# Comparative Interaction of n-dodecylguanidine Acetate With Four Plant Pathogenic Fungi

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ABSTRACT

The relative tolerance of *Venturia inaequalis, Blumeriella jaapii, Fusarium solani* f. sp. *phaseoli,* and *Colletotrichum orbiculare* in continuous exposure (fungistatic test) to *n*-dodecylguanidine acetate (dodine) was in the ratio 1:4:14:30, respectively, determined as $ED_{50}$ doses. The difference in reaction of *F. solani* and *C. orbiculare* was in part due to the time required for toxic dose to be sorbed. *F. solani* was more sensitive to short exposures than was *C. orbiculare*. The time course of sorption varied among the organisms. The amount taken up increased continually with *B. jaapii* and *C. orbiculare,* increased to a plateau with *V. inaequalis,* and increased to a peak followed by an apparent decrease in sorbed material with *F. solani.* The latter may involve a detoxification that would explain the relative tolerance of this organism to dodine. Phytopathology 60:345-349.

The sorption of *n*-dodecylguanidine acetate (dodine) by fungal conidia has attracted attention in studies of the mode of action of the compound because of its marked surfactant properties (6, 9, 10, 11). It has been proposed that the plasmalemma of the cell is one of the sites of action of dodine (4, 5, 6, 7, 10), but a later report suggested that the principal effect of dodine on the plasmalemma was to allow more molecules to move into the cell where the ultimate toxic reaction occurred (11). In either case, the action of dodine appeared to be closely related to its surfactant properties.

Miller (9) first reported differences in sensitivity of fungal conidia to dodine in studies primarily concerned with determining uptake and innate toxicity. In these tests, *Venturia inaequalis, Neurospora sitophila,* and *Glomerella cingulata* were more sensitive to dodine than were the other fungi tested. Sensitivity to dodine was not correlated with rapid uptake of the chemical, as *G. cingulata* conidia sorbed dodine more slowly than even some of the more resistant fungi.

Somers & Pring (11) reported that *Neurospora crassa* and *Alternaria tenuis* differed in sensitivity to dodine with $ED_{50}$'s of 10,000 and 24,000 μg/g spore wt, respectively. Cell-free walls of *A. tenuis* had a greater affinity for dodine than those of *N. crassa,* although intact *N. crassa* conidia sorbed more dodine than intact *A. tenuis* conidia. It was suggested that the binding to the cell wall was a type of detoxification of dodine, and that this provided an explanation for the differences in sensitivity.

The following study examines the toxicity of dodine to four different plant pathogenic fungi and relates the uptake of dodine-$^{14}C$ by the four fungi to their sensitivity to that chemical.

MATERIALS AND METHODS.—The fungi used were *Venturia inaequalis* Cke. (Wint.), *Blumeriella jaapii* v. Arx (*Higgensia hiemalis*), *Fusarium solani* f. sp. *phaseoli* (Burk.) Snyd. & Hans., and *Colletotrichum orbiculare* (Berk. & Mont.) v. Arx. Conidia of *C. orbiculare* and *F. solani* were collected from cultures grown for 6-12 days on potato-dextrose agar (PDA) slants. Conidia were washed by centrifugation and resuspended in sterile distilled water. The suspensions were quantified by turbimetry using a Bausch and Lomb Spectronic 20 colorimeter set at 425 mμ. Conidia of *B. jaapii* were harvested and prepared as described above from 10- to 14-day-old cultures. Conidia of *V. inaequalis* were harvested from lesions on heavily infected apple leaves collected from an unsprayed orchard, and were stored as a frozen aqueous suspension at −23 C. Concentration of both *B. jaapii* and *V. inaequalis* spores was determined by means of a haemocytometer.

