Environmental Influences on the Sensitivity of Fusarium oxysporum f. sp. lycopersici to Methylisothiocyanate

Klaus H. Domsch and Malcolm E. Corden

Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331. Present address of senior author: Institut für Bodenbiologie Forschungsanstalt für Landwirtschaft, Braunschweig-Völkenrode, Germany.

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ABSTRACT

In shake culture, *Fusarium oxysporum* f. sp. *lycopersici* produces microconidia from phialides. A rapid decrease in pH of the culture medium from 6.0 to 4.2, followed by an almost equally rapid increase to about 8.0, is correlated with microconidia production. The pH increase is accompanied by decreased sensitivity to methylisothiocyanate (MIT). This decreased sensitivity to MIT is caused neither by the basic pH of the culture nor by morphologic changes in the spores as they age, but is associated with physiological maturity of the spores as demonstrated by the reduced biosynthetic activity of older spores. Reduced toxicity of MIT to *Fusarium* microconidia in potassium phosphate as compared to water at the same pH is not due to pH control during the assay, but is the result of phosphate ion interference with MIT toxicity. Phytopathology 60:1347-1350.

Methylisothiocyanate (MIT) is a highly fungitoxic component in the soil fumigant Vorlex, and is generally believed to be the primary decomposition product accounting for fungitoxicity from Vapam. The action of MIT on fungi is poorly understood, and can be greatly influenced by environmental factors. For example, toxicity to microconidia of *Fusarium oxysporum* f. sp. *lycopersici* is significantly reduced in potassium phosphate buffer and in spores collected during the late stage of logarithmic growth when the pH of the culture medium becomes basic (2). The ultimate cause of these shifts in sensitivity to MIT is unknown.

The present study was initiated to examine critically the influence of potassium phosphate and the pH and age of the fungal inoculum on toxicity of MIT to *F. oxysporum* f. sp. *lycopersici*. An understanding of these factors is necessary for development of valid experimental designs to study toxicity, uptake, and mode of action of MIT, and may eventually aid in determining the optimum conditions for control of plant diseases with fumigants containing or yielding MIT.

MATERIALS AND METHODS.—*Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyd. & Hans. strain R5-6 was grown in shake culture on a wrist action shaker at 25 C with 260 ft-c of artificial light for 16 hr a day. One liter of the liquid medium contained: 25 g glucose, 2.5 g KNO_3, 500 mg $MgSO_4 \cdot 7HOH$, 310 mg K_2HPO_4, 940 mg KH_2PO_4, 10 mg $ZnSO_4 \cdot 7HOH$, 10 mg $FeCl_3 \cdot 6HOH$, 1.25 mg H_2BO_3, 1.0 mg $CaCl_2 \cdot 2HOH$, 0.9 mg $MnCl_2 \cdot 4HOH$, 0.275 mg $CuCl_2 \cdot 2HOH$, and 0.01 mg MoO_3; the initial pH was about 6.1. Two hundred ml of the medium in a 500-ml Kjeldahl flask was inoculated with a 7.0-mm disc cut from the margin of an actively growing *Fusarium* colony on potato-dextrose agar (PDA). At various intervals, the microconidia were separated from the mycelium by filtering the shake culture through cheesecloth and Whatman No. 4 filter paper, then centrifuging at 5,860 *g* for 10 min. The microconidia were resuspended in sterile, deionized water, buffer, or salt solutions and counted in a hemacytometer, and sufficient spores were added to 12-cc glass vials to give 500,000 spores/ml in the final test solution. The vials were sealed with serum caps, MIT solutions were injected into the spore suspensions to give a final volume of 10 cc, and the serum caps were momentarily punctured to equilibrate the pressure within the vials.

MIT solutions were prepared in deionized water immediately before each experiment, and the concn was checked by the absorption max at 235 mμ. Stability of MIT during the assays was verified by periodic measurements of the absorption spectrum between 210-340 mμ.

