Inhibitory Activity of Sulfur Dioxide on the Germination of Spores of *Botrytis cinerea*

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We thank the California Table Grape Commission for financial assistance. We also thank Barry Gump and Carlos Muller for advice and critical reading of the manuscript.

Accepted for publication 28 August 1989.

ABSTRACT

Smilanick, J. L., Hartsell, P. L., Henson, D., Fouse, D. C., Assemi, M., and Harris, C. M. 1990. Inhibitory activity of sulfur dioxide on the germination of spores of *Botrytis cinerea*. Phytopathology 80:217-220.

Spores of gray mold (*Botrytis cinerea*) were exposed to sulfur dioxide for 4 hr in dilute, buffered grape juice at pH 3.0, 3.5, and 4.0, acidities approximating those of mature table grapes. Concentrations of 2.8, 9.6, or 33.8 μg/ml total SO_2, respectively, were required to kill 99% of the spores. The concentration of sulfur (IV) species (SO_2 and HSO_3^-) at each acidity was calculated using the Henderson-Hasselbalch equation from the total sulfur dioxide concentration that killed 50% (LD_{50}) of the spores. "Molecular" sulfur dioxide (SO_2) was present at 0.10–0.11 μg/ml, whereas bisulfite (HSO_3^-) ranged from 1.6 to 17.1 μg/ml at LD_{50}. This confirmed that the "molecular" form of SO_2 was the primary toxic species. Spores were exposed to SO_2 in dilute, buffered grape juice at pH 4 for 4 hr at 0, 10, 15, 20, 25, and 32 C. Concentrations of 886, 343, 300, 57.8, 49.3, and 25.8 μg/ml total SO_2 were required, respectively, to kill 99% of the spores. The concentration and distribution of total SO_2 residues within fumigated grapes were determined by ion chromatography. After a 30-min fumigation with 5,000 or 10,000 ppm SO_2, the skin and pulp contained 7.6–16.5 and 0.7–0.9 μg/g SO_2, respectively. Residues on or near the surface of fumigated grapes approached the toxic dosage above 20 C, whereas residues in the pulp were insufficient to kill the spores in dose-mortality tests.

Table grapes (*Vitis vinifera* L.) in California can be stored several months or more after harvest if refrigerated and periodically fumigated with sulfur dioxide (10,18). Sulfur dioxide fumigation inhibits decay by *Botrytis cinerea* Pers.:Fr. by killing the spores and mycelia of the fungus on the surface of the fruit, but infections established before harvest continue to develop within individual berries, making periodic refumigation necessary (17). Sulfur dioxide is typically applied to packaged grapes for 30 min at 5,000–10,000 parts per million (ppm) for the first fumigation, followed by weekly fumigations of 2,500 ppm (10,18,23). Fumigations with lower concentrations of sulfur dioxide at shorter intervals than those schedules usually employed by industry have been employed successfully (15).

The concentration of sulfur dioxide gas used in commercial fumigations exceeds that shown to kill *B. cinerea* in chamber studies. Death of the spores and mycelium of *B. cinerea* occurs after about 20 min of exposure to 100–200 ppm sulfur dioxide at 0–1 C under the high humidity conditions under which grapes are stored (7,16). In a constant atmosphere, only 5–10 ppm are required to inhibit fungal growth at 0–1 C (6,20).

The potential health risk that ingestion of sulfur dioxide poses for sulfite-sensitive individuals has recently been recognized, and many uses of this chemical have become regulated. The sulfur dioxide residue tolerance for table grapes is 10 ppm (or μg/g)(3). This tolerance is occasionally exceeded in commercial practice. More information on how sulfur dioxide fumigation reduces postharvest decay is needed, since new fumigation schemes may be needed to reduce residues on grapes. Although it is known that the gas kills the spores and mycelium of the fungus on the fruit surface but does not eliminate the infection, the role of residues on decay control has not been addressed experimentally. The toxicity of sulfur dioxide to *B. cinerea* in an environment such as grape flesh, where the molecule exists in both ionic and neutral forms, has not been determined. Furthermore, the concentration and location of sulfite residues in grape tissue after fumigation is not known. The purposes of this work were to establish the toxicity of sulfite solutions to *B. cinerea* and to quantify and localize sulfite residues in grapes after fumigation.

