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Station Bulletin 656
January 1982

Agricultural Experiment Station
Oregon State University, Corvallis

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ABSTRACT

Sixty-five compounds, including general microbicides, fungicides, antibiotics, and fumigants were screened for their sporicidal effect on Ascosphaera aggregata. The chalkbrood spore, the only readily accessible stage of the fungus, was unaffected by most of the materials tested. Only the halogenated general microbicides, iodine, hypochlorite, and stabilized dry chlorine, demonstrated consistent sporicidal activity at low concentrations. Suggestions are made as to possible disease control strategies.

ACKNOWLEDGMENTS

The Multistate Chalkbrood Project is supported by contributions from grower groups and industry representatives in Idaho, Nevada, Oregon, and Washington; the Idaho, Nevada, and Washington Alfalfa Seed Commissions; Universities of Idaho and Nevada-Reno; Oregon and Washington State Universities, and by a cooperative agreement with the USDA/SEA Federal Bee Laboratory, Logan, Utah.

The authors thank Dr. J. Corner and Mr. D. McCutcheon, British Columbia Ministry of Agriculture and Food, Vernon and Surrey, B.C., for help with the ETO tests; Dr. G. Puritch, Canadian Forestry Service, Pacific Forest Research Center, Victoria, B.C., for supplying the fatty acid derivatives; and Dr. D. Coyier, USDA/SEA/AR Ornamental Plants Laboratory, Corvallis, Ore., for assistance with fumigation studies.

INTRODUCTION

Ascosphaera aggregata Skou is the causative agent of chalkbrood in larvae of the alfalfa leafcutting bee, *Megachile rotundata* (Fabricius) (Vandenberg and Stephen, 1982). Since chalkbrood was first identified in *M. rotundata* in 1971 (Thomas and Poinar, 1973), this larval disease has caused severe bee losses in most areas of the world where the bee is propagated (Stephen and Undurraga, 1978; Stephen *et al.*, 1981).

The alfalfa leafcutting bee is an important commercially domesticated pollinator of alfalfa in western North America. Bee populations are maintained in or near alfalfa fields in domiciles provided with various types of reusable nesting materials, the most common of which are drilled or laminated wood (Stephen and Every, 1970). Female bees construct linear series of leaf-lined cells in individual tunnels. Each cell is provisioned with alfalfa pollen and nectar and topped by a single egg before being capped with circular leaf pieces.

In areas of high chalkbrood incidence, emerging adults must frequently chew through sibling larval cadavers and thereby become covered with massive numbers of spores (Vandenberg *et al.*, 1980). Adults may then contaminate their mates and eggs, as well as their pollen provisions. Developing larvae are subsequently invaded by spores germinating in the gut with host death and fungal sporulation occurring before the prepupal stage (Vandenberg and Stephen, 1982).

Efforts to control the disease have been confined to general prophylaxis including removing cells from the nesting medium, phasing bees out of contaminated solid nesting boards, and providing clean sterilized nesting media (Stephen and Undurraga, 1978; Kish *et al.*, 1981). Although

these practices have arrested the disease they have not achieved the level of control sought by alfalfa seed producers. Our objectives in this paper are to investigate the sporicidal effects of various chemical and physical treatments of A. aggregata and to determine how promising agents may be incorporated into a chalkbrood control program. As virtually no information is available on the sensitivity of the sporulating stage of the fungus to microbicides, the screening was restricted to compounds representative of general groups of microbicides, fungicides, antibiotics, and fumigants which have proven to be effective against one or more Ascomycetes (Table 1).

MATERIALS AND METHODS

Inoculum Preparation: Chalkbrood cadavers were collected from field populations of Megachile rotundata near Ontario, Oregon (OR-1, OR-3), Nampa, Idaho (ID-5, ID-18), Lovelock, Nevada, and Corning, California, in 1979. Spore cysts were scraped from the body surfaces of 50 cadavers from each area into separate sterile glass vials and stored at 2°C until used. Unless otherwise noted, all tests were conducted on inoculum from Nampa, Idaho (ID-5).

Basic Procedure: The standard procedure used to germinate spores was modified from that reported by Kish (1980). Medium composed of 50% V-8® juice (Campbell Soup, Inc., Camden, N.J.), 2.5% glucose, 2.0% yeast extract, and 2.0% agar was used for all tests. The pH was adjusted to 7.8 with 1 N NaOH before sterilization and was 7.0 after autoclaving. Spore cysts were suspended in saline buffer (pH 7.15) composed of 0.02 M BES (N,N-bis(2- hydroxyethyl)-2-aminoethane sulfonic acid), 0.154 M NaCl, and 0.0075 g/l DOSS (dioctyl sulfosuccinate, sodium) (M. E. Martignoni,

personal communication). The cysts were ground gently in buffer between glass slides to suspend the spores. Additional buffer was added following hemacytometer counts to obtain a final inoculum concentration of 10^7 - 10^8 spores/ml.

Blocks of V-8 agar were cut ca 1 cm^2 and placed on sterile microscope slides. The blocks were inoculated in one of several ways described below and covered with a glass cover slip. Inoculated agar blocks were incubated in a 2 liter chamber purged with CO_2 to a final CO_2 concentration of 60 $\pm$ 5% (Blood Micro System, BMS-3-Mk-2 Radiometer, Copenhagen). Germination was evaluated after 3 days at 30°C and again after an additional 2 days. Each test was replicated from 2 to 4 times.

Exposure to Sporicides: Two methods were employed to test the sporicidal efficacy of the water or ethanol soluble general microbicides, iodine, and chlorine compounds.

1. One ml of suspended spores was loaded into a 10 ml syringe subsequently fitted with a 25 mm Swin-Lok membrane holder (#420200, Nuclepore Corp.) containing a 25 mm diameter polycarbonate filter (pore size 0.8 µm). The spores were collected on the polycarbonate filter by expelling the contents of the syringe. Each test material was loaded into an appropriately sized syringe and slowly and continuously expelled through a spore-laden filter for the prescribed time.
2. One ml of spore suspension was drawn into a 10 ml syringe, following which 1 ml of the test material (at twice the desired concentration) was added. The contents of the syringe were agitated continuously for the prescribed exposure time and the spores collected on a polycarbonate filter.

After spore collection the filter was removed and air dried in a sterile petri dish for ca 15 minutes. Inoculation of the agar block was achieved by inverting the filter and pressing it lightly against the agar surface.