Concentrations of spores used in these tests varied from organism to organism because of production and handling problems. To facilitate comparison of the sensitivity of the four fungi to dodine, the mean dosage of dodine/spore required for a specific response is expressed as picograms ($10^{-12}$ g) per spore (pg/sp). The procedure used in any series of tests was completely standardized with regard to number, type, and size of pipettes, beakers, and other containers used. Since free toxicant in test solutions varied somewhat between test series due to adsorption to glass or plastic surfaces or to materials present in the germination medium, comparisons of values given are valid only within the confines of a particular series of tests.

The germination medium was a potato extract broth (PEB) made by diluting (1:10) a clear decoction obtained by autoclaving 100 g of potato slices in 1 liter of water. This was used in all bioassays, as it supported more vigorous germination of conidia of all four species of fungi used than did distilled water alone, 0.5% glucose, or either of two synthetic media.

Two types of bioassay procedures were used. When continuous contact between spores and a dodine solution was desired, 0.5 ml of a suspension of washed conidia was added to 4.5 ml of the test solution of dodine dissolved in PEB. All tests were made in 60-mm plastic petri plates. A second bioassay procedure determined the effect of short exposures of spores to dosages of dodine above the $ED_{99}$ level. Spores suspended in

5 ml distilled water were added to 95 ml of a dodine-PEB solution to give the desired spore:toxicant ratios. After specific periods of exposure with constant agitation, 2 ml of the suspension were removed by a glass syringe, and the suspending medium was expelled through a 1.2-μ Millipore filter in a 13-mm Swinny adaptor. Metal screens were placed on both sides of the filter so that after each volume of liquid was expelled, a fresh volume could be drawn through the filter resuspending the conidia. After three washes with water, the spores were resuspended in 0.5 ml water and added to 4.5 ml potato extract.

Pure dodine melting at 136-137.5 C dissolved in either PEB or sterile distilled water was used for the toxicity studies. The uptake of dodine was studied using dodine-$^{14}$C (specific activity 125 μc/mM) that was synthesized in a refluxing mixture of dodecylamine, cyanamide-$^{14}$C, acetic acid, water, and isopropanol, and purified by diethyl ether washes (1, 2).

Radioactivity was assayed in a Packard Inc. Tri-Carb Scintillation Spectrometer. The scintillation fluid was composed in 5 g 2,5-diphenyloxazole (PPO), 0.1 g 1,4-bis-[2-(5-phenyloxazole)]-benzene (POPOP), and 66.7 g "Carb-O-Sil" thixotropic gel/1,000 ml of toluene. The gel present at 3.5% w/w held solid materials in suspension and allowed more efficient counting of radioactivity present.

Uptake of dodine-$^{14}$C was measured by counting the $^{14}$C bound to intact conidia collected on a Millipore filter. Conidia were exposed to dodine-$^{14}$C in distilled water, and after predetermined time intervals, aliquots were removed and washed on the filter with five 2-ml volumes of water. Finally, a 2-ml volume of air was forced through the adaptor to remove free water and settle the conidia firmly against the filter. The filter was then removed and placed into a polyethylene vial containing scintillation fluid, along with a small bit of tissue used to carefully wipe the interior of the filter adaptor.

Viability of the spores during the uptake studies was determined by adding a drop of the spore-toxicant mixture to 5 ml of PEB in plastic petri plates. The unsorbed dodine was diluted at least 100 times, so the concentration of toxicant remaining was insignificant for the purposes of these tests. In all bioassay procedures incubation was at 24 ± 2 C; the average germination of 200 spores in each of two replicate plates was determined. Values reported are percentages of control germination.