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MATERIALS AND METHODS

Sulfur dioxide toxicity assays. Freshly harvested Thompson Seedless grapes grown in central California were obtained and stored at 0 C before use. Grape juice was prepared by macerating whole, stemmed grapes in a centrifugal juicer with filter (Olympic Products, San Dimas, CA), then clarified further by passage through a Whatman No. 1 filter. The juice contained 14–15% soluble solids. The buffered assay solution employed for sulfur dioxide spore germination experiments contained 20 mM each of glycylglycine and HCl disodium phosphate plus 10% (v/v) grape juice and was adjusted to pH 3.0, 3.5, or 4.0 with 1 N KOH or HCl. The acidities selected approximate that of mature table grapes. Sodium bisulfite (assay 61.1% sulfur dioxide) was used for sulfur dioxide solutions that were prepared in water immediately before use.

Seven- to 10-day-old cultures of single-spore isolates of *B. cinerea* from Thompson Seedless grapes were used in spore germination assays. The fungus was cultured on potato-dextrose agar (PDA) at 20 C and was subcultured no more than twice from original single-spore isolates preserved on silica gel before use. Spores were obtained from culture plates by agitation of the colony surface with a glass rod with 5 ml of 0.05% Triton X-100, and the solution was filtered through two layers of cheesecloth followed by low-speed centrifugation, decanting of the Triton X-100 solution, and resuspension of the spores in sterile distilled water. Cultures designated BCG6, BCG8, and BCG11 were isolated from California grapes, whereas BCG14 was isolated from a package of grapes imported from Chile.

The spore germination assay procedure used 3 ml of buffered assay solution, 0.5 ml of *B. cinerea* spores at a concentration of 10^6 spores per milliliter, and 0.5 ml of an appropriately diluted sulfur dioxide solution. After 4 hr at 25–27 C, the assay solution was decanted and replaced with sterile distilled water, and 0.5 ml of the solution was plated on PDA. To determine the influence of temperature on sulfur dioxide toxicity, a water bath was employed to maintain temperatures of 0, 10, 15, 20, 25, and 32 C during the sulfur dioxide exposure when the assay solution was decanted, and the spore pellet was rinsed twice with sterile distilled water of the same temperature as the assay before the solution

was plated on PDA. After 24 hr at 20 C, the proportion of germinated spores was determined by examining 200–300 spores by light microscopy (100×). Spores were counted as germinated if the germ tube length exceeded the diameter of the spore. A minimum of 10 sulfur dioxide concentrations were used to develop a mortality curve at each acidity for each isolate. The control treatments were 4-hr exposures of spores in the assay solution at each pH without sulfur dioxide. Germination of spores from control treatments exceeded 95%. The LD_{50} and LD_{95} sulfur dioxide concentrations and 95% confidence intervals were estimated using Finney's probit analysis (9).

The concentration of sulfur dioxide in assay solutions was determined by the method of Beutler (5) using a sulfite oxidase-NADH peroxidase assay kit sensitive to about 0.5 μg/ml sulfur dioxide (Boehringer Mannheim Biochemicals, Mannheim, Germany). In aqueous solution, sulfur dioxide is present as several species according to the following equilibria:

$$H_2SO_3 + H_2O \overset{pK_1}{\rightleftharpoons} H^+ + HSO_3^- \overset{pK_2}{\rightleftharpoons} 2H^+ + SO_3^= \ (SO_2).$$

Although the undissociated free dibasic acid (H_2SO_3) is predicted by this equilibrium, evidence shows that the free acid does not occur in aqueous solutions (28). Raman and infrared spectra of sulfur dioxide solutions indicate that "molecular" SO_2 is present in place of the free acid (H_2SO_3)(28).

The concentrations of the "molecular" SO_2, bisulfite (HSO_3^-), and sulfite ($SO_3^=$) species in solutions were calculated from the solution pH using the Henderson-Hasselbalch equation with pK_1 and pK_2 values of 1.81 and 7.18 (28).

