The Systemic Fungicidal Effect of Benzimidazole Derivatives and Thiophanate Against Cercospora Leaf Spot of Sugarbeet

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ABSTRACT

Thiabendazole, 2-(4′-thiazolyl) benzimidazole (TBZ); a dichloro derivative of thiabendazole, 2-(4′-thiazolyl) dichlorobenzimidazole (ClTBZ); benomyl, methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate; and thiophanate, 1,2-bis-(3-ethoxycarbonyl-2-thioureido)-benzene, applied systemically, decreased infection by *Cercospora beticola*. Leaves were completely protected from infection when their opposite surface was prophylactically treated. Treatments 5 days after inoculation cured the disease by limiting fungus colonization. Application to infected leaves suppressed sporulation on both treated and untreated surfaces. Benomyl and TBZ applied either to the soil or to a part of the plant protected all leaves. An effect restricted to benzimidazole derivatives was an inhibition of spore germination on the opposite, untreated leaf surface. All those results were attributed to penetration of the fungicides into leaves or roots and to their translocation across the leaf, from root to leaf and from leaf to leaf. ClTBZ was fungicidal at 100 ppm, in vitro. At sublethal concentrations of benzimidazole derivatives, spore germination was abnormal, and numerous initial and distorted germ tubes were observed. Phytopathology 60:1186-1190.

Research on systemic fungicides for the control of *Cercospora* leaf spot in sugarbeet was begun by Staron & Allard (14), who in 1964 found that thiabendazole (TBZ), 2-(4′-thiazolyl) benzimidazole, was highly inhibitory to growth of *Cercospora beticola* Sacc. in culture, and that it moved upward systemically in plants. Later it was found that postinoculation application of TBZ prevented infection of sugarbeet, and that TBZ controlled the disease in the field (2, 6). Recently, the ability of three other experimental systemic fungicides to control *Cercospora* leaf spot of sugarbeet has been suggested. These are: benomyl, methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (3); a dichloro derivative of TBZ (ClTBZ), 2-(4′-thiazolyl) dichlorobenzimidazole; and thiophanate, 1,2-bis-(3-ethoxycarbonyl-2-thioureido)-benzene. The first two and TBZ are related by being derivatives of benzimidazole.

The concept of systemic activity was stated by Dimond et al. (4) as follows: "The systemic fungicide has a known and restricted mode of action: it enters the plant, is translocated as far as the locus of infection (or the potential locus) and then acts directly upon the pathogen by virtue of its fungitoxic properties." Sharvelle (8) made a clear distinction between (i) chemical prophylaxis which prevents disease; and (ii) chemotherapy in which a disease already established is cured. Thus, performance of a systemic fungicide might be determined by several factors such as activity of the fungicide in vitro against a specific pathogen, and the rate of absorption and translocation in the host plant.

The objectives of this research were to investigate the fungitoxicity and comparative performance of these fungicides, their systemic movement in the sugarbeet plant, and the consequent effect on disease development.

MATERIALS AND METHODS.—*Fungicides tested and rate of application.*—The tested formulations were: TBZ—WP 75% and technical, produced by Merck & Co., Inc., Rahway, N.J., USA; ClTBZ—soluble powder, prepared by T. Staron, INRA, Versailles, France; benomyl (Benlate)—WP 50%, produced by E.I. Du Pont de Nemours, Wilmington, Delaware, USA; thiophanate (Cercobin)—WP 50%, produced by Nippon Soda Co., Ltd., Ohtemachi, Tokyo, Japan. For comparison, Brestan 60, containing 60% triphenyl tin acetate and 20% maneb (WP), produced by Farbwerke Hoechst AG, Frankfurt (M)—Hoechst, W. Germany, one of the best protective fungicides for controlling *Cercospora* leaf spot (10, 12) was used. Surfactants were added at the rate of 500 ppm to aqueous suspensions of TBZ and benomyl. When applied to leaves, suspensions were sprayed to runoff with a DeVilbiss No. 15 atomizer. For soil application, the fungicides, suspended in water, were drenched on the soil of 20-cm pots which had been planted with sugarbeets.

