1985

Potential for Development of Tolerance by *Penicillium digitatum* and *Penicillium italicum* after Repeated Exposure to Potassium Sorbate

Lisa L. Schroeder
University of Nebraska-Lincoln

Lloyd B. Bullerman
University of Nebraska-Lincoln, lbullerman1@unl.edu

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1985, p. 919–923
0099-2240/85/100919-05$02.00/0

Vol. 50, No. 4

Potential for Development of Tolerance by *Penicillium digitatum* and *Penicillium italicum* after Repeated Exposure to Potassium Sorbate†

LISA L. SCHROEDER AND LLOYD B. BULLERMAN*

Department of Food Science and Technology, University of Nebraska—Lincoln, Lincoln, Nebraska 68583-0919

Received 22 February 1985/Accepted 2 July 1985

Two strains of *Penicillium digitatum* and one strain of *Penicillium italicum* were exposed to various levels of sorbic acid and potassium sorbate, and the MICs were determined. Selected strains of the molds were then repeatedly exposed to subinhibitory levels of the compounds to determine whether increased tolerance might develop. The MIC of sorbic acid (pH 4.75) to *P. digitatum* was between 0.02 and 0.025%. The MIC of sorbate (pH 5.5) to two strains of *P. digitatum* and *P. italicum* was found to be between 0.06 and 0.08%. Increasing levels of sorbate resulted in increasing growth suppression of the molds. Populations of *P. digitatum* were tested for increased tolerance to sorbic acid, and none was found. Individual molds that started from the same parent colony were examined for increased tolerance to potassium sorbate. Two *P. digitatum* strains developed no observable increased tolerance, but *P. italicum* developed a slight increase in tolerance to sorbate. When spores of *P. italicum* and *P. digitatum* were exposed to higher levels of sorbate for prolonged times, the fungicidal or fungistatic activity of the inhibitor was dependent upon pH, length of exposure time, level of sorbate, and the mold strain.

Mold growth on citrus fruits during storage is a continuing problem that results in economic loss. Although several fungal species have been reported to be involved in the spoilage of citrus products, *Penicillium digitatum* (green mold) and *Penicillium italicum* (blue mold) are the primary organisms involved. Control of postharvest mold spoilage of citrus fruits by the use of benomyl and thiabendazole has been standard procedure in many citrus-producing areas since the early 1970s (4, 12). However, there have been reports that *P. digitatum* and *P. italicum* can develop resistance or tolerance to these fungicides (4, 6, 8). Harding (4) reported that benzimadazole-resistant isolates of *P. digitatum* and *P. italicum* have also been found in orchards and citrus packing-houses in which benzimadazole fungicides had never been used.

In an effort to find a new fungicide to effectively combat these resistant strains, Smoot and McCornack (9) investigated the use of potassium sorbate for citrus decay control. They reported that a 2% aqueous solution of potassium sorbate, applied as a dip, effectively reduced postharvest decay in several citrus products. Although not as effective as benomyl or thiabendazole, Smoot and McCornack (9) found that strains of *P. digitatum* resistant to these fungicides were sensitive to potassium sorbate. The question has remained, however, whether molds common to citrus products might also develop resistance to sorbic acid and potassium sorbate on prolonged usage.

The purpose of this investigation was to determine the inhibitory effects of sorbic acid and potassium sorbate on *P. digitatum* and *P. italicum* and to study the possibility of development of tolerant strains upon repeated exposure to subinhibitory levels of these compounds.

* Corresponding author.

† Published as paper no. 7607, Journal Series, Agricultural Research Division, Lincoln, NE.

MATERIALS AND METHODS

Study I: effects of sorbic acid on potential tolerance of *P. digitatum*. (i) Organism. *P. digitatum* WSQ1, isolated from a citrus packing plant, was obtained from P. McDonald, Monsanto Industrial Chemicals Co., St. Louis, Mo. The culture was maintained on potato dextrose agar (PDA) slants at 25°C. Spore suspensions were obtained by transferring spores from slants or culture agar plates to a tube containing Butterfield phosphate buffer (10) plus 0.05% Tween 80, using an inoculating needle wetted with Tween 80. The final concentration of the spores was adjusted to ca. 10^6 spores per ml with a Petroff-Hausser counting chamber.