Sulfur dioxide fumigation and residue analysis. Grapes were fumigated at 0 C in a 117-L volume chamber. The sulfur dioxide concentration during fumigation was monitored by constantly pumping the chamber atmosphere through a nondispersive, infrared analyser (Horiba PIR-2000, Kyoto, Japan). A 15-cm diameter, four-blade fan moving 50 m/min in the chamber provided uniform distribution of sulfur dioxide during treatment. The gas was dispensed into a gas-tight syringe from a lecture bottle of SO_2 (Matheson Gas Products, Secaucus, NJ) and injected into a port in the fumigation chamber; concentrations used were 10,000 and 5,000 ppm and did not decrease more than 500 ppm during the 30-min fumigation period. The grapes were aerated 30 min after fumigation, frozen in liquid nitrogen, and stored at −15 C before analysis.

The enzymatic assay employed for spore germination assays in buffered sulfite solutions was unsatisfactory with the grape tissue because of turbidity and the rapid sulfur dioxide oxidation that occurs when grape tissue is macerated (20). To localize residues in grape tissues, the "skin" of frozen grapes was isolated by removing the cuticle, exocarp, and 1–2 mm of subsurface tissue with a scalpel. The "pulp" was the remaining tissue. Sulfur dioxide residues in grapes were quantified by macerating 50 g of frozen grape tissue under liquid nitrogen for 2 min at high speed in a Waring blender in 100 ml of 150-mM glycylglycine and 5% (v/v) isopropanol (pH 8.9), filtering through a syringe filter (0.45 μm pore size), and injecting into an ion chromatograph (Waters Chromatography Div., Millipore Corp., Milford, MA; model 590 pump, model 460 electrochemical detector). The filtration-injection procedure was completed in 1 min; after longer periods the recovery of sulfur dioxide was low. The chromatograph employed a 0.04% phosphoric acid mobile phase pumped at 1 ml/min flow rate, Water's "fast fruit juice" column, and an electrochemical detector operating in the oxidative mode at 0.7 V. A sulfite peak appeared 3.0–3.3 min after injection. The minimum sensitivity of this system for residues in grapes was 0.1 μg of sulfur dioxide per gram of fresh tissue.

RESULTS

Acidity and concentration of sulfur dioxide did not change measurably in the assay solutions over the 4-hr incubation period. The germinability of spores incubated in the control buffer solutions at the pH values tested exceeded 95%. The toxicity of sulfur dioxide solutions was greatly influenced by acidity (Fig. 1, Table 1). The LD_{50} and LD_{99} concentrations of "molecular" SO_2 to *B. cinerea* spores were about 0.1 and 0.2 μg/ml, respectively (Table 2). The amount of "molecular" SO_2 present at LD_{50} and LD_{99} concentrations was similar to that at the three pH levels tested, although the calculated amount of bisulfite ion (HSO_3^-) increased about 10 times between pH 3 and 4. The concentration of sulfite ($SO_3^=$) was insignificant (≤0.001 μg/ml) over the pH range tested.

Temperature had a profound influence on sulfur dioxide toxicity. Concentrations of 886, 343, 300, 57.8, 49.3, and 25.8 μg/ml of sulfur dioxide were required to kill 99% of the spores at 0, 10, 15, 20, 25, and 32 C, respectively (Table 3).

More than 90% of the total sulfite residues were located in the grape "skin," where sulfite residues averaged 7.6 or 16.5 μg/g 30 min after 5,000 or 10,000 ppm SO_2 fumigations, respectively (Table 4). The "pulp" contained less than 1 μg/g of sulfite. The pH of the "skin" and "pulp" were 4.1 and 4.2, respectively. Whole grape, "skin," and "pulp" sulfite residues of unfumigated grapes were below detectable levels.

DISCUSSION

We report that "molecular" SO_2 was equally present at LD_{50} concentrations for *B. cinerea* spores at pH 3–4, whereas the concentrations of bisulfite changed 10-fold and sulfite was not present. We conclude that the "molecular" SO_2 is the primary contributor to the toxicity of sulfur dioxide solutions. This interpretation is not supported by Babich and Stotzky (4), who

