Tolerance of *Mycosphaerella citri* to Benomyl in Florida Citrus Groves

J. O. WHITESIDE, Plant Pathologist, University of Florida, Agricultural Research and Education Center, Lake Alfred, FL 33850

ABSTRACT

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Benomyl-tolerant isolates of the citrus greasy spot fungus (*Mycosphaerella citri*) were defined as those showing little or no reduction in growth on agar media containing 100 μg/ml of benomyl. Sensitive isolates were completely inhibited by 1 μg/ml of benomyl. Postinoculation foliar sprays of benomyl at 150 μg/ml reduced greasy spot severity only on leaves inoculated with sensitive isolates. Tolerance in the population of *M. citri* in citrus groves was estimated by ejecting ascospores from perithecia-bearing fallen citrus leaves onto water agar containing 100 μg/ml of benomyl and observing the ascospores for distorted or normal germ tubes. Eleven of 12 groves never treated with benomyl showed no tolerance among the $1-3 \times 10^4$ ascospores examined from each sample; in the remaining grove, about 0.01% of ascospores were tolerant. In 21 of 37 treated groves sampled, more than 0.1% of the ascospores were benomyl-tolerant, but only where more than two sprays had been applied. Thirteen groves had received one benomyl treatment per year for 5 yr; tolerant ascospores exceeded 1% in 11, ranging between 10 and 20% in four and exceeding 80% in three.

Published reviews on tolerances of plant pathogens to fungicides (1,2) contain many examples of loss of sensitivity of fungi to benomyl. Mostly, this problem develops only after 3 yr or more of continuous use of benomyl and where several treatments have been applied each year.

Commercial use of benomyl in Florida citrus groves to control greasy spot caused by *Mycosphaerella citri* Whiteside began in 1974. A serious tolerance problem was not anticipated for many years because citrus trees seldom receive more than one benomyl treatment a year and because only partial, though generally adequate, greasy spot control is achieved. This presupposed that selection pressure on the inoculum supply, mostly ascospores released from decomposing citrus leaves on the grove floor (3), would be light, thereby permitting much of the benomyl-sensitive population to survive and predominate, even if some tolerance were present initially in the wild population.

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First indications that a tolerance problem might already exist came from fungicide evaluation plots at Lake Alfred, Florida, during the 1978–1979 season, when, for the first time, benomyl provided significantly less control of greasy spot than a standard copper fungicide treatment.

This paper reports the methods used to confirm the presence of benomyl-tolerant strains of *M. citri* in the fungicide testing site and the results of subsequent surveys to estimate the amount of tolerance to benomyl in current populations of the fungus in commercial groves.

MATERIALS AND METHODS

Fungus isolation and culture media. Ascospores of *M. citri* were ejected from decaying citrus leaves onto water agar and transferred 24 hr later as single germinating spores to Difco potato-dextrose agar (PDA), on which the cultures were maintained.

To isolate the fungus from infected grapefruit rind, an area showing greasy spot rind blotch was wiped with 95% ethanol and flamed. Under a stereoscopic microscope, a shallow tangential cut was made to reveal the localized necrosis that occurs beneath stomata invaded by *M. citri.* The dead tissue was lifted out with a pointed scalpel and transferred to PDA.

Water agar (2%) was used for ascospore germination tests, and PDA was used for growth studies and production of inoculum for pathogenicity tests. Benomyl-amended water agar and PDA were prepared by incorporating a commercial formulation, Benlate 50W (E. I. du Pont de Nemours & Co., Inc., Wilmington, DE), as a water suspension in the medium immediately before autoclaving.

Inoculation technique. Inoculum consisted of a suspension of mycelial fragments prepared by grinding 10-day-old colonies of *M. citri* on PDA in a Waring blendor for 5 sec and straining the material through cheesecloth. The inoculum was sprayed onto the lower surfaces of recently expanded leaves on container-grown rough lemon (*Citrus jambhiri*) plants in the greenhouse. This host was used because greasy spot has an incubation period of only 6–8 wk on it, compared with 4 mo or longer on other cultivars. The plants were placed in a damp chamber each night for 12 days after inoculation to encourage a copious growth of mycelia on the leaf surface, which increases the chances for multiple penetrations that are essential to induce disease (4,7).

Estimation of tolerance to benomyl of potential ascospore supply. Fallen decomposing citrus leaves bearing perithecia of *M. citri* were collected from at least 10 locations in each grove sampled. The leaves were agitated in water to remove soil particles, then air-dried. If perithecia were immature, the leaves were wetted and dried daily for 1–2 wk to hasten perithecial development. Leaves with mature perithecia were held above water in a closed chamber (modified desiccator) for 3–7 days to promote ascospore production.