(ii) Substrate. Yeast extract sucrose agar (2% yeast extract, 15% sucrose, 1% agar) (YESA) containing increasing concentrations of sorbic acid (Monsanto) was used as the substrate. Sorbic acid was aseptically added to hot sterilized YESA from a 10% stock solution of sorbic acid in ethanol. Sorbic acid levels used were 0.000, 0.005, 0.010, 0.015, 0.020, 0.025, and 0.030%. After the addition of the sorbic acid, the pH of the agar was adjusted with 1 N HCl to obtain a final pH of 4.75 ± 0.05. This pH was used because it is near the pK_a of sorbic acid, which is where the greatest amount of sorbic acid would be in the active or undisassociated form without going to an extremely low pH. The agar was then poured into sterile disposable plastic petri dishes.

(iii) Culture conditions. Spore suspensions were spread on the surfaces of YESA plates and then incubated at 25°C. Colony counts were made after 3 to 5 days of incubation; the plates were then incubated until well sporulated. These spores were used as an inoculum for the next generation.

(iv) Experimental design. A flow diagram of the transfer scheme is shown in Fig. 1. Spores were harvested, transferred, and exposed to sorbic acid through 10 generations. The spores used for inoculum at the A1 level were always taken from spores that grew at the 0.000% sorbic acid

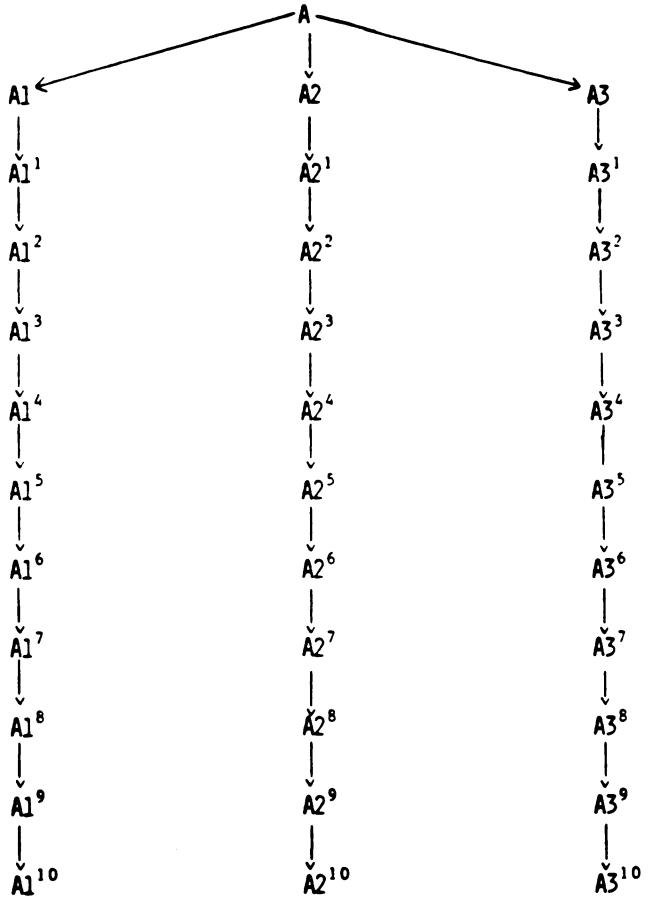

FIG. 1. Transfer scheme of 10 generations of *P. digitatum* exposure to sorbic acid. A represents the starting stock culture. The A1 series represents spore inoculum always taken from the zero level of sorbic acid on the previous plate. The A2 level represents spore inoculum always taken from the 0.01% level of sorbic acid on the previous plate. The A3 level represents spore inoculum always taken from the 0.02% level A of sorbic acid on the previous plate. Growth did not occur at the 0.03% level of sorbic acid.

concentration on the previous transfer. Thus, the inoculum for each transfer or generation in this series had no prior exposure to sorbic acid. At the A2 level, the spore inoculum was always taken from the spores that were produced from growth occurring at the 0.010% sorbic acid level. At the A3 level, the spore inoculum was taken from the 0.020% sorbic acid level. The scheme was replicated three times, and the results are reported as the mean log counts for each spore inoculum source.