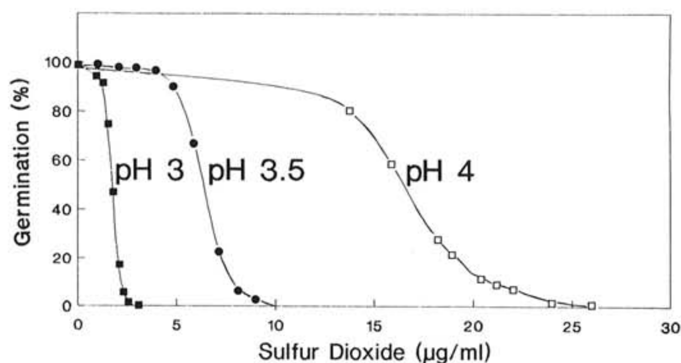

Fig. 1. Germination of *Botrytis cinerea* (isolate BCG8) spores after 4 hr at 25–27 C in dilute, buffered grape juice containing various amounts of sulfur dioxide at various pH levels, followed by 24-hr incubation on potato dextrose agar. For each value, 300 spores were examined.

TABLE 1. Estimated LD_{99} and LD_{50} concentration (μg/ml) of (plus 95% confidence intervals [CI]) for spores of four isolates of *Botrytis cinerea* incubated in buffered dilute grape juice containing sulfur dioxide for 4 hr at 25–27 C

Isolate	LD_{50} (95% CI)			LD_{99} (95% CI)		
	pH = 3.0	pH = 3.5	pH = 4.0	pH = 3.0	pH = 3.5	pH = 4.0
BCG6	1.68 (1.77, 1.57)	6.3 (6.5, 6.1)	16.5 (16.7, 16.2)	2.66 (3.12, 2.41)	9.0 (9.6, 8.5)	25.2 (25.9, 24.5)
BCG8	1.64 (1.76, 1.49)	5.6 (6.0, 5.2)	16.5 (17.0, 16.0)	2.44 (3.08, 2.19)	9.2 (10.9, 8.4)	29.7 (31.9, 27.6)
BCG11	1.74 (1.83, 1.64)	5.4 (5.8, 4.9)	18.9 (19.7, 18.1)	3.28 (3.63, 2.96)	10.8 (13.2, 9.5)	43.2 (51.0, 36.6)
BCG14	1.68 (1.77, 1.58)	5.7 (6.0, 5.3)	16.9 (17.5, 16.4)	2.63 (3.04, 2.40)	9.5 (11.4, 8.5)	33.0 (36.3, 30.0)

reported that bisulfite was the primary sulfur dioxide species toxic to *B. cinerea* at pH 4.0. They stated that "molecular" SO_2 was not present in sufficient quantity above pH 3.5 to provide germicidal activity. Conversely, we report that equal "molecular" SO_2 concentrations were present when inhibition of spore germination occurred at these acidities. The toxicity to many microbes of the neutral "molecular" SO_2 compared to the ionic species has long been recognized (8) and has been attributed to the more rapid, specific incorporation of this species over the bisulfite and sulfite ions (11,13). For the control of yeast growth in wine musts, which range in pH from 3.0 to 4.0, about 0.8 μg/ml "molecular" SO_2 (19) or 75–270 μg/ml total sulfur dioxide (1) are recommended. In the present study, *B. cinerea* was more sensitive than most microorganisms (12,22,27) and required only about 0.2 μg/ml of "molecular" SO_2 to effect control.

B. cinerea spores showed a two- to fourfold increase in sulfur dioxide sensitivity for each 10 C increase from 0 to 32 C at pH 4. Similarly, Schmiz (24) reported a four- to fivefold increase in the sulfur dioxide sensitivity of *Saccharomyces cerevisiae* at 28 C, compared to 18 C at pH 3.6. The increased sulfur dioxide toxicity may be due to the influence of temperature on sulfur dioxide incorporation. In fungi, sulfur dioxide is transported by a specific permease at neutral acidities (26), but under more acidic conditions neutral "molecular" SO_2 passively diffuses very rapidly through the plasma membrane, where it is ionized in the near-neutral acidity of the spore or hyphal cytosol and trapped, since the sulfite ions cannot pass through the hydrophobic plasma membrane (11,25). The intracellular sulfur dioxide concentration can exceed that of the extracellular medium up to 60-fold, until acidification of the cytosol occurs and ionization-trapping of intracellular sulfur dioxide ceases (11,14). Incorporation of sulfur dioxide under acid conditions (<pH 4) by *S. cerevisiae* is sensitive to temperature; Q_{10} values of 2.5 were reported between 20 and 40 C (13,25). We suggest that sulfur dioxide incorporation into *B. cinerea* spores is similarly temperature sensitive and is the mechanism for the variable toxicity we observed.

