Endogenous Germination Inhibitors in Teliospores of the Wheat Bunt Fungi

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ABSTRACT

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Teliospores of *Tilletia* spp. contain endogenous germination inhibitors. There was a decrease in percent germination as the spore population increased. Germination inhibitors were extracted from teliospores with warm methanol. The inhibitor complex from dwarf bunt teliospores was partially purified by adsorption chromatography on silicic acid. Six or more inhibitory substances were detected. After one of the inhibitors was purified and crystallized from cold hexane, it was found to have a melting point of 51 C and a maximum absorbance at 218 nm. The ED_{50} of the purified inhibitor was 7 μg/ml. *Tilletia* teliospores contain about 2 to 4 mg trimethylamine/g spores, but this was not enough to account for the observed self inhibition.

Additional key words: common bunt, *Tilletia caries*, dwarf bunt, *Tilletia controversa*, trimethylamine, chromatography.

Wheat bunt is one of the most destructive of all wheat diseases in the northwestern United States. The contamination with bunt spores of wheat shipments recently caused international concern and the halting of all wheat shipments from the U.S. Pacific Coast states to mainland China. Common bunt of wheat, *Tilletia caries* (DC.) Tul., has been effectively controlled in the United States by the combined use of resistant wheat cultivars and a seed-treatment fungicide, hexachlorobenzene. There is concern, however, that the use of hexachlorobenzene on wheat may soon be prohibited. In contrast, dwarf bunt of wheat, *Tilletia controversa* Kühn, is not controlled by any of the presently registered seed treatment chemicals (5). Consequently, new virulent races of *T. controversa* pose serious threats to the production and marketing of wheat in the northwestern United States (5) and more specific and effective controls for the bunt diseases of wheat are needed.

Knowledge of factors affecting teliospore germination is important in any study of the early interaction between the bunt pathogen and the wheat plant. Teliospores of the common bunt fungus, *T. caries*, require 3 to 5 days for germination, whereas teliospores of the dwarf bunt fungus, *T. controversa*, require up to 3 mo under optimal conditions of moisture, oxygen, light, and temperature. It has not been possible to enhance germination of these spores either by the addition of exogenous nutrients or the germination stimulants which are effective on some dormant bacterial and fungal spores (15). Germination of fungal spores may be controlled by endogenous inhibitors that must be removed or counteracted before germination can proceed (9). There are several reports of endogenous inhibitors associated with bunt teliospores. Unidentified water-soluble inhibitors of germination were detected in dwarf bunt teliospores but not in those of common bunt teliospores (8), and trimethylamine was reported to be an endogenous germination inhibitor of the latter (1). The purpose of the research reported here was to extract and purify the germination inhibitors found in teliospores of the wheat bunt fungi.

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MATERIALS AND METHODS

Teliospores of T-races of the common bunt fungus were obtained from infected wheat plants in the experimental field plots of R. J. Metzger, geneticist, U.S. Department of Agriculture (USDA), Corvallis, OR. Teliospores of D-races of the dwarf bunt fungus were obtained from J. A. Hoffmann, plant pathologist, USDA, Logan, UT. Spores were shaken from the broken sori, air-dried, and passed through a series of standard sieves to remove host-plant residues. Unless described differently, the agar media, incubation temperature, and method of counting germinated spores were as explained earlier (15).

For moisture determinations, 1-g quantities of teliospores were hydrated in aqueous solutions containing 2 mg penicillin G, 2 mg streptomycin sulfate, and 0.6 mg albamycin per 20 ml water in 50-ml Erlenmeyer flasks. The flasks were placed on a rotary shaker at 22 C. At designated times the spore suspensions were vacuum-filtered through tared filter disks (8-μm pore size). The spores were air-dried in vacuum for 30 min to remove the surface moisture and then weighed (wet weight); then they were dried in a vacuum oven at 80 C for

24 hr and the dry weight was determined. The diameters of 100 teliospores of each sample were measured to the nearest 0.5 μm, and these values were used to calculate the average volume changes during hydration.