Spores were treated with sterile distilled water as controls for water soluble test solutions. Controls for ethanol-solubilized materials were ethanol treated.

Fatty acids and their derivatives, water or ethanol insoluble fungicides, and antibiotic suspensions were either applied directly with the inoculum to the surface of the agar block and spread evenly over its surface with a sterile glass rod, or the material was incorporated into the medium. In the latter procedure, 1 ml of acetone-solubilized antibiotic or fungicide (or acetone alone for controls) was added to 5 ml warm agar (45-50°C). The agar was poured and the acetone allowed to evaporate for 24 hours before inoculation.

Fumigants and high temperatures were tested against ca 1 mg aliquots of fresh spores and spore cysts placed in the bottom of glass vials (8 ml capacity). Open vials were exposed to the fumigants and high temperatures. Inoculum in tightly closed vials as well as untreated cysts were used as controls. Spores and cysts were heat-treated in a laboratory oven (Imperial II, Lab-Line Instruments, Melrose Park, IL) at 70, 80, and 90°C for periods of 12 and 24 hours.

Estimation of Germination: Germination was estimated by counting the number of germinated and ungerminated spores in four quadrats on each replicated agar block after 72 hours of incubation. Based on the inoculum volume and concentration described above (2 1 x 10^7 - 10^8 spores/ml), ca 25 - 250 spores/0.08 mm^2 microscope field (500X) were counted. Thus

100 to 1,000 spores were counted per replicate. Spores were reincubated in CO_2 for an additional 48 hours and re-examined for germ tube growth. Statistical analyses of variance of the percentage of germinated spores were conducted using the Dunnet and Student-Newman-Keuls tests for multiple comparisons of treatment means (Steel and Torrie, 1960, p. 110).

RESULTS

Amphotericin B at 1% was the only fungicide applied to the agar block surface and incubated with spores to cause significantly reduced germination (Table 2). Additional tests with amphotericin B at 0.1 and 0.01% did not reduce germination below the levels of the control. Readings of the treated blocks were made 72 and 120 hours after the onset of incubation and the efficacy of the compounds as sporicides (i.e., the absence of germination) was of primary interest. With many of the fungicides there was no further growth after the spores had swollen significantly (Table 2, Growth; Figure 1). As the spores were in continuous contact with the test materials, we concluded that the swelling (germ tube formation) resulted in spore wall changes, and the effect of the fungicide was principally against growth rather than germination. Miconazole and miconazole nitrate at 0.1% incorporated into the medium were sporicidal (Table 2).

The highest concentration (1%) of the even-numbered short chain fatty acids (C-6 and C-8) significantly reduced spore germination but never below 20% (Table 3). However, the C-6, C-8, and C-10 acids at both 0.1 and 1.0% caused abnormal germination patterns in which no growth occurred after the initial spore swelling.

Odd numbered fatty acids from C-7 through C-11 were completely sporicidal at 1% (Table 4). Both the C-11 (undecanoic acid) and C-11:1 (undecylenic acid) were the most effective fatty acids causing significant reductions in germination at 0.1%. The unsaturated undecylenic acid is the active ingredient in several commercial antifungal preparations (Windholz et al., 1976). The C-7, C-9, and C-11 fatty acids caused abnormal germination at concentrations of 0.1 and 1.0%, whereas, undecylenic acid prevented growth beyond germination at 0.01%, the lowest concentration tested.

Several of the 10 fatty acid derivatives known to have sporicidal properties caused significant reductions in spore germination at 1.0% (Table 5). Of these only the designate GP-28 was completely effective at 1.0%. GP-28, GP-912, PH-118, and PH-252 caused abnormal germination and germ tube death at both 0.1 and 1.0%.

None of the 11 non-halogenated general microbicides tested were found to be effective sporicides at the dosages employed (Table 6). Only two of the compounds significantly reduced germination: 0.5% Kleen-Quat® at 1 minute and 10% formalin at 20 minutes. All germ tubes from treated spores, except those exposed to thimerosal, appeared normal and growth continued with further incubation. Spores treated with thimerosal assumed early germination characteristics but no further growth occurred.

Spores continously agitated in a 0.5% solution of sodium hypochlorite (NaOCl) for 0.5 to 3 minutes all had significantly reduced germination (Table 7). Comparable treatment in a 5.0% NaOCl solution resulted in complete spore kill. In tests of the efficacy of low concentrations of NaOCl (0.01, 0.05, and 0.10%) for 30 and 60 seconds, only the 60 second exposure to the 0.1% solution resulted in sporicidal activity (Tables 8 and 14). The effect of pH modification of NaOCl solutions on spore germination

is shown in Table 9. Agitation of spores for 1 minute in 0.1 and 1.0% solutions at pH 5 and 7 and in a 1.0% solution at pH 9 resulted in significant reductions in germination. An increase in the pH for a given concentration of NaOCl yielded lower sporicidal activity.

The combination of 35% sucrose with 0.5% NaOCl, a solution used in our laboratory to surface sterilize leafcutting bee eggs and larvae without adversely affecting the osmotic balance, proved to have no sporicidal activity on A. aggregata (Table 10).

Free iodine in a solution of potassium iodide (I in KI) affected spore germination significantly at 0.01 and 0.1% when spores from two sources were exposed for 5 and 60 seconds (Table 11). The 1% free iodine gave complete spore kill at both 5 and 60 seconds. Potassium iodide (KI) was not effective in reducing spore germination at concentrations to 1.0% (Table 12). Free iodine on the other hand gave significant control at 0.1 and 1.0%.

The povidone-iodine preparations (Betadine® and Bovidone) in concentrations of 0.01 to 1.0% did not significantly reduce germinability of treated spores (Table 13).

Spores and spore cysts exposed to combinations of pH-modified (pH 7) NaOCl and I at 0.1 and 0.05%, 0.1 and 0.1%, 0.01 and 0.1%, and 0.05 and 0.1%, respectively, suffered near complete (0.05 and 0.1%) mortality. The combination of these two compounds was more sporicidal to A. aggregata than either compound alone at comparable concentrations (Table 14).

Stabilized dry chlorine (sodium dichloro-s-triazinetrione dihydrate, Georgia-Pacific), a product used to control the growth of microorganisms in swimming pools, gave significant and consistent control at concentrations of 0.5 and 1.0%. Even brief agitation of spores (15 and 30 sec) resulted in complete or near complete mortality (Table 15).

