Tolerance to Dodine in Venturia inaequalis

K. S. Yoder and E. J. Klos

Former Graduate Assistant and Professor, respectively, Department of Botany and Plant Pathology, Michigan State University, East Lansing 48824. Present address of senior author: Biochemicals Department, E. I. duPont de Nemours and Company, Wilmington, Delaware 19898.

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ABSTRACT

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Monoconidial isolates of *Venturia inaequalis* from Michigan were about half as tolerant to dodine as the least-sensitive New York isolate as determined by zone inhibition, germination in water solutions, and growth inhibition in broth. The most tolerant isolate crossed with normal isolates produced tetratype asci having four levels of tolerance suggesting additive action of at least two major independent genes for dodine tolerance. Testing of progeny from crosses of a green color mutant with isolates representing intermediate and high levels of tolerance indicated no close linkage between the genes for dodine tolerance and the green color gene of a known *V. inaequalis* linkage group. Inoculation experiments showed that isolate tolerance was a factor in scab control on trees treated with dodine at 4 μg/ml but not at 10 μg/ml. An ascospore abortion factor arising from the most tolerant isolates was not caused by the genes that conditioned higher levels of tolerance to dodine.

Tolerance of *Venturia inaequalis* (Cke.) Wint. to dodine, first appearing in 1969 (21), has been the subject of recent reports (7, 22, 23, 24). Tolerance was shown to be controlled by at least two genes (18, 19, 25, 26). Ultraviolet-induced resistance to dodine in *Nectria haematococca* var. *cucurbitae* (8) was controlled by four mutant genes which had an additive effect on the degree of resistance when combined in progeny (9).

Fungal species differ greatly in sensitivity to dodine (1, 4, 15). In *Fusarium solani* f. sp. *phaseoli* a terminal decrease in retention of ^{14}C-labeled dodine was attributed to a modification of the dodine compound and subsequent release of a less-toxic labeled compound (2). Although a slight terminal decrease in retention was also noted in *V. inaequalis,* evidence explaining the loss was lacking.

In 1969, failure of dodine to control apple scab in certain areas of New York State (while other standard fungicides gave adequate control) was attributed to fungal resistance acquired during a 10-year exposure to dodine (21). Because growing conditions and practices in Michigan are similar to those of New York State, this study was undertaken to determine the dodine tolerance levels of a group of natural isolates of *Venturia inaequalis*. Additional tests on the inheritance of dodine tolerance, and the effects of this tolerance on control of the apple scab fungus were conducted.

MATERIALS AND METHODS

Isolation from leaves.—Scab-infected apple leaves were collected in Michigan from unsprayed orchards, orchards with a long history of dodine usage, from orchards in which scab control with dodine seemed unusually difficult, and from wild crabapples. Samples also were collected from the Poray Orchard, Sodus, New York, where the tolerance problem was first discovered.

Monoconidial cultures were isolated by rubbing detached lesions across the surface of Difco potato-dextrose agar (PDA, pH 5.6) and transferring single germinating conidia to fresh PDA in petri plates. Stock cultures were maintained at 1-5 C in screw-cap tubes.

Inoculum production.—Inoculum was produced on cheesecloth wicks in 230-ml prescription bottles containing 30 ml 4% Difco malt extract broth. Spores were collected by rinsing the nutrient medium from the bottles, adding 30 ml of sterile deionized water, shaking the bottles vigorously to loosen the conidia, and straining the conidia through the two layers of cheesecloth. Spore suspensions which were standardized turbidimetrically by a modification of Kirkham's method (11), contained about 4-6 $\times$ 10^5 conidia/ml.

Testing of isolates.—Cultures were tested for dodine tolerance using a disk assay, spore germination, and growth inhibition in liquid culture. The standard spore suspension was stirred into PDA at 42 C at the rate of 1 ml per 10 ml PDA for the disk assay. Twenty-two ml of the seeded agar was immediately poured into 9-cm diameter plastic petri dishes and allowed to solidify. Two hours after the agar had hardened, a 12.7-mm diameter assay disk (VWR Scientific, S and S No. 740E) was saturated with 0.163 ml of a 50 or 300 μg/ml dodine solution and

immediately placed on the seeded agar. The plates were incubated 1 week at 19 C before zones of inhibition were measured. Two or three replicate plates were measured per test in three or four tests for each isolate.