Study II: effects of increasing levels of sorbate on growth and potential tolerance of *P. italicum* and *P. digitatum*. (i) Organisms. *P. italicum* NRRL 1293 was obtained from the Northern Regional Research Center, Peoria, Ill. *P. digitatum* WSQ1 and *P. digitatum* BR (benomyl resistant; isolated from a citrus packing plant) were obtained from P. McDonald, (Monsanto). The organisms were maintained on PDA slants at 25°C. Transfers were made by using a wetted inoculating needle and touching it to the surface of the slants or petri dishes to make single-point inoculations in the center of an appropriate petri dish.

(ii) Substrate. PDA containing increasing concentrations of potassium sorbate (Monsanto) was used as the substrate. Sorbate levels were 0.000, 0.005, 0.010, 0.020, 0.040, 0.060, and 0.080%. The sorbate was added aseptically to sterilized PDA in appropriate amounts from a 10% stock solution of sorbate in sterile distilled water, and the pH of the agar was adjusted to 5.5 ± 0.1 with sterile 1 N HCl or 1 N NaOH. This pH was used because it is the pH of citrus peel and is near the pH of the surface of citrus fruit. A new stock solution of sorbate was prepared at the beginning of each transfer period.

(iii) Culture conditions. Single-point inoculations were made from stock slants on the first transfer, and the plates were incubated at 25°C for 15 days. Every 3 days, the minimum and maximum diameter of the colony was measured (in centimeters) with a vernier caliper (6, 11). Colony diameter was used to calculate the area of the mold colony with the formula: area = $[\pi(\min)(\max)]/4$.

(iv) Experimental design. A flow diagram of the transfer scheme is shown in Fig. 2. Spores were transferred from the previous plate by making direct single-point inoculations in the center of the petri dishes. The molds were subcultured three consecutive times at a given level of sorbate and then shifted to the next highest level of sorbate. The procedure was carried through 22 transfers, or until the molds beginning at the 0.000% sorbate level had been exposed to three transfers on each subinhibitory level and transferred to the next highest subinhibitory level. Each mold was treated in four to six replicates, and the values used are expressed as means of those replicates.

Study III: effect of prolonged exposure to sorbate on spore viability. (i) Organisms. *P. digitatum* WSQ1 and *P. italicum* NRRL 1293 were used as the test organisms. Stock cultures were maintained on PDA slants at 25°C. Spore suspensions were prepared from 12-day-old well-sporulated slants by adding 10 ml of PO_4 buffer (10) plus 0.05% Tween 80 to the slant and gently dislodging the spores with a flamed wire loop. The suspension was then filtered through several layers of sterile cheesecloth, and the spores were counted with a Petroff-Hausser Counting chamber and then adjusted to ca. 5.0×10^4 spores per ml.

(ii) Exposure medium. Potato dextrose broth (PDB) (Difco Laboratories, Detroit, Mich.) was used as the exposure medium. The broth was dispensed in amounts of 100 ml in 250-ml Erlenmeyer flasks and sterilized at 121°C for 15 min. Sterile 10% potassium sorbate stock solution was then added to the autoclaved media to give concentrations of 0.05, 0.15, 0.25, 0.5, 0.75, and 1.00% sorbate. Sterile 1 N HCl or 1 N NaOH was used to adjust the final pH of the broth to either 4.7 ± 0.1 or 5.5 ± 0.1.