The effects of the wetting agents Tween 20 and Triton X-100®, alone and in combination with 0.5% stabilized dry chlorine, are summarized in Table 16. Neither of the adjuvants alone (0.05%) had any sporicidal effect in the 30 and 60 second exposures. Their incorporation into 0.5% solutions of stabilized dry chlorine resulted in complete spore kill at both agitation times.

Of the fumigants tested, chloropicrin + DMSO, chloropicrin + methyl bromide, EL-228®, and 1% ozone lowered germination significantly. However, none gave practical control. KWG-0599® and ethylene oxide (test b) enhanced germination over that of the controls, but none of the treated spores showed further growth (Table 17).

The effects of high temperature treatments of fresh spores and spore cysts from two sources (Idaho-18 and California-1) are summarized in Table 18. Analysis of variance showed no significant differences between the 12 and 24 hour treatments at 50° and 70°, and 90°C. Germination below that of untreated controls occurred at all temperatures. Complete inactivation of both inocula occurred after treatment for 24 hours at 90°C, but some germination was recorded in the CA-1 sample kept at 90° for 12 hours.

Spores from three of six sources were completely inactivated when exposed to 90°C for 12 hours (Table 19). Inoculum from one source (ID-5) germinated at ca 5% following heat treatment and those from two other sources at less than 1%.

DISCUSSION

General prophylactic programs to reduce the impact of the disease (see Introduction) were developed at a time when there was uncertainty as to the causative organism(s) and when the most commonly associated fungus could

not be germinated or cultured in the laboratory (Stephen and Undurraga, 1978). The ability to culture the fungus (Kish, 1980) coupled with evidence that it is the causative organism of the disease (Vandenberg and Stephen, 1982) has permitted this systematic survey of fungicidal and general microbicidal agents.

A wide spectrum of chemicals was screened in this study partly because of the broad resistance shown by the pathogen and partly because of the different points in the life cycle of the host at which different sporicides may be integrated into a disease control program. For these preliminary screenings the initial concentrations of all chemicals tested was from 2 to 10 times the highest label recommendations. Promising sporicides and fungicides were subsequently retested to determine minimum effective concentrations.

There are two stages in the fungus life cycle during which it may be attacked: the vegetative growth stage (germ tubes and mycelia), and the reproductive-dispersal stage (spores). The vegetative stage is usually more susceptible to fungicides, but in nature spores of A. aggregata germinate only in the gut of the larva growing in a cell located in an inaccessible nesting tunnel. Of the fungicides and general antibiotics screened, only miconazole and miconazole nitrate exhibited sporicidal properties, whereas, most others were effective after spore germination. A number of fatty acids and their derivatives exhibited sporicidal activity at intermediate concentrations (0.10% - Tables 3, 4, 5), but only undecylenic acid inhibited growth at 0.01%. To be effective, such fungicides would have to be incorporated into the pollen masses of the bee and ingested by the larva as it consumes the spores. Work along these lines has not been pursued because of the logistical problems of having the bee

collect and transport such foreign materials into the nest. Should it be possible to "contaminate" the pollen provisions with a sporicide or fungicide, testing for its effect on larval development and survival would be necessary.

The pathogen is most accessible as a spore during its dispersal phase. In heavily contaminated leafcutting bee populations large numbers of spores can be found on the leafy material from which the cells are constructed, on and about the nesting domiciles of the bee, in tunnels of the medium from which bees have emerged, and on the body surface of newly emerged bees (Stephen et al., 1981; Vandenberg et al., 1980). The latter two serve as the greatest inoculum source. At least three different tactics may optimize disease control: 1) surface sterilization of the walls and the floors of field domiciles before and during the bee flight period using a sporicidal, economical, aqueous chemical, 2) sterilization of nesting media each year with a sporicidal rinse, fumigation, or heat treatment, and 3) surface sterilization of the body of the bee before its release in the field using a sporicidal aqueous chemical which is non-toxic to the bee.

To successfully employ Tactics 1 and 3, the sporicide(s) should be rapid acting, easy to formulate, economical to use, and preferably should have residual properties non-toxic to the bee. Of the compounds tested, the halogenated general microbicides come closest to satisfying the above criteria. Sodium hypochlorite was effective at concentrations of 0.5% and higher. Although the adjustment of the pH of the NaOCl solution to 5.0 increased the sporicidal properties, the acidification caused a rapid release of Cl and shortened its effective life. Free iodine appeared to have significant sporicidal effect at lower concentrations (0.1% for 5 sec). We recognize that iodine precipitates from solutions in the presence

of NaOCl, yet the sporicidal effects of the combination were greater than those of either of the compounds alone at equivalent concentrations. Iodine appeared to enhance the effect of NaOCl at very low concentrations (Table 14). Stabilized dry chlorine showed consistent sporicidal activity at 0.5 and 1.0% even at the lowest exposure times (15 seconds). Dry chlorine solution lacks the wettability of NaOCl, but addition of the surfactants Tween 20 or Triton X-100® enhanced its efficacy.

All three halogens exhibited properties which could make them candidates for incorporation into disease control programs. For the surface sterilization of field domiciles or of unoccupied bee nesting media, caution should be exercised in selecting a concentration well above the minimal effective dosage to insure complete control. Both chlorine compounds are inexpensive and readily degradable, thus solutions of less than 3% may be only partially effective. The use of either chlorine or iodine materials for surface sterilization of emergent bees will require further testing to determine the optimal material and dosage. These studies are currently underway in this laboratory.

Considerable variation was recorded in the percent germination of comparably treated samples from the same spore source. The principal variation can be attributed to different techniques used in exposing spores to test materials (see Methods). Most of the iodine tests were run by passing the solution continuously over a sample of spores collected on a polycarbonate filter (Tables 11-13). It is suspected that the higher germination recorded is a result of an accumulation or clumping of the spores and spore balls on the filter which may have protected some from the full effects of the test solutions. The sporicidal effects of the same

materials were significantly greater when the spores were kept in suspension and shaken continuously for the prescribed exposure times (Table 14). Other minor variation can be attributed to the presence of unruptured spore balls in each sample at the time of treatment. The effects of lower concentrations of the microbicides may have been limited to spores on the periphery of the ball, for upon plating, spore germination was usually evident only in and about the ruptured spore balls.