Spore suspensions containing MIT were incubated in the sealed vials at 25 C on a device that tumbled them 18 times/min. After 2 hr, the spore suspensions were diluted, and 3-ml aliquots were placed in 30 ml of PDA at 41 C adjusted to pH 3.5 with lactic acid. The PDA was distributed in three petri plates; after 4 days the number of *Fusarium* colonies was counted. Dilutions were made so that each control plate contained at least 100 *Fusarium* colonies. Each experiment was repeated at least 3 times and analyzed statistically when applicable. The effectiveness of MIT was expressed as percentage inhibition of colony formation.

RESULTS AND DISCUSSION.—*Development of F. oxysporum f. sp. lycopersici in shake culture.*—Microscopic examinations of *Fusarium* shake cultures were made periodically to determine the extent to which morphologic changes in microconidia may be correlated with sensitivity to MIT. The number of spores produced and the pH of the culture medium were concomitantly measured to provide a full description of growth in shake culture.

Following germination of the inoculum (Fig. 1-A), hyphae are produced with relatively few branches (Fig. 1-B). Phialides develop extensively along the hyphae and many microconidia are produced (Fig. 1-C). These spores from shake cultures are often mis-

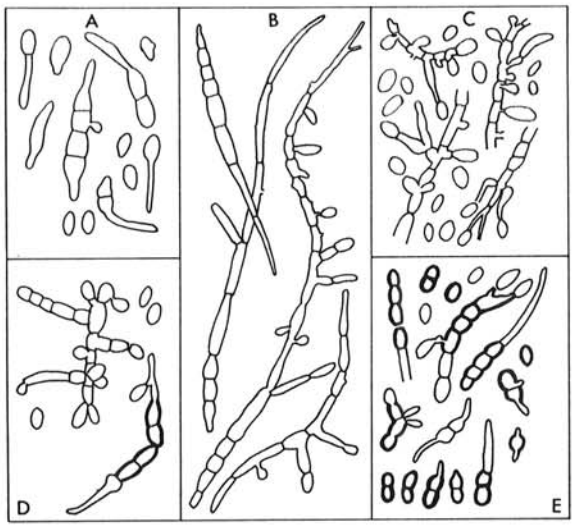

Fig. 1. Development of *Fusarium oxysporum* f. sp. *lycopersici* in shake culture. **A)** Germination of the inoculum (after 20 hr, pH of medium 6.1). **B)** Formation of long hyphae with few branches, initiation of phialides (30 hr, pH 6.1). **C)** Development of many phialides along the hyphae, production of microconidia beginning (40 hr, pH 6.1). **D)** Heavy production of microconidia, beginning of fragmentation of hyphae, and occurrence of thick-walled cells (60 hr, pH 6.2). **E)** Mycelial fragmentation and transformation of older spores into chlamydospores almost complete (90 hr, pH 4.2).

takenly called bud-cells (e.g., 4, 5), but there is no evidence of budding and they should be termed phialospores. Finally, most of the hyphae fragment (Fig. 1-D) and the hyphal cells and microconidia become thick-walled chlamydospores (Fig. 1-E).

The pH of the *Fusarium* shake culture medium changes drastically during the logarithmic phase of growth (2). This pH change is correlated with spore production (Fig. 2), and is a more accurate index of the morphological development of a particular culture than is age. When the microconidia reach a concn of about 10^7/ml, the pH of the medium rises slightly to about 6.3, then drops sharply to 4.2 at about 95 hr. At this point, the glucose in the medium is consumed completely and the morphological development of the fungus is almost completed (Fig. 1-E). Subsequently, spore production slows, and the pH of the medium increases to about 8.0 in 170-hr cultures. In cultures of pH 4.2 to 8.0, there is no significant increase in the number of thick-walled spores; thus, it is doubtful that changes in sensitivity to MIT are caused by an increased proportion of thick-walled resistant spores in the older basic cultures.