RESULTS.—*Toxicity of dodine to conidia.*—The relative toxicity of dodine to conidia of *F. solani, C. orbiculare, V. inaequalis,* and *B. jaapii* was determined by the continuous exposure bioassay procedures described above. The initial dosage of dodine required to prevent germination of 95% of the conidia of *C. orbiculare* was nearly 30 times that required for a similar response by conidia of *V. inaequalis* (Fig. 1). Germination of the former was inhibited 50% by 159 pg/sp, while only 3.5 pg/sp were required for 50% inhibition of the latter. Conidia of *C. orbiculare, F. solani, B. jaapii,* and *V. inaequalis* required initial

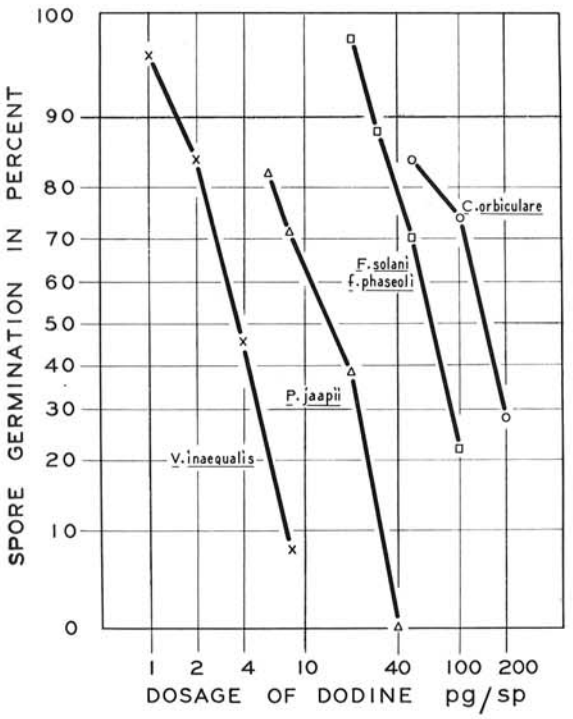


**Fig. 1.** Germination of spores of *Venturia inaequalis, Blumeriella jaapii, Fusarium solani,* and *Colletotrichum orbiculare* at various dosages of dodine.

dosages of 290, 135, 40, and 10 pg/sp, respectively, for 95% inhibition of germination.

Since metabolic processes in inactive spores frequently differ from those of the germinating spore, there was a possibility that the sensitivity to the toxicant might also differ. To explore this point, the time of exposure to the toxicant that was required for lethal action to occur with freshly harvested (inactive) and preincubated (activated) spores of *F. solani* was determined. For the preincubation treatment, the spores were suspended in germination medium for approximately 60% of the time that would be required for 50% of the spores to begin to germinate (4.5 hr for *F. solani*; 5.5-6 hr for *C. orbiculare*). The conidia were then washed and exposed to hyperlethal doses of dodine (200-800 pg/sp) for periods ranging from 2-64 min.

The response of the two species differed strikingly (Fig. 2). Germination of *F. solani* was completely inhibited after 4-min exposure at 400 pg/sp. When the dosage was 200 pg/sp, the germination of conidia of *F. solani* decreased with time of exposure to about 10% at 16 min. The preincubation treatment decreased the sensitivity somewhat at longer exposures. Freshly harvested spores of *C. orbiculare* were not affected at 400 pg/sp after 60-min exposure, and were much more sensitive to a dosage of 800 pg/sp than were the preincubated spores.

It was apparent that the spores of the four species differed substantially in their sensitivity to applied doses, with *V. inaequalis* being the most sensitive and *B. jaapii* next, followed by *F. solani* and *C. orbiculare*