For spore germination, 0.05 ml of the desired dodine concentration was pipetted onto ceramic-ringed glass slides and allowed to dry. An equal volume of the standard spore suspension was pipetted onto the same area of the slides. After 24 hours of incubation at 20 C, a minimum of 50 spores per ×10 field were counted in each of the three fields of three or four replications.

Inhibition of growth of *V. inaequalis* was determined for several rates of dodine in 250-ml Erlenmeyer flasks containing 50 ml of 4% malt extract broth amended with the dodine. Three milliliters of a standard spore suspension was added to the medium which then was incubated at 19 C for 2 weeks. The mycelium was removed by filtration, oven-dried at 60 C, and weighed.

The initial tests were conducted with the Cyprex 65W formulation of dodine. Subsequent tests used an equivalent rate of the technical grade of dodine (American Cyanamid Co., Princeton, N.J.) from an ethanol stock solution that was diluted with water. Appropriate ethanol controls were included.

Crossing of isolates and isolation of ascospores.—Isolates were mated in 9-cm diameter plastic petri dishes according to the methods of Keitt and Langford (10). The medium contained 0.5% malt extract and 2.5% Difco agar amended with apple leaf decoction. Aqueous spore suspensions of two single-spore isolates were mixed with the agar in the petri dish. The dishes were incubated at 19 C for 2 weeks and then transferred to 8 C. After 6 months, the ordered or unordered ascospore tetrads were isolated with a micromanipulator or by hand with a glass needle. Random ascospores were asceptically isolated from five or six perithecia crushed in water. Single discharged spores were picked up with a small-diameter capillary tube. Spores were germinated and cultured on PDA.

Greenhouse studies.—Greenhouse inoculations were conducted on 2-year-old McIntosh apple trees. Actively growing trees were sprayed with Cyprex 65W applied to the runoff point with a paint sprayer (DeVilbiss Type GD3). When the trees were dry, they were spray-inoculated with spore suspensions of selected isolates of *V. inaequalis* containing approximately 2×10^5 conidia/ml. Immediately after inoculation the trees were placed in a moist chamber at 18-23 C for 2-4 days and 3 weeks later lesions on the one or two of the most heavily infected leaves of each shoot were counted and the data were calculated as percent of those of the control treatment.

Statistical analysis.—Significance of disk assays was determined by analysis of variance in a completely randomized design. After conversion to percent of control and application of the arcsine transformation where that was appropriate, spore germination and greenhouse test data were analyzed in completely randomized or random-block designs.

RESULTS

Screening of field isolates.—The data in Table 1 are representative of those found in the disk-assay screening of 144 isolates from apple leaves. Isolate SR4, from the Poray Orchard, New York, where dodine tolerance was first noted, was the most tolerant field isolate collected and S1, from a dodine-treated commercial orchard, was the most tolerant Michigan isolate. Although zones ranging from 1 to 3 cm were noted with 50 μg/ml, average zone diameters greater than 2.4 cm were typical of isolates from untreated areas and were considered to represent the normal dodine sensitivity level.

The most tolerant isolates came from orchards in which dodine was used extensively, but the tolerance level of the isolate was not always correlated with the length of dodine usage or with the apparent degree of control by dodine. Isolate S1 came from an orchard with excellent commercial control, whereas normal isolates L2, L4, and V2 were from orchards with scab control problems.

Isolates SR4, S1, and 04, representing three significantly different levels of tolerance, were chosen to

TABLE 1. Inhibition of *Venturia inaequalis* in potato-dextrose agar by dodine diffusing from paper assay disks

Isolate	Origin	Inhibition zone diameter (cm)[a]	
		50 μg/ml	300 μg/ml
C2	Unsprayed tree, Michigan	3.05	3.75
L4	Commercial orchard, Michigan	3.03	3.60
V2	Commercial orchard, Michigan	3.00	3.67
B4	Unsprayed tree, Ohio	2.83	3.27
G5	Unsprayed tree, New York	2.73	3.53
04	Abandoned orchard, Michigan	2.68	3.63
CR2	Unsprayed crabapple, Michigan	2.60	3.38
S4	Commercial orchard, Michigan	2.58	3.38
H6	Unsprayed tree, Maryland	2.58	3.05
L2	Commercial orchard, Michigan	2.40	3.30
S1	Commercial orchard, Michigan	2.18	3.18
SR7	Poray Orchard, New York	1.70	2.58
SR4	Poray Orchard, New York	0.83	1.63
LSD ($P = 0.05$)		0.48	0.34
LSD ($P = 0.01$)		0.65	0.46

