

# The Effect of Thiabendazole on Spore Germination

David Gottlieb and Krishna Kumar

Professor of Plant Pathology and Research Assistant, respectively, Department of Plant Pathology, University of Illinois, Urbana 61801. Present address of junior author: Department of Botany and Plant Pathology, University of Maine, Orono 04473.

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## ABSTRACT

Thiabendazole, 2-(4'-thiazolyl) benzimidazole (TBZ), inhibited germination of *Penicillium atrovenerum* and *Aspergillus oryzae* 80% at 20 µg/ml and slightly more at higher concn. Complete inhibition did not occur even at 100 µg/ml. As little as 2 µg/ml prevented elongation or growth of germ tubes but did not inhibit spore germination. Growth of mycelium of *P. atrovenerum* and *P. oxalicum* was also inhibited by 2 µg/ml. TBZ is fungicidal as shown by the inability of spores to germinate after being removed from TBZ solution, washed, and placed in Czapek's medium. The fungicide is very readily absorbed by spores, and absorption is

directly related to concn of TBZ and to time of incubation. At 100, 200, and 400 µg/20 ml TBZ, uptake by 200 g spores was 75, 140, and 260 µg, respectively. Spore concn of 10, 25, 50, and 100 µg and 200 µg TBZ/20 ml remove 5.0, 4.6, 3.0, and 2 µg/mg spores, respectively. TBZ was found mainly in the cell fluids, from which it was readily removed by adsorption on sand during preparation of cell-free spore extracts. The remainder was distributed among various particulate fractions obtained from ground spores: the cell wall and nuclear 37%, mitochondrial 42%, and ribosomal 20%. *Phytopathology* 60:1451-1455.

*Additional key words:* *Penicillium atrovenerum*, *Aspergillus oryzae*, *Penicillium oxalicum*, fungicide.

The antihelminthic agent, Thiabendazole (TBZ), has been widely used for treatment of animal infections (2, 4, 7, 9, 10). Recently, TBZ engaged the attention of plant pathologists as an antifungal agent and is of special interest because of its reported systemic activity (6, 13, 14). Ascomycetes, except for yeasts, and Fungi Imperfecti examined thus far are generally sensitive to TBZ, but relatively few Basidiomycetes are inhibited at low concn. Control of a number of diseases by TBZ has also been reported (1, 3, 5, 8, 11); however, we found no published data on the inhibitory effect of TBZ on spore germination and very little on the mechanism of disease control (12, 13). The current report deals with various aspects of TBZ; e.g., its effects on spore germination, its fungicidal properties, its effect on spore morphology, its uptake, and its distribution within fungus spores.

**MATERIALS AND METHODS.**—*Penicillium atrovenerum* G. Smith, *P. oxalicum* Currie & Thom, and *Aspergillus oryzae* (Ahlb) Cohn were obtained from the Commonwealth Mycological Institute and maintained on potato-dextrose agar (PDA) slants. For the production of spores, the fungi were grown on slants of 100 ml malt agar in 500-ml flasks for 21 days at 26 C. The spores were harvested by gently rubbing the surface of the cultures with a long-handled camel's-hair brush, then inverting and tapping the flasks over glazed weighing papers.

To obtain mycelium, 5 ml of sterile Czapek's liquid medium were added to cultures maintained on test tube slants of PDA, and the mycelium was suspended by scraping the surface with an inoculating needle. Five ml of this suspension were used to inoculate 25 ml Czapek's medium in a 125-ml Erlenmeyer flask. The fungus was incubated on a reciprocal shaker for 36 hr, then homogenized for 10 sec in a Waring Blender. Two ml of the homogenate was added to a similar flask with medium, grown for 24 hr, then homogenized

as before. Two ml of homogenate was used to inoculate each of a series of similar flasks for various experiments. The cultures were grown on a reciprocal shaker for 96 hr, after which the mycelia were collected by filtration on tared No. 1 Whatman filter paper, dried in a vacuum oven at 90 C for 24 hr, and weighed.

