Purification and Partial Characterization of a Factor in Cotton Wax Stimulating the Germination of Self-Inhibited Wheat Stem Rust Uredospores

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Summary. Filter paper, nonabsorbent cotton, and cotton wax were found to be progressively richer sources of germination-stimulatory activity effective in counteracting the self-inhibition of Puccinia graminis var. tritici Erikss. and E. Henn uredospores. The major stimulatory component of cotton wax was purified and partially characterized. It was catalytically effective in stimulating germination and oxygen consumption of uredospores and appeared to be as active as pelargonialdehyde. Unlike most of the previously reported chemical stimulants, however, it was not active across an air gap.

Although the active compound was not identified, both the ultraviolet spectrum and the nonionic and solubility properties of the active fractions were consistent with the infrared spectrum which indicated a relatively long-chained, \( \alpha, \beta \) unsaturated carbonyl compound such as a ketone or possibly an ester.

The purification procedure involved deionization of ethanolic extracts from cotton or cotton wax on Dowex 50 (H\(^+\)) and Dowex 1 (OH\(^-\)) columns followed by chromatography on neutral alumina using ethylene dichloride as the developing and eluting solvent.

The wheat stem rust fungus is an obligate parasite the growth of whose mycelium is inseparably associated with the tissues of its hosts. Physiological investigations of this important pathogen are therefore restricted to studies with the readily available uredospores. Such studies have been seriously restricted however, not only with this but also with other rust fungi, because potent self-inhibitors normally prevent the rapid germination of uredospores en masse (1, 7, 15, 16, 17, 23, 24).

Numerous diverse compounds (1, 2, 6, 7, 8, 10, 11, 21), as well as prefloating techniques (1, 22), have been used with varying success to induce the germination of self-inhibited wheat stem rust uredospores. To date, catalytically effective chemical stimulants, notably pelargonialdehyde (6, 8, 11) and other terpenoid compounds (8) have proved the most effective in inducing, immediately, the desired rapid and synchronous germination. Certain fatty acids, while active at substrate concentrations, are not satisfactory for studying the normal metabolism of germinating spores because they serve as exogenous substrates (6, 18, 19).

The present investigation stemmed from the observation (P. J. Allen, unpublished) that only stimulatory activity could be recovered from developed paper chromatograms of crude preparations of the germination self-inhibitor (2). The finding that chromatography paper contained a potent germination stimulant led in turn to the discovery that cotton, from which Whatman paper is manufactured (4), and cotton wax are progressively richer sources of this or a similarly active compound. The purification, partial characterization, and biological activity of the major stimulatory component of cotton wax is reported here.

A preliminary account of these studies was presented to the American Society of Plant Physiologists (3).

Materials and Methods

Production and Storage of Spores. Uredospores produced on greenhouse-grown Atlas 46 barley (Hordeum vulgare L.) and originating from a single-pustule isolate of Race 56, Puccinia graminis var. tritici Erikss. and E. Henn. were used in assaying for germination-stimulating activity. Daily collections of spores were placed in gelatine capsules and stored as reported previously (1). Before use the spore collections were bulked and freed of debris by passing them through a 200-mesh sieve.

All spores used were stored for at least 2 or 3 weeks before use and exhibited complete self-inhibition (less than 1% germination when tested en masse). However, in no instance were spore lots used that showed less than 80% germination in trace quantities.
Spore Germination Procedures. Germination tests were routinely carried out on 0.5 ml of buffered solution in the center well of Pyrex micro-Conway vessels (2) covered with petroleum-sealed glass lids. All tests were carried out on 0.01 M potassium phosphate buffer (pH 6.2) containing 10^{-4} M CaCl_2 to counteract the inhibitory effect of potassium ions (2).

When testing for stimulatory activity, spores were dispersed over the entire surface of the test solution with an inoculating loop and a uniform en masse load obtained by gently blowing excess spores off the surface film. For testing the germinability of spores only trace quantities were utilized. Trace amounts were conveniently obtained by forming a uniform film of spores on the surface of a small beaker of water and then transferring an inoculating loopful to each test vessel.

Bioassay. The stimulatory activity of cotton and cotton wax extracts was followed through the purification procedure by testing germination for the counteraction of germination self-inhibition by spores en masse. Accurately measured volumes (500 µl or less) of the various fractions were dispensed into the center well of the test vessels and the solvent evaporated in a vacuum desiccator. One-half ml of buffer was then added to the resulting residue and the solution was covered with spores. After a 100-minute incubation in the dark at 18°C, inoculating loopfuls of spores were spread on glass slides in a drop of dilute detergent and percent germination estimated under the low-power field of a microscope.

At each step in the purification procedure, the active fractions were tested at various dilutions and the germination results recorded. For purposes of estimating the degree of purification achieved and the percentage of the original activity recovered in the isolation procedure, a unit of activity was taken as that amount of material required per ml of buffer to induce 50% germination in the standard bioassay. Estimates of percent germination are probably more accurate at this than at higher or lower levels.