[a]Mean of four tests. Inhibition by dodine diffusing from 12.7-mm diameter paper assay disk in potato-dextrose agar after 7 days of incubation at 19 C. Dodine concentration with which paper assay disk was saturated.

determine their ability to grow in the presence of dodine in malt extract broth. Growth of 04, a normal isolate, was almost completely inhibited by a dodine concentration of 1 μg/ml; S1, the most tolerant Michigan isolate, grew well at 1 μg/ml; and SR4, the most tolerant New York isolate collected, grew well at 2 μg/ml, but was inhibited by 5 μg/ml.

Crossing of isolates.—Initial crosses of the most tolerant isolates with normal isolates were made to determine if the level of dodine tolerance is heritable. Test crosses of F_1 progeny with the most tolerant parent and with normal isolates, and intercrosses of F_1 progeny were done to test the two-major-gene hypothesis. Finally, two isolates having different levels of tolerance were crossed with 2295-2, a green-color mutant from D. M. Boone, Dept. of Plant Pathology, University of Wisconsin, to study possible linkage between genes for tolerance and the green-color gene of a known *V. inaequalis* linkage group.

Progress of this study was impeded by an ascospore abortion factor arising from the most tolerant isolate, SR4. Only about 2% of the asci produced by crosses of SR4 with normal isolates had eight spores. This greatly impaired the isolation of ascospore tetrads and further evidence was needed regarding this factor before firm conclusions about inheritance of dodine tolerance could be drawn.

Testing of F_1 progeny.—Progeny of the initial crosses were tested for tolerance by disk assay and germination of conidia in dodine (Table 2). In both tests differences in results with two members of the same spore pair were not significant but differences between members of different

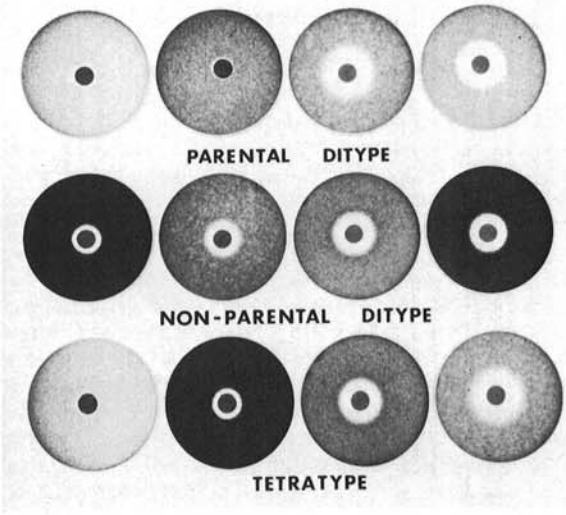

Fig. 1. Inhibition of isolates from ascus tetrads of SR4 × normal crosses by dodine diffusing through potato-dextrose agar from paper assay disk.

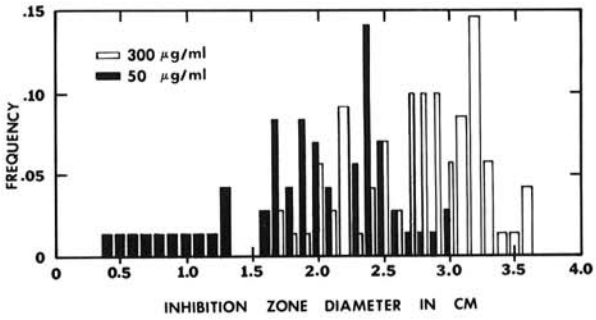

Fig. 2. Frequency distribution of dodine inhibition zones of 71 progeny of cross 04SR4-1-2×04SR4-6-1 isolates representing the two intermediate tolerance levels in tetratype asci. Assay disks were saturated with indicated dodine concn. Four replications measured after 1 week of incubation at 19 C. LSD ($P = 0.05$): 300 μg/ml (0.63 cm); 50 μg/ml (0.47 cm). Zone diameter of parents in 50 and 300 μg/ml tests: 04SR4-1-2 (1.80 and 2.68 cm); 04SR4-6-1 (2.38 and 3.20 cm).