Influence of spore age, culture pH, and metabolic activity on sensitivity of Fusarium spores to MIT.—*Fusarium* microconidia collected from cultures at pH 4.2 and 7.5 were treated with various concn of MIT at pH 5.8 in water or in 0.1 M potassium phosphate buffer to determine the influence of the pH of the culture at the time the spores were collected on susceptibility of the spores to MIT. The pH of the spore

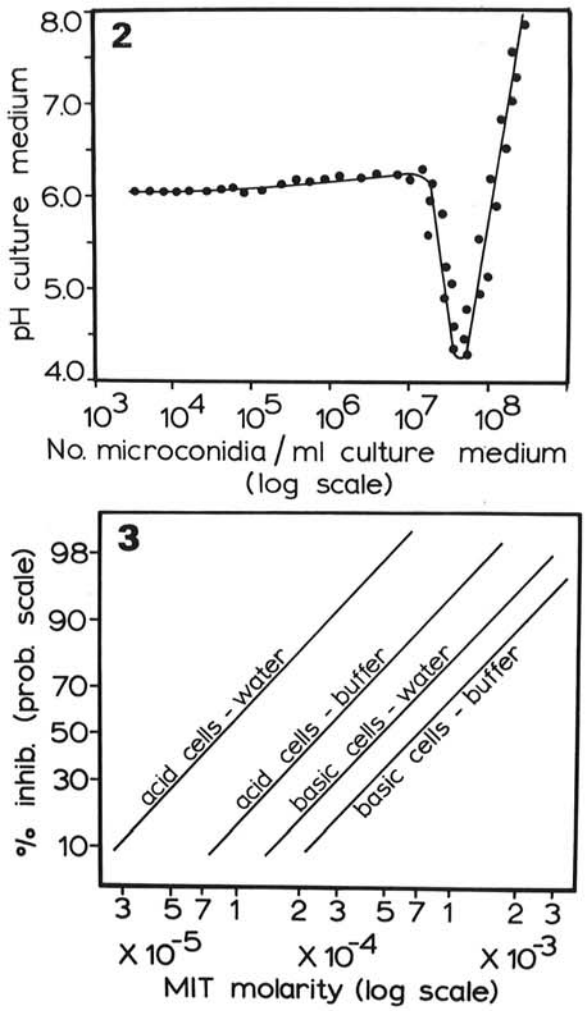

Fig. 2-3. **2)** Changes in the pH of a shake culture medium correlated with microconidia production by *F. oxysporum* f. sp. *lycopersici*. Microconidia were collected at 10-hr intervals during a 7-day period. **3)** Toxicity of methylisothiocyanate (MIT) at pH 5.8 in water and 0.1 M potassium phosphate buffer to microconidia of *F. oxysporum* f. sp. *lycopersici* from acid and basic shake cultures. Toxicity was measured as inhibition of colony formation by treated spores. The dosage-response lines represent the means of at least three experiments.

suspension could affect MIT toxicity by influencing the uptake of the toxicant through differentially charged cell membranes.

The slopes of the dosage-response curves for MIT are similar irrespective of the pH of the culture from which the spores were obtained and the composition of the assay medium (Fig. 3). But microconidia from the acid medium are significantly more sensitive to MIT than those from a basic medium, and MIT is significantly more toxic in water than in phosphate buffer at the same pH.

Experiments were designed to determine the influence of the ambient medium on MIT toxicity to spores independent of their age. Equal portions of microconidia from a pH 4.2 culture (4-day-old) were

TABLE 1. Sensitivity of microconidia of *Fusarium oxysporum* f. sp. *lycopersici* to methylisothiocyanate (MIT) in water and potassium phosphate buffer following incubation in acid or basic shake culture medium

Reaction of culture medium during:		Inhibition of colony formation by microconidia treated with MIT in:[c]		
Growth[a]	Pretreatment[b]	Water	Phosphate buffer	L.S.D. ($P = .05$)
		%	%	
Acid	Acid	86	74	9
Acid	Basic	91	72	6
Basic	Basic	43	38	9
Basic	Acid	38	26	18
L.S.D.	($P = .05$)	14	16	

[a] H+-ion reaction of the shake culture medium when the microconidia were harvested (acid = pH 4.2; basic = pH 7.5).

[b] Microconidia were suspended in acid (pH 4.2) or basic (pH 7.5) cell-free culture media for 90 min prior to treatment with MIT.