Contrary to the effects of many of the fungicides and fatty acids, no abnormal growth was observed in any spores treated with halogenated compounds. The materials were either sporicidal, in which case the spore did not change shape or size, or their growth patterns were normal.

Fumigants were tested to find a simple means of decontaminating wooden nesting media which are adversely affected by repeated exposure to aqueous sterilants. None of the fumigants proved to be sporicidal although KWG-0599® and ethylene oxide treated spores did not grow beyond the initial swelling (Table 12). However, in these tests spores were exposed in open vials and may have been much more vulnerable than spores concealed by pollen and leafy residues in the nesting tunnel. Further tests under conditions approximating those in the field are warranted with both of these materials.

"Solid" boards (i.e., those with holes drilled into, but not completely through a wooden block) are the most widely used nesting medium in the Pacific Northwest. In the process of recycling, the boards are "redrilled" to remove most of the residue, but because of the blind tunnels neither liquid nor fumigants can readily penetrate. Heat sterilization of the medium has been recommended and widely used for the past several years even though definitive information on heat susceptibility of the spores was

unavailable (Stephen and Undurraga, 1978; Kish et al., 1981). These studies showed significant variability in the heat sensitivity of the fungus with 3 of the 6 samples having viable spores after 12 hours at 90°C. Although the spore kill was not complete, germinability was sharply reduced indicating that heat may be used as a means of controlling rather than eliminating the disease. Again it should be noted that in these tests the spores were completely exposed rather than covered by residue in a tunnel of a solid board.

Several possible strategies are presented as means of reducing the inoculum reservoir of Ascosphaera aggregata spores associated with the leafcutting bee. The halogens appear to be the most effective, economical, and available of the sporicides tested and can be utilized to control spores on and in nesting media, field domiciles, and on the surface of loose cells. High temperatures may be effective for decontamination if the temperature of the spore can be maintained above 90°C for at least 12 hours.

The principal inoculum source is that found on the body surface of adult bees emerging from contaminated materials (Vandenberg et al., 1980). Methods of decontaminating bees upon their emergence must be developed before a satisfactory control program can be established.

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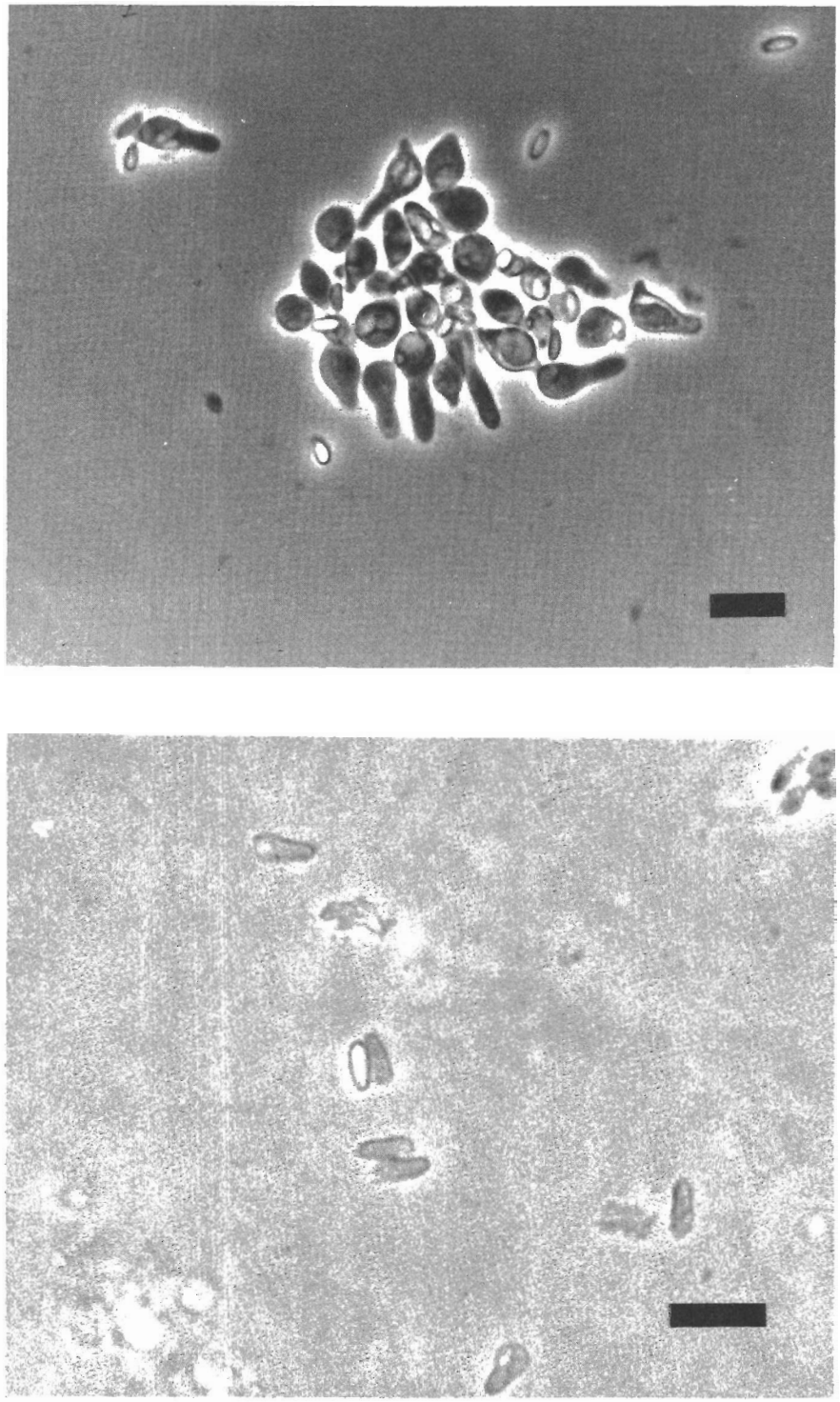

Figure 1. Normal germ tubes and spores of Ascosphaera aggregata (top), and abnormal germ tubes produced following exposure to certain fungicides (bottom), after 3 days incubation. Bar = 10 µm.