All studies on the effect of the fungicide on spores were made with 200 g fresh wt of *P. atrovenerum* or *A. oryzae* spores, suspended in 100 ml Czapek's medium containing 0.05 ml Tween 80 (polyoxyethylene sorbitan monooleate). Ten ml of this suspension was placed in 20-ml test tubes, and aliquots of TBZ in 0.01 N HCl were added in amounts needed to give the range of concn used in the various experiments. Controls consisted of solutions of TBZ without spores and of spore suspensions without the fungicide. Germination counts were made by placing triplicate drops of spore suspension on slides in a moist chamber and incubating for 12 hr for *P. atrovenerum* and 18 hr for *A. oryzae*. Four drops of spore suspension containing a min of 200 spores were counted at each TBZ concn. Procedures for carrying out experiments on spore germination other than those involving standard procedures are given in the section on results.

Qualitative and quantitative studies on the uptake and distribution of TBZ by spores were made with a Gilford 2000 spectrophotometer. The TBZ was determined either on aqueous solutions containing the fungicide or on methanol extracts of spores or their organelles. Apparently, methanol extractable components of the spores did not interfere with the quantitation of the fungicide (Fig. 1-C). The absorbance at 298 mµ was directly related to concn of TBZ (Fig. 1-B). Uptake of fungicide was measured by decrease in absorbance of TBZ solutions of known initial concn. Spores suspended in the solutions and incubated for various periods of time were removed by filtration on a UF

sintered glass filter. "Free" TBZ on the spores was removed by repeated washes with distilled water. Fungicide in the spores was extracted with methanol.

The distribution of absorbed TBZ in spores was determined by disruption of the spores and differential sedimentation of the spore fractions by centrifugation. The spores were ground with acid-washed sand, 400 mg spores to 1 mg sand, in 20 ml of Czapek's medium added in small amounts during the disruption of the spores. All separations of cell fractions were made by centrifugation at various gravitational forces. The sediments from centrifugations were suspended in 10 ml methanol, placed on the reciprocal shaker for 10 min, then filtered through the UF filter. This process was repeated until no more TBZ could be extracted and the extracts were combined.

**RESULTS AND DISCUSSION.**—TBZ at low concn inhibited both spore germination and mycelial growth. In no case, however, was germination of *P. atrovenetum* or *A. oryzae* spores inhibited completely, even when high concn of fungicides were used (Fig. 1-A). The min time for max-percentage germination of *A. oryzae* in Czapek's medium was 18 hr. In the absence of TBZ, spores of both species germinated 90%. The graph for inhibition of *P. atrovenetum* (Fig. 1-A) shows a small decrease in germination at between 2 and 7  $\mu\text{g/ml}$  TBZ, a sharp rise at 10  $\mu\text{g}$  to 30% inhibition, then a leveling off at 20  $\mu\text{g}$  with 82% inhibition with no further inhibition even at 100  $\mu\text{g}$ . Similar leveling off of inhibition was obtained with *A. oryzae*, even though this fungus seemed more sensitive to the fungicide (Fig. 1-A). The per cent inhibition of germination at 2, 5, 10, 20, and 50  $\mu\text{g/ml}$  were 17, 61, 76, 82, and 88%, respectively.

Preventing spore germination is not the only criterion for the effectiveness of a fungicide. A material may not inhibit germination yet prevent further elongation and growth. Whether the inhibition is of germination or elongation of the germ tube is often a function of the concn of fungicide that is used. In both *P. atrovenetum* and *A. oryzae*, the effective concn for preventing elongation was much lower than for preventing germ tube formation. Two  $\mu\text{g/ml}$  TBZ was always sufficient to prevent germ tube elongation after germ tubes equal to the diam of the spore had been formed. Incubation periods in the presence of the fungicide for as long as 20 hr for the *Penicillium* and 24 hr for the *Aspergillus* did not allow further elongation.

