Deleterious Effects of Tobacco Smoke on Germination and Infectivity of Spores of Puccinia graminis tritici and on Germination of Spores of Puccinia striiformis, Pyricularia oryzae, and an Alternaria species

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ABSTRACT

Uredospores of *Puccinia graminis tritici* and *P. striiformis* and conidia of *Pyricularia oryzae* and an *Alternaria* sp. were unable to germinate on 1.25% H_2O agar when 6,000 μliters of cigarette smoke/liter of air was in the incubation chamber. In control chambers without cigarette smoke the germination percentages for these organisms on agar plates ranged from 42 to 91. Decreased concns of smoke down to about 300 μliters inhibited germination; the magnitude of this effect varied with spore lot and with species. An additional incubation period in smoke-free air, following the smoke exposure, resulted in higher germination percentages if the original exposure to smoke was not greater than about 3,000 μliters. Exposure of agar to smoke before seeding with spores and incubation in a smoke-free atmosphere also inhibited or prevented germination, depending on the concn of smoke and the duration of exposure. Smoke from cigar and pipe tobacco had essentially the same effect as that described above. With *P. graminis tritici*, decreased germination at inhibitory smoke concns was because the rates of germ tube emergence and elongation during the first 2 h of incubation were greatly reduced. When the incubation period was increased from 2 h (the time usually sufficient for uninhibited spores to achieve maximum germination) to 6 h, mildly inhibited spores caught up with the no-smoke control spores in percentage germination values, but the average length of their germ tubes was one-half that of the control spores. Exposure of dry uredospores to 6,000 μliters of cigarette smoke/liter of air did not affect their subsequent germination on agar or their ability to infect wheat seedlings when used as inoculum. However, when agar plates were seeded and wheat was inoculated with spores of proven infectivity and incubated in dew chambers in 3,000 μliters of smoke/liter of air, germination was decreased and essentially no infection occurred.

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The percentage germination of individuals within spore populations under various test conditions has been used as a measure of relative vigor among different lots of spores and occasionally as a rough indicator of potential infectivity. Although standardization of well recognized factors affecting biological processes (light, temp, moisture, nutrition, etc.) is attempted in germination tests, variation in results due to factors other than these often occur (9, 13, 16). Some of these differences, particularly with spores of obligate parasites, are due to variation in conditions during spore production, collection, and pretest handling (9, 11). Significant fluctuations in germination of uredospores of *Puccinia striiformis* West. have been associated with low ratios of small:intermediate air ions during the incubation period, which correlate with reduced rate of germination and reduced total germination (14). This study concerns the effect of tobacco smoke from different sources on germination (and in one instance infectivity) of spores of several species of fungi. Some preliminary results from these tests were previously reported (12).

MATERIALS AND METHODS.—Uredospores of *Puccinia graminis* Pers. var. *tritici* Eriks. & E. Henn., races 15B and 56, and of *P. striiformis* West. were produced on greenhouse-grown wheat (cultivar Baart, C.I. 1697), harvested with a cyclone collector (1), and either used within 2 h or stored in liquid nitrogen (7). Prior to use, spores from storage were heat-shocked at 40 C for 6 min, then hydrated at 21 C (*P. graminis tritici*) or at 12 C (*P. striiformis*) for 16 h in the dark. Conidia of *Pyricularia oryzae* Cavara and of the *Alternaria* sp. were grown on supplemented agar plates and harvested in sterile, distilled H_2O for test use. The germination substrate was water agar, 1.25% Difco Bacto.

Agar plates were seeded with uredospores in a settling tower and with conidia by spreading a drop of the spore suspension over the agar surface. Inoculum was adjusted to provide an average of 10 spores/mm^2 on each plate in each test. Seeded plates were placed in 8-liter glass desiccators or in 146-liter plastic isolation chambers, and smoke was introduced into these chambers by methods described for the specific experiments. All tobacco products were purchased on the open market in the vicinity of Frederick, Md. At the end of specified incubation periods, the spores on agar plates were placed immediately into a desiccator in the vapor phase above 37% formaldehyde; this killed the spores quickly, sharply delineating the end of the incubation period, and preserved the spores for up to several weeks in a condition suitable for microscopic observation. A spore was considered germinated if it produced a germ tube as long as or longer than the minor diam of the spore.

Each germination percentage figure reported is based on 1,800-2,200 observed spores in three or more replicate plates. Each type of study was repeated three or more times over a 2-yr period, and data presented are from experiments representative also of others yielding similar results.