For extraction of trimethylamine from teliospores, the spores were broken by placing 0.5 g of spores, 6.5 ml of 2-methoxyethanol, 50 μliters 12 N HCl, and 15 g of glass beads (4-mm diam) in a small closed glass vial and shaking it vigorously for 16 hr at 4 C. This treatment ruptured all of the spores. The resulting mixture of spore debris then was extracted with 50 ml 2-methoxyethanol by stirring for 4 hr. The solvent was removed by centrifugation and the residue was extracted as before with 50 ml of 2-methoxyethanol. These extracts were combined, 125 mg $Ba(OH)_2$ was added, and the solution was stirred and heated gently until trimethylamine distilled over into a cold trap. The amount of trimethylamine in the distillate was determined by the method of Ruch and Critchfield (14). The recovery of trimethylamine by this procedure ranged from 93 to 100% (average = 95.6%).

The effects of pH and trimethylamine concentrations on the percent germination of teliospores of *T. caries*, race T-5, were studied. Trimethylamine hydrochloride crystals were weighed, dissolved in water, and added to a benzenepentacarboxylic acid buffer (10) at the desired pH. These buffered trimethylamine solutions were filter-sterilized and mixed thoroughly with warm sterile agar to achieve a 3% agar preparation containing the buffer at 3 mM and trimethylamine at 1, 10, 100, or 1,000 μM concentration. Teliospores were seeded on the surface of the agar at approximately 20 spores per square millimeter. The seeded plates were incubated at 17 C for 84 hr and the percent germination was determined microscopically.

Germination inhibitors were isolated from dwarf bunt teliospores, race D-2, by refluxing 250 g of teliospores (2% moisture) in 2 liters of absolute methanol. The spores were removed by filtration and re-extracted, as above, four more times. The filtrates were combined and vacuum evaporated to 100 ml. One-half of this total extract was placed on a 50 × 1,000-mm column containing Cellex-Mx (microcrystalline cellulose). Solutes were eluted with ethanol:water (95:5, v/v) at a flow rate of 1 ml/min, and collected in 160 test tubes (20 ml per tube). Alternate fractions were bioassayed for inhibitor activity as described below. The fractions containing the germination inhibitors were combined, concentrated in vacuum, and further purified by passage through a 50 × 800 mm silicic acid (Bio Sil A) column.

This column was eluted with a step-wise gradient starting with benzene and progressing through a series of solvents of increasing polarity including benzene, ether, acetone, and methanol (Fig. 2). The solutes eluted from this column, at a flow rate of 1 ml/min, were collected in 450 tubes (20 ml per tube) and every other fraction was assayed for inhibitory activity as described below. Fractions 245 through 325, representing the central portion of the largest inhibitory zone, were combined, concentrated to a low volume, and further purified on a silicic acid (Bio Sil HA) column, 25 × 900 mm. This column was eluted with a linear gradient of increasing polarity employing hexane, ether, and acetone (Fig. 3).

After the chromatographic separation of the germination inhibitors, every fraction or alternate fraction was sampled (5% of fraction volume) and bioassayed for inhibitory activity. The samples were placed in 30 ml beakers, the solvent was evaporated quickly with an air stream at room temperature, and 10 ml of warm (40-50 C) 3% water agar was added to each 30-ml beaker. After vigorous swirling and agitation the agar solutions were poured into small petri dishes and, after cooling, seeded with teliospores of *T. caries*, race T-5. The percent germination was estimated to the nearest value in 10% units after incubating 72-84 hr at 17 C.

RESULTS

The amount of water available greatly influences teliospore germination. Teliospores of common or dwarf bunt germinate poorly or not at all when bathed in an aerated aqueous solution. A thin film of water covering the teliospores on an agar surface is sufficient to prevent germination, whereas teliospores scattered on the agar surface without free water germinate well. Germination of dry teliospores proceeds normally on 2% water agar, but on water agar media containing 1% or less agar, germination is inhibited strikingly.

The rate of water uptake in both common and dwarf bunt teliospores was very slow during the first 24 hr, even when the spores were stirred in aqueous solution (Table 1). During the second 24-hr period, the rate of water uptake increased markedly.