(iii) Exposure conditions. One milliliter of the stock spore suspension was added to each of the treatment flasks to give ca. 10^4 spores per ml. At 1-day intervals, 1 ml was aseptically withdrawn from each treatment flask and filtered through a 0.45-μm membrane filter (Gelman Sciences, Inc., Ann Arbor, Mich.) to collect the spores. The membrane was rinsed with two 1-ml samples of sterile distilled water, transferred to a tube containing 10 ml of PO_4 buffer plus 0.05% Tween 80, and mixed at low speed on a vortex-type mixer for 60 s, and serial dilutions were made. A three-tube most probable number (MPN) technique was used to determine the number of viable CFU (1, 5). Culture vessels for the MPN were 4-dram vials containing 4 ml of extra-strength PDB adjusted to pH 5.5. (Extra-strength PDB was used so that when 1 ml of spore suspension was added to the vessel, the broth was at normal strength.) Samples (1 ml) of the appropriate dilution were transferred to the MPN vials, agitated briefly, and incubated at 25°C for 14 days, at which time the viable CFU count was determined with a three-tube MPN table.

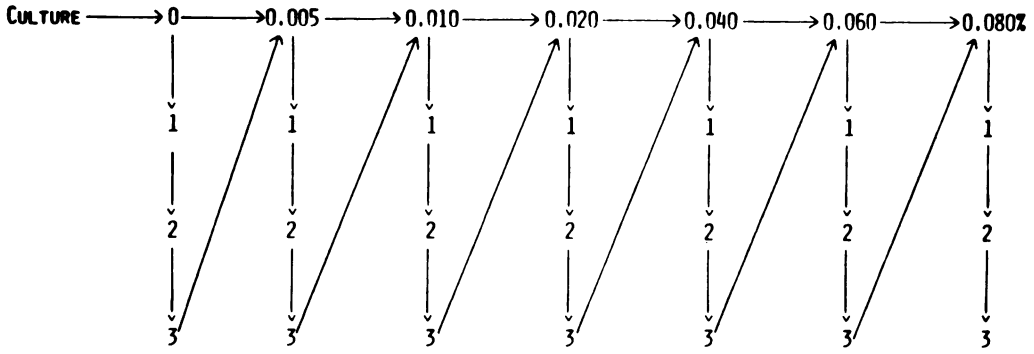

FIG. 2. Design used in study II to determine whether resistance to potassium sorbate might be acquired after repeated exposure to gradually increasing levels of sorbate.

(iv) Experimental design. The overall scheme was a 7 by 8 factorial design, with seven different treatment levels of sorbate, incorporating two different pH levels, studied for the effects of prolonged exposure to sorbate on spores of two organisms. The spores were left in the presence of sorbate for up to 7 days, and each day samples were taken to determine the number of viable spores. The experiment was replicated twice, and the data are reported as the mean log counts of each sorbate treatment.

RESULTS AND DISCUSSION

Study I. When spores of *P. digitatum* (without prior subculturing on media with or without sorbic acid) were plated on YESA containing sorbic acid, the MIC was found to be between 0.020 and 0.025% (Fig. 3). When spores of *P. digitatum* without prior exposure to sorbic acid were plated through 10 subsequent generations, no apparent increase in tolerance to sorbic acid was observed. Likewise, no increase in tolerance was seen when spores exposed to either 0.010 or 0.020% sorbic acid were transferred through the 10-generation scheme.

