromatographed immediately above spot A (spot B, blue-fluorescent, R_f 0.66-0.75), also contained some inhibitory activity, allowing 35-40% germination. The spots above R_f 0.8 and below 0.4 contained no inhibitory activity. Rechromatography of each of these two inhibitor spots appeared to result in some partitioning of inhibitory activity between the two spots. Perhaps the two compounds interconvert during purification or upon exposure to light (6, 7, 8).

The purified germination self-inhibitor caused inhibition of initial germ tube emergence (Fig. 3). However, by 10 minutes after the initiation of

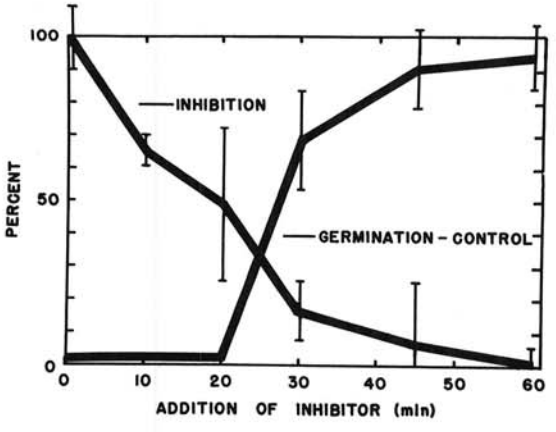

Fig. 3. Time of action of germination self-inhibitor extracted from *Cronartium comandrae* aeciospores. Vertical bars indicate one standard deviation.

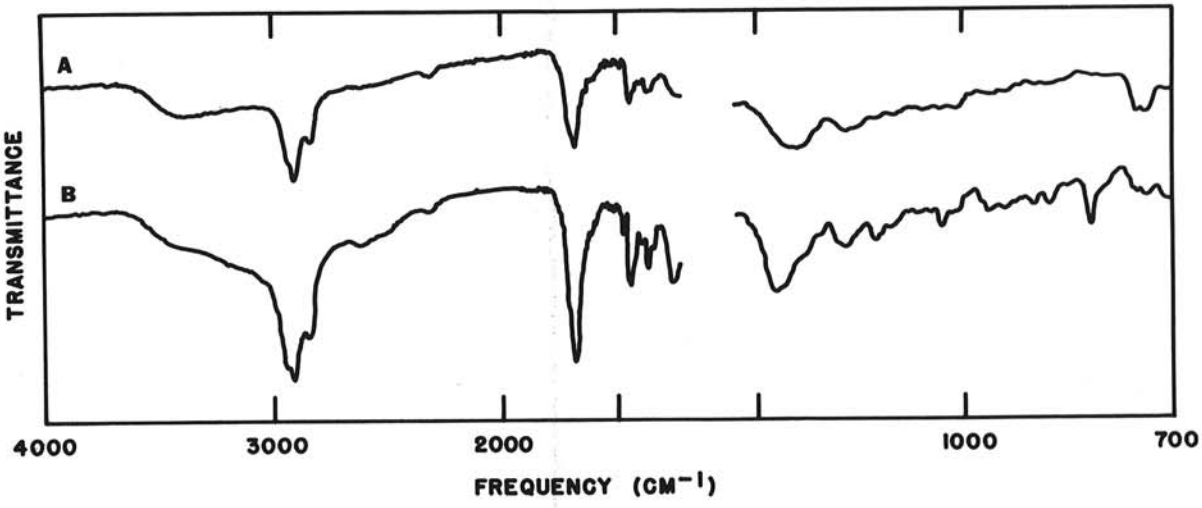

Fig. 4. Infrared spectra of self-inhibitor isolated from *Cronartium comandrae* aeciospores and purified by thin-layer chromatography (spots A and B).

germination (but before visible emergence of the germ tube), addition of the inhibitor resulted in only 65% inhibition compared with nearly 100% inhibition when the same inhibitor concentration was added at the start of germination. When the inhibitor was added after 20 minutes (still no visible germ tube emergence), only 49% inhibition was observed. Thus, it would appear that, within 10-15 minutes preceding visible germ tube emergence, the germinating spore already had progressed past the stage at which the self-inhibitor was effective. The self-inhibitor had no visible effect on rate of germ tube elongation or morphology.

Gas-chromatographic analyses of the active spots showed one main peak with a retention time of 14.5 minutes.

The IR spectra of the two active TLC spots were similar (Fig. 4) with strong peaks at 2,920, 2,850 (C-H stretch), 1,690 (C-O stretch), and 1,250 cm^{-1} (spot A), or 1,270 cm^{-1} (spot B) (C-O stretch); medium peaks at 1,440 (OH deformation), 1,370, and 1,175 cm^{-1}; and weak peaks at 2,630 and 2,330 and between 1,175 and 878 cm^{-1}. The spectrum of spot B had a very broad O-H stretch band (3,600-2,500 cm^{-1}), whereas that of spot A had a slightly narrower O-H stretch band (3,600-3,200 cm^{-1}). The spectra of the spots suggest a medium- or long-chain, saturated, nonaromatic carboxylic acid.

DISCUSSION

Cronartium comandrae must be added to the list of rust fungi which contain germination self-inhibitors. It appears that the self-inhibitor of *C. comandrae,* and perhaps that of *Cronartium fusiforme* Hedg. & Hunt (4), is structurally different from the cinnamic acid derivatives responsible for self-inhibition in *Puccinia* (6) and *Uromyces* (7). Although we did not test the effect of these derivatives against *C. comandrae,* certain of them were only slightly effective against uredospores of *Hemileia vastatrix* Berk. & Br., race 2 (9). With the coffee rust, available evidence indicates that the active compound may be a free organic acid (9). Possibly it is structurally related to the inhibitor of *C. comandrae.* Identification of these inhibitors and a comparison of their modes of action with that of fungi that utilize the cinnamic acid derivatives for self-inhibition (5) should prove invaluable in understanding the phylogeny of the rusts.

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