The Adaptability of the Benomyl-Resistant Population of Cercospora beticola in Northern Greece

C. Dovas, G. Skylakakis, and S. G. Georgopoulos

Hellenic Sugar Industry S.A., Salonica; Eli Lilly S.A., Athens office, Aghia Paraskevi; and Department of Plant Pathology, Athens College of Agriculture, Votanikos, Athens 301, Greece.

Accepted for publication 11 June 1976.

ABSTRACT

DOVAS, C., G. SKYLAKAKIS, and S. G. GEORGOPOULOS. 1976. The adaptability of the benomyl-resistant population of Cercospora beticola in northern Greece. Phytopathology 66: 1452-1456.

In a field comparison of a benomyl-sensitive and a benomyl-resistant strain of *Cercospora beticola*, the relative proportion of the resistant strain increased after applications of benomyl, benomyl in alternation with triphenyltin hydroxide, or a tank mixture of the two fungicides. The increase in the proportion of the resistant strain was followed by a pronounced reduction of effectiveness of benomyl for leaf spot control. Triphenyltin was equally effective for control of both strains. In the unsprayed control the benomyl-resistant strain appeared more adaptable than the particular sensitive strain with which it was compared. Extensive sampling of leaf spot for three consecutive years after the cessation of benomyl usage for control of *C. beticola* in Greece showed that the frequency with which benomyl-resistant strains were isolated tended to remain constant for a given area, including fields that were sprayed with triphenyltin fungicides. Thus, the benomyl-sensitive and benomyl-resistant portions of natural populations apparently are equal in fitness for survival under selection pressures imposed by various field conditions.

Additional key words: sugarbeet, leaf spot, fungicides.

The development of resistance by target fungi is a serious threat to the continued disease control effectiveness of some systemic fungicides. The benzimidazole group of fungicides is particularly vulnerable since several fungal species have developed resistance to these fungicides under different environmental conditions (2, 3, 6, 8). Often sudden failures in disease control have had dramatic consequences as happened in the summer of 1972 when benomyl suddenly failed to control Cercospora leaf spot of sugarbeets in northern Greece (7). Surveys in 1972 and early 1973 of the major sugarbeet-growing districts of Greece indicated widespread occurrence of benzimidazole-resistant strains of *Cercospora beticola* Sacc. This forced a complete change from the use of benomyl for the control of leaf spot in 30,000 hectares of sugarbeets and a return to use of protective sprays of triphenyltin fungicides. It was important to determine whether the proportion of resistant strains in the problem areas would decline after benzimidazole usage had ceased. If so, benzimidazoles could be used again for leaf spot control. For this reason, a large experiment was conducted in the summer of 1973 in which one sensitive and one resistant strain of *C. beticola* were compared as to their fitness for survival under various fungicide regimes. The frequency of occurrence of benomyl-resistant strains was determined by periodic surveys of pathogen populations in selected areas during the 1973, 1974, and 1975 growing seasons. The results of these studies are presented in this paper.

MATERIALS AND METHODS

A field in the experimental farm of the Platy sugar factory, near Salonica, that had not been planted in beets for the previous 3 years was chosen for the experiment. The area around this field was kept fallow in 1972 and was cropped with cereals in 1973. To avoid introduction of pathogen biotypes on seed, a 4-year-old sugarbeet seed, of cultivar Polybeta 13, was used. The field was divided in three parts, designated as S (sensitive), R (resistant), and SR (mixture), which were separated by vegetation-free zones 8 m wide. In each of the three parts a randomized complete-block design with four replications of each treatment was used. No leaf spot was noticed in the plots prior to 23 May 1973 when the plots were inoculated with artificially-grown inoculum which was applied during the evening, immediately after a sprinkler irrigation. The two strains, one benomyl-sensitive and the other benomyl-resistant, which were compared have been deposited with the American Type Culture Collection (accession numbers ATCC 24888 and ATCC 24889, respectively). To obtain conidia the fungus was grown in plates of 70% tomato-juice agar for 1 week, as described by Yong Joon (10).

Inoculation was carried out by spraying a spore suspension (approximately 300 conidia per milliliter in water) with a precision sprayer. Spraying pressure was maintained at four atmospheres and the volume delivered was approximately 600 liters per hectare. Each plot received approximately 600,000 conidia. Only benomyl-sensitive conidia were applied in plots of part S and only benomyl-resistant in part R. A mixture of sensitive and resistant conidia, in the ratio of 9:1, were applied in part

