Identification of the Self-Inhibitor and Some Germination Characteristics of Peanut Rust Uredospores

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ABSTRACT

The native germination self-inhibitor of *Puccinia arachidis* uredospores has been identified as methyl *cis*-3,4-dimethoxycinnamate. Uredospores of the peanut rust fungus were more sensitive to the self-inhibitor than any other rust whose self-inhibitor has been identified (ED_{50} = 8 picograms per milliliter). During germination, peanut rust uredospores, as is typical of uredospores having the dimethoxycinnamate self-inhibitor, required collodion-paraffin oil membranes for induction of infection structures, while heat-shock treatments were ineffective. Freezing the spores in liquid nitrogen did not induce cold-dormancy, and a brief exposure to heat was unnecessary to initiate germination.

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Germination or rust uredospores begins with imbibition of water followed by protrusion of a germ tube. Eventually, sequential formation of infection structures is initiated beginning with the appressorium. Germ tube protrusion is controlled by endogenous germination inhibitors, whereas formation of the infection structures is triggered by contact of the germ tube with a stoma of the leaf (1).

Recently several aspects of the germination process have been clarified. The self-inhibitor of bean, sunflower, corn, and snapdragon rust uredospores was identified as methyl *cis*-3,4-dimethoxycinnamate, and that from wheat stem rust uredospores as methyl *cis*-4-hydroxy-3-methoxycinnamate (10, 11, 12). Furthermore, differentiation of the germ tube can be induced on collodion membranes, by heat shock, or by chemicals derived from the uredospores (1, 13). It has been shown that even in closely related species, differences can exist in the chemical identity of the native self-inhibitors, and in other germination characteristics such as cold-induced dormancy, response to differentiation stimuli, and response to germination stimulators (9). However, the germination process has been characterized in too few species of Pucciniaceae to determine whether any characteristic pattern of germination exists in the family.

Uredospores of peanut rust fungus were chosen for the present studies. Peanut rust is endemic to the Western Hemisphere and has been reported in many peanut growing regions (5). Since 1965 there has been an increase in the occurrence of this rust in the United States, causing serious economic losses to the peanut growers in the southern and south-central states (5, 7). Recently a widespread outbreak of this rust in the Eastern Hemisphere was recorded (K. R. Bromfield, *personal communication*). Yet the biology of this pathogen is poorly understood, and only limited information is available about the germination of the uredospores.

We have studied the germination of peanut rust uredospores in order to increase our knowledge about this important plant pathogen. For this purpose we have identified the germination self-inhibitor of peanut rust uredospores and studied its biological activity. As a necessary part of these studies we have elucidated the conditions conducive to differentiation of the germ tube into infection structures in order to provide a comparison of several parameters of germination of these spores with those for bean and wheat stem rust uredospores.

MATERIALS AND METHODS.—*Uredospore production*.—Initial uredospore inoculum of the peanut rust fungus (*Puccinia arachidis* Speg.) was obtained from Donald Smith, Texas A&M University Plant Disease Research Station, Yoakum, Texas. Uredospores were increased by spraying a spore suspension in water (1.0 mg/ml) containing 0.001% Tween 20 (polyoxyethylene

sorbitan monolaurate) on Spanish-type peanut plants (*Arachis hypogaea,* L. 'Starr'). The inoculated plants were grown at 20 C in controlled environment chambers on a 16-h day. After 10 to 12 days the uredospores were collected from infected plants by shaking them over enamel pans. The dry spores were sieved through a 0.149 mm (100-mesh) screen and stored in stoppered glass vials at −5 C. No significant loss in viability was noted in spores stored in this manner over a 3-mo period.

Uredospores of bean rust fungus [*Uromyces phaseoli* (Pers.) Wint.] and wheat stem rust fungus (*Puccinia graminis tritici* Eriks. & Henn., race 56) were obtained from stock supplies (12).

Purification of self-inhibitor.—Each step in the purification process was monitored by a germination bioassay. Two to five mg of freshly collected spores were washed 10 min in 200 ml of water and used for the assay immediately in cylindrical glass microdishes (12).

The self-inhibitor from uredospores of the peanut rust fungus was extracted and purified essentially as described for the bean rust fungus (11). Uredospores were extracted three times with water. The water extracts were repeatedly partitioned with peroxide-free diethyl ether, and the ether fractions were spotted onto preparative silica gel thin-layer plates. To prevent *cis-trans* isomerization, the extraction and purification procedures and subsequent germination bioassays were carried out in darkness with occasional use of a Kodak Wratten No. 1 red safelight (Eastman Kodak, Rochester, N.Y.) when necessary.

Synthesis of inhibitory compounds.—The *cis*- and *trans*-isomers of methyl 4-hydroxy-3-methoxycinnamate (methyl ferulate) and methyl 3,4-dimethoxycinnamate were synthesized and quantified as previously described (12). Purity of the isomers was checked by thin-layer chromatography before each assay.