Forty leaves with abundant perithecia were selected from each sample for testing. A disk was cut with a 16-mm diameter cork borer from the most productive perithecia-bearing area of each leaf. After wetting, 20 leaf disks were placed in each of two spore discharge towers to eject the ascospores downward onto four-compartment 90-mm diameter petri dishes containing 0, 1, 10, and 100 μg/ml of benomyl in water agar. The discharge towers consisted of 65-mm tubes of Plexiglas with 95 mm i.d. A platform of 6-mm mesh hardware cloth was located 5 mm beneath the top of the tube. The wetted leaf disks were placed on the platform with their lower, predominantly perithecia-bearing surface downward. The tower was covered during ascospore discharge with the top of a 14-cm diameter petri dish. To ensure representative dispersal of ascospores over all compartments of the dish, the tower was rotated 90° every 2 min. After 45 min, or sooner if sufficient ascospores had been deposited, the petri dish was covered with its lid and incubated at 25 C for 24 hr. The ascospores were examined at × 400 magnification along two radial transects in each compartment. If no normal germ tubes were found, the whole compartment was searched for tolerant ascospores.

RESULTS AND DISCUSSION

Nearly 100% germination of benomyl-sensitive ascospores occurred on benomyl-amended agar, even at 100 μg/ml, but the germ tubes were short and distorted. Sensitive populations sometimes produced a few normal germ tubes at 0.1 μg/ml of benomyl but never at 1 μg/ml. The effect of benomyl on germ tube growth was fungistatic. After germinating ascospores were transferred from water agar containing 100 μg/ml of benomyl to benomyl-free PDA, growth became normal, even after a 3-wk exposure to benomyl. Most tolerant isolates grew as rapidly at 100 μg/ml of benomyl as on PDA alone, whereas some grew normally at 10 μg/ml but were inhibited at 100 μg/ml.

Both sensitive and tolerant isolates produced symptoms of greasy spot. A foliar application of 150 μg/ml of benomyl 14 days after inoculation reduced disease severity only on plants inoculated with the sensitive isolate. Benomyl did not even reduce symptom severity on plants inoculated with isolates that tolerated 10 μg/ml but not 100 μg/ml of benomyl in PDA.

Benomyl-tolerant strains of *M. citri* at Lake Alfred fungicide testing site. This site was isolated from other benomyl-treated groves. Each year since 1970, 12–30% of the trees had been sprayed with benomyl once in June or July, but, because of rerandomization each year, relatively few trees had received benomyl in two or more consecutive years.

A sample of perithecia-bearing fallen leaves collected randomly over the whole site in February 1979 yielded 2% ascospores tolerant to 100 μg/ml of benomyl in water agar, whereas a sample collected at the same time from beneath individual trees sprayed with benomyl in July 1978 yielded 28% benomyl-tolerant ascospores. A sample of leaves picked from the 1978 spring flush of these trees in February 1979 was induced to produce perithecia by first drying the leaves and then alternately wetting and drying them for 7 wk. These leaves yielded 69% benomyl-tolerant ascospores. The percentage of benomyl-tolerant ascospores from the naturally fallen leaves was lower than this, presumably because the sample included some leaves that originated in 1977 and some that had blown in from neighboring untreated trees.

Tolerance of *M. citri* to benomyl in commercial citrus groves. The oil spray commonly applied to Florida citrus groves every year in the summer has many purposes, one of which is to control greasy spot. Oil is not fungicidal or fungistatic to *M. citri* (5). Apparently, its main action in controlling greasy spot is reduction of host cell predisposition to the invading intercellular hyphae (5). Benomyl, or alternatively a copper fungicide, is added to the oil spray only in case disease pressure is heavy. Unless greasy spot is severe, therefore, even a substantial increase in tolerance of *M. citri* to benomyl would not necessarily be revealed because of masking by an effective oil spray.

Grapefruit was envisaged as the cultivar most likely to reveal any reduction in efficacy of benomyl against greasy spot. Grapefruit leaves are highly susceptible to greasy spot. In addition, a conspicuous rind blemish follows infection by *M. citri* that oil alone can fail to control (7). In the search for tolerant strains of *M. citri*, therefore, attention was given initially to two benomyl-treated grapefruit groves that showed an unusually high incidence of greasy spot rind blotch. One grove was near Bradenton and the other was 80 miles away near Lake Wales. Both had been sprayed with benomyl once a year for five consecutive years. All isolates of *M. citri* obtained from the 11 fruit sampled from the Bradenton grove were tolerant to benomyl, and a sample of fallen leaves yielded 96% benomyl-tolerant ascospores. Eight of 18 isolates from fruit from the Lake Wales grove were benomyl-tolerant, as were 10% of ascospores released from fallen leaves.