In our estimates, we assumed that the sulfur dioxide added to the dilute Thompson grape juice assay medium was "free" and not "fixed" in addition compounds. We did not attempt to determine whether the sulfur dioxide added was "free" or "fixed" because the lower dosages were below the sensitivity of available analytical methods, Thompson Seedless grape juice lacks high concentrations of acetaldehyde or anthocyanins primarily responsible for the rapid fixation of sulfur dioxide in other grape products (1), and concentrations in fresh grape juice of total and free sulfur dioxide usually are not different (2).

Sulfur dioxide residues approaching the lethal dosage to spores were found in the surface tissue of the grapes but not in the pulp. Doubling the dosage of gas approximately doubled the surface residue but only slightly increased those in the pulp. This result corroborates the work of Nelson (17), who showed that *B. cinerea* remained viable inside grapes after fumigation. Fumigation affects disease control only by periodic elimination of surface mycelial growth of infected grapes and not by eradication of the fungus in the grape tissue. We believe residues left after fumigation probably are too low in concentration to have a role in disease control. This contrasts with the suggestion of Peiser and Yang (20) that high dosages are needed during fumigation to leave sufficient residues to impart 5–7 days of fungal inhibition, and the recommendations of early workers that 20 μg/g or more of residue was needed for decay control (21). The important inhibition of the pathogen is in the air spaces between grapes, which explains why very low dosages of sulfur dioxide gas, shown to suppress mycelial growth and spore germination (7,16,20), also can retard decay development (6,15). The residues remaining after low-dosage fumigations (<1,000 ppm) are very low and proportionately less than those from higher-dosage fumigations (5,000 ppm) (Smilanick and Hartsell, *unpublished*). A reduction in the amount of sulfur dioxide employed by industry in commercial fumigations would probably maintain decay control efficacy and eliminate undesirably high residues (<10 μg/g) if the gas was uniformly distributed, penetrated the packages, and was removed promptly after fumigation.

TABLE 2. Calculated amounts of bisulfite and molecular sulfite present at the combined LD_{50} and LD_{99} (±SD) concentrations for spores of four isolates of *Botrytis cinerea* incubated in sulfur dioxide-containing solutions of buffered dilute grape juice for 4 hr at 25–27 C

	Bisulfite (μg/ml)		"Molecular" (SO_2 (μg/ml)	
pH	LD_{50}	LD_{99}	LD_{50}	LD_{99}
3.0	1.6 ± 0.1	2.6 ± 0.1	0.10 ± 0.002	0.16 ± 0.020
3.5	5.7 ± 0.4	9.2 ± 0.3	0.11 ± 0.008	0.19 ± 0.016
4.0	17.1 ± 1.1	29.3 ± 3.9	0.11 ± 0.007	0.21 ± 0.045

TABLE 3. Estimated LD_{50} and LD_{99} concentrations of sulfur dioxide (μg/ml) (plus 95% confidence intervals [CI]) for spores of *Botrytis cinerea* isolate BCG8 incubated in buffered dilute grape juice containing sulfur dioxide at pH 4 for 4 hr at 0–32 C

	Sulfur dioxide (μg/ml, 95% CI)	
Temperature	LD_{50}	LD_{99}
0	336 (357, 315)	886 (1,057, 742)
10	160 (171, 147)	343 (446, 293)
15	104 (120, 88)	300 (581, 222)
20	41.0 (43.9, 34.8)	57.8 (79.2, 52.3)
25	27.5 (28.3, 26.7)	49.3 (51.9, 46.9)
32	11.6 (12.2, 11.0)	25.8 (29.0, 23.0)

TABLE 4. Total sulfite residues determined by ion chromatography in "skin," "pulp," and whole berries of Thompson seedless grapes frozen after 30-min aeration following 30-min fumigation with 5,000 or 10,000 ppm sulfur dioxide at 0 C

	Sulfur dioxide (μg/g) after fumigation with	
Tissue	5,000 ppm SO_2	10,000 ppm SO_2
"skin"	7.6	16.5
"pulp"	0.7	0.9
whole	4.5	9.9
LSD		
0.05	3.0	5.5

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