Preliminary studies of self-inhibition in teliospores of four races of common bunt and one race of dwarf bunt supported the hypothesis that water-soluble substances from the spores contain inhibitory activity. The relationship of germination and concentration of

TABLE 1. Effect of hydration time on moisture content and volume of teliospores of the common (race T-5) and dwarf bunt (race D-2) pathogens

Hydration time (hr)	Moisture (%)		Increase in volume (%)	
	Race T-5	Race D-2	Race T-5	Race D-2
0	2.2	2.5	0	0
0.16	3.7	4.2		
0.50	4.3	4.7	95	125
1.66	4.7	5.2		
5.00	5.5	7.2	104	122
24.00	8.5	9.9	108	131
50.00	28.3	42.7	117	143

TABLE 2. The effects of water-soluble substances from common bunt teliospores, race T-5, upon the germination percentage of race T-5 teliospores (two replications)

Extract	Dilution of extracts			
	1:2	1:4	1:6	1:10
First	13[a]	30	44	49
Second	17	38	53	62
Third	23	53	72	81
Fourth	24	63	79	79
Fifth	36	64	80	81

[a]Control plates had 79% germination.

common bunt teliospores (race T-5) per unit surface area of 3% water agar medium was studied. The results indicated that percent germination decreased as the number of spores per square millimeter increased: 79% at three spores/mm^2, 77% at nine, 62% at 30, 34% at 100, 12% at 302, and 0% at 1,000.

Results of another experiment demonstrated that aqueous extracts of common bunt teliospores (race T-5) inhibited germination. Teliospores were hydrated in an aqueous solution containing 5 g spores per 100 ml. The suspension was stirred for 30 min at 22 C and then filtered through an 8-μm (pore size) filter. The teliospores were re-extracted in the same way four additional times. The filtrates were vacuum-evaporated to dryness at 30 C. A portion of each filtrate was bioassayed by incorporating it into a warm agar solution and germinating teliospores of the common bunt fungus (race T-5) upon the agar surface. The results (Table 2) indicated that water-soluble inhibitory substances were present in common bunt teliospores and that these solutes were released from the spores slowly.

On water agar containing high dilutions of the aqueous extracts of teliospores, the germination of teliospores was abnormal even though percent germination was close to control values. Germination on such media often proceeded only to the formation of a germ tube, and when growth of promycelia did occur it was abnormal; i.e., stunted, irregularly branched, and disorganized. Repeated extraction of the teliospores with water continued to remove inhibitors (Table 2), but the teliospores that had been extracted five times germinated precisely as did nonextracted spores. This observation and interpretation of the data in Table 2 suggested that endogenous nonwater-soluble inhibitors may be present and that these might be extracted more efficiently by a polar solvent capable of penetrating the plasma membrane. Solvents less polar than ethanol do not readily penetrate to the plasma membrance of the teliospores (7, 15).

Teliospores of the dwarf bunt fungus were extracted with methanol and the solutes were separated on a microcrystalline cellulose column which was eluted with ethanol. Substances present in fractions 56 through 85 blocked germination completely (Fig. 1).

The fractions containing the inhibitors were further purified on a silicic acid column (Fig. 2). At least six strongly inhibitory substances were eluted from this column. Fractions 245-340, which represented the central portion of the largest inhibitory zone, were pooled and purified further on a Bio Sil HA column. Elution with a linear gradient of increasing polarity (Fig. 3) clearly separated two zones of inhibition. The fractions (90-100) in the first zone were combined, concentrated, and further purified on silicic acid thin-layer chromatographic (TLC) plates using hexane:acetone (7:3, v/v) as the solvent. Compounds were detected under ultraviolet light and in iodine vapors, eluted with ethyl acetate, concentrated, and assayed for inhibitory activity. Only one area, at R_f 0.35, contained a germination inhibitor. Except for a fluorescent substance at R_f 0.38, six contaminating substances were well separated from the inhibitor. The

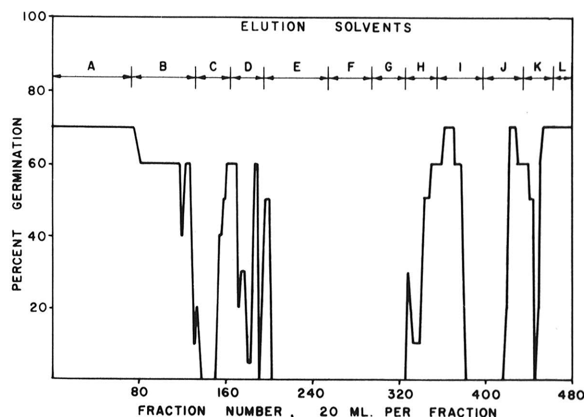

Fig. 2. Teliospore germination inhibitory activity of fractions eluted from the Bio Sil A column. The column was eluted in a stepwise gradient with: (A) benzene; (B) benzene:ether, 99:1; (C) benzene:ether, 97:3; (D) benzene:ether, 90:10; (E) benzene:ether, 70:30; (F) benzene:ether, 65:35; (G) benzene:ether:acetone, 55:35:10; (H) benzene:ether:acetone, 40:30:30; (I) benzene:ether:acetone:methanol, 30:30:30:10; (J) benzene:ether:acetone:methanol, 10:30:30:30; (K) acetone:methanol, 50:50, and (L) methanol.