Inoculation procedures.—Sugarbeet plants (cv. Zwaanesse III), raised singly in 20-cm pots, were inoculated by spraying with aqueous suspensions of *C. beticola* spores collected from infected leaves. A standard amount of the suspension was evenly atomized on marked circles (28 mm diam) on the upper leaf surface, after which the plants were kept in a humid chamber for 4 days. Lesions began to appear a few days later, and severity of infection, expressed as percentage of the controls, was recorded 2 weeks later, when no additional spots appeared.

Bioassay procedures.—For spore germination tests, *C. beticola* spores were collected from infected leaves, washed twice with water, and centrifuged. Then the spore suspensions were mixed together with the fungicide suspension or solution and placed on a concave slide; germination rate was determined after 24 hr at 25 C. For inhibition tests of mycelium growth, fungicides were added to potato-dextrose agar (PDA), and

TABLE 1. Severity of infection of sugarbeet leaves inoculated on their upper surface by *Cercospora beticola* spores and treated with fungicides on either leaf surface before or after inoculation

Fungicide	Concn in spray (ppm)	Severity of infection (% of check)[a]			
		3 Days preinoculation		5 Days postinoculation	
		Upper[b]	Lower[c]	Upper[b]	Lower[c]
TBZ	120	0.0a[d]	0.0a	21.2bc	8.7b
ClTBZ	120	0.0a	0.0a	4.8b	1.2b
Benomyl	120	0.0a	0.0a	2.3b	0.0a
Thiophanate	120	0.0a	0.0a	44.1c	28.8b
Brestan	360	0.0a	72.7d	100.8d	106.4d

[a] Severity of infection was calculated from the number of spots per inoculated leaf circle of 28-mm diam.
[b] Spray was applied to the upper leaf surface.
[c] Spray was applied to the lower leaf surface.
[d] Values followed by the same letter do not differ significantly from each other at the 5% level.

TABLE 2. Effect of fungicides on in vivo and in vitro germination of *Cercospora beticola* spores

Fungicide	Concn in spray (ppm)	Spore germination on leaves[a]			LD_{95}[d] (ppm)
		On treated side[b]		On opposite side	
		% Germination	Length of germ tubes (μ)	% Germination[c]	
TBZ	120	58.6	<10	11.1	55
ClTBZ	120	51.4	<10	10.9	8
Benomyl	120	46.7	<10	5.0	7
Thiophanate	120	53.4	<10	72.0	10
Brestan	360	1.8	<10	69.8	0.01
Tween 20	1000	91.7	>120	84.3	>1000
Check		92.1	>120	86.2	

[a] Leaves were inoculated 1 day after treatments with fungicides.
[b] Figures are an average of five leaves on different plants, with 100 spores/leaf.
[c] Only spores with germ tubes longer than 10 μ were considered as germinating. Figures are an average of the results from three trials, ten replications of 100 spores each on different plants.
[d] Figures are an average of the results from four experiments, with 500 spores each.

8-mm discs of *C. beticola* cultures were placed on 15 ml of the medium in petri dishes. Inhibition percentage was calculated from the diam of the colonies 8 days later.

The fungus in the leaves was examined after bleaching with chloral hydrate and staining with Trypan blue.

RESULTS.—*Penetration and translocation across the leaf.*—All the fungicides were applied prophylactically to the lower leaf surface. When leaves were inoculated 3 days later on the upper surface no infection occurred except on leaves treated with Brestan (Table 1). A repeated trial showed similar results. Examination of the inoculated leaf surface revealed that spore germination was significantly inhibited on leaves treated with the benzimidazole fungicides, whereas thiophanate did not affect germination (Table 2).

The therapeutic effect (expressed as relative severity of infection) of TBZ and Brestan when applied 1 to 7 days after inoculation is shown in Table 3. Both compounds reduced the severity of *Cercospora* leaf spot if applied up to 3 days after the inoculation. In three repeated trials, application on the 5th day showed a marked decrease in efficacy of Brestan, while TBZ clearly exhibited therapeutic activity. In two trials, application of TBZ on the 6th or 7th day after inoculation still considerably reduced the infection rate.