RESULTS.—Freshly collected uredospores of *P. graminis tritici*, races 15B and 56, were seeded on agar plates, placed in plastic (polyvinyl) chambers, and exposed to tobacco smoke from a pipe (Sail Aromatic

TABLE 1. Percentage germination[a] of freshly-harvested spores of four different fungi incubated in various concns of cigarette smoke (Lucky Strike plain tip) and of spores incubated first in smoke and then in smoke-free air[b]

	μliters smoke/liter air					
	0	300	600	1,200	3,000	6,000
P. graminis tritici, 15B						
2-h incubation at indicated concn	77	24	6	0	0	0
Additional 2-h incubation, smoke-free	82	44	45	12	0	0
P. graminis tritici, 56						
2-h incubation at indicated concn	77j	...	65	33	2	0
Additional 2-h incubation, smoke-free	78j	...	77	71	45	0
Pyricularia oryzae						
2-h incubation at indicated concn	8	...	4	2h	1h	0
Additional 2-h incubation, smoke-free	41	...	19	14	6	0
Alternaria sp.						
4-h incubation at indicated concn	48	21i	20i	13	4	0
Additional 4-h incubation, smoke-free	69	49	30	25	16	6

[a]Each figure is a mean value of ca. 2,100 observed spores, >400 in each of five replicate plates. Conversion of raw data for analysis was done by adding "1" to each value and performing the arcsine transformation. Within each row, all means with different values are significantly different from each other ($P = 0.01$) using Duncan's multiple range comparison, except for the pair followed by "h" (significantly different, $P = 0.05$) and the pair followed by "i" (no significant difference). Within organisms and treatments, all increases in germination with additional smoke-free incubation were significant ($P=0.01$), except for the pair followed by "j" (not significant).

[b]Incubation temp was 21 C for *P. graminis tritici*, races 15B and 56, and for the *Alternaria* sp., and was 25 C for *Pyricularia oryzae*; all were dark incubation periods.

tobacco, Theodorus Niemeyer, Ltd., Netherlands), Phillies Cheroot cigar (Bayuk Cigars, Inc., Philadelphia, Pa.), or a Lucky Strike plain tip cigarette (The American Tobacco Co., Richmond, Va.). The smoke was introduced by a smoker blowing one "puff" into each chamber; the chambers were then immediately sealed for the 2-h incubation period at 21 C in the dark. With race 15B, mean germination percentages were 72, 43, 0, and 4 for the no smoke, cigarette, cigar, and pipe treatments, respectively. For race 56, the germination percentages were: no smoke, 67; cigarette, 26; cigar, 0; pipe, 0.

A similar study was done with both freshly harvested and stored uredospores of *P. graminis tritici* race 56 and of *P. striiformis* in an 8-liter glass incubation chamber. No germination occurred in either organism in cigarette smoke (Lucky Strike plain tip), whereas spores in the no-smoke, control chambers germinated at 50% or more.

To quantitate the smoke in subsequent experiments, a 13-gauge hypodermic needle was fitted tightly to a polyethylene tube that served as a cigarette holder. The cigarette was inserted into the tube, the hypodermic was attached to a glass syringe, and the cigarette was lighted and "smoked" by pulling air through the lighted cigarette at an average rate of 16 cm/sec into the calibrated syringe. After two 25-ml "puffs" were drawn and ejected, the smoke required for a specific study was drawn into the syringe and measured volumes were immediately injected through the side-arm ports of the glass desiccators (used as incubation chambers), which then were immediately sealed gastight.

Using the above procedure, 1,200, 3,000, or 6,000 μliters of smoke/liter of air completely prevented germination of spores of *P. graminis tritici* race 15B (Table 1). At 600 and 300 μliters, 6 and 24% germination, respectively, occurred. The 0 μliter (no smoke) control consisted of a desiccator receiving 50 ml of air that had been drawn through the unlighted cigarette; here germination was 77%. When selected plates were incubated for an additional 2 h in smoke-free air, additional germination occurred except in the 3,000- and 6,000-μliter smoke treatments.

With *P. graminis tritici* race 56, spores germinated in the 3,000- and 1,200-μliter treatments. Compared with race 15B, race 56 is significantly less sensitive to a given concn of smoke (Melching, Stanton and Koogle, *unpublished*). Additional incubation without smoke resulted in increased germination percentages, except in the 6,000-μliter smoke treatment, where no germination occurred, and in the 0-μliter control.

With *Pyricularia oryzae*, the 2-h incubation period was too short, as indicated by the large increase in germination in the 0-μliter treatment after an additional 2 h. Nevertheless, significant increases in inhibition that correlated positively with increases in smoke concn were observed. Definite increases in germination within treatments occurred (except in 6,000-μliter of smoke) during the smoke-free incubation.