The hypothesis behind the design of this study was that if samples of a homogenous spore suspension were simultaneously plated on media containing increasing concentrations of sorbic acid, it would be expected that the spore counts would decrease as the sorbate concentration increased. This was as expected when *P. digitatum* was plated on sorbate-containing media. The resulting curve showed a concentration (0.015% sorbic acid) at which a cut off point was observed, i.e., a point at which the majority of the spores were tolerant to the sorbic acid but were not tolerant at a higher concentration (>0.020% sorbic acid). If spores at the higher concentrations were able to grow as the result of a genetic difference or change, it would be logical to assume that their progeny would also survive the higher concentrations. Thus, after exposure of the molds to subinhibitory levels of sorbate, a repeat of the same experiment utilizing the most resistant spores should yield a cut off concentration somewhat higher than in the first experiment and a concomitant shifting of the curve to the right if a genetic change or selection toward resistance had occurred. However, if those spores were not genetically different, i.e., not more resistant, they would be expected to produce offspring exhibiting the same growth pattern as the majority of spores in the first experiment and yield a cut off concentration similar to that in the first experiment and a similar curve. After 10 generations, the spores from all sources (with and without prior exposure to sorbic acid) yielded mean curves similar to that of the first experiment. This indicated that a genetic difference or change had not occurred and that the mold did not develop increased tolerance to sorbic acid.

Study II. The objective of this study was to determine whether individual molds which started from the same parent stock colony might develop increased tolerance to sorbate upon repeated subculturing at low levels of the inhibitor. The MIC of sorbate was found to be between 0.06 and 0.08% for *P. italicum* and both strains of *P. digitatum*. Growth of *P. digitatum* strains was more strongly inhibited

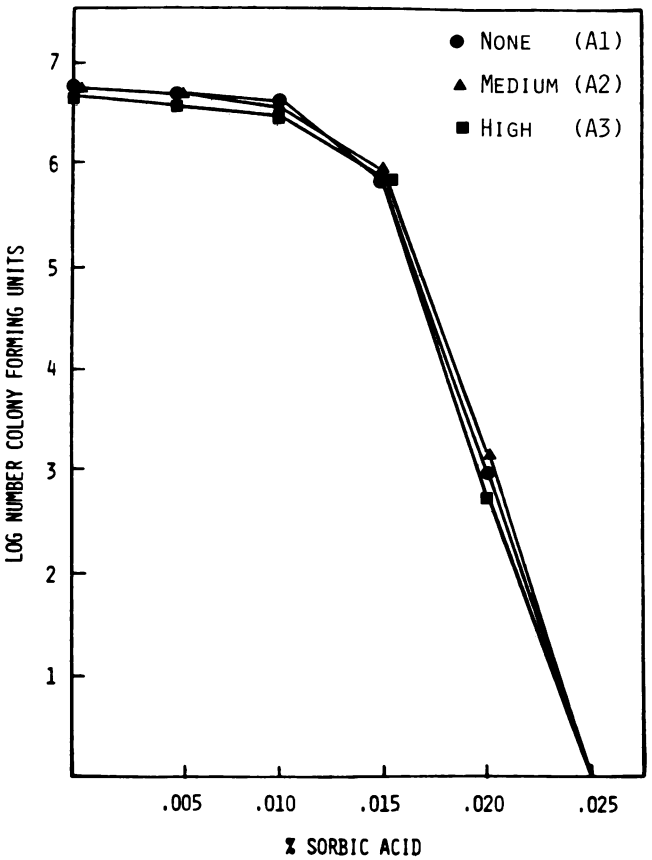

FIG. 3. Mean exposure curves of 10 generations of *P. digitatum* WSQ1 to sorbic acid, with inoculum produced in the absence (A1) of sorbic acid and in the presence of 0.01% (A2) and 0.02% (A3) sorbic acid.