A more extensive search for benomyl-tolerant *M. citri* was made between February and July 1979 in many other plantings, including orange as well as grapefruit groves. The results (Table 1) indicated that the overall percentage of ascospores tolerant to benomyl was greater where benomyl had been applied for 3 yr or more than where it had been applied less often or not at all. In some orange and grapefruit groves, 1–9% of the ascospores were tolerant after only three benomyl treatments. Of 13 groves sprayed with benomyl once a year for five consecutive years, 11 had more than 1% of ascospores that were tolerant; three of these had 88, 90, or 96% benomyl-tolerant ascospores (Table 1).

One of the 12 groves never treated with benomyl yielded 0.01% benomyl-tolerant ascospores (Table 1). In the other 11, no tolerance was observed among the 1–3 $\times 10^4$ ascospores ejected from each sample. The incidence of benomyl tolerance in wild populations of *M. citri* is open to question because too few ascospores were observed.

M. citri usually requires almost a year to complete its life cycle. Leaves that become infected during the major infection period of June through August do not generally drop from the tree until the following winter or spring. Ascospores are not released in large numbers until early summer (3). Some infected leaves stay on the tree for as long as 2 yr. In spite of this long life cycle, annual benomyl treatment must have caused a rapid reduction in the benomyl-sensitive component of the original fungus

Table 1. Survey for tolerance of *Mycosphaerella citri* to benomyl in Florida citrus groves

Number of years benomyl applied	Number of groves sampled	Number of groves with percentage of ascospores tolerant to 100 μg/ml of benomyl in range of: 0	0.01–0.09	0.1–0.9	1.0–9.0	10–100
0	12	11	1	0	0	0
1	4[a]	4	0	0	0	0
2	4[a]	3	1	0	0	0
3	11[b]	6	1	1	3	0
4	5[a]	3	0	0	2	0
5	13[a]	0	1	1	4	7[c]

[a] These groves received only one benomyl treatment each year.

[b] In the group that showed no tolerance, one grove received two benomyl treatments each year, one was sprayed twice in 1978 only, and one was sprayed twice in 1977 and 1978. All other groves received only one treatment per year.

[c] Tolerance percentages were 10, 12, 14, 19, 88, 90, and 96.

population. Furthermore, and even more surprisingly, the tolerant fungus propagules must have multiplied rapidly to have caused such heavy infection in some groves by 1978.

Benomyl sprays against *M. citri* act partly on the fallen leaves. Ground spraying of leaf litter with benomyl has reduced ascospore production to very low levels for 4–6 wk after treatment (6). Some recovery has occurred later, and eventually more ascospores may be released from treated leaves than from untreated leaves (6). If treated leaves decompose rapidly, however, thereby eliminating the perithecial substrate, the supply of ascospores can virtually cease for the rest of the season.

Another stage of the life cycle of *M. citri* that is very vulnerable to benomyl treatment is the epiphytic growth the fungus produces on the host surface after ascospore germination (4,7). Benomyl also provides a long kickback action against fungal penetration that may have occurred before application. Progress of hyphae into the internal tissue of the leaf is slow, and benomyl applied as long as 6 wk after inoculation can still control greasy spot (7). Thus, much of the fungus that has reached the leaf before the benomyl spray is applied can be eliminated.

After elimination of benomyl-sensitive epiphytic growth, any tolerant fungus on the leaf surface would, presumably, multiply more rapidly because of reduced competition for substrate. This could increase the chances for leaf penetration by tolerant strains and lead to greater production of benomyl-tolerant ascospores the next year.

The selection pressure imposed by benomyl on *M. citri* must vary greatly from grove to grove. The rapid reduction in the sensitive part of the population that occurred in some groves would have required excellent spray coverage of the lower leaf surface and, probably, much wetting of the leaf litter as well. It may be no coincidence that all cases of detected high tolerance to benomyl occurred in groves sprayed with dilute preparations (high volume) by air-blast sprayers. Such sprayers are known to provide good coverage of the lower leaf surface and to cause much wetting of fallen leaf litter by direct spray contact and by heavy runoff.

Results of the survey raised serious doubts about continued reliance on benomyl to control citrus greasy spot. In some groves, tolerance of *M. citri* to benomyl increased rapidly and after relatively few treatments. Monitoring of leaf litter for ascospore tolerance to benomyl is theoretically feasible but hardly practical, except on a limited scale. Tests would have to be repeated in each grove every year. Furthermore, because ascospores are airborne, it would be necessary to assure that the populations of *M. citri* in neighboring groves had not become tolerant to benomyl.

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