TABLE 2. Inhibition by dodine of germination and growth of conidia of the ordered monoascosporic cultures of a tetratype ascus of *Venturia inaequalis* cross 04 × SR4

Isolate	Position in ascus	Inhibition zone diameter (cm)[a] 50 μg/ml	300 μg/ml	Inhibition of germination[b] (%)
04SR4-1-2	1	1.80	2.75	73.5 ab
04SR4-2-2	2	1.90	2.60	79.4 b
04SR4-3-2	3	1.23	2.33	65.9 a
04SR4-4-2	4	1.13	2.23	63.9 a
04SR4-5-2	5	2.30	3.15	94.7 cd
04SR4-6-2	6	2.27	3.25	94.0 c
04SR4-7-2	7	2.98	3.62	97.9 de
04SR4-8-2	8	2.70	3.30	98.1 e
04	parent	2.68	3.63	...[c]
SR4	parent	0.83	1.63	...
LSD ($P = 0.05$)		0.41	0.35	
LSD ($P = 0.01$)		0.56	0.48	

[a]Mean of four tests. Inhibition by dodine diffusing from 12.7-mm diameter paper assay disk in potato-dextrose agar after 7 days of incubation at 19 C. Dodine concentration with which paper assay disk was saturated.

[b]Inhibition in 1 μg/ml dodine in water. Generally 4 μg/ml completely inhibited germination of the most tolerant isolates. Means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

[c]Parent isolates not tested same as offspring.

pairs usually were significant ($P = 0.05$).

In these tetratype asci, one pair of spores gave rise to cultures having a tolerance level similar to that of the normal parent (04), one pair was similar to the more tolerant parent (SR4), and two pairs had intermediate levels of tolerance. Tetratype asci characteristic of a two-gene interaction (5, 6) were found in crosses of SR4 with several unrelated normal isolates, although the intermediate levels were not always significantly different. In a survey of these crosses 12 of 20 asci tested were considered to be tetratypes based on the presence of one pair of cultures having a level of tolerance similar to SR4. Usually the tolerance level of the more sensitive parent also was discernible in one pair of cultures from a tetratype ascus, although this level depended somewhat on the isolate that was crossed with SR4. Two of these asci were thought to be nonparental ditypes having only intermediate levels of tolerance and one was a parental ditype ascus. Five of the 20 asci did not fit clearly any of the above tetrad types owing to experimental error. Figure 1 illustrates dodine tolerance levels demonstrated by types of asci in the disk assay at 50 μg/ml.

The two most tolerant isolates, SR4 and SR7 (Table 1), were crossed to determine whether recombination of these two tolerance levels would yield progeny with dodine tolerance levels greater than SR4. None of the 39 progeny was more tolerant than SR4 (25). Two isolates were slightly more sensitive than SR7, but not as sensitive as normal isolates. Apparently, there was n recombination of major tolerance genes in this cross.

Crosses of F_1 progeny.—Some progeny of a cross of isolates 04SR4-6-1 and 04SR4-1-2, which represented the two intermediate tolerance levels of tetratype asci, demonstrated inheritance of the tolerance levels of 04 and SR4 when tested with dodine at 50 μg/ml (Fig. 2). This indicated a recombination of two major genes conditioning dodine tolerance. Fewer significant deviations from the tolerance levels of the intermediate parents were seen with 300 μg/ml, although eight of the 71 progeny were significantly more tolerant than the more tolerant parent, 04SR4-1-2.

In a backcross of SR4 with 04SR4-3-2, representing the highest dodine tolerance level in a tetratype ascus (Table 2), none of 30 progeny was significantly more sensitive than the more tolerant parent.

In a test cross of normal isolate C2 with 04SR4-5-2 [intermediate in tolerance in a tetratype ascus (Table 2)] none of 31 isolates was more sensitive than C2 to dodine. Although seven progeny had slightly smaller inhibition zones than 04SR4-5-2 in the 300 μg/ml test, none was significantly more tolerant than this parent at 50 μg/ml.