Analytical techniques.—Preparative gas chromatography was performed on a Hewlett Packard model 5700 A with a 1.5 m×6.3 mm (o.d.) copper column of 1% FFAP on chromosorb W (Varian Aerograph Co.,

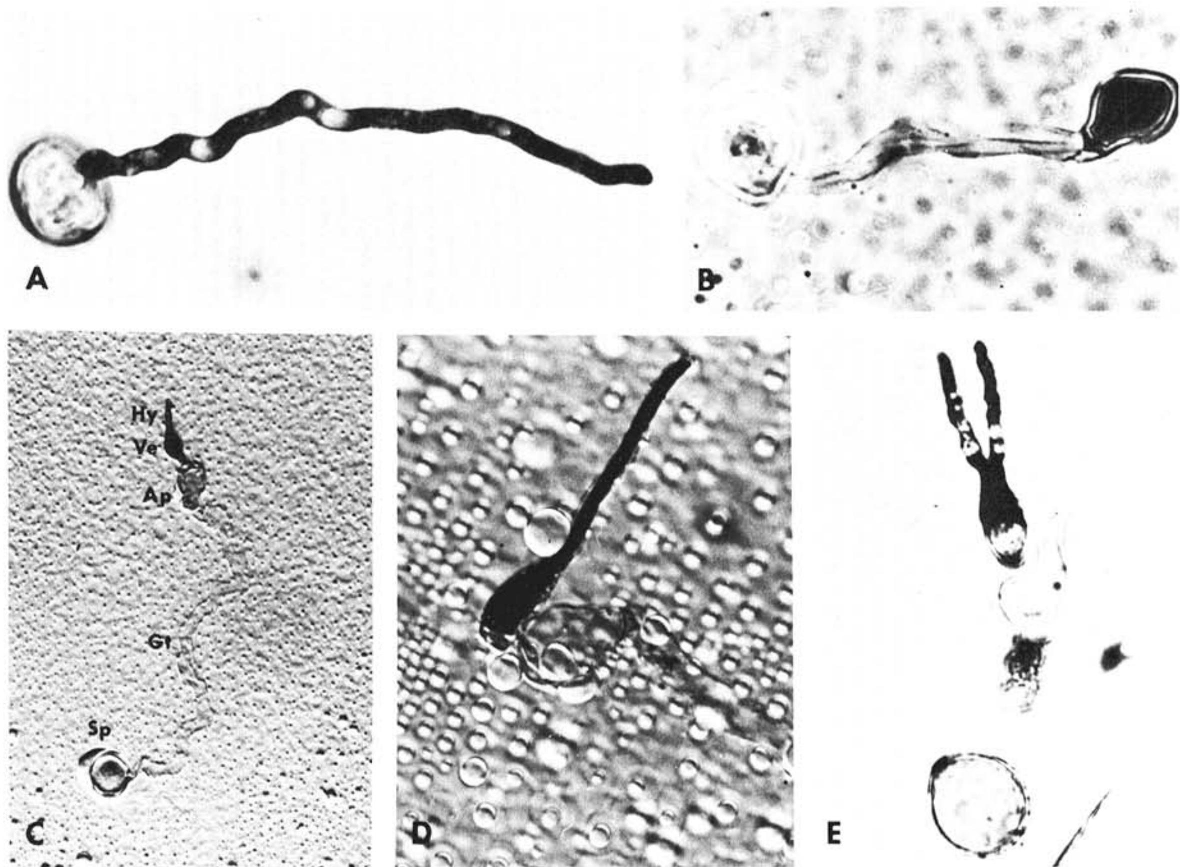

Fig. 1-(A-E). A comparison of the germination of uredospores of *Puccinia arachidis* and *Uromyces phaseoli* on collodion-oil membranes. **A)** *P. arachidis.* Oil omitted from membrane and germ tube has not differentiated. **B)** *P. arachidis.* Membrane contains 0.125 μl paraffin oil/cm^2 which shows as a mottled background; only the appressorium has developed with this concn of oil. **C)** *P. arachidis.* Membrane contains 0.375 μl paraffin oil/cm^2, and germ tube has differentiated completely as shown by Nomarsky optics. **D)** *P. arachidis.* Membrane contains 0.375 μl paraffin oil/cm^2. The portion of germ tube shown has the infection structures with an elongated hypha. **E)** *U. phaseoli.* The membrane contains only 0.06 μl paraffin oil/cm^2 yet there is complete differentiation. (Sp = spore; Gt = germ tube; Ap = appressorium; Ve = vesicle; Hy = hypha). The diam of the uredospores in A, B, C, and E is approximately 26 μ.