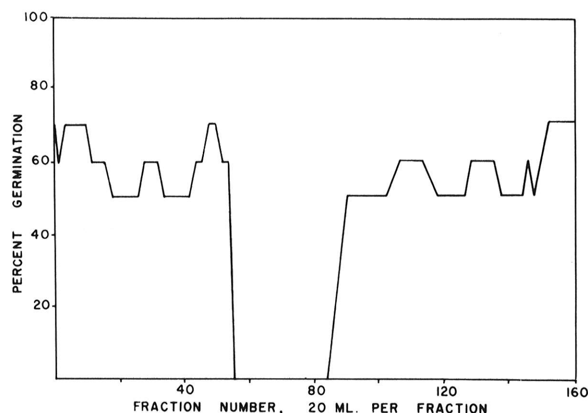

Fig. 1. Teliospore germination inhibitory activity of fractions eluted with ethanol:water (95:5, v/v) from the microcrystalline cellulose column.

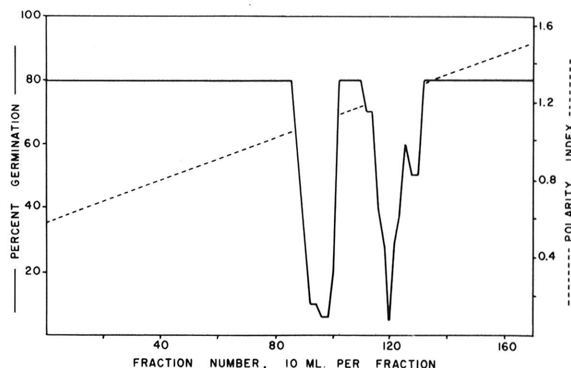

Fig. 3. Teliospore germination inhibitory activity of fractions eluted from the Bio Sil HA column. Polarity index of the eluant is plotted as a dotted line.

area of the TLC plate containing the inhibitor was eluted with ethyl acetate, concentrated, and placed on another silicic acid TLC plate. When developed with 100% acetone, the inhibitor had an R_f of 0.65, and was clearly separated from the fluorescent contaminant. The inhibitory area was eluted with ethyl acetate, evaporated to dryness, and taken up in a small volume of hexane.

This technique of purifying the inhibitor from peak 1 (see Fig. 3) was used to accumulate about 15 mg of the inhibitor. The inhibitor was readily soluble in hexane at 20 C, but crystallized at −15 C. The teliospore germination inhibitor had a melting point of 51 to 51.5 C, an absorbance maximum at 218 nm, and an extinction coefficient (1% solution, 1 cm light path) of 347 in hexane. The molecular structure of this inhibitor is being studied.

The purified inhibitor, at 7 μg/ml in agar, reduced germination to 50% of the control and blocked germination completely at 30 μg/ml. The inhibitor has lipid-like characteristics and is only slightly soluble in the agar medium. The active concentraction of the inhibitor at the site of the germinating spore is probably far less than 7 μg/ml. Purification of the other germination inhibitors from dwarf bunt teliospores is in progress.

Trimethylamine has been reported to be an endogenous inhibitor of *Tilletia* teliospores (1, 4, 18). We determined the amount of trimethylamine in teliospores of four races of common bunt and one race of dwarf bunt (Table 3). These data indicate that dry teliospores contain 2 to 4 mg trimethylamine per gram of spores. Since trimethylamine is a strongly basic substance, highly volatile in alkaline conditions, the effect of pH on its inhibitory characteristics was tested. Table 4 indicates that trimethylamine, even at 1 mM, was only weakly inhibitory and that pH values between 5 and 7 did not greatly influence this activity.

