Germinability of Teliospores of *Tilletia indica* after Hot Water and Sodium Hypochlorite Treatments

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ABSTRACT

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Hot water and sodium hypochlorite (NaOCl) were evaluated to eradicate teliospores of the Karnal bunt fungus, *Tilletia indica*, for the purpose of decontaminating grain storage and handling equipment. The germinability of free teliospores and teliospores within the sori of infected wheat was assessed. Temperatures of 25, 60, and 80°C, NaOCl concentrations (wt/vol, pH 11.5) of 0, 0.53, and 1.60%, and immersion periods of 1, 5, 15, and 30 min were evaluated. In other tests, the influence of pH on NaOCl potency and of a delay between treatment and water rinsing were evaluated. Immersion at 80°C in water alone or with NaOCl killed both free teliospores and those within the sori of infected seeds within 1 min. NaOCl at 1.60% at 25°C killed teliospores suspended in water within 15 min, but some teliospores inside sori survived 30 min of this treatment. NaOCl adjusted to pH 8 before use was superior to NaOCl at pH 11.5. An application of 1.60% NaOCl at 25°C for 5 min followed by a 10-min delay before the seeds were rinsed in fresh water killed free teliospores but not all teliospores within sori. This treatment was more effective than the 5-min treatment alone but inferior to the 15-min treatment with NaOCl at a concentration of 1.60%. Because teliospores within the sori of infected seeds are partially protected and much more resistant to NaOCl, we recommend the removal and disposal of seeds from equipment before the treatments are applied. NaOCl radically altered the appearance of the teliospores, leaving a persistent visual indication that they had been treated, while hot water treatment alone did not. Therefore, it is beneficial to add NaOCl to hot water, although the improvement in the sporicidal efficacy was often small.

Karnal bunt of wheat, caused by *Tilletia indica* Mitra (syn. *Neovossia indica* (Mitra) Mundk.), a seed- and soilborne flower-infecting bunt of wheat, was first reported from northwest India in 1931 and has spread since that time and become the object of intensive quarantine efforts (5,13). Contaminating teliospores can reside within the sori of Karnal bunt-infected wheat seeds or on the surfaces of healthy seeds, bins, combines, railcars, and elevators. Seeds infected with *T. indica* were found in the state of Arizona in the United States in March 1996 (17). This occurrence, the first report of Karnal bunt in the United States, elicited an effort by regulatory agencies to establish emergency measures to contain the distribution of teliospores on contaminated seeds, field equipment, and grain-processing facilities.

A decontamination treatment should be fast, effective, inexpensive, and readily available. It should pose minimal hazards to workers and the environment, and it should leave a persistent indication that a treatment has been applied (e.g., bleaching of the teliospores) so that compliance with treatment protocols is evident. Because the unruptured periderm of the sori protects teliospores from many chemical treatments (12,15), infected seeds should be included in efficacy tests of decontamination treatments. Among decontamination treatments issued by the U.S. Department of Agriculture's Animal Plant Health Inspection Service are (i) immersion in a 30% (vol/vol) solution of laundry bleach (5.25% [wt/vol] NaOCl) for 15 min followed by a fresh water rinse; and (ii) hot water or steam applied with a point-of-contact temperature of 80°C. The efficacy of these treatments has not been evaluated.

The temperatures required to kill teliospores under moist or dry conditions can be estimated from prior studies in which the objectives were to control teliospore germination in soil or seeds (6,9,10,12). The moisture status of the teliospores at the time of treatment greatly influences the temperatures at which mortality occurs. In water or moist soil heated to 50 to 55°C, mortality of *T. indica* teliospores occurs

after about 0.5 to 5 h (6,9,10,12). Teliospores are much more resistant to heat under dry conditions. In dry air heated to 100, 110, 125, or 140°C, teliospores survived 88 h, 12 h, 1 h, or 10 min, respectively (4,12). The susceptibility of teliospores to heat within unruptured sori is difficult to predict, because their moisture content during a moist or wet treatment would be increasing, thereby decreasing their sensitivity to heat.

NaOCl potency is improved by heating, increasing its concentration, and by decreasing solution pH before use (2,8). Chastain (1) and Smilanick and coworkers (11) reported that heating the NaOCl accelerated the mortality of teliospores of *Tilletia* species. For example, teliospores of *T. controversa* were killed by an 18-min treatment with 0.25% NaOCl at 23°C, while at 50°C, a treatment of only 3 min was required (11). Reducing the pH of NaOCl solutions increases the proportion of hypochlorous acid, the germicidal component of free hypochlorite (16), and improves NaOCl effectiveness for cereal seed disinfection (8). Smilanick and coworkers (12) reported that germination levels of teliospores of *T. indica* after immersion for 10 min in 0.25% NaOCl at pH 11 and pH 6 were 22.9 and 0%, respectively. However, hypochlorite solutions below pH 6 or those heated at neutral pH can decompose to release chlorine gas, which is a safety hazard and also more corrosive to equipment (16).