The question as to whether a compound is fungistatic or fungicidal depends on the definition of these terms on morphological bases and on the parameters of concn and time that are chosen. Furthermore, the definition must make clear whether one is referring to the concn within the spore or in the medium. Despite these limitations, it is useful to know whether an antifungal agent binds so tightly to the metabolic substrate or functional entity that, for practical purposes, it prevents germination and is thus fungicidal. On the other hand, the compound might be so loosely bound that the agent could diffuse away from its site of action, when the spores are removed and placed in a

TABLE 1. Fungicidal action on germination of spores of *Aspergillus oryzae* suspended in various concn of thiabendazole for different periods of time, washed, then incubated for 20 hr in Czapek's medium containing no fungicide

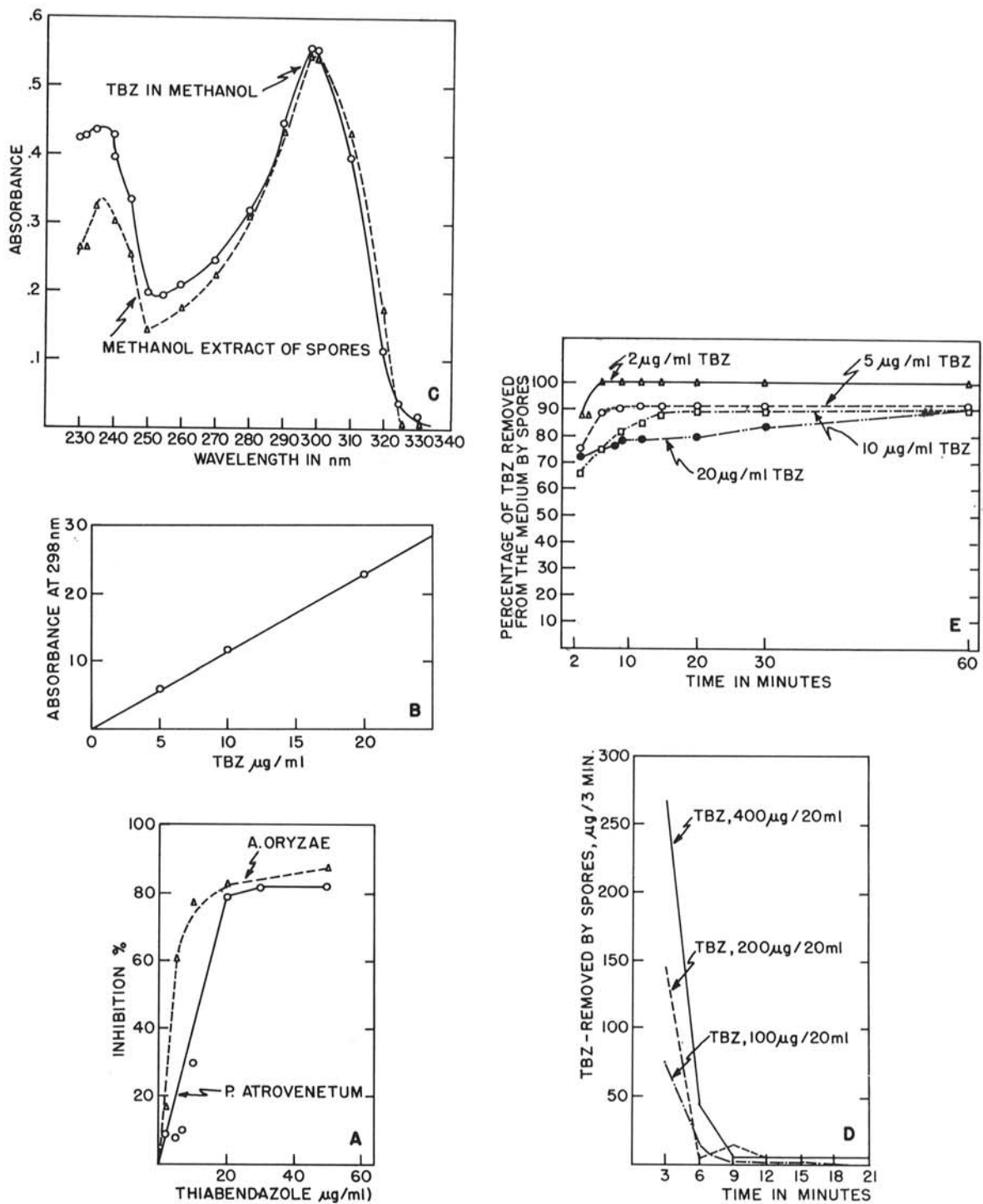
$\mu\text{g/ml}$ TBZ	% Germination after exposure in TBZ			
	30 min	60 min	240 min	480 min
0	91	90	89	90
10	56	14	12	11
20	22	13	11	10
40	13	13	10	10