Microscopic examination of the leaves treated 5 days after inoculation showed numerous stomatal penetrations by germ tubes; many of them colonized the mesophyll and caused necrosis. The percentage of penetrated germ tubes which colonized the leaf tissue was significantly lower with TBZ than with Brestan, which differed only slightly from the control (Fig. 1). The classification of necrotic spots according to their relative size showed a higher percentage of small necrotic spots resulting from application of TBZ.

Comparison of the therapeutic activity of the fungi-

TABLE 3. Severity of the infection of sugarbeet leaves inoculated on their upper leaf surface by *Cercospora beticola* spores and sprayed with fungicides at various intervals after their inoculation on the same leaf surface

Interval between inoculation and treatment	Severity of infection (% of check)[a]	
	TBZ 120 ppm	Brestan 360 ppm
days	%	%
1	0.2	2.4
3	2.2	9.6
5	11.1[b]	69.3[b]
6	35.1[c]	97.4
7	37.1[c]	109.5

[a] Severity of infection was calculated from the number of spots per inoculated leaf circle of 28 mm diam. Figures of each trial are an average of four plants, with 40 circles each.
[b] Average of three trials.
[c] Average of two trials.

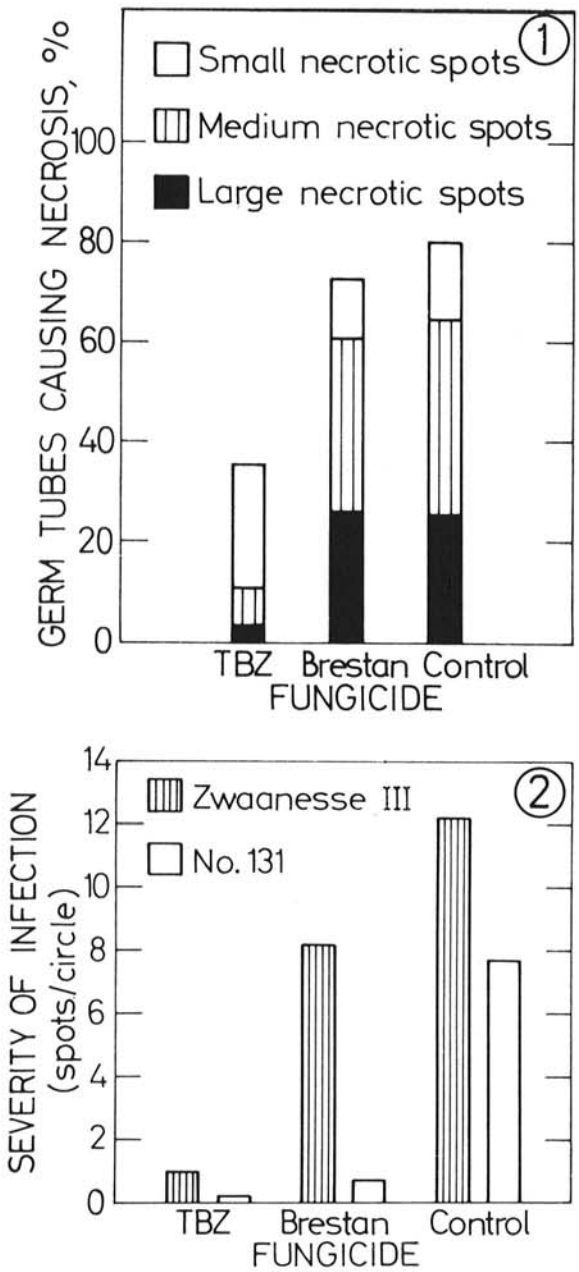

Fig. 1-2. **1)** Development of *Cercospora beticola* infection on sugarbeet leaves treated with suspensions of thiabendazole (TBZ) (120 ppm) and Brestan (360 ppm) 5 days after inoculation. Colonization of the leaf tissue is expressed as percentage of germ tubes which penetrated into stomata and caused necrosis, out of total penetrations; necrosis is classified as small, medium, and large necrotic spots according to diam of 0.3, 0.6, and greater than 0.6 mm, respectively. **2)** Severity of *Cercospora beticola* infection on sugarbeet cv. Zwaanesse III and 131 treated with suspension of thiabendazole (TBZ) (120 ppm) and Brestan (360 ppm) 5 days after inoculation.

cides applied to leaves 5 days after inoculation showed that all but Brestan were effective (Table 1). Benomyl was somewhat superior to TBZ and ClTBZ, and thiophanate was inferior to them. Application to the lower leaf surface seemed more efficient than treatment to the upper, inoculated leaf surface.