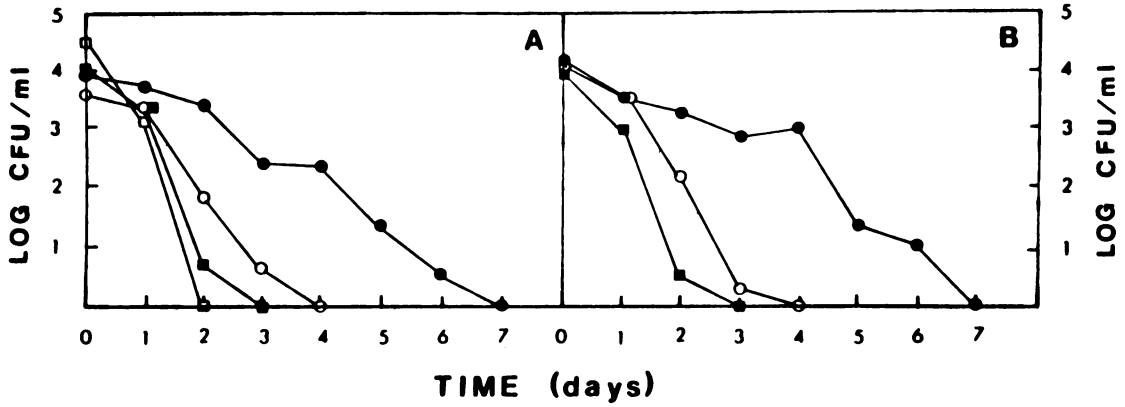

FIG. 4. Effect of prolonged incubation in the presence of sorbate on the spore viability of *P. digitatum* WSQ1. (A) Incubation medium contained sorbate at pH 5.5 at a concentration of 0.25 (●), 0.50 (○), 0.75 (■), or 1.0% (□). (B) Sorbate at pH 4.7 at a concentration of 0.05 (●), 0.15 (○), or 0.25% (■).

by levels of 0.06 and 0.08% sorbate than was *P. italicum* at all incubation times. Smoot and McCornack (9) reported 100 ppm (0.01%) of potassium sorbate caused a 20% inhibition of *P. italicum* and 83% inhibition of *P. digitatum* on PDA (pH 4.5), and that 1,000 ppm (0.1%) of potassium sorbate totally inhibited both molds. This is in agreement with the findings of this study, as the MICs were found to be between 0.06 and 0.08% sorbate for both molds. Smoot and McCornack reported that 100 ppm (0.01%) of potassium sorbate inhibited *P. digitatum* by 83%. In the present study, 0.01% showed little or no inhibition after 15 days. This difference can be attributed to the difference in pH of the agar, since this study was done at a higher pH, thereby lowering the antifungal activity of the sorbate.

The results of carrying the molds through the transfer scheme for this study are shown in Tables 1 and 2. After 10 transfers, beginning at the 0.02% level of sorbate, *P. italicum* was able to grow at the previously inhibitory level of 0.08% sorbate. After 19 transfers, the mold was still able to grow at the 0.08% sorbate level but not at the 0.10% level. The greatest number of transfers was 22, beginning at the 0.00% sorbate level and going through the 0.10% level. The molds that went through the scheme were not able to grow at the 0.10% sorbate level. Thus, after repeated subculturing of *P. italicum* on low levels of sorbate, this mold developed a slight increase in tolerance to potassium sorbate, since after 10 transfers, starting at 0.02% sorbate, it was able to grow at the previously inhibitory level of 0.08% sorbate.

When the two *P. digitatum* isolates were taken through the same experimental design, it was found that the two strains behaved very similarly in their response to sorbate. After 19 transfers, beginning at the 0.00% sorbate level, the two strains were able to grow at the 0.06% sorbate level; however, they were not able to grow at any higher levels. Neither strain of *P. digitatum* developed increased tolerance to sorbate through repeated subculturing in the presence of subinhibitory levels.

Study III. In study II, it was determined that the MIC of sorbate was between 0.06 and 0.08% for *P. digitatum* and *P. italicum*. In the third study, an attempt was made to determine whether this range was fungistatic or fungicidal. When *P. digitatum* was incubated in the presence of 0.1% sorbate in PDB for up to 3 days, there was no decrease in the number of viable CFU. Therefore, it was concluded that the lower concentration of 0.08% was exerting a fungistatic rather than a fungicidal action. To determine the level at which sorbate is fungicidal, higher sorbate levels (0.25, 0.50, 0.75, and 1.00%) were employed.