Preparation of Active Extracts. Initially, stimulatory preparations were obtained by exhaustively extracting nonabsorbent plugging cotton (Rock River Cotton Company, Janesville, Wisconsin) by repeated steepings of several hours each in 95% ethanol at room temperature. Because of the bulkiness of cotton this method of extraction yielded large volumes of extract that had to be concentrated before attempting isolation of the active component(s).

The bulked crude extracts were concentrated on a rotary flash evaporator at about 30°C until appreciable quantities of solids accumulated in the flask. These inactive solids were removed after allowing precipitation to continue in the cold (6°C). The supernate was then further concentrated on the evaporator. This process of evaporation and precipitation was repeated several times. Because the solids were at first flocculent and difficult to remove by filtration, the supernate was initially siphoned off. Later the solids precipitated well and were easily removed on a Buchner funnel. All but traces of stimulatory activity were removed from the solids by washing them on the filter several times with cold ethanol. Proceeding in this manner ethanolic extracts of cotton were concentrated to the equivalent of 3 to 5 g of cotton per ml of solution. These concentrated, golden-yellow extracts were used in preliminary attempts to develop an isolation procedure.

Later, a small sample of cotton wax obtained from the Southern Utilization Research and Development Division, U.S.D.A., was used as a more concentrated and convenient source of active material. The wax sample we used had been prepared from the fraction remaining in solution at room temperature after hot alcoholic extraction of cotton and constituted about 57% of the total extracted solids. One to 2 g of this wax were exhaustively extracted with 6 successive 50-ml aliquots of ethanol and samples of the bulked extracts subjected to the isolation procedure without further processing. About 75% of the wax went into solution.

Additional methods are given later.

Results

Numerous preliminary experiments led to the development of a 2-phase isolation procedure involving deionization of the crude cotton or cotton wax extracts followed by chromatography of the deionized sample on neutral alumina. Figure 1 is a schematic
outline of the steps involved in this purification procedure. The quantities shown are representative of those obtained in processing the cotton wax sample.

**Preliminary Purification by Deionization.** Deionization was accomplished by passing the extract through columns of Dowex 50-X2 (H+) and then Dowex 1-X2 (OH-) ion exchange resins (50-100 mesh) from which the water had first been displaced by 95% ethanol. Application of the sample to the cationic column was followed by additions of 95% ethanol and effluent was collected until it no longer showed an acid reaction. This effluent was concentrated on a rotary evaporator to the volume of the original sample and then passed through the anionic column, again followed by ethanol. To ensure that no activity was discarded, effluent equal to twice the volume of the applied sample was collected.

Preliminary experiments had shown that up to three-quarters of the dissolved solids in concentrated ethanolic extracts from cotton could be removed by deionization without apparent loss of germination-stimulating activity. The cotton wax extracts behaved similarly except that only about 55% of the dissolved solids were removed (fig 1). Visual evidence of the purification effected was provided by the appearance of variously colored adsorption bands near the top of each column and the virtually complete decolorization of the golden-yellow extracts by Dowex 1 resin.

**Chromatography on Alumina.** In preparation for chromatography on alumina, the deionized extract was reduced to dryness at about 34° on a rotary evaporator and the residue extracted several times with petroleum ether. This solvent had first been extracted with water, dried with calcium chloride, and redistilled (B.R. 40-60°). Although some residue remained undissolved (fig 1), bioassays showed that it did not contain any germination-stimulating activity.

The petroleum-ether solution was applied in successive small aliquots to the top of a column dry-packed with neutral alumina (BioRad, 100-200 mesh). When the last of the sample had been added the chromatogram was developed with ethylene dichloride, which previously had been extracted with water, dried with calcium chloride, and redistilled.

Bioassay of successive 5-ml fractions of the alumina column eluate revealed several activity peaks. However, most of the germination-stimulating activity was associated with a group of fractions having a single major absorption peak at 285 mp (fig 2).

**Purification and Recovery of Activity.** Bioassay results, considered in terms of activity units as defined earlier, provide a basis for roughly estimating the degree of purification achieved and the amount of activity recovered in the purification procedure. The data presented in table 1 indicate that deionization of the original cotton wax extract gave a 4-fold increase in specific activity with almost a doubling of total activity. This apparent gain in total activity was presumably due to the removal of inhibitory components in the crude extract. The further increase in specific activity achieved in chromatographing the deionized extract represents a 28-fold purification over the starting material. Based on the activity shown by the partially purified deionized extract, only 3.3% of the total activity was recovered in fractions 11 and 12. However, estimates of the recovery achieved in this procedure are complicated not only by the apparent presence of inhibitory as well as stimulatory activity but also by the demonstrated occurrence of more than one stimulatory component in the original extract.