Table 1. Materials and treatments tested for sporicidal activity on Ascosphaera aggregata

General Microbicides: (non-halogenated)
- Chloramphenicol
- Copper sulfate
- Formalin
- 8-Hydroxyquinoline
- Hyamine-10X[R1]
- Kleen-Quat
- Merthiolate tincture
- Methyl-p-hydroxybenzoate
- Thimerosal

General Microbicides: (halogenated)
- Betadine[R7] (povidone-iodine)
- Bovidone (povidone-iodine)
- Iodine (free I in KI)
- Iodine + sodium hypochlorite
- Potassium Iodide (KI)
- Sodium hypochlorite
- Sodium hypochlorite + sucrose
- Stabilized Dry Chlorine (Stabil Clor 56)[R10] (sodium dichloro-s-triazinetrione dihydrate)
- Stabilized dry chlorine + Tween-20
- Stabilized Dry chlorine + Triton X-100[R1]

Fungicides and Antibiotics:
- Amphotericin B
- Benomyl (Benlate)[R4]
- CGA-64251[5]
- Ferbam (Carbamate)
- Clotrimazole
- Captafol (Difolatan-4FL)[R6]
- Gentamycin sulfate
- Griseofulvin
- Miconazole[9]
- Miconazole nitrate[9]
- Natamycin (Delvocid)
- Captan (Orthocide-50W)[R6]
- Folpet (Phaltan-50W)[R6]
- Sulfathiozole
- Thiobendazole (Mertect-340F)[R7]
- Tolnaftate

Fumigants:
- Chloropicrin
- Chloropicrin + DMSO
- Chloropicrin + Methyl bromide
- EL - 288[R3]
- Ethylene
- Ethylene oxide
- KWG-0599[R3]
- Ozone

Fatty Acids:
- Caproic
- Heptanoic
- Caprylic
- Nonanoic
- Capric
- Undecanoic
- Undecylenic
- Lauric
- Tridecanoic
- Myristic
- Palmitic
- Stearic
- Arachidic

Fatty Acid Derivatives:[11]
- GP-28
- GP-900
- GP-912
- MT-208
- PH-118
- PH-219
- PH-252
- RR-53
- RR-73
- RR-75
- CPS

Heat Treatment:
- 30-90°C for 12 & 24 h.

1. Rohm and Haas, Philadelphia, PA.
2. Unit Chemical, Los Angeles, CA.
3. Eli Lilly, Indianapolis, IN.
4. E. I. Du Pont, Wilmington, DE.
5. Ciba-Geigy, Greensboro, NC.
6. Chevron Chemical, Fresno, CA.
7. Purdue Frederick, Norwalk, CN.
8. Merck Chemical, Rahway, NJ.
9. Janssen R & D Inc., New Brunswick, NJ
10. Georgia- Pacific, Los Angeles, CA.
11. Dr. G. Puritch, Pacific Forest Research Center, Victoria, B.C., Canada

Table 2. Germination of Ascosphaera aggregata spores during exposure to fungicides and antibiotics [a]

Compound	% A.I.	Dose	Germ.[b]	Control Germ.[b]	Significance[c]	Growth[d]
Cycloheximide	1.0	(2 µl/cm^2 medium)	19.3 ± 0.6	21.3 ± 2.9 (2)	NS	N
Amphotericin B	1.0		2.3 ± 3.2		S	A
Gentamycin sulfate	1.0		82.0 ± 5.4	86.9 ± 4.7 (4)	NS	N
sulfathiozole	1.0		45.8 ± 14.1	52.2 ± 10.5 (4)	NS	N
Clotrimazole	1.0		33.9 ± 4.0	35.9 ± 6.8 (4)	NS	N
Natamycin (Delvocid)	0.5		50.0 ± 8.0	48.0 ± 4.0 (2)	NS	A
Benomyl (Benlate[R1])	2.7	(3 µl/cm^2 medium)	21.5 ± 12.9	61.4 ± 16.2 (2)	NS	A
Ferbam (Carbamate)	4.1		49.0 ± 0.1		NS	A
Captafol (Difolaton-4FL[R2])	3.9		62.6 ± 18.4		NS	A
Captan (Orthocide-50W[R2])	2.7		47.0 ± 1.4		NS	A
Folpet (Phaltan-50W[R2])	2.7		55.3 ± 18.4		NS	A
Thiabendazole (Mertect-340F[R3])	2.3		72.4 ± 1.0	76.9 ± 5.8 (2)	NS	N
(Confidential) (CGA-64251 10W-G[R4])	0.5		74.3 ± 3.6		NS	N
Tolnaftate	0.01	(in the medium)	50.0 ± 4.0	50.9 ± 3.4 (3)	NS	N
Griseofulvin			48.1 ± 6.0		NS	N
Miconazole			35.6 ± 7.2		NS	A
Miconazole nitrate			35.3 ± 10.9		NS	A
Griseofulvin	0.10	(in the medium)	43.3 ± 15.6		NS	A
Miconazole			0.0 ± 0.0		S	A
Miconazole nitrate			1.3 ± 1.2		S	A

R. 1. Dupont. 2. Chevron. 3. Merck. 4. Ciba-Geigy.

b. In percent ± standard deviation. Number of replicates in parentheses.

c. Dunnet's multiple comparison, $p < 0.01$.

d. N = normal germ tube formation. A = abnormal, no growth after initial spore swelling.

Table 3. Germination of Ascosphaera aggregata spores in response to exposure to fatty acids with an even number of carbon atoms

Fatty Acid	Concentration[a]	Germination[b]	Significance[c]	Growth[d]
Caproic (C-6)	0.01	47.7 ± 1.2	NS	N
	0.10	46.4 ± 10.8	NS	A
	1.00	20.4 ± 1.2	S	A
Caprylic (C-8)	0.01	47.8 ± 0.1	NS	N
	0.10	40.5 ± 3.7	NS	A
	1.00	23.7 ± 24.0	S	A
Capric (C-10)	0.01	38.5 ± 7.1	NS	N
	0.10	38.9 ± 1.5	NS	A
	1.00	27.2 ± 1.9	NS	A
Lauric (C-12)	0.01	53.1 ± 0.4	NS	N
	0.10	52.2 ± 0.9	NS	N
	1.00	38.7 ± 3.8	NS	A
Myristic (C-14)	1.00	65.3 ± 8.6	NS	N
Palmitic (C-16)	1.00	73.4 ± 4.1	NS	N
Stearic (C-18)	1.00	67.2 ± 15.1	NS	N
Arachidic (C-20)	1.00	62.4 ± 4.5	NS	N

a. Percent solution. C-6 to C-12 solubilized in 50% ethanol (EtOH); C-14 to C-20 solubilized in 95% EtOH; 3 µl added to each inoculated agar block.

b. In percent ± standard deviation, 2 replicates. 50% EtOH control germ.: 62.8 ± 0.7%. 95% EtOH control germ.: 61.6 ± 1.4%.

c. Dunnet's multiple comparison, $p<0.01$.

d. N = normal germ tube formation. A = abnormal; no growth after initial spore swelling.