As the sorbate concentration in the exposure medium increased, the length of exposure time to obtain fungicidal action against *P. digitatum* increased (Fig. 4). After 1 day of exposure to sorbate, all levels reduced the number of recoverable CFU. Beyond 1 day, the number of viable CFU rapidly decreased in 0.50, 0.75, and 1.00% sorbate. No viable spores were recovered after 7, 4, 3, and 2 days at the 0.25, 0.50, 0.75, and 1.00% sorbate levels, respectively, when the pH of the exposure medium was 5.5 (Fig. 4A). When the pH of the exposure was lowered to 4.7, the maximum amount of sorbate that would go into solution was 0.25%. Therefore, levels of 0.05, 0.15, and 0.25% sorbate

TABLE 1. Growth of *P. italicum* NRRL 1293 on PDA containing sorbate after 15 days at 25°C

Transfer	Growth with the following sorbate concn:							
	0.000	0.005	0.010	0.020	0.040	0.060	0.080	0.100
1	+	+	+	+	+	+	−	
4	+	+	+	+	+	−	−	
7	+		+	+	+	+	−	
10	+			+	+	+	+	
13	+				+	+	+	−
16	+					+	+	−
19	+						+	−
22	+							−

TABLE 2. Growth of *P. digitatum* WSQ1 and *P. digitatum* BR on PDA containing sorbate after 15 days at 25°C[a]

Tranfer	Growth with the following sorbate concn:						
	0.000	0.005	0.010	0.020	0.040	0.060	0.080
1	+	+	+	+	+	+	−
4	+	+	+	+	+	−	−
7	+		+	+	+	+	
10	+			+	+	+	−
13	+				+	+	−
16	+					+	−
19							−

[a] Data are the same for both organisms.

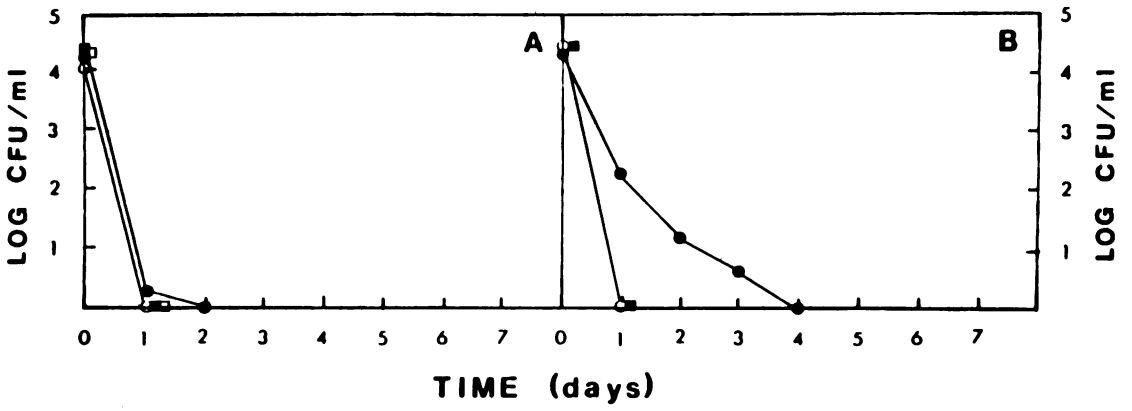

FIG. 5. Effect of prolonged incubation in the presence of sorbate on the spore viability of *P. italicum* NRRL 1293. (A) Incubation medium contained sorbate at pH 5.5 at a concentration of 0.25 (●), 0.50 (○), 0.75 (■), or 1.0% (□). (B) Sorbate at pH 4.7 at a concentration of 0.05 (●), 0.15 (○), or 0.25% (■).

were used with pH 4.7. The lines of the graph of the viable CFU of 0.25, 0.50, and 0.75% at pH 5.5 were very similar to those of 0.05, 0.15, and 0.25% at pH 4.7, respectively (Fig. 4). This agrees with the observations that at lower pH values, the effectiveness of sorbate increases (2, 3). This is evidenced by the fact that at pH 5.5, three to five times as much sorbate was required to get the same antifungal effects as at pH 4.7. Przybylski and Bullerman (7) reported concentrations of 0.25 and 0.5% sorbic acid in YESA (pH 4.75) showed marked decreases in the viability of spores of *Aspergillus parasiticus*. This is similar to the effects observed on *P. digitatum* when the pH of the exposure medium was adjusted to pH 4.7.