**Purity of Isolated Fractions.** The small quantity of material isolated in fractions 11 and 12 (fig 1) precluded comprehensive purity tests. However, ultraviolet absorption spectra of each of the fractions

![Graph](image-url)
collected from the alumina column were determined and these data provide some basis for judging the relative purity of fractions 11 and 12. The ultraviolet absorption curves were obtained with a Beckman Model DU spectrophotometer after first removing the eluting solvent under reduced pressure and replacing it with redistilled 95% ethanol.

The absorption curves illustrated in figure 2 show that fractions 11 and 12 are both characterized by a single major absorption peak at 285 m\(\mu\) and a minimum at 245 m\(\mu\). Another less well-defined peak occurs at about 230 m\(\mu\). Active fractions separated during preliminary trials using extracts from cotton also showed these ultraviolet absorption characteristics.

OD at 285 m\(\mu\) and the spectrophotometric ratio (OD 285 m\(\mu\)/OD 245 m\(\mu\)) were used to plot the alumina column elution pattern (fig 3). The closeness of the spectrophotometric ratios, 2.0 and 2.2 for fractions 11 and 12 respectively, indicates the similarity of these fractions and suggests that they contain primarily one component. They were therefore used to determine the infrared absorption spectrum.

**Infrared Spectrum.** A Baird-Atomic Inc. Model No. 4-55 spectrophotometer was used to determine the infrared spectrum (fig 4).

The well-defined absorption peak at 5.8 \(\mu\), when considered together with the peak at 6 \(\mu\) and the shoulder just above 3.4 \(\mu\) strongly suggests an \(\alpha, \beta\) unsaturated ketone or possibly an ester. Apart from this functional group, the relatively strong C-I1 stretching peak just below 3.4 \(\mu\) indicates that the compound consists essentially of a long saturated chain.

**Stimulation of Germination.** The impressive germination-stimulating activity of the cotton factor was evident from the ease with which it could be followed through the purification procedure by bioassay. Factor-induced germination of wheat stem rust uredospores was so immediate and synchronous and the resultant germ tube growth so rapid that short (100 min) incubation periods were required to avoid mycelial-like entanglement of the spores.

Because most of the purified sample was used in an unsuccessful chemical characterization study, the concentration-activity relationships of this material are not accurately known. The best available data indicate that the purified cotton factor was fully effective at concentrations of approximately 25 mg per liter. At least with fresh spores, considerable activity was apparent at concentrations one-tenth of this (2-3 mg per liter). The results presented in figure 5 demonstrate that the substance is more effective than 2,4-dinitrophenol or coumarin and has maximal effectiveness over a wide range of concentrations.

**Volatility.** Cotton factor added to the annulus of micro-Conway vessels was ineffective in inducing the germination of self-inhibited spores floated on buffer in the inner compartment.
stimulator was not effective across an air gap. The compound would undoubtedly have been lost if it did possess appreciable volatility since organic solvents were removed under reduced pressure at various stages in the purification procedure and in preparation for all bioassays.

Our results (figs 5, 6) agree with Farkas and Ledingham’s (6) finding that the more effectively a chemical stimulant counteracts the germination self-inhibition of uredospores, the more effectively it stimulates their respiration. Thus, like pelargonaldehyde (6), the cotton factor is considerably more effective than coumarin and DXP in increasing both the germination and the QO₂ of self-inhibited wheat stem rust uredospores. Although the absolute QO₂ values induced by the cotton stimulant were lower than those reported for pelargonaldehyde, a lower level of activity is not necessarily indicated. In this study stored spores were used whereas Farkas and Ledingham (6) used freshly harvested spores, which have a higher respiratory rate. When compared on the basis of the respective QO₂ levels obtainable with coumarin, the respiration rates induced by the cotton factor and pelargonaldehyde appear to be of the same order of magnitude.

Because of the variety of compounds that he found to be stimulatory, French concluded that the acceleration of germination of uredospores is non-specific as far as chemical reactivity is concerned (8). This view seems inconsistent with his suggestion that the stimulators could act by functioning as substrates for specific enzyme systems. Indeed, the fact that coumarin, the cotton factor, and most of the stimulatory compounds reported by French possess a carbonyl group suggests the importance of chemical specificity.

Subsequent reports (9, 20) have demonstrated that wheat stem rust uredospores can convert certain exogenously supplied aldehydes to the corresponding alcohols. French (9) has speculated that the relatively nonspecific dehydrogenases responsible for this conversion normally prevent the accumulation of effective levels of the endogenously produced pelargonaldehyde. He also suggests that exogenously supplied chemical stimulants are effective because the concentrations supplied are normally great enough to inhibit the aldehyde-destroying enzymes. One could also speculate that a variety of compounds are effective because they are first transformed by the spores into compounds with the specific configuration necessary for stimulatory activity. Indeed, it has recently been reported that the nongerminating or dormant spores of many filamentous fungi can carry out various types of oxidative transformations (5, 12, 13, 14).

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Literature Cited