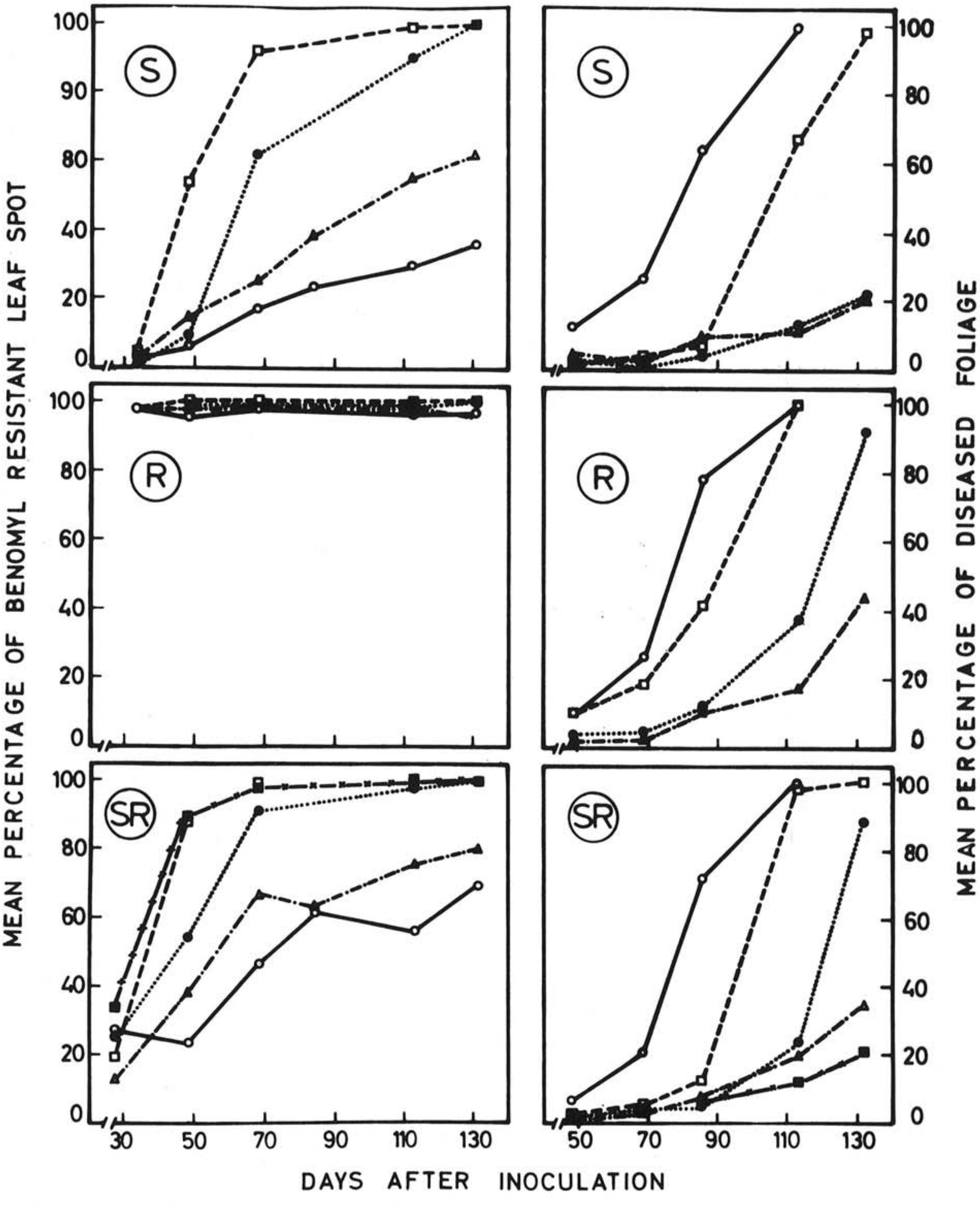

Fig. 1. Effect of fungicide applications on the frequency of benomyl-resistant strains of *Cercospora beticola* (left) and on the severity of disease (right) in plots artificially inoculated with benomyl-sensitive (S) or resistant (R) or a mixture (SR) of sensitive and resistant conidia. Legend: □– – –□, benomyl alone; Δ—●—●Δ, triphenyltin hydroxide alone; ●· · ·●, alternate applications of benomyl and triphenyltin hydroxide; ■– × –■, tank mixture of benomyl and triphenyltin hydroxide; and o——o, untreated control.

SR. Changes in the proportion of benomyl-resistant leaf spot in each plot during the growing season were determined by periodic sampling. At least 50 individual spots were excised from leaves in each plot early in the season and as many as 200 at later dates. Every effort was made to use newly-formed conidia. Four days before each sampling the plots were sprinkler-irrigated to remove old conidia and to assure adequate humidity for the production of new conidia. In addition, samples were taken only from young leaves whenever possible. From each spot a single conidium was isolated and tested for resistance to benomyl as previously described (7). Before each sampling, leaf spot incidence (% of total leaf area spotted) was visually assessed and recorded.

The plots were sprayed on 30 June, 13 and 27 July, 25 August, and 17 September. The treatments were: benomyl, 150 g active ingredient per hectare; triphenyltin hydroxide, 300 g active ingredient per hectare; alternation of the two fungicides starting with triphenyltin; a mixture of the two fungicides (each at the above indicated dose); and a nonsprayed control. A Holder mechanical sprayer was used and approximately 500