Table 4. Germination of Ascosphaera aggregata spores in response to exposure to fatty acids with an odd number of carbon atoms

Fatty Acid	Concentration[a]	Germination[b]	Significance[c]	Growth[d]
Heptanoic (C-7)	0.01	64.3 ± 15.1	NS	N
	0.10	62.1 ± 2.6	NS	A
	1.00	0.0 ± 0.0	S	-
Nonanoic (C-9)	0.01	54.5 ± 9.3	NS	N
	0.10	41.0 ± 21.0	NS	A
	1.00	0.0 ± 0.0	S	-
Undecanoic (C-11)	0.01	38.7 ± 12.4	NS	N
	0.10	20.0 ± 3.1	S	A
	1.00	0.0 ± 0.0	S	A
Tridecanoic (C-13)	0.01	47.0 ± 19.0	NS	N
	0.10	45.2 ± 5.1	NS	N
	1.00	46.1 ± 2.6	NS	N
Undecylenic (C-11:1)	0.01	30.3 ± 2.1	NS	A
	0.10	10.2 ± 2.2	S	A
	1.00	0.0 ± 0.0	S	A

a. Percent solution in 50% ethanol (EtOH). 3 µl added to each inoculated agar block.

b. In percent ± standard deviation, 2 replicates. EtOH control germ.: 68.6 ± 11.4%.

c. Dunnet's multiple compariosn, $p<0.01$.

d. N = normal germ tube formation. A = abnormal; no growth after initial spore swelling.

Table 5. Germination of Ascosphaera aggregata spores in response to exposure to some fatty acid derivatives

Compound	Concentration[a]	Germination[b]	Significance[c]	Growth[d]
GP-28	0.01	52.2 ± 0.1	NS	N
	0.10	28.3 ± 1.8	S	A
	1.00	0.0 ± 0.0	S	-
GP-900	0.01	54.5 ± 4.4	NS	N
	0.10	40.5 ± 1.3	NS	N
	1.00	7.0 ± 9.0	S	A
GP-912	0.01	51.3 ± 11.9	NS	N
	0.10	41.2 ± 3.0	NS	A
	1.00	34.3 ± 7.8	NS	A
MT-208	0.01	59.8 ± 0.6	NS	N
	0.10	48.8 ± 0.8	NS	N
	1.00	38.7 ± 3.4	NS	A
PH-118	0.01	59.7 ± 3.2	NS	N
	0.10	43.3 ± 8.6	NS	A
	1.00	15.5 ± 2.3	S	A
PH-219	0.01	55.3 ± 3.3	NS	N
	0.10	57.0 ± 0.1	NS	N
	1.00	46.4 ± 0.1	NS	A
PH-252	0.01	59.4 ± 3.8	NS	N
	0.10	48.8 ± 17.7	NS	A
	1.00	21.3 ± 6.2	S	A
RR-53	0.01	61.4 ± 1.8	NS	N
	0.10	58.2 ± 7.8	NS	N
	1.00	42.4 ± 13.4	NS	A
RR-73	0.01	63.1 ± 0.2	NS	N
	0.10	40.3 ± 0.4	NS	N
	1.00	28.6 ± 0.2	NS	A
RR-75	0.01	58.4 ± 8.1	NS	N
	0.10	46.5 ± 3.1	NS	N
	1.00	40.5 ± 5.1	NS	A
CPS	0.01	45.0 ± 5.1	NS	N
	0.10	47.5 ± 8.6	NS	N
	1.00	49.2 ± 4.8	NS	N

a. Percent aqueous solution. 3 µl added to each inoculated agar block.

b. In percent ± standard deviation, 2 replicates. Control germ.: 59.1 ± 6.4%.

c. Dunnet's multiple comparison, $p<0.05$.

d. N = normal germ tube formation. A = abnormal; no growth after initial spore swelling.

Table 6. Effect of non-halogenated general microbicides on Ascosphaera aggregata spore germination

Compound	Dose[a]	Germination[b]	Control Germination[b]	Significance[c]	Growth[d]
Kleen-Quat R1	0.005	73.2 ± 4.3	67.2 ± 2.4 (2)	NS	N
	0.05	70.2 ± 4.8		NS	N
	0.5	37.4 ± 9.8		S	N
Methyl-p-hydroxybenzoate	0.25	48.0 ± 6.5	44.6 ± 3.2 (4)	NS	N
Thimerosal	1.0	50.1 ± 5.0			A
Sorbic acid	1.0	41.2 ± 8.2	52.2 ± 10.5 (4)	NS	N
Merthiolate tincture	0.1	53.7 ± 5.0	43.8 ± 11.0 (4)	NS	N
Chloramphenicol	1.0	42.8 ± 11.0	50.0 ± 3.4 (4)	NS	N
Thymol	1.0	61.7 ± 5.5			N
8-Hydroxyquinoline	1.0	39.5 ± 2.8	52.4 ± 9.5 (2)	NS	N
Copper sulfate	1.0	29.5 ± 5.7			N
Hyamine-10X R2	4.0	26.4 ± 5.3	21.5 ± 0.3 (2)	NS	N
Formalin, buffered pH 7	1.0	77.1 ± 0.8	79.3 ± 4.7 (2)	NS	N
	10.0	27.8 ± 0.7		S	N

R 1. Unit Chemical. 2. Rohm and Haas.

a. In percent. Spores exposed for 1 min at 25 ± 2°C except formalin exposure for 20 min, on the filter.

b. In percent ± standard deviation. Number of replicates in parentheses.

c. Dunnet's multiple comparison, $p<0.01$.

d. N = normal germ tube formation and growth. A = abnormal; no growth after initial spore swelling.

Table 7. Effect of sodium hypochlorite on *Ascosphaera aggregata* spore germination

Compound	Time[a]	Germination[b]
H_2O	0.5	11.5 ± 3.2
	1.0	12.9 ± 3.7
	2.0	16.7 ± 7.2
	3.0	15.2 ± 3.2
0.5% NaOCl	0.5	5.1 ± 8.7
	1.0	0.6 ± 0.6
	2.0	0.5 ± 0.7
	3.0	3.2 ± 1.9
5.0% NaOCl	0.5	0.0 ± 0.0
	1.0	0.0 ± 0.0
	2.0	0.0 ± 0.0
	3.0	0.0 ± 0.0

a. Exposure time on the filter in minutes at 25°C. pH 11-11.5

b. In percent ± standard deviation, 4 replicates.