When *P. italicum* was used as the test organism the results were very different from those observed with *P. digitatum*. *P. italicum* appeared to be much more sensitive to prolonged exposure to sorbate. After 1 day of exposure, viable spores were recovered only at levels of 0.25 (pH 5.5) and 0.05% (pH 4.7) sorbate (Fig. 5). After 4 days of exposure time, no viable spores were recovered at any sorbate level at either pH value. In this study, *P. italicum* appeared to be more sensitive to higher levels of sorbate than did *P. digitatum*, whereas in study II, in which the molds were exposed to lower (subinhibitory) levels of sorbate, *P. italicum* was observed to gain a slight increase in tolerance to sorbate, while *P. digitatum* did not. This study indicates that the conditions which govern the fungistatic or fungicidal action of sorbate include pH, length of exposure time of the spore to the sorbate, level of sorbate employed, and the individual mold strain itself.

ACKNOWLEDGMENTS

The technical assistance of K. S. Przybylski and Keith Fraka is gratefully acknowledged.

Research was conducted under project 16-029 and was partially supported by a grant from Monsanto Co., St. Louis, Mo.

LITERATURE CITED

1. **Adams, G. H., and Z. J. Ordal.** 1976. Effects of thermal stress and reduced water activity on conidia of *Aspergillus parasiticus*. J. Food Sci. **41**:547–550.
2. **Bandelin, F. J.** 1958. The effect of pH on the efficiency of various mold inhibiting compounds. J. Am. Pharm. Assoc. Sci. Ed. **47**:691–694.
3. **Bell, T. A., J. L. Etchells, and A. F. Borg.** 1959. Influence of sorbic acid on the growth of certain species of bacteria, yeasts, and filamentous fungi. J. Bacteriol. **77**:573–580.
4. **Harding, P. R., Jr.** 1972. Differential sensitivity to thiabendazole by strains of *Penicillium italicum* and *P. digitatum*. Plant Dis. Rep. **56**:256–260.
5. **Koburger, J. A., and A. R. Norden.** 1975. Fungi in foods. VII. A comparison of the surface, pour plate, and most probable number methods for enumeration of yeasts and molds. J. Milk Food Technol. **38**:745–746.
6. **McDonald, R. E., L. A. Risse, and B. M. Hillebrand.** 1979. Resistance to thiabendazole and benomyl of *Penicillium digitatum* and *P. italicum* isolated from citrus fruits from several countries. J. Am. Soc. Hortic. Sci. **104**:333–335.
7. **Przybylski, K. S., and L. B. Bullerman.** 1980. Influence of sorbic acid on viability and ATP content of conidia of *Aspergillus parasiticus*. J. Food Sci. **45**:375–376, 385.
8. **Smoot, J. J., and G. E. Brown.** 1974. Occurrence of benzimidazole-resistant strains of *Penicillium digitatum* in Florida citrus packinghouses. Plant Dis. Rep. **58**:933–934.
9. **Smoot, J. J., and A. A. McCornack.** 1978. The use of potassium sorbate for citrus decay control. Proc. Fla. State Hortic. Soc. **91**:119–122.
10. **Speck, M. L. (ed.).** 1976. Compendium of methods for the microbiological examination of foods, p. 79–80. American Public Health Association, Washington, D.C.
11. **Troller, J. A., and R. A. Olsen.** 1967. Derivatives of sorbic acid as food preservatives. J. Food Sci. **32**:228–231.
12. **Wild, B. L.** 1974. Pathogen resistance to citrus postharvest fungicides. Food Technol. Aust. **Nov**:505–508.