On the tolerant sugarbeet cultivar 131, Brestan as well as TBZ controlled the disease even if applied 5 days after inoculation (Fig. 2), because of the slower development of the infection process on this cv. (11).

When the upper surface of leaves was sprayed after symptom appearance but before sporulation, the fungicides reduced sporulation on both leaf surfaces. In two trials, the average reduction ranged between 88 and 98%, benomyl being the most effective. Brestan prevented sporulation on the upper leaf surface of 96% of the spots, but on the lower surface the reduction was only 12%.

Translocation.—When TBZ and benomyl were applied (3 mg/3 kg oven-dry wt soil) to sugarbeet plants grown in pots and the plants were inoculated 9 days later, benomyl reduced severity of infection by 88.9%, whereas TBZ did not affect infection significantly. In three trials, inoculation 20 days after treatments with TBZ and benomyl resulted in an average reduction of severity of infection by 89.2 and 97.5%, respectively.

When untreated leaves were inoculated 3 days after spraying the remaining leaves in the plant with suspensions of 120 ppm benomyl (3 trials) and TBZ, severity of infection was diminished by 75.3 and 61.7%, respectively. Rate of spore germination on those leaves was very low, and germ tubes did not exceed 60 μ. This effect is similar to that found on the opposite surface of treated leaves.

Fungitoxic activity of the tested fungicides.—Fungitoxicity was evaluated in vitro by determining the LD_{95} for spore germination (Table 2). Brestan inhibited spore germination very markedly, at a concentration far below that of the other fungicides, while TBZ was the least inhibitory. At sublethal concentrations of TBZ, ClTBZ, and benomyl, numerous initial and distorted germ tubes were found (Fig. 3).

Mycelial growth of three isolates of *C. beticola* was completely inhibited by TBZ, ClTBZ, benomyl, and Brestan at concn of 2.5 ppm. At 0.1 ppm, mean inhibition percentages of the three equally sensitive isolates with the four materials were 15, 20, 25, and 9, respectively, with no significant difference among them.

In order to determine whether ClTBZ is fungitoxic or fungistatic, spores of *C. beticola* were soaked for 24 hr in solutions of ClTBZ. Results of 11 consecutive trials with spores soaked in ClTBZ, rinsed with water, and placed overnight for germination showed that at concn of 100-1,000 ppm, rate of germination was low and development of germ tubes was poor. When treated at 20 C, the average germination rate was 8.2% with germ tubes in the range of 10-30 μ, whereas at 4 C, a temp below min for germination, the average germination rate was 32% with germ tubes in the range of 10-60 μ. It appears that ClTBZ was fungicidal.

Results of the in vivo experiment, where spore germination was determined on a previously treated leaf surface, are given in Table 2. Brestan prevented germination almost completely, but the other fungicides were only partially effective. When germ tubes under 10 μ in length were considered as ungerminated (12),