[c] Treatment with MIT (5.0×10^{-4} M) at pH 5.8 was made in water or in 0.1 M potassium phosphate buffer.

resuspended in either their own medium or in the cell-free medium from a pH 7.5 culture (about 7 days old). Two corresponding treatments were prepared with spores collected from a pH 7.5 culture. The four spore suspensions were incubated for 90 min on a shaker; then the microconidia were collected and exposed to MIT in the standard assay.

Incubation of microconidia from an acid culture in a basic medium or vice versa failed to influence their response to MIT significantly in either water or potassium phosphate buffer (Table 1). Spores from the acid medium maintain their high sensitivity to MIT in water and phosphate buffer.

Additional evidence on the influence of the pH of the ambient medium and age of the inoculum on MIT sensitivity was obtained. Beginning when the *Fusarium* shake culture pH had dropped to 4.2, sufficient 0.1 N HCl was added to maintain the pH at 4.2 until the 7th day, when the nonacidified culture reached 7.2. The microconidia were then collected and exposed to MIT in the standard assay.

There was no significant difference in the sensitivity of spores from acidified and basic cultures to two concn of MIT (Table 2). Thus, microconidia of the same age have a similar sensitivity to MIT regardless of the pH of their culture medium.

TABLE 2. The effect of methylisothiocyanate (MIT) on microconidia of *Fusarium oxysporum* f. sp. *lycopersici* from 7-day-old acid and basic shake cultures

pH of shake culture	MIT concn	Inhibition of colony formation from treated microconidia
	M	%
4.2[a]	2.0×10^{-4}	14
7.2	2.0×10^{-4}	11
4.2[a]	5.0×10^{-4}	31
7.2	5.0×10^{-4}	28

[a] The pH of the shake culture was maintained at 4.2 by the addition of HCl from the time the pH reached 4.2 (4th day) through the 7th day of growth.

As noted earlier, differences in the MIT sensitivity of *Fusarium* microconidia from 4- and 7-day-old shake cultures are not due to morphologic changes that occur as the spores age. But the metabolism of the young and older spores is probably quite different and may account for the differences in MIT sensitivity. Accordingly, an experiment was designed to determine the degree to which utilization of a carbohydrate might be correlated with sensitivity to MIT. In a typical experiment, one ml of a glucose-1-^{14}C solution (0.3 μc/ml) was added to 19 ml of a *Fusarium* spore suspension (2×10^6 spores/ml). Microconidia from 4- and 7-day-old shake cultures (pH 4.2 and 7.2, respectively) were used. Starved spores from the younger cultures were obtained by a 24-hr incubation in a glucose-free medium prior to the experiment. Utilization of glucose at 25 and 500 μg/ml was determined in a modified respirometer according to the method of Wang & Krackov (7). The CO_2 produced metabolically was collected hourly in 10 ml of a solution containing one part absolute ethanol and two parts 2-aminoethanol. The trapping solution was diluted to 15 ml with absolute ethanol, and a 5-ml aliquot was mixed with 10 ml of a scintillation mixture containing 3.0 g terphenyl and 30 mg of 1,4-bis-2′(5′-phenyloxazolyl) benzene/liter of toluene. The scintillation mixture was placed in 20-ml glass vials and counted in a Packard Tri-Carb Model 314-DC liquid scintillation counter.

After 5-hr incubation, the microconidia and media were separated by centrifugation, and aliquots were counted in a thixotropic gel (8). The amount of $^{14}CO_2$ and radioactivity in spores was expressed as a percentage of radiochemical activity originally administered.

Young and older microconidia metabolize a low concn of glucose similarly, but with more glucose available, the young spores incorporate a greater proportion of the substrate than do the older ones (Table 3). This indicates that biosynthetic processes predominate in the young spores, particularly when they are starved immediately before the experiment, and sug-

TABLE 3. Utilization of glucose-1-^{14}C by microconidia of *Fusarium oxysporum* f. sp. *lycopersici* during 5-hr incubation on labeled glucose

Age of cultures[a]	Substrate concn	Recovery of radioactivity	
		In microconidia	As $^{14}CO_2$
days	*μg/ml*	%	%
4	25	28	67
4[b]	25	75	20
7	25	27	63
4	500	48	48
7	500	26	70

[a] Microconidia were collected after 4 and 7 days from shake cultures with final pH values of 4.2 and 7.2, respectively.