Table 8. Effect of low sodium hypochlorite concentrations on germination of Ascosphaera aggregata spores

Time[a]	Concentration[b]	Germination[c]
30	0.00	61.1 ± 3.1
	0.01	57.2 ± 7.2
	0.05	58.5 ± 4.7
	0.10	43.7 ± 4.2
60	0.00	54.1 ± 6.4
	0.01	60.4 ± 3.5
	0.05	50.8 ± 7.6
	0.10	0.0 ± 0.0

a. Exposure time in seconds on filter.

b. In percent pH 11-11.5

c. In percent ± standard deviation, 2 replicates.

Table 9. Effect of pH and concentration of NaOCl on germination of Ascosphaera aggregata spores

pH	NaOCl[a]	Germination[b]	Significance[c]
5.0	0.01	57.2 ± 0.2	i
	0.10	3.9 ± 2.5	k
	1.00	0.0 ± 0.0	k
7.0	0.01	49.3 ± 4.6	ij
	0.10	3.4 ± 4.5	k
	1.00	5.2 ± 7.2	k
9.0	0.01	61.2 ± 4.1	i
	0.10	35.0 ± 7.0	j
	1.00	8.9 ± 9.0	k

a. Percent solution, spores exposed 1 min. at 25°C., on the filter.

b. In percent ± standard deviaion, 2 replicates. Untreated control germ.: 61.0 ± 3.6%.

c. A-N-K multiple comparisons test, $P<0.01$. Means followed by the same letter are not significantly different.

Table 10. Effect of sucrose on sodium hypochlorite efficacy toward spores of Ascosphaera aggregata

Compound	Time[a]	Germination[b]
35% sucrose (w/v)	0.5	52.9 ± 4.3
	2.0	55.8 ± 6.8
	5.0	51.2 ± 8.0
35% sucrose + 0.5% NaOCl	0.5	57.1 ± 4.2
	2.0	50.8 ± 10.0
	5.0	51.6 ± 6.3

a. Exposure time in minutes at 25°C., on the filter.

b. In percent ± standard deviation, 4 replicates.

Table 11. Effect of iodine solutions on Ascosphaera aggregata spore germination

Time[a]	Concentration[b]	Germination[c] ID inoculum	CA inoculum
5	0.00	64.1 ± 5.9 i	40.4 ± 1.1 x
	0.01	30.2 ± 3.4 j	27.3 ± 5.6 y
	0.10	19.9 ± 5.3 jk	21.3 ± 3.2 y
	1.00	0.0 ± 0.0 k	0.0 ± 0.0 z
60	0.00	51.2 ± 13.8 i	52.8 ± 4.3 w
	0.01	22.4 ± 2.1 j	16.0 ± 3.8 y
	0.10	11.0 ± 4.4 jk	17.6 ± 1.1 y
	1.00	0.0 ± 0.0 k	0.0 ± 0.0 z

a. Exposure time in seconds at 25°C., on the filter.

b. Percent iodine (I) in final solution; 0.00 = water control.

c. In percent ± standard deviation, 2 replicates. For each inoculum, means followed by the same letter are not significantly different; S-N-K multiple comparisons, $p<0.01$.

Table 12. Effect of iodine solutions on Ascosphaera aggregata spore germination

Compound	Concentration[a]	Germination[b]
H_2O (control)	--	82.9 ± 3.2 i
KI	0.01	81.8 ± 3.4 i
	0.10	79.0 ± 4.3 i
	1.00	70.4 ± 5.4 i
I in KI	0.01	50.2 ± 10.7 j
	0.10	18.9 ± 18.8 k
	1.00	1.6 ± 1.4 k

a. Percent solution. Spores exposed on the filter 1 min at 25°C. 4 replicates.

b. In percent ± standard deviation. Means followed by the same letter are not significantly different, $p<0.01$. S-N-K multiple comparisons test.

Table 13. Effect of iodine formulations on Ascosphaera aggregata spore germination

Concentration[a]	Iodine	Betadine[Rb]	Bovidone[c]
0.00	67.5 ± 10.9[d]	69.9 ± 3.4	66.8 ± 4.0
0.01	39.3 ± 14.7	34.1 ± 3.3	59.1 ± 20.6
0.10	25.1 ± 18.3	28.1 ± 7.7	41.7 ± 1.4
1.00	3.3 ± 0.1[e]	23.4 ± 9.0	32.0 ± 3.0

a. Percent (I) in final solution. 0% = water control. Spores exposed 1 min at 25°C., on the filter.

b. Purdue Frederick; povidone-iodine.

c. Povidone-iodine.

d. In percent ± standard deviation, 2 replicates.

e. Significantly different from other treatment means. S-N-K test, $p<0.05$.

Table 14. Effect of sodium hypochlorite and iodine solutions on Ascosphaera aggregata spore germination

NaOCl[a]	I[a]	Germination[b]	Control Germination[b]	Significance[c]
0.10	--	19.6 ± 24.2	61.1 ± 1.5	S
--	0.01	42.9 ± 1.7		NS
--	0.05	22.3 ± 5.4		NS
--	0.10	5.9 ± 5.0		S
0.10	0.01	23.3 ± 12.4		NS
0.10	0.05	0.5 ± 0.1		S
0.10	0.10	0.5 ± 0.7		S
--	0.10	0.7 ± 1.0	62.8 ± 0.4	S
0.01	--	59.0 ± 13.6		NS
0.05	--	66.8 ± 11.4		NS
0.10	--	9.5 ± 12.0		S
0.01	0.10	0.9 ± 1.2		S
0.05	0.10	0.0 ± 0.0		S
0.10	0.10	1.4 ± 0.6		S

a. Percent solution, pH 7.0, spores exposed 1 min. at 25°C., on on the filter.

b. In percent ± standard deviation, 2 replicates

c. Dunnet's multiple comparison, $p<0.01$.