Fourteen of 32 progeny of cross :C2 × 04SR4-7-2 were significantly more tolerant at 50 μg dodine/ml than either parent (25) and at 300 μg/ml zones for seven of the isolates were significantly smaller than those for either parent but not significantly smaller than those of 04, the normal parent of 04SR4-7-2 (Table 2).

In these three crosses, SR4 × 04SR4-3-2, C2 × 04SR4-5-2, and C2 × 04SR4-7-2, there was no apparent recombination of major tolerance genes. The presence of some progeny slightly more tolerant than either of the two parents C2 and 04SR4-7-2, but not significantly more tolerant than 04, may indicate a recombination of minor genes that affect the level of tolerance to dodine.

Significance of ascospore abortion in inheritance of dodine tolerance.—The ascospore progenies whose test results are reported above were all isolated from crosses with a high frequency of ascospore abortion. Because the causal nature of this phenomenon is not known, conclusions regarding frequencies of different levels of tolerance in these crosses must be restricted. Linkage of the ascospore abortion and dodine tolerance factors cannot be excluded on the basis of present evidence. However, all tolerance levels found in tetratype asci also were found among progeny isolated from asci with less than eight spores. Also, the discovery of several monoascosporic isolates similar to SR4 in dodine tolerance but not exhibiting ascospore abortion indicates that the two phenomena are not controlled by the same factor or factors.

Ascospore abortion did not occur in a later cross of a normal isolate, S4, with C2SR4-7-4, an F_1 progeny as tolerant as SR4. The frequencies of isolates at different tolerance levels in the 50 μg/ml and the 300 μg/ml tests

TABLE 3. Control of infection by *Venturia inaequalis* isolates 04 (normal dodine sensitivity) and SR4 (high dodine tolerance) on trees treated with dodine (Cyprex 65W)[a]

Isolate	Dodine (μg/ml)	Lesions/leaf[b]	Control[c] (%)	Shoots with infection (%)
04	unsprayed	12.9	0.0 b	100.0 a
04	200	1.5	88.3 a	37.5 b
04	300	0.2	98.4 a	16.7 bc
04	600	0.0	100.0 a	0.0 cd
SR4	unsprayed	7.7	0.0 b	100.0 a
SR4	200	5.3	31.2 b	72.2 a
SR4	300	0.8	89.6 a	19.4 bc
SR4	600	0.1	98.7 a	8.3 cd

[a]Completely randomized design, three replications. Trees held in moist chamber 4 days at 19-22 C.
[b]Lesions per single most heavily-infected leaf per shoot.
[c]Percent control = $100 - \frac{\text{lesions per leaf, sprayed}}{\text{lesions per leaf, unsprayed}} \times 100$.
Column means followed by the same letter are not significantly different ($P = 0.05$).

were similar in 40 progeny of this cross and in the progeny of 04SR4-1-2 × 04SR4-6-1 illustrated in Fig. 2. This suggests that ascospore abortion probably did not affect the frequency distribution of dodine tolerance levels in the earlier crosses.

Nonlinkage of dodine tolerance genes and green-color gene.—Isolates representing two significantly different levels of tolerance were crossed with 2295-2, a green-color mutant with normal dodine tolerance, to study possible linkage of the genes for dodine tolerance with the green-color gene of an identified *V. inaequalis* linkage group (3). Distribution of the tolerance levels of 20 wild-type and 24 green progeny was not significantly different throughout the tolerance range demonstrated. The tolerance levels of 27 progeny of 2295-2 and C2SR4-7-3, an F_1 progeny similar to SR4 in tolerance, also were distributed throughout the tolerance range of their parents. This random distribution of tolerance levels of green and wild-type progeny suggests free recombination and nonlinkage of the genes for dodine tolerance and the green-color gene.

Inoculation studies.—In an initial inoculation experiment (Table 3) significantly greater control by 200 μg/ml dodine was achieved on trees inoculated with normal isolate 04 than on trees inoculated with the most tolerant isolate, SR4, although differences in control of the two isolates by higher concentrations were not significant. A qualitative difference also was noted in symptoms caused by the two isolates on the unsprayed trees: 04 had normal heavily-sporulating lesions, but SR4 produced lesions which were more chlorotic with sporulation somewhat reduced.