[b] Starved microconidia were obtained by incubation in a glucose-free medium for 24 hr before addition of glucose-1-^{14}C.

gests that sensitivity to MIT is related to enhanced biosynthetic activity.

Microconidia from a 7-day-old shake culture (pH 8.2) were incubated in a glucose-free medium for 24 hr before treatment with MIT to determine if MIT sensitivity might be induced in resistant spores in which biosynthetic activity was stimulated by starvation. Correspondingly nonstarved spores were produced by incubation in a complete medium for the 24 hr immediately before MIT treatments.

Colony formation by nonstarved and starved microconidia is inhibited 4 and 30% respectively by MIT at 5×10^{-4} M. Similar results were obtained at other MIT concn; thus, sensitivity to MIT is correlated with, if not causally related to, a high biosynthetic activity in *Fusarium* microconidia.

The influence of potassium phosphate on fungitoxicity of MIT.—It is doubtful that decreased toxicity of MIT in potassium phosphate buffer as compared to activity in water at a comparable pH is attributable to maintenance of a constant H^+-ion concn during the assay. The pH shift of MIT in water is negligible during a 2-hr assay, and inhibition of colony formation from *Fusarium* microconidia produced at pH 4.2 and treated with MIT (5×10^{-4} M) in 0.1 M potassium phosphate is relatively constant ($46 \pm 3\%$) over the pH range 4.0-7.0. The latter was unexpected considering the pH dependence of MIT inhibition of acetate respiration (3).

The independence of MIT toxicity and the assay pH was further evident in the differential suppression of toxicity by other buffers. For example, 0.1 M potassium citrate buffer at pH 5.8 fails to suppress MIT (5×10^{-4} M) toxicity significantly relative to water, but potassium acetate buffer under comparable conditions reduces toxicity (69%) significantly more than does the phosphate buffer (29%). The influence of these buffers on fungitoxicity suggests that they may be important confounding factors in studies on the mode of action of MIT.

In standardized spore germination assays (1) in the absence of MIT, 0.1 and 0.01 M potassium phosphate at pH 5.8 inhibit germination of *Fusarium* microconidia 73 and 12%, respectively; thus, the influence of potassium phosphate concn on inhibition of colony formation by MIT (5×10^{-4} M) was determined. Between 0.03 and 3.0 M, the buffer was equally effective in reducing MIT toxicity. The failure of potassium phosphate concn to differentially influence MIT toxicity may occur because in this experimental design the microconidia were germinated in an agar medium in which the toxicant and buffer had been greatly diluted.

The influence of various potassium and phosphate salts on MIT toxicity was determined. Microconidia from a pH 4.2 shake culture of *Fusarium* were incubated in MIT (5×10^{-4} M) with various equimolar salt solutions (Table 4). The three phosphates uniformly reduced MIT toxicity, while the other potassium salts failed to lower toxicity significantly. Thus, the phosphate ion appears responsible for interference with MIT toxicity.

TABLE 4. The effect of various salts on toxicity of methylisothiocyanate (MIT) to microconidia of *Fusarium oxysporum* f. sp. *lycopersici*

Salts[a]	Inhibition of colony formation from microconidia treated with MIT at 5.0×10^{-4} M
	%
$KH_2PO_4 + K_2HPO_4$	42
Na_2HPO_4	44
$(NH_4)_2HPO_4$	41
KCl	66
K_2SO_4	65
KNO_3	67
Control (water)	70

[a] Microconidia were treated with MIT for 2 hr in the presence of 0.1 M salt solutions adjusted to pH 5.8 with HCl or NaOH.

MIT destroys the cytoplasmic membrane of *Saccharomyces cerevisiae*, possibly through specific reaction with the phospholipid portion of the membrane (6). Similar action of MIT on *Fusarium* may account for the sparing action of the phosphate ion on MIT toxicity.

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