Walnut Creek, Calif.) at 190 C. Analytical gas chromatography was carried out using a Varian Aerograph model 1200 with a 1.5 m × 3 mm (o.d.) stainless steel column of 1% FFAP at 190 C.

Mass spectra of the natural and synthetic inhibitors were obtained using a Varian Aerograph model 200 gas chromatograph with the 1.5 m × 3 mm (o.d.) stainless steel column of 1% FFAP connected through a jet separator to a Hitachi-Perkin Elmer model RMU-6E mass spectrometer.

Differentiation of uredospores.—Differentiation experiments were performed using collodion membranes and heat-shock treatment. Collodion membranes containing paraffin oil were atomized with spores, sprayed with double-distilled water, then incubated in the dark at 18 C for 24 h (13). The spores were fixed and stained for light microscopical examination with 0.02% trypan blue in lactophenol.

Spores to be heat-shocked for the induction of differentiation were germinated on water at 18 C for the period of time required to produce germ tubes of the length two to four times the diam of the spore (approx. 2 h). Spores were then transferred to a 30 C incubator for 120 min and then returned to 18 C for 12 h (13).

Cold-induced dormancy.—Uredospores in 5-ml glass vials were stored in liquid nitrogen for 10 days to test for cold-induced dormancy (4, 8).

RESULTS.—*Identification of the self-inhibitor.*—Over 99% of the inhibitory activity of the crude extract was recovered from a single band with a chromatographic mobility (R_f) of 0.6 on thin-layer plates. Gas chromatography of the active zone from the plate showed only one peak (retention time, 4.5 min). The mass spectrum of this peak showed a parent peak P (mass to charge, m/e) of 222 (base peak) and diagnostic peaks at 207 (P-CH_3), 191 (P-OCH_3), and 163 (P-$COOCH_3$). The total spectrum, as well as R_f and retention time, was identical with that of synthetic methyl *cis*-3,4-dimethoxycinnamate.

Experiments on the activity of the self-inhibitor.—A crude extract of the inhibitor from peanut rust uredospores prepared by extracting the spores with water and partitioning into ether was tested for its potential to inhibit germination of peanut, bean, and wheat stem rust uredospores. The potency of the inhibitor was expressed in spore weight equivalents (2).

The microgram equivalents (micrograms of rust spores required to yield sufficient self-inhibitor in 1 ml test solution to give the ED_{50} value in germination bioassay) for peanut, bean, and wheat stem rust were 2.5 to 5.0 μg, 500 to 1,000 μg, and 2,000 plus μg, respectively, when each rust was assayed with peanut rust self-inhibitor. The value for bean rust (500 to 1,000 μg) was the same as that obtained when bean rust spores were assayed with its own self-inhibitor, in agreement with the fact that the self-inhibitors of bean and peanut rust are identical.

The ED_{50} for synthetic methyl *cis*-3,4-dimethoxycinnamate was 8 pg/ml. This value is considered reliable since ED_{50} values for methyl *cis*-ferulate and methyl *cis*-3,4-dimethoxycinnamate tested against bean and wheat stem rust compared favorably with their previously determined ED_{50} values (12). The comparison of the microgram equivalents of bean and peanut rusts with the relative biological activity of synthetic methyl *cis*-3,4-dimethoxycinnamate indicated that both rusts contain approximately the same quantity of extractable self-inhibitor (1 to 5 ng/mg spores). Apparently peanut rust is about 300 times more sensitive than bean rust to its native self-inhibitor.

Germination and differentiation of peanut rust.—Uredospores of both peanut and bean rust germinated and produced nondifferentiated germ tubes on collodion membranes without paraffin oil (Fig. 1-A). However, only 6% of the germinated peanut rust spores formed complete infection structures on collodion membranes with 0.125 μl paraffin oil/cm^2, while 63% of the germinated spores formed appressoria only, and did not differentiate further (Fig. 1-B). By increasing the amount of paraffin oil in the collodion membrane to 0.375 μl/cm^2, 36% of peanut rust spores differentiated completely (Fig. 1-C, D). This increase is attributed to the completion of infection structures by spores which previously had formed only appressoria. On collodion membranes containing 0.06 μl paraffin oil/cm^2, 95% of the germ tubes of bean rust uredospores were differentiated (Fig. 1-E). Above 0.250 μl paraffin oil/cm^2 the percent of bean rust spores that differentiated decreased, although germination was decreased only slightly. While the initial differentiation stimulus of peanut rust and bean rust is similar, peanut rust uredospores require more than five times the oil to induce the appressorium.