DISCUSSION

Trimethylamine has been reported to be involved in the dormancy of *Tilletia* teliospores (1, 4, 18). Hanna et al. (4) reported about 40 to 120 μg of trimethylamine per gram of common bunt teliospores. The trimethylamine content of common bunt teliospores was estimated by von Kamienski (18) to be 400 to 500 μg per ear; this is equivalent to about 800 μg per g teliospores. Based on our estimate of 4 mg trimethylamine per gram of spores (Table 3) there would be a concentration of about 44 μM trimethylamine in a petri dish containing a spore density of 1,000 spores/mm^2. This assumes that all of the trimethylamine in the spores diffuses uniformily into the agar. At a density of 1,000 spores/mm^2 on agar at pH 6.5 the teliospores are self-inhibited and will not germinate. This inhibition must therefore be due to other endogenous factors because the spores are inhibited only slightly, if at all, by trimethylamine at that concentration (Table 4).

The nature of the extreme dormancy of dwarf bunt teliospores appears to be a complex of many factors (2, 8, 12). The group of endogenous germination inhibitors, including trimethylamine, discussed in this paper surely contributes to this dormancy, but other unusual metabolic patterns also have been detected. The imbalance and extremely low levels of some of the free amino acids in these teliospores, compared to common bunt teliospores, suggest an impairment of essential protein synthesis in the early stages of germination (16). These teliospores contain about 35% lipids, of which free fatty acids account for about one-half and bound fatty acids about one-fourth of the total lipids (17). It is unusual that free fatty acids account for such a high percentage of the total lipids. Some individual fatty acids and mixtures of fatty acids have been found to inhibit fungal spore germination (6, 11, 13). Some of the endogenous germination inhibitors extracted from these spores are probably a part of this large lipid complex, and preliminary evidence supports the concept that the endogenuous inhibitory activity is greater in dwarf bunt than in common bunt teliospores. The very low moisture content in dry dwarf bunt teliospores and the slow rate of water uptake (Table 1) may contribute to the dormancy,

TABLE 3. Trimethylamine in teliospores of the common bunt [*Tilletia caries* (T races)] and dwarf bunt [*T. controversa*, (D-race)] pathogens

Trial	Trimethylamine (milligrams per gram) in teliospores of wheat bunt races:				
	T-1	T-5	T-10	T-15	D-2
Trial 1	4.4[a]	3.2	3.4	3.5	2.7
Trial 2	3.5	2.4	2.4	2.7	2.1

[a]Average of four analyses.

TABLE 4. Effects of pH and trimethylamine on the percent germination of teliospores of *Tilletia caries*, race T-5

pH	Control	Germination (%) of teliospores exposed to trimethylamine concentrations of:			
		1 μM	10 μM	100 μM	1,000 μM
5.0	65[a]	61	52	62	58
5.5	58	63	50	60	49
6.0	50	48	60	48	42
6.5	66	59	61	57	48
7.0	66	55	50	48	41

[a]Average of four experiments.

but these spores share these characteristics with common bunt teliospores which germinate in 3 days instead of 3 mo.

Germination is commonly regulated in fungal spores so that it occurs primarily in instances that favor outgrowth and survival of the organism (3, 9). Examples of self-inhibition involving decreased germination as the spore population is increased must be related to water-soluble or polar substances. This should not imply that all (or even most) of the germination inhibitor can be removed by washing the spores with water. There may be many types of dormant spores that contain endogenous germination inhibitors that are not readily soluble in water or that possess permeability barriers to prevent leaching of the inhibitor. Such spores would not exhibit an inverse relationship between crowding and germination, because they probably have sufficient inhibitor within each spore to cause dormancy. Teliospores of the dwarf bunt fungus may be an example of the latter type.

The germination inhibitors separated on the Bio Sil A column (Fig. 2) block or disrupt the delicate (but essential) postgermination developmental stages of the bunt fungi. It would be helpful to know if one of these development stages is a "weak link"; i.e., much more susceptible to inhibition than earlier or later stages.

As the world becomes more concerned about the use of broad-spectrum, highly-toxic pesticides to control insects and diseases, more research should be focused on the natural physiological and biochemical regulatory mechanisms of these pest organisms. The great progress made in the last few years in elucidating insect hormones and the use of these highly specific and potent compounds for insect control demonstrates the relevance of this research approach. Similarly, the impact of highly specific, potent, naturally occurring fungal inhibitors should assume greater significance in plant pathology.

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