medium containing no antimicrobial agent, and be only fungistatic. Table 1 shows the effect of exposing spores of *A. oryzae* to various concn of TBZ, washing 3 times with distilled water (after which further washing removed no more TBZ), then placing them into Czapek's medium. Incubation at 10  $\mu\text{g/ml}$  and a 30-min exposure to the antifungal agent were not completely fungicidal. But exposures to TBZ for 60 min or longer were fungicidal to all spores. Incubation in increasing concn of TBZ was increasingly fungicidal, until, at 40  $\mu\text{g/ml}$ , no germ tubes were formed even from spores that had been in contact with TBZ for only 30 min. Thus, under the conditions used, TBZ was primarily fungicidal.

Adding the fungicide after germination began inhibited further development. Spores of *P. atrovenetum* were first incubated in Czapek's medium for 8 hr so that germ tube development began. After this period, these spores were placed in Czapek's medium containing TBZ at 10  $\mu\text{g/ml}$  for 24 hr. Under these conditions, normal elongation occurred but the germ tubes became malformed (Fig. 2).

TBZ also inhibited mycelial growth of *P. atrovenetum* and *P. oxalicum* that were grown in shake flasks from blended mycelium. Mycelial growth was more sensitive to the fungicide than was spore germination, and growth was completely prevented at 2  $\mu\text{g/ml}$ . In flasks containing no fungicide, the dry wt for *P. atrovenetum* and *P. oxalicum* averaged 286 and 302 mg, respectively; in the presence of concn of 2 to 10  $\mu\text{g}$  TBZ, the wt varied from 5.2 to 5.7 mg.

TBZ was readily absorbed from Czapek's medium by spores of *A. oryzae*. The other two species were also examined, but could not be used because a soluble pigment diffused from the spores into the medium and interfered with the TBZ spectrum. No such pigment was present in *A. oryzae*, and the height of the peak absorption of TBZ at 298 nm was proportional to the amount of TBZ in the medium. The absorption curve for TBZ in methanol is given in Fig. 1-C, and the direct relationship between absorbance and concn of the fungicide is given in Fig. 1-B. The graphs in Fig. 1-E relate the absorption of TBZ from Czapek's medium to both the original concn of TBZ and to the length of time the spores were incubated in the TBZ medium. The greatest percentage of fungicide was removed at the lowest concn. After 3 min at concn of 2, 4, 10, and 20  $\mu\text{g/ml}$ , the removal was 87.5, 75, 72.5, and 66%, respectively. A similar relationship held for periods of incubation up to 20 min. At 2  $\mu\text{g/ml}$  and incubation times of 6 min or longer, all the



**Fig. 1.** A) Inhibition of germination of spores of *Penicillium atrovenetum* and *Aspergillus oryzae* by thiabendazole. B) Relation of ultraviolet absorbance of thiabendazole. C) Ultraviolet spectrum of thiabendazole in methanol and of a difference spectrum of extracts taken from spores of *A. oryzae* treated with the fungicide. D) Uptake of thiabendazole by spores of *A. oryzae* with increasing time of incubation. E) Uptake of thiabendazole by spores of *A. oryzae* as a function of original concn of fungicide and of time of incubation.

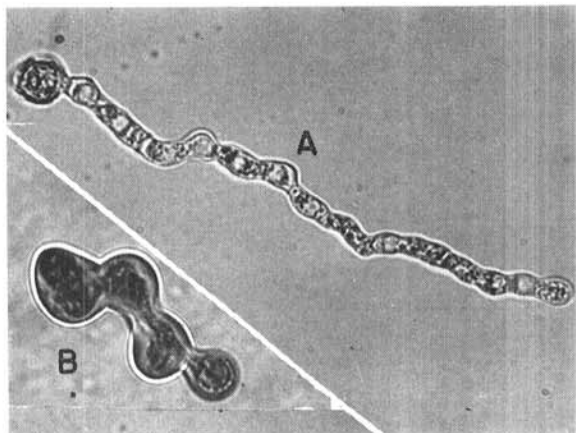


Fig. 2. The effect of thiabendazole on germinating spores of *Penicillium atrovolutum*. Germ tubes after 8-hr incubation in Czapek's medium followed by A) 16-hr incubation in 0.01 N HCl; B) 16-hr incubation in 0.01 N HCl containing 10 µg/ml thiabendazole.