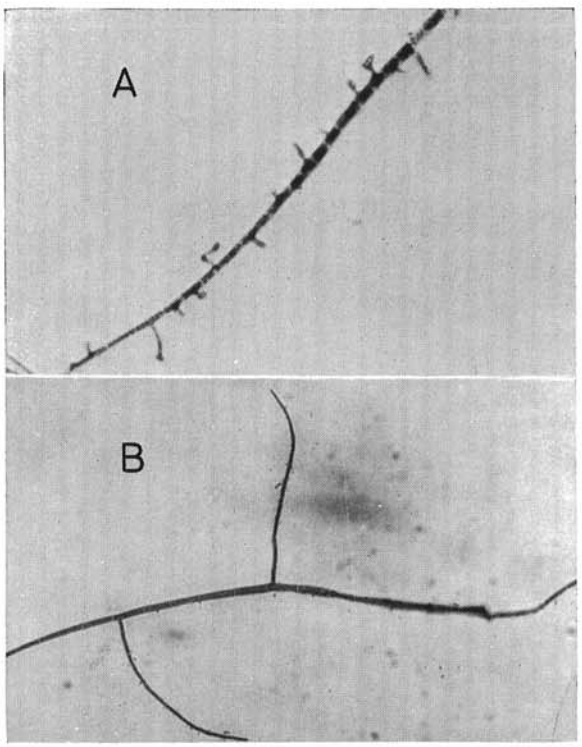

Fig. 3. Germination of *Cercospora beticola* spores in concave slides. **A)** A spore with abnormal initials of germ tubes, germinated in suspension of thiabendazole (10 ppm). **B)** Normal germination of a spore in water.

the systemic fungicides were equal to Brestan as protectants. The growth of germ tubes was extremely inhibited, and they rarely reached a stoma. The effect on spore germination is well reflected in infection results where all the fungicides conferred complete protection to leaves (Table 1). TBZ and Brestan persisted under greenhouse conditions for 45 days without loss of leaf protection. Tween 20 (polyoxyethylene sorbitan monolaurate) was nontoxic in the concn used in these experiments.

Phytotoxicity.—When the tested fungicides were sprayed on leaves in concentrations of 1,200 ppm, no visible phytotoxic symptoms appeared. When applied as a drench to soil in pots at 6 mg/3 kg soil per plant, treatment with TBZ caused chlorosis in the margin of blades on leaves of some of the mature plants. With 3 mg/plant, chlorosis was rare. On seedlings, TBZ at both dosages caused burns of the leaf blade, advancing from the margin of the leaves and from the distal towards the proximal end.

DISCUSSION.—The effect of the fungicides on the development of the disease led to some hypotheses on the systemic movement. The disease-controlling action of the fungicides was ascribed to their innate toxicity against the pathogen, possibly combined with systemic movement in plants. The possibility that the chemical structure of the fungicides might have been altered in the sugarbeet plants or that the metabolism of the host was altered by the fungicides was not excluded by experiments. Protection and curing of both treated and untreated surfaces of leaves, as well as the suppression of sporulation, indicated penetration of the fungicides into the leaves (depth action). Protection of untreated leaves by benomyl and TBZ applied either to the soil or to a part of the plant suggested their translocation in the plant. The movement was either upward in root uptake, or initially downward out of the leaf blade in leaf uptake. Findings on upward movement of TBZ agree with those obtained when sugarbeet roots were immersed in a nutrient solution supplemented with TBZ (15). Furthermore, control of the disease was achieved by drenching the soil with benomyl and TBZ, without interference of adsorption or degradation in the soil. Similar results with TBZ drenched on soil were reported by Stallknecht & Crane (13). Recent investigations showed that benomyl in an aqueous solution breaks down rapidly to the benzimidazole carbamic acid methyl ester (BCM), a stable fungitoxic compound (1). This substance was shown to be taken up and translocated unaltered by cotton plants, and is the one responsible for the antifungal action on leaves of cotton plants grown in benomyl-treated soil (9).

The fungitoxic effect of the benzimidazole derivatives was manifested also upon spores on leaves either when the opposite leaf surface was treated or when the fungicide was translocated from another part of the plant. A description of such an effect has not been found in the literature on systemic fungicides, although in the case of rust-inoculated bean leaves treated with antibiotics on their opposite surface, stomatal exclusion of germ tubes was reported (7). The conclusion that CITBZ is fungicidal seems to disagree with the reported views on TBZ, which was considered fungistatic (5, 14). This controversy might arise from the variations between the chemical structure of the two compounds or, more probably, from the different methods of testing. With the method used in this study, the contact of the spores with the fungicide was over all its external surface, whereas the other workers placed mycelium or spores on solid media, thus forming only partial contact, in addition to using a relatively insoluble toxicant.

A comparison of the performance of systemic fungicides showed that benomyl was slightly superior in chemotherapeutic and genestatic activities and in inhibition of spore germination on the untreated leaf surface, whereas thiophanate was inferior as a chemotherapeutant and did not affect spore germination on the untreated leaf surface. Since the fungicides did not differ considerably in their fungitoxicity, the differences in their performance might be derived from differences in the rates of their systemic movement.

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