To test the response of peanut rust uredospores to differentiation by heat-shock, germinated spores were exposed to 30 C for 2 h as described in Materials and Methods. Unlike wheat stem rust uredospores (13), peanut rust uredospores failed to form differentiation structures under these conditions, nor did uredospores of peanut rust exhibit cold-induced dormancy or any loss of viability after storage in liquid nitrogen. Bean rust uredospores also failed to become dormant when frozen in liquid nitrogen (4). In contrast, wheat stem rust spores had to be heat-shocked at 40 C for 3-5 min to obtain normal germination following cryogenic storage (4, 8).

DISCUSSION.—The addition of the peanut rust fungus to the list of spores which have methyl *cis*-3,4-dimethoxycinnamate as a self-inhibitor emphasizes the potential biological and physiological significance of this compound. Presently there are five rusts in the family Pucciniaceae which have methyl *cis*-3,4-dimethoxycinnamate as their natural self-inhibitor: *U. phaseoli*, *P. helianthi*, *P. antirrhini*, *P. sorghi*, and *P. arachidis*. Only *P. graminis tritici* has methyl *cis*-ferulate as its native self-inhibitor (12). Maheshwari et al. (13) observed that wheat stem rust would differentiate following heat-shock but would not differentiate on collodion-oil membranes, whereas the reverse was true for bean, sunflower, and snapdragon rust. Recently we found that corn rust spores also differentiate on collodion-oil membranes (Macko, *unpublished*).

Information on self-inhibitors together with other physiological characteristics of uredospore germination may be useful in the phylogenetic classification of rust fungi. Presently peanut and bean rust fungi, which share the physiological characteristics of membrane differentiation, are classified in different genera based

upon the number of cells in the teliospore (3, 6). In contrast, wheat stem rust which differentiates by heat-shock and becomes dormant in the cold, is classified in the same genus as peanut rust which cannot respond to either stimulus. It appears now as if the rust fungi that have methyl *cis*-3,4-dimethoxycinnamate as self-inhibitor are closely related to each other and more distantly related to the rust fungi which contain other self-inhibitors. As the germination of more species of rust fungi is characterized, it will be possible to state this more clearly and with more certainty (9).

Methyl *cis*-3,4-dimethoxycinnamate offers the opportunity to examine critically the primary physiological and biochemical events in the initiation of germination in rust uredospores. The native self-inhibitor apparently inhibits one of the initial steps in the germination process, for once germination has started the self-inhibitor is no longer effective. Until germ tube protrusion begins, however, inhibition by the chemical is fully reversible (1, 9).

LITERATURE CITED

1. ALLEN, P. J., and L. D. DUNKLE. 1971. Natural activators and inhibitors of spore germination. Pages 23-58 *in* S. Akai and S. Ouchi, eds. Morphological and biochemical events in plant-parasite interaction. The Phytopathological Society of Japan, Tokyo. 415 p.
2. ALLEN, P. J., R. N. STRANGE, and M. A. ELNAGHY. 1971. Properties of germination inhibitors from stem rust uredospores. Phytopathology 61:1382-1389.
3. ARTHUR, J. C. 1929. The Plant Rusts. John Wiley & Son, Inc., New York. 446 p.
4. BROMFIELD, K. R. 1964. Cold-induced dormancy and its reversal in uredospores of Puccinia graminis var. tritici. Phytopathology 54:68-74.
5. BROMFIELD, K. R. 1971. Peanut rust: a review of literature. J. Am. Peanut Res. Educ. Assoc. 3:111-121.
6. CUMMINS, G. B. 1959. Illustrated genera of rust fungi. Burgess Publishing Co., Minneapolis. 129 p.
7. HARRISON, A. L. 1973. Control of peanut leaf rust alone or in combination with Cercospora leaf spot. Phytopathology 63:668-673.
8. LOEGERING, W. Q., and D. L. HARMON. 1962. Effect of thawing temperature on urediospores of Puccinia graminis f. sp. tritici frozen in liquid nitrogen. Plant Dis. Rep. 46:299-302.
9. MACKO, V., and R. C. STAPLES. 1973. Regulation of uredospore germination and germ tube development. Bull. Torrey Bot. Club 100: 223-229.
10. MACKO, V., R. C. STAPLES, P. J. ALLEN, and J. A. A. RENWICK. 1971. Identification of the germination self-inhibitor from wheat stem rust uredospores. Science 173:835-836.
11. MACKO, V., R. C. STAPLES, H. GERSHON, and J. A. A. RENWICK. 1970. Self-inhibitor of bean rust uredospores: Methyl 3,4-dimethoxycinnamate. Science 170:539-540.
12. MACKO, V., R. C. STAPLES, J. A. A. RENWICK, and J. PIRONE. 1972. Germination self-inhibitors of rust uredospores. Physiol. Plant Pathol. 2:347-355.
13. MAHESHWARI, R., P. J. ALLEN, and A. C. HILDEBRANDT. 1967. Physical and chemical factors controlling the development of infection structures from urediospore germ tubes of rust fungi. Phytopathology 57:855-862.