TBZ was removed. With higher concn and even longer times of incubation, the removal of TBZ from solution was never complete. At 5 to 20 µg and 60-min incubation, the uptake was only about 90%. When calculated in absolute quantities per 200 mg of spores, it is evident that the greatest amount of TBZ was taken up in the first 6 min, with lesser amounts removed per unit time as the time of incubation increased (Fig. 1-D). The permeation apparatus of the spore apparently was saturated rapidly at these concn of TBZ. The higher the concn of TBZ, the more rapid was the uptake of the fungicide by the same wt of spores (Fig. 1-D).

Methanol was used to extract TBZ from spores, and the concn of the fungicide in the extract was determined by the absorption of the methanol solution at 298 nm. Because materials other than TBZ were extracted by methanol and absorbed at this wavelength, they would interfere with a direct measurement of TBZ. A difference spectrum was performed on the extract; one cuvette contained the extract from untreated spores and the other cuvette the extract from spores treated with TBZ. The resulting difference spectrum of the extracts was very similar to that of TBZ alone in methanol (Fig. 1-C), indicating that we probably were extracting the unaltered fungicide by these procedures and that the difference of absorbance at 298 nm would reflect the amount of TBZ being extracted. Eighty per cent of the TBZ that was originally removed by the spores from Czapek's medium could be recovered. Two hundred mg of spores of *A. oryzae* removed 180 of the 200 µg of TBZ in solution, 90% of that originally present. Washing the spores twice with distilled water removed all the free fungicide, 14 µg, from the surface of the spores; further washings were ineffective. Four subsequent extractions, each with 20 ml methanol, removed 146 µg TBZ, presumably that which was tightly bound on or inside the spores.

TBZ was distributed among many of the spore cell

TABLE 2. Distribution of thiabendazole in the components of spores incubated in the fungicide<sup>a</sup>

Fraction	Gravitational forces, g	TBZ µg/400 mg spore
Removed by spores		190
Water washes		13
Sand	Decantation	106
Cell wall and nuclei	2,900 (15 min)	16
Mitochondria	29,000 (30 min)	18
Ribosome	100,000 (90 min)	9
Total		162

<sup>a</sup> A solution of TBZ in methanol was used to make a concn of TBZ in Czapek's medium of 200 µg/20 ml. Spores, 400 mg, were suspended in the 20 ml on a reciprocal shaker for 30 min, removed by filtration and washed with water; then the cell-free extract was prepared. All subsequent extractions were with methanol.

components (Table 2). It is probable that most of the TBZ was free in the cell fluid. When the acid-washed sand that had been used to grind the spores was separated by allowing the sand to settle for 30 min at 4 C followed by decantation of the supernatant and washing the sand with methanol, 106 µg of TBZ were extracted from the sand. Subsequent experiments showed that sand itself also readily adsorbs TBZ from Czapek's medium. It is reasonable to assume that when the sand was ground to a fine powder during cell breakage, it adsorbed the TBZ from the cell fluid. Extraction of the particles that were recovered after centrifugation at 2,900 g for 15 min yielded 16 µg TBZ. This fraction contains cell wall and probably nuclei. The mitochondrial fraction that sedimented on centrifugation at 30,000 g for 30 min contained 18 µg of recoverable TBZ. The ribosomal fraction, removed at 100,000 g, had only 9 µg. Since the total amount of TBZ in the spores after water washes was 177 µg, and total 162 µg were recovered in the various extractions, the complete recovery from the spore fractions was 91%. The recovery from similar extractions from whole cells was 88%.

The distribution of TBZ in or on the various spore fractions probably indicates that the fungicide reacts with some generally distributed component of living cells. It thus furnishes no definite clue to the specific physiological mechanism being attacked by the fungicide. Though effects of TBZ on general physiological functions of whole cells such as the inhibition of growth and oxygen consumption can be guides on mechanism of action of a fungicide, more definitive statements on its mechanism of action must await studies with cell-free systems. These are now under way.

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