Conidia of the *Alternaria* sp. were able to germinate after a 4-h smoke-free incubation that followed a 4-h incubation in 6,000-μliters of smoke. With this exception, the response of this organism to the various treatments was essentially similar to that of the other fungi tested.

To determine if the inhibitory effects of smoke exposure could be cancelled by a sufficiently long incubation period in smoke-free air, spores of *P. graminis tritici* race 56 on agar were exposed to smoke for 2 h and then incubated for 3 or 15 h without smoke. In another test, agar was exposed to the various concns of smoke before it was seeded with spores.

Spores did not germinate during 3- or 15-h incubation periods in smoke-free air following a previous 2-h exposure to 6,000 μliters of smoke/liter of air (Table 2). Germination did occur during smoke-free periods following exposure to the 3,000-, 1,200-, and 0-μliter treatments, attaining 13, 83, and 76%, respectively, after 15 h. The difference between 83 and 76% was barely

TABLE 2. Percentage germination[a] of uredospores of *Puccinia graminis tritici* race 56 first incubated in various concns of cigarette smoke (Lucky Strike plain tip), and then in smoke-free air, or incubated in smoke-free air on agar plates previously exposed to smoke

Incubation conditions[b]	μliters smoke/liter air			
	0	1,200	3,000	6,000
2 h at indicated concn	67	0	0	0
2 h at indicated concn plus 3 h smoke-free	67	46	8	0
2 h at indicated concn plus 15 h smoke-free	76h	83h	13	0
Agar exposed to indicated concn for 2 h, seeded with spores, and incubated 15 h smoke-free	63i	66i	36	4

[a]Each figure is a mean value of ca. 1,850 observed spores, > 450 in each of four replicate plates. Within each row, all means with different values are significantly different from each other using Duncan's multiple range test ($P = 0.01$), except for the pair followed by "h" (significantly different, $P = 0.05$) and the pair followed by "i" (not significant). Within treatments, where increases in germination with additional smoke-free incubation occurred, all mean increases were significant, $P = 0.01$.

[b]Incubations were at 21 C in the dark; spores were from liquid nitrogen storage.

significant ($P = 0.05$) in this test. By itself, this result might suggest a slight stimulatory effect when the long (15-h) smoke-free incubation followed the 2-h exposure to 1,200 μliters of smoke/liter of air. However, in two separate tests done to clarify this point, significant differences did not occur between these two treatments.

When agar after exposure to smoke was seeded with spores, germination was 4 and 36% at 6,000 and 3,000 μliters, respectively, whereas the control germination was 63% (Table 2). No inhibition occurred on agar that had been exposed to 1,200 μliters of smoke/liter of air before seeding with spores.

Uredospores of *P. graminis tritici* race 56 were exposed to smoke from the following sources: Camel filters (R. J. Reynolds Tobacco Co., Winston-Salem, N.C.); Tareyton filters (The American Tobacco Co., Reidsville, N.C.); Kent micronite filter, King size (Lorillard Co., Greensboro, N.C.); Lucky Ten, filter (American Tobacco Co., Richmond, Va.); Chesterfield filter (Liggett-Myers Tobacco Corp., Richmond, Va.); Muriel cigar (Consolidated Cigar Corp., New York, N.Y.); White Owl cigar (General Cigar Co., New York, N.Y.); Borkum Riff pipe tobacco (Swedish Tobacco Co., Stockholm, Sweden); and Amphora (regular) pipe tobacco (Douwe Egberts Royal Factories, Utrecht, Holland). The incubation period was for 2 h in 6,000 μliters of smoke/liter of air; a no-smoke control treatment was included. The cigarettes were used both with filters in place and with filters removed. Smoke from all sources prevented germination, with the exception of the Borkum Riff pipe tobacco, where germination was 16%. Germination of spores in the no-smoke chamber was 57%.

In another study, agar plates seeded with uredospores of *P. graminis tritici* race 56 were placed in a room where workers smoked cigarettes regularly (and which was occupied by a smoker during the experimental period) and in an adjacent room where workers did not smoke. The plates were in glass containers that were wrapped in aluminum foil to exclude light, but the tops were propped open slightly to permit passage of air. The temp in both rooms was 25-26 C. Spores on control plates were also incubated for the same 4-h period in a smoke-free chamber at 18 C in the dark. The results in terms of average germination percentages were as follow: smoker's room, 76%; non-smoker's room, 88%; control chamber, 89%. This degree of inhibition was significant at $P = 0.05$ (using "Students" method of paired comparisons) and was similar to that observed with the 600-μliter concn of smoke used in the previously described studies.