Table 15. Germination of *Ascosphaera aggregata* spores following exposure of Dry Chlorine[a] solutions

Time[b]	Percent Dry Chlorine	Germination[c]	Significance[d]
15	0	48.8 ± 4.3	ij
	0.1	61.3 ± 15.6	ij
	0.5	1.9 ± 0.9	k
	1.0	0.0 ± 0.0	k
30	0	43.8 ± 3.0	ij
	0.1	24.2 ± 18.4	jk
	0.5	0.0 ± 0.0	k
	1.0	0.0 ± 0.0	k
45	0	57.2 ± 7.2	ij
	0.1	49.4 ± 11.3	ij
	0.5	0.0 ± 0.0	k
	1.0	0.0 ± 0.0	k

a. Dry Chlorine = sodium dichloro-s-triazinetrione dihydrate (Georgia-Pacific).

b. Exposure time in seconds at 25°C., on the filter.

c. In percent ± standard deviation, 2 replicates.

d. S-N-K multiple comparisons, $p<0.01$. Means followed by the same letter are not significantly different.

Table 16. Germination of Ascosphaera aggregata spores following exposure to Dry Chlorine and surfactants

Time[a]	Compound[b]	Germination[c]	Control Germination[c]
30	Tween 20	64.2 ± 0.2	69.7 ± 11.6
	Triton X-100[R]	72.5 ± 13.6	
	Dry Chlorine	2.4 ± 2.1	
	Tween 20 + Dry Chlorine	0.0 ± 0.0	
	Triton X-100 + Dry Chlorine	0.0 ± 0.0	
60	Tween 20	76.5 ± 16.0	65.3 ± 3.2
	Triton X-100	56.8 ± 0.8	
	Dry Chlorine	0.0 ± 0.0	
	Tween 20 + Dry Chlorine	0.0 ± 0.0	
	Triton X-100 + Dry Chlorine	0.0 ± 0.0	

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a. Exposure time in seconds at 25°C., by continuous agitation.

b. Tween 20 and Triton X-100 at 0.05% in solution, Dry Chlorine at 0.5%.

c. In percent ± standard deviation, 2 replicates.

Table 17. Germination of Ascosphera aggregata spores in response to fumigant exposure

Fumigant	Dose	Germination[a]	Control Germination[a]	Significance[b]	Growth[c]
Chloropicrin[d]	33 ml/m^3	14.7 ± 5.9	20.4 ± 2.1 (3)	NS	N
Chloropicrin + DMSO[e]		4.5 ± 5.6		S	N
Chloropicrin + CH_3Br[f]	17 ml/m^3	10.6 ± 3.7	20.1 ± 4.6 (4)	S	N
Chloropicrin + Ch_3Br[f]		17.9 ± 3.1	31.2 ± 3.6 (4)	S	N
EL-228[R g]	1.8 mg/m^3	23.1 ± 4.6	19.3 ± 3.6 (4)	NS	N
		18.1 ± 2.9	18.9 ± 8.4 (4)	NS	N
	18. mg/m^3	25.8 ± 3.6	43.7 ± 5.9 (4)	S	N
		24.2 ± 3.9	43.3 ± 4.1 (4)	S	N
KWG-0599[R g]	18. mg/m^3	48.3 ± 12.3	26.3 ± 5.1 (4)	S	A
		60.1 ± 8.9	31.7 ± 6.6 (4)	S	A
Ethylene[h]	2000 ppm	48.9 ± 1.6	35.5 ± 4.7 (2)	NS	N
	10000 ppm	18.9 ± 4.7		NS	N
Ethylene oxide[i]	a) 138 g/m^3,12 h	16.3 ± 3.3	26.3 ± 11.8 (4)	NS	A
	,24 h	6.7 ± 1.1	14.3 ± 7.9 (4)	NS	A
	b) ,12 h	68.6 ± 15.1	30.5 ± 6.4 (4)	NS	A
	,24 h	46.9 ± 6.8		NS	A
Ozone[j]	1%, 15 sec	47.2 ± 1.0	58.3 ± 5.4 (4)	NS	N
	30	45.3 ± 13.4		NS	N
	60	36.3 ± 11.5		S	N

a. In percent ± standard deviation, number of replicates in parentheses.
b. Dunnet's multiple comparison, $p<0.01$.
c. N = normal germ tube formation and growth, A = abnormal; no growth after initial swelling.
d. All chloropicrin exposures for 24 h at 35°C.
e. DMSO = dimethyl sulfoxide, 16 g/m^3 volatilized at 95°C over 24 h.
f. CH_3Br = methyl bromide, 24 g/m^3 for 24 hrs at 35°C.
g. R. Ely Lilly, volatilized at 65°C over 24 h.
h. 24 h at 25°C.
i. Exposed at 40°C.
j. Exposed at 25°C.

Table 18. Effect of high temperature treatment on germination of Ascosphaera aggregata spores [1]

Temperature	Exposure Time	Germination[a] ID-18 inoculum	Germination[a] CA-1 inoculum
30°C[b]	12 h	58.8 ± 7.1 i	52.6 ± 3.4 w
	24	61.2 ± 10.8 i	42.1 ± 2.0 x
50	12	54.9 ± 0.5 i	34.5 ± 1.1 xy
	24	48.2 ± 1.0 i	32.1 ± 5.0 y
70	12	16.2 ± 4.2 j	6.1 ± 1.4 z
	24	13.8 ± 0.4 j	7.4 ± 0.8 z
90	12	0.0 ± 0.0 j	0.9 ± 1.2 z
	24	0.0 ± 0.0 j	0.0 ± 0.0 z

a. In percent ± standard deviation, 2 replicates. For each inoculum means followed by the same letter are not significantly different, S-N-K multiple comparison, $p<0.01$. Each inoculum analyzed separately. Untreated control germination: ID-18 84.4 ± 3.7; CA-1 64.4 ± 5.8%.

b. Relative humidity ca 25%.

Table 19. Effect of high temperature treatment on germination of *Ascosphaera aggregata* spores from various sources

Inoculum[a]	Germination[b]	Control Germination[b]
CA-1	0.7 ± 0.6	47.5 ± 5.1
ID-5	4.7 ± 1.5	72.9 ± 5.7
ID-18	0.0 ± 0.0	59.5 ± 5.0
NE-5	0.0 ± 0.0	50.3 ± 3.5
OR-1	0.2 ± 0.3	84.1 ± 5.0
OR-3	0.0 ± 0.0	62.6 ± 9.0

a. All inocula exposed to 90°C for 12 h.

b. In percent ± standard deviation, 4 replicates.