The objective of this work was to determine the efficacy of hot water and NaOCl, alone or in combination, to kill teliospores of *T. indica* suspended in water, on the surface of healthy seeds, or within the unruptured sori of infected seeds.

MATERIALS AND METHODS

Two-year-old *T. indica*-infected wheat seeds were obtained from a variety trial in a containment facility located in Ft. Detrick, MD. (All experiments were conducted there under permit). Infected seeds classified 1 to 2 (point infections) by the definition of Warham, Mujeeb-Kazi, and Rosas (14) were selected from seeds threshed by hand from infected spikes. Only seeds with unruptured sori, with the periderm intact, were used in the experiments with infected seeds. The sori of seeds of this infection class are more difficult to rupture by mechanical pressure and often

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remain intact after passing through commercial combines.

Free teliospores were prepared by gently crushing severely infected seeds, classified 3 to 5 (14), with a mortar and pestle and passing the teliospores through a 60- μ m-pore-size sieve to remove debris. For each treatment, a suspension of about 1,000 teliospores in 200 μ l of water and three infected seeds with unruptured sori were immersed in 40 ml of the test solutions in capped, presterilized, 50-ml-capacity conical centrifuge tubes. NaOCl solutions of 0, 0.53, or 1.60% were prepared immediately before use and preheated to the exposure temperature. The tubes were suspended in a water bath at 25, 60, or 80°C and incubated for 1, 5, 15, or 30 min. The temperature in the solutions was monitored with 1-mm-diameter type T probes (Gaffney Engineering, Gainesville, FL) attached to a thermocouple thermometer (model HH11, Omega Engineering, Stamford, CT). Free hypochlorite concentrations in treatment solutions were measured spectrophotometrically at 528 nm by using *N,N*-diethyl-*p*-phenylenediamine (16).

To terminate each treatment, free teliospore solutions and infected seeds with intact sori were placed on an 8- μ m-pore-size filter and rinsed three times with 5-ml volumes of 20 to 25°C sterile water. The infected seeds were removed from the filter; and the filter was removed, inverted, and applied to and removed from the surface of a petri dish containing 2% (wt/vol) agar amended with 100 mg each of ampicillin and streptomycin sulfate per liter to minimize contamination. The three infected seeds with intact sori retained from each treatment were transferred to a sterile petri dish, and the sori were excised with a scalpel and crushed in a small volume of sterile water. This teliospore-containing solution was poured through a second filter apparatus, and a 5-ml volume of sterile water was applied to rinse and disperse the teliospores on the filter. The spores were transferred to an agar plate as previously described. Each agar plate had about 300 to 500 teliospores on the agar surface.

The agar plates were incubated under optimal conditions for germination (15°C, 12-h light-dark cycle), and all were examined after 34 to 38 days to determine the percentages of germinated teliospores. In each experiment, 300 teliospores were examined from each treatment. Each experiment was conducted three times. Teliospores were classified as germinated if any germination products, such as promycelia or sporidia, were present. For statistical analysis, germination percentages were arcsine transformed before an analysis of variance was applied followed by Duncan's new multiple range test ($P \leq 0.05$) or orthogonal comparisons to separate effects. To compare some treatments, the time at which 99% teliospore mortality occurred was calculated by using Finney's probit analysis (3).

RESULTS

The temperature of the water and the NaOCl solutions did not change during the treatments. Solutions of 1.60% NaOCl at pH 11.5 at 25, 60, and 80°C were stable, while those at pH 8 were stable only at 25°C and quickly decomposed at 60°C. Therefore, NaOCl solutions adjusted to pH 8 were evaluated only at 25°C. The free hypochlorite concentration did not change during most tests; the greatest loss, a decrease of 20%, occurred during treatment

of seeds and teliospores with 1.60% NaOCl at 80°C for 30 min.