The effect of exposure of "dry" uredospores of *P. graminis tritici* race 56 to smoke (Lucky Strike plain tip) upon their subsequent germinability and infectivity on wheat plants was investigated. After a 2-h exposure to 0, 1,200, 3,000, or 6,000 μliters of smoke/liter of air, the dry spores were immediately discharged into a turntable tower (10) to inoculate wheat seedlings and to seed agar plates. After inoculation, the plants were placed in dew chambers (6) for 16 h at 21 ± 1 C, then put in the greenhouse; the agar plates were incubated as usual. Neither germination nor infectivity was affected by the previous exposure of dry spores to smoke. Control agar plates seeded with spores and incubated in the chambers with the dry spores during the exposure to smoke showed the typical inhibition in germination response: 0 μliters, 78%; 1,200 μliters, 32%; 3,000 μliters, 0%; 6,000 μliters, 0%.

Untreated, dry uredospores of *P. graminis tritici* race 56 were used to inoculate wheat seedlings and seed plates in the turntable tower. Forty plants and six plates (with covers propped open to facilitate air exchange) were placed into each of two dew chambers. Smoke (Lucky Strike plain tip) was introduced into one chamber to provide 3,000 μliters of smoke/liter of air; no smoke was in the second chamber. Temperatures in both chambers were 21 ± 1 C. After 16 h, plants were removed to the greenhouse and germination percentages on the agar plates were determined. Fourteen days after inoculation, disease signs and symptoms were tabulated for each leaf on each plant. Three separate tests were done. The results of all were similar and were averaged.

Spore germination was 60% in the dew chamber with smoke and was 84% in the no-smoke chamber, a mean difference which was significant ($P = 0.01$). The wheat plants from the smoke chamber had an average of 0.2 flecks/primary leaf (no sporulation was evident), and those from the no-smoke chamber had an average of 13.6 sporulating pustules/primary leaf.

The average rate of growth of the primary germ tube as well as the total germination percentage attained by

uredospores of *P. graminis tritici* race 56 were affected by smoke (Fig. 1). The most striking effect of the 600- and 900-μliter concns was the delay in the onset of germination; at the end of 1.5 h of incubation, essentially no germination was observed, whereas the uredospores in the control plates and those in the noninhibited 300-μliter smoke treatment had essentially reached their maximum percentage germination with an average germ tube length of 80 μm. From 1.5 through 6.0 h incubation, the inhibited spores in smoke showed progressive increases in percentage of spores germinated and in germ tube elongation, but even at 6 h the spores in the 900-μliter smoke chamber had not achieved as high a percentage germination as the control spores had in 1.5 h, 59 and 85%, respectively. At the end of 6 h of incubation, the average length of the germ tubes in the inhibited smoke treatments was one-half or less than that of the control spores. No differences were noted in spore responses between the no-smoke control and the 300-μliter smoke treatment. No germination occurred at any of the incubation times in spores exposed to 1,200 μliters of smoke.

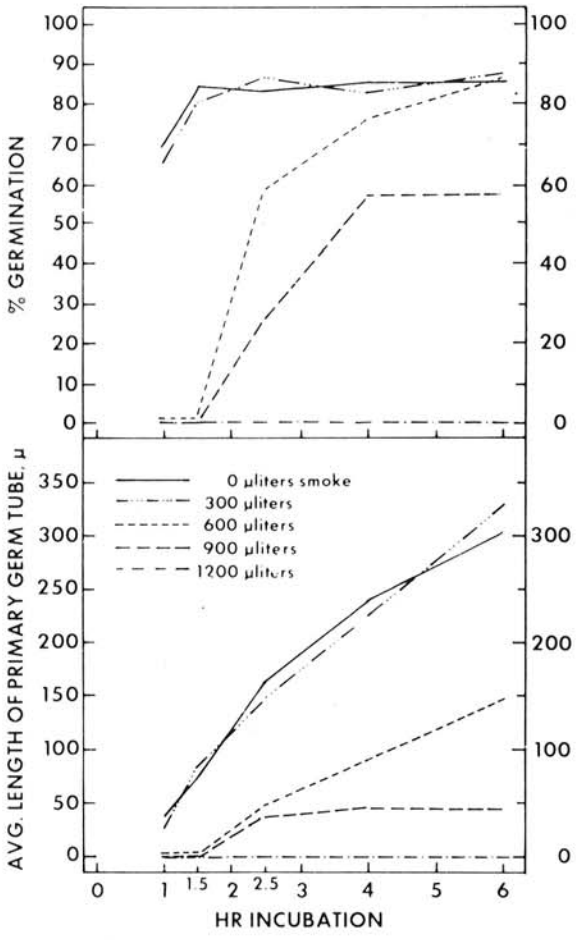

Fig. 1. Growth of primary germ tubes from uredospores of *Puccinia graminis tritici* race 56 incubated at 22 C in the dark for 1, 1.5, 2.5, 4, or 6 h in the presence or absence of smoke. Smoke source was a Lucky Strike plain tip cigarette. Data points for germ tube lengths are averages of measurements from 50 germ tubes in each instance.