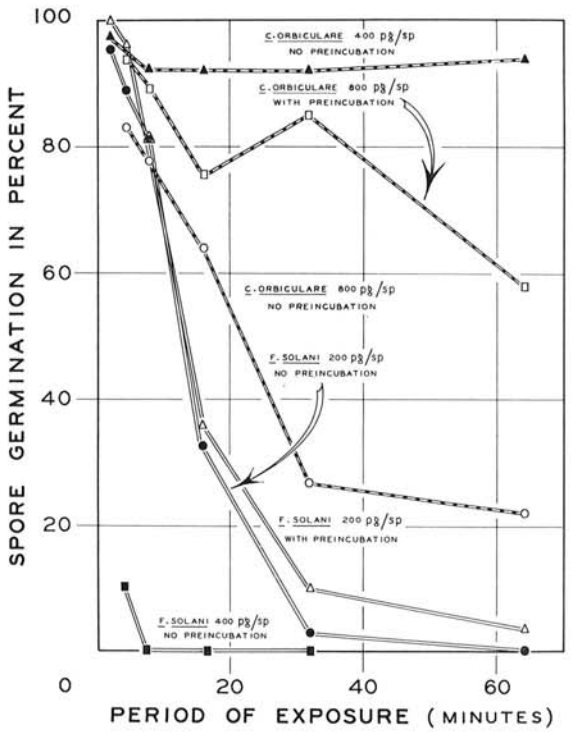


**Fig. 2.** The effect of dosage of dodine and preincubation in germination medium before exposure to toxicant on germination of conidia of *Fusarium solani* and *Colletotrichum orbiculare*.

as the least sensitive. Such differences in sensitivity could reflect differences in rate of uptake or in inherent toxicity of the chemical to the spores of the various fungi.

*Sorption of dodine-$^{14}$C by conidia.*—The rate of uptake of dodine-$^{14}$C by spores of the four fungi and the concurrent effect on germinability of the spores was determined to differentiate effects due to uptake from those on inherent toxicity. Spore concentrations of $1 \times 10^7$ spores/ml were used with *F. solani* f. *phaseoli* and *C. orbiculare*. *V. inaequalis* and *B. jaapii* were used at 5 and $1 \times 10^6$ ml, respectively. Samples of spores were withdrawn at designated intervals, washed to remove unsorbed material, and the amount of radioactive label was retained by the spores determined. Viability of spores was determined at each sampling time by methods noted earlier. The time required for withdrawing and washing the samples was approximately 30 sec. The experiments with each of the fungi were repeated many times; typical data are presented in Fig. 3.

The spores of the four fungi differed with respect to the rate of sorption, the amount of labeled material sorbed, and the concurrent effect on germinability. *C. orbiculare* sorbed the least dodine-$^{14}$C of any of the fungi tested, and was inhibited the least. The other three organisms all sorbed between 1.5 and 2 pg/sp when supplied with a 4-5 pg/sp dose. *V. inaequalis* sorbed 55 and 61% of the maximum amount ultimately sorbed from the 5 and 10 pg/sp doses, respectively, within the first 2-min exposure. The amount retained by the spores increased during the next 60 min, and then declined by 90 min. This decline did not always occur. The initial (2-min) sorption by *B. jaapii* was similar to that with *V. inaequalis*, except that the sorption from the 2 and 4 pg/sp dosage was proportionately greater, representing 71 and 75% of that sorbed at 96 min. The amount retained increased steadily throughout the exposure period, and no maximum was reached. The initial sorption by *C. orbiculare* was small, and there was little increase during the subsequent 16 min. The amount of label retained then increased steadily during the remainder of the exposure period. Sorption by conidia of *F. solani* exposed to the 4 pg/sp dose was similar to that of *V. inaequalis*. The immediate sorption amounted to 1.1 pg/sp, and this increased to 1.6 pg/sp by 8 min. Maximum sorption was reached at 48 min, after which the amount of label retained decreased during the remainder of the experiment to a level equal to the 8-min value. At the low dosage of 1 pg/sp, sorption was 0.2 pg/sp at zero time, reached a peak of 0.55 pg/sp at 36 min, and decreased to 0.1 pg/sp at 96 min. This pattern of label retention was consistent in all experiments.

The comparative effect on the viability of the spores was also determined; the results are indicated in Fig. 3. Inhibition of spore germination did not reach 50% during the period of the exposure at the lower dosages used with *C. orbiculare* and *F. solani*. At the 4 pg/sp dosage, the $ED_{50}$ was 1.65 pg/sp for *F. solani* and 0.67 for *C. orbiculare*. The situation is complicated with the other two organisms, where both dosages resulted in inhibition exceeding 50%, and more than one $ED_{50}$ value resulted. $ED_{50}$ values of 1.75 and 2.35 pg/sp were obtained with *V. inaequalis* at the 5 and 10 pg/sp dosage, respectively. With *B. jaapii*, $ED_{50}$ values of 0.83 and 1.53 were noted at the 2 and 4 pg/sp doses, respectively. When the dry wt of the spore preparations used was determined and the $ED_{50}$ calculated on a spore wt basis, the results (Table 1) indicated that for equal dosage the $ED_{50}$ values were quite similar for all of the organisms except *V. inaequalis*. The fact that the spores of this fungus had been collected from leaves of orchard trees and contained a small percentage of contaminants may account for the decreased values.

The relation between dosage of dodine to response of *F. solani* was studied further by altering the dosage in two ways: (i) by changing the amount supplied while maintaining a constant spore density; and (ii) by maintaining the concentration of toxicant constant, but altering the spore density. In one such experiment, suspensions containing $10^3$, $10^4$, and $10^5$ spores/ml of germination medium were exposed to dodine concentrations of 2, 4, 6, and 8 μg/ml. The specific dosages and the resulting germination data are given in Table 2. A 10-fold decrease in spore concentration effectively increased the dose per spore by a comparable amount. The change in germination with a 10-fold increase in effective dosage achieved by diluting the spore suspension was comparable to that obtained by doubling the dosage at the original spore concentration. It is ap-

parent from this that there was an interaction between spore density and response to a given dosage with *F. solani.* There is no apparent explanation for this effect. It may be that a proportion of the material supplied is sorbed in a way that is not related to a physiologically active site in the cell.

DISCUSSION.—The reaction of conidia of the four plant pathogenic fungi to dodine differed in three ways. First, in a fungistatic test the parallel dosage-response curves were displaced along the dosage axis. The relative tolerance, as indicated by $ED_{95}$ values, was in the ratio of 1:4:14:30 for *V. inaequalis, B. jaapii, F. solani,* and *C. orbiculare,* respectively. This seemed to reflect a fundamental difference in sensitivity.

A second difference in type of reaction to dodine was noted when the latter two fungi were exposed to high doses for short periods of time. When conidia were exposed to 400 pg/sp for 8 min, subsequent germination of *F. solani* was completely inhibited, whereas that of *C. orbiculare* was not affected by exposure to this dosage for 64 min. Preincubation of the conidia of these two fungi before exposure to toxicant for periods just short of that required for germination had little effect on the toxicity of dodine to *F. solani,* but resulted in an increase in the germination of *C. orbiculare* from 27 to 85% when spores were exposed to 800 pg/sp for 32 min. The germinating spores of the latter fungus were less sensitive to the toxicant than were the inactive spores. While the volume of the conidia undoubtedly changed somewhat during this period, the amount would not have been enough to significantly affect the results.

A third means used for comparing differences in the sensitivity of organisms to a chemical is the innate toxicity of the latter to each of the organisms. McCallan & Miller (8) have defined innate toxicity as the amount of the chemical that must be taken up by the fungus to achieve a given degree of physiological effect (e.g., $ED_{50}$). The results reported in Fig. 3 relating the uptake of the chemical by the four organisms to the corresponding decrease in germination suggested that with dodine, at least, the length of exposure required for germination to be reduced 50% was determined by the dosage as well as the spore characteristics. When

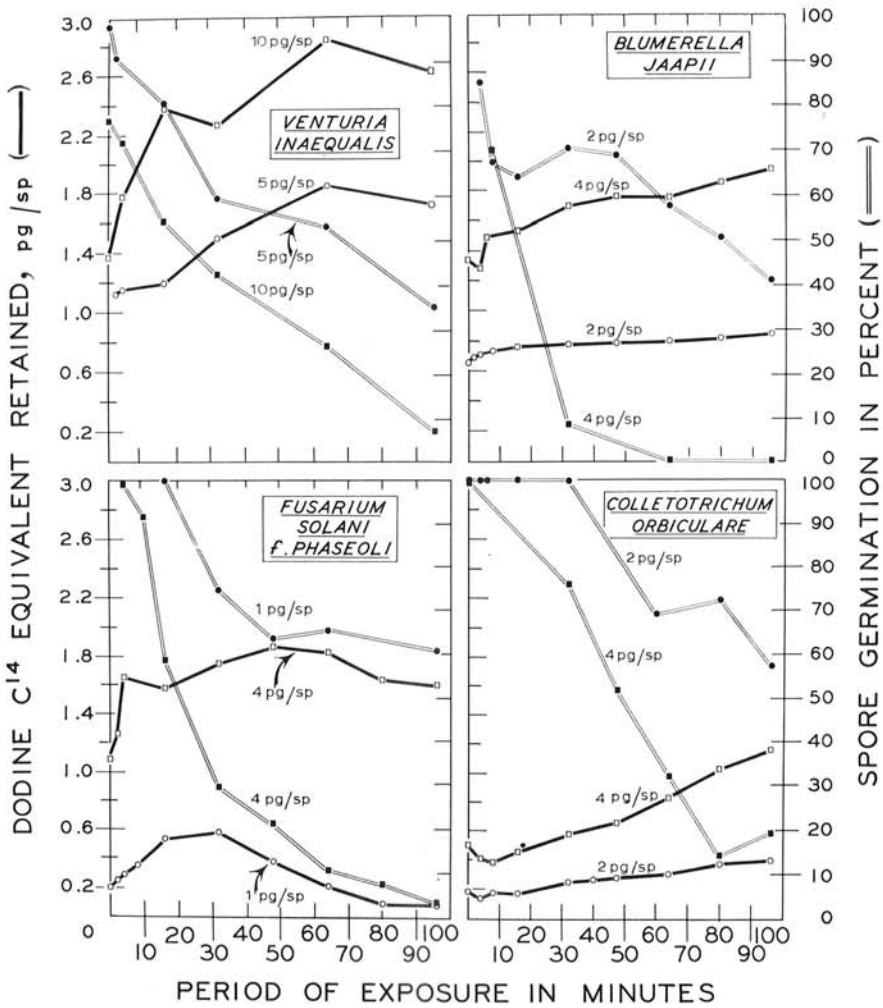


**Fig. 3.** The effect of time of exposure to two dosages of dodine on the viability of spores of four plant pathogenic fungi and on their sorption and/or retention of dodine-$^{14}$C or its labeled metabolite.

TABLE 1. The relation between inherent toxicity values, applied dosages, and time of exposure of dodine to spores of four fungi

| Test organisms | Dry wt/ conidium (pg) | Dosage applied (pg/sp)[a] | Time of exposure (min)[b] | $ED_{50}$ expressed as pg dodine/ | |
|---|---|---|---|---|---|
| | | | | spore | pg dry wt |
| *Blumeriella jaapii* | 0.26 | 2 | 80 | 0.83 | 3.2 |
| | | 4 | 16 | 1.53 | 6.0 |
| *Colletotrichum orbiculare* | 0.11 | 4 | 44 | 0.67 | 5.8 |
| *Fusarium solani* f. sp. *phaseoli* | 0.32 | 4 | 20 | 1.65 | 5.1 |
| *Venturia inaequalis* | 0.73 | 5 | 69 | 1.75 | 2.4 |
| | | 10 | 22 | 2.35 | 3.2 |

[a] pg/sp picogram ($10^{-12}$ g) per spore.
[b] Time of exposure to designated dosage for germination to be reduced to 50%.

TABLE 2. The effect of concentration of conidia of *Fusarium solani* on the toxicity of an applied dosage of dodine

| Dodine applied µg/ml suspension | Concn of spores/ml | | | | | |
|---|---|---|---|---|---|---|
| | $10^5$ | | $10^4$ | | $10^3$ | |
| | Dosage pg/sp[a] | Germination % | Dosage pg/sp | Germination % | Dosage pg/sp | Germination % |
| 2 | 20 | 82 | 200 | 27 | 2,000 | 16 |
| 4 | 40 | 26 | 400 | 9 | 4,000 | 3 |
| 6 | 60 | 10 | 600 | 1 | 6,000 | 0 |
| 8 | 80 | 0 | 800 | 0 | 8,000 | 0 |

[a] pg/sp picogram ($10^{-12}$ g) per spore.

the response of the various fungi was compared on the basis of the dosage per spore, *C. orbiculare* was the most sensitive ($ED_{50}$, 0.67), but it sorbed the chemical more slowly, and was thus affected less than were the spores of the other species. *V. inaequalis,* on the other hand, was the least sensitive ($ED_{50}$, 1.75-2.35), but sorbed the required dose quickly and, thus, was affected most in the tests. The other two organisms were intermediate in this respect, but showed the same relationship. The behavior of *F. solani* was unique, in that the net amount of label retained by the spores consistently decreased after a relatively brief exposure period. Evidence that this represents a modification of the dodine molecule involving a loss of toxicity is presented in a subsequent paper (3). No such evidence was found for *V. inaequalis,* and the terminal decrease in retention of $^{14}C$ by this fungus must be discounted until studied further.

The differences in sensitivity of the fungi used in this study are related to rates of sorption of the chemical as well as to differences in inherent sensitivity. More work with these and other plant pathogens is needed in order that the pertinent reactions of the organisms, for which fungicides may be applied, can be defined.

LITERATURE CITED


1. AMERICAN CYANAMID CO. 1951. Allylol-substituted alkyl or alkaryl guanidines. Brit. Pat. 650, 820 7 Mar. Abstr. Chem. Abstr. 45:10626.
2. BARTZ, J. A. 1968. Investigations on the differences in the sensitivity of four plant pathogenic fungi to n-dodecylguanidine acetate, dodine. Ph.D. Thesis, Univ. Wis. 96 p.
3. BARTZ, J. A., & J. E. MITCHELL. 1970. Evidence for metabolic detoxification of *n*-dodecylguanidine acetate by ungerminated macroconidia of *Fusarium solani* f. sp. *phaseoli.* Phytopathology 60:350-354.
4. BROWN, I. F., JR., & H. D. SISLER. 1959. Mechanism of action of n-dodecylguanidine acetate (Cyprex). Phytopathology 49:534 (Abstr.)
5. BROWN, I. F., JR., & H. D. SISLER. 1960. The effect of dodine on enzymes in *Saccharomyces pastorianus.* Phytopathology 50:569 (Abstr.)
6. BROWN, I. F., & H. D. SISLER. 1960. Mechanisms of fungitoxic action of n-dodecylguanidine acetate. Phytopathology 50:830-839.
7. KOTTKE, MARGARET, & H. D. SISLER. 1962. Effect of fungicides on permeability of yeast cells to the pyruvate ion. Phytopathology 52:959-961.
8. MC CALLAN, S. E. A., & L. P. MILLER. 1958. Innate toxicity of fungicides, p. 107-134. *In* R. J. Metcalf [ed] Advances in pest control research, Vol. II. Interscience Publishers Inc., N.Y.
9. MILLER, L. P. 1960. Uptake and innate toxicity of dodine (n-dodecylguanidine acetate) to fungus conidia. Phytopathology 50:646 (Abstr.).
10. SOMERS, E. 1963. The uptake of dodine acetate by *Neurospora crassa.* Meded. Landbhoogesch. Opzoekstns. Gent 28:580-589.
11. SOMERS, E., & R. J. PRING. 1966. Uptake and binding of dodine acetate by fungal spores. Ann. Appl. Biol. 58:457-466.