Germination of free teliospores treated with water at 25°C was $34.7\% \pm 5.0\%$ (Table 1). At 25 and 60°C, the inhibition of germination of free teliospores by NaOCl at 1.60% was significantly ($P = 0.02$) superior to NaOCl at 0.53%. The time to kill 99% of the free teliospores at 25°C with 0.53 or 1.60% hypochlorite was 24.2 and 7.1 min, respectively. The time to kill 99% of the free teliospores at 60°C with 0.53 or

Table 1. Teliospore germination 34 to 38 days after treatment with water or NaOCl for 1 to 30 min at 25, 60, or 80°C

Temperature (°C)	NaOCl (%)	Exposure (min)	Teliospore germination (% $\pm$ SD) ^y	
			Free	From sori
25	0	1	28.0 $\pm$ 10.4	39.7 $\pm$ 22.6
	0	5	33.8 $\pm$ 9.1	69.6 $\pm$ 6.8
	0	15	38.5 $\pm$ 15.3	47.7 $\pm$ 13.0
	0	30	38.5 $\pm$ 9.5	36.0 $\pm$ 19.0
	0.53	1	16.6 $\pm$ 9.5	50.5 $\pm$ 15.6
	0.53	5	13.1 $\pm$ 14.2	33.8 $\pm$ 30.4
	0.53	15	1.3 $\pm$ 1.3	27.5 $\pm$ 20.4
	0.53	30	0.3 $\pm$ 0.3	29.2 $\pm$ 24.4
	1.60	1	9.1 $\pm$ 1.9	37.3 $\pm$ 30.6
	1.60	5	0.8 $\pm$ 0.6	41.4 $\pm$ 12.6
	1.60	15	0.0	10.8 $\pm$ 4.4
	1.60	30	0.0	7.6 $\pm$ 5.4
60	0	1	4.2 $\pm$ 1.7	21.3 $\pm$ 15.3
	0	5	1.7 $\pm$ 0.3	15.0 $\pm$ 6.3
	0	15	0.3 $\pm$ 0.3	0.1 $\pm$ 0.2 ^z
	0	30	0.1 $\pm$ 0.2 ^z	0.0
	0.53	1	0.4 $\pm$ 0.5	32.1 $\pm$ 28.3
	0.53	5	0.2 $\pm$ 0.2 ^z	8.3 $\pm$ 13.4
	0.53	15	0.0	1.3 $\pm$ 2.3
	0.53	30	0.0	0.0
	1.60	1	0.1 $\pm$ 0.2 ^z	55.7 $\pm$ 11.2
	1.60	5	0.1 $\pm$ 0.2 ^z	6.9 $\pm$ 9.9
	1.60	15	0.0	0.0
	1.60	30	0.0	0.0
80	0	1	0.0	0.0
	0	5	0.0	0.1 $\pm$ 0.2 ^z
	0	15	0.0	0.0
	0	30	0.0	0.0
	0.53	1	0.0	0.0
	0.53	5	0.0	0.1 $\pm$ 0.2 ^z
	0.53	15	0.0	0.0
	0.53	30	0.0	0.0
	1.60	1	0.0	0.0
	1.60	5	0.0	0.0
	1.60	15	0.0	0.0
	1.60	30	0.0	0.0

^y Each value is the mean of three experiments in each of which 300 teliospores were examined.

^z One teliospore germinated on one agar plate in one experiment.

Table 2. Teliospore germination after immersion in NaOCl, pH 8 or 11.5, for 5 min and a rinse in fresh water after a delay of 10 min or immersion in NaOCl (1.60%), pH 11.5, for 5 or 15 min followed by an immediate rinse in fresh water

NaOCl (%)	pH	Temperature (°C)	Exposure (min)	Delay ^y	Teliospore germination (% $\pm$ SD) ^z	
					Free	From sori
1.60	11.5	25	5	0	0.8 b	41.4 b
1.60	11.5	25	5	10	0.0 a	41.7 b
1.60	8.0	25	5	10	0.0 a	19.0 c
1.60	8.0	25	15	0	0.0 a	10.8 d
Water alone	...	25	5	0	33.8 c	69.6 a
Water alone	...	25	15	0	38.5 c	47.7 b

^y Delay in minutes before the seeds were rinsed in fresh water.

^z Values followed by different letters are significantly different ($P \leq 0.05$) according to Duncan's new multiple range test.

1.60% NaOCl was 2.7 and 0.2 min, respectively.

Germination of teliospores from within sori treated with water at 25°C was 48.3% $\pm$ 15.0% (Table 1). Germination of teliospores from sori was greater than that of free teliospores, probably because when the free teliospores were first prepared by crushing sori, many of the teliospores were cracked. Survival of teliospores within sori was significantly ($P < 0.001$) greater after heat and NaOCl treatments than that of free teliospores at 25 and 60°C. At 25°C, NaOCl did not kill all teliospores within sori after 30 min of treatment. Control of teliospores within sori by 0.53 or 1.60% NaOCl was not significantly different. No teliospores survived any 80°C treatment, with the exception of two teliospores from sori, one of which survived a 5-min treatment in water and the other a 5-min treatment in 0.53% NaOCl.

Treatment with 1.60% NaOCl at 25°C for 5 min followed by a delay of 10 min before rinsing with fresh water killed free teliospores but not all teliospores within sori (Table 2). This treatment was better than a 5-min treatment alone but significantly inferior to a continuous 15-min treatment with 1.60% NaOCl. The efficacy of NaOCl solutions adjusted to pH 8 before use was significantly superior to that of solutions at pH 11.5, but some teliospores within sori survived.

The color of most of the free teliospores

and many of the teliospores from within sori was bleached, and the spore wall and sheath were greatly expanded after NaOCl treatments (Fig. 1). Completely decolorized, expanded teliospores did not germinate, but some partially decolorized teliospores did germinate.

DISCUSSION

The primary findings of this work are (i) 80°C water kills nearly all teliospores of *T. indica* after 1 min of contact, even if they reside inside unruptured sori at the time of treatment; (ii) the addition of NaOCl to water kills more free teliospores than hot water alone; and (iii) the altered appearance of teliospores usually leaves a persistent indication that they have been treated with NaOCl. The addition of NaOCl to hot water adds both confidence that fewer teliospores will survive treatment and a visual indication that they have been treated. Teliospores within sori, however, resisted NaOCl treatments, were irregularly bleached, and were reliably controlled only at 80°C. Therefore, we recommend the removal of as many seeds as possible from equipment or facilities to be treated before the treatments are applied. With appropriate worker safety and environmental precautions, a combination of hot water and NaOCl could be applied by adding NaOCl to the water heated in steam or hot water cleaners or a NaOCl solution could be injected into the steam or water stream afterward. The pH of the NaOCl solution should be high (about 11.5), because decomposition to chlorine gas will occur if solutions of lower pH are heated (16). A rinse with fresh water after the treatments are applied is recommended to reduce corrosion damage to equipment and painted surfaces, although lengthening the period between application of the NaOCl and rinsing will improve the efficacy of the treatment. Treated equipment should be placed on wash-containment mats before treatment so that runoff NaOCl and rinse water do not contaminate soil.

The heat and NaOCl treatments we report that killed teliospores of *T. indica* within sori are too harsh for wheat seeds to survive without injury. Teliospores contaminating the surfaces of wheat seeds, however, can be greatly reduced by hot water and NaOCl at rates that do not injure seeds. For example, water alone at 54°C for 20 min killed about 90% of the teliospores of *T. indica* on seed surfaces without inhibiting germination of wheat cultivar Wanser seeds (11). At ambient temperatures, 1.60% NaOCl for 10 min killed about 96% of teliospores of *T. indica* on seed surfaces without inhibiting germination of wheat cultivar Wanser seeds (11). Harsher treatments injure wheat seed; Chastain (1) reported that wheat cultivars Daws and Stephens survived 15- or 30-s treatment with 1% NaOCl at 55 to 60°C without injury, although 60-s treatments

reduced seed germination and vigor. Approaches to improving the efficacy of NaOCl sanitation of seeds have been incompletely investigated. Sauer and Burroughs (8) reported that treatment with ethanol before NaOCl immersion improved the disinfection of wheat seeds contaminated with several *Aspergillus* spp.; this approach probably would improve NaOCl efficacy against teliospores of *T. indica* as well. Adding ethanol to NaOCl probably would improve its effectiveness. The toxicity of formaldehyde to teliospores of *T. indica* was enhanced by ethanol, and ethanol, particularly at a concentration of 40%, was itself an effective sporicide at ambient temperature (12).

The combination of heat and NaOCl achieves most of the attributes required of a feasible sanitation treatment: (i) it is effective against free teliospores and teliospores within sori, although every effort to remove infected seeds before treatment should be made; (ii) it is inexpensive and readily available; (iii) worker and environmental hazards can be managed; and (iv) the teliospore appearance is altered, usually leaving a persistent indication that teliospores have been treated so that compliance with treatment orders is evident.

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Fig. 1. (A) Untreated teliospores of *Tilletia indica* and (B) teliospores treated for 30 min with 1.6% NaOCl. The brown to dark brown color is absent after treatment, and the teliospore wall becomes enlarged and disorganized. Bar = 30 μ m.

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