Smoke from burning wood matches, cigarette paper, newspaper, or shredded cigarette tobacco also prevented germination of spores of *P. graminis tritici* race 15B (Melching, *unpublished*).

DISCUSSION.—Tobacco smoke from cigarettes, cigars, or pipes inhibited the germination process in the spores of several genera of fungi; inhibition in some instances was significant at concns as low as 300 μliters of smoke/liter of air. The effect was greatest when the smoke exposures occurred during the incubation period, when the spores were on the moist agar surface. In contrast, dry uredospores of *P. graminis tritici* race 56 showed no decrease in germinability when tested following their exposure to the highest smoke concn. It is probable that spores on agar are more susceptible than dry spores to chemical injury because of their higher water content, greater permeability, and increased metabolism (3, 8). However, the data indicated that damage to spores on agar during exposure to smoke was only partly responsible for their decreased germination during subsequent smoke-free incubation. The agar also accumulated substances from smoke that inhibited germination during the following smoke-free period, as shown by the decreased germination observed when seeding occurred following exposure of agar to smoke.

Uredospores of *P. graminis tritici* and *P. striiformis* reacted essentially the same whether they were freshly harvested or brought from storage in liquid nitrogen for testing.

At the lower and intermediate concns of smoke used in these studies, different species of fungi and different lots of spores within a species varied in the degree of inhibition caused by specific exposures, but no spores germinated when exposed to the 6,000 μliter/liter concn. When spores on agar plates were given additional incubation periods in smoke-free air after their exposure to smoke, additional germination sometimes occurred. The magnitude of this "recovery" was dependent upon the smoke concn during the initial exposure.

When several different cigarette, cigar, and pipe tobaccos were used as sources of smoke, all prevented or drastically reduced spore germination; the presence or absence of the filter on the cigarettes did not affect the results. However, only one species of organism and one smoke concn were used in these tests.

Inhibitory smoke concns, below those that completely prevented germination, affected the rate at which uredospores of *P. graminis tritici* germinated primarily by delaying the process during the first 1-2 h of incubation. Once initiated, the number of germinated spores and the elongation of the germ tubes progressively increased with time at rates inversely related to the amount of smoke present during the incubation period.

The virtually complete failure of uredospores of *P. graminis tritici* to infect wheat when smoke was present during the dew period, seemed surprising in view of the fairly high percentage germination (60%) of spores from the same lot in the same chamber. The effect of smoke at sublethal concns upon rates of germination and germ tube development, as previously discussed, might provide the explanation. A uredospore with a 200-μ-long germ

tube can theoretically (based on the spatial distribution of stomates on a leaf of Baart wheat) contact 10 times as many stomates as a spore with a 50-μ-long germ tube, yet both spores are by definition "germinated." Whether this occurred is speculation because, unfortunately, the germ tube lengths were not measured on plates or leaves in the infection studies. The possibility that smoke affects further steps in the establishment of infection cannot be discounted.

Tobacco smoke is a complex aerosol in which more than 1,200 components have been identified (15). Among the toxic substances found were nicotine, carbon monoxide, pyridine, phenol, and hydrogen cyanide. In some preliminary tests, these toxic compounds were introduced singly into incubation chambers to provide concns that, calculated from values given in the literature (5, 15), approximated that of each compound in the experimental smoke treatments. Results to date support the tentative conclusion that none of these compounds alone can account for the degree of inhibition noted in the various smoke concns (Melching, *unpublished*). Tests for additive or synergistic effects among these or other substances in the smoke have not been done.

Regardless of the specific compounds responsible, tobacco smoke must be considered as a possible cause of variation in spore germination tests and infection studies. Although the concns of tobacco smoke in the air in laboratories where workers smoke would probably be lower than the intermediate levels used in these studies, such concns might be high enough to affect critical evaluations. Other factors seldom measured or controlled influence spore germination and disease development, i.e., volatile chemicals in low concn (2), ozone (4), charge and size ratios of ions (14), etc.

In spore germination studies, the composition of the air in the experimental area must be defined and controlled if results among different experiments and laboratories are to be valid and comparable.

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