Because of the difference in macroscopic symptoms, experiments were repeated with tolerant and normal isolates selected for uniform symptoms on the unsprayed trees. Dodine at 4 μg/ml gave significantly less suppression of sporulating lesions of the more tolerant isolate, H6SR4 6-1, than of normal isolate H6, but there was no difference in control by 10 and 200 μg/ml.

DISCUSSION

Dodine sensitivity of field isolates tested in this study varied somewhat, but the differences in tolerance to dodine concentrations were in the order of a factor of five, and are comparable to those noted previously (7, 14, 22, 25). They are less than those reported for phenyl mercury acetate, similar to those reported for dichlone and sulfur, and about two times the natural variation in sensitivity reported for glyodin, captan, copper sulfate, and Paris green (17, 20).

The degree of natural variation in sensitivity to dodine by *V. inaequalis* in this study is similar to, or slightly less than, that expressed by the dodine-resistant mutants in *Nectria haematococca* var. *cucurbitae* developed by Kappas and Georgopoulos (9). The variation in tolerance in *V. inaequalis* shown here is about 1,000 times less than that of the antimycin A-resistant ultraviolet mutant developed by Leben et al. (12).

The pattern of inheritance of tolerance to dodine found in this work appears to follow more closely that proposed by Kappas and Georgopoulos for two *N. haematococca* var. *cucurbitae* mutants (9) than that suggested by Polach for *V. inaequalis* (19). In the system proposed for *Nectria*, each of two genes conditioned different levels of tolerance when inherited separately, and were additive when inherited together. In the system suggested by Polach (19), a nontolerant isolate would not grow at 0.25 μg/ml, one gene would allow growth at 0.25 μg/ml, and a second gene, effective only when the first was in dominant form, allowed growth at 0.5 μg/ml dodine. In the present study, disk assay of asci of crosses of the most tolerant isolate with normal isolates has shown four distinct levels of tolerance (Table 2). An explanation for this phenomenon is the independent action of two separate genes, each conditioning distinct levels of tolerance, having an additive effect when inherited together (5, 6). This has been confirmed by a cross of the two intermediate types to yield recombinant progeny more tolerant or more sensitive than either of the intermediate parents.

Further testing must be conducted to assure that recombinant progeny more tolerant than SR4 cannot arise from crosses of S1 with isolates having tolerance levels similar to SR4. If the gene conditioning the tolerance level of S1 is the same as one of the genes in SR4 then there should be no build-up in tolerance from this cross. However, if three separate genes are involved, then recombinant progeny should appear with no tolerance genes or with all three tolerance genes. The reaction of the progeny having three tolerance genes would depend on whether the action of the third gene is additive or whether its action is masked by the other two genes. In a cross of S1 with a highly tolerant progeny of SR4, none of 10 progeny was as tolerant as SR4, nor did any have the normal level of sensitivity which would indicate a recombination of tolerance genes.

Inheritance of tolerance to dodine in *V. inaequalis* has been clearly demonstrated. However, one must assess experimentally the importance of fungal sensitivity to dodine as a factor in apple scab control to place it in proper perspective with other factors such as weather, inoculum density, proper coverage, timing, and rate of dodine application (13). A natural mutation rate to dodine tolerance as low as 1 in 10^6 (14) should be high enough to assure a constant population with this level of tolerance in the orchard if the selection pressure of dodine is the major factor inhibiting survival of the fungus.

The limited emergence of populations sufficiently tolerant to dodine to have notable economic importance in spite of a relatively high mutation frequency has been attributed to "overkill" in commercial practice (14). The ability of dodine to prevent sporulation undoubtedly has limited the population screened for dodine tolerance in nature. Additional factors limiting the success of strains with high levels of dodine tolerance may be reduced pathogenicity or reduced sporulation. Isolate SR4 sporulated well in culture, but sporulation was limited on plants in the greenhouse.

The majority of the cases of poor scab control by dodine investigated in Michigan demand an alternate explanation because isolates collected from heavily-infected orchards have shown a normal level of tolerance to dodine whereas the most tolerant Michigan isolates have come from orchards with good control. Frequently poor control in mid-season can be attributed to an early build-up of inoculum due to late timing of an early application, poor coverage due to improper pruning, or careless spraying.

Since inhibition of spore germination is dependent upon the amount of dodine available per spore (16), an early season build-up of inoculum means the rate of application must be increased or the spray interval reduced, or both, to maintain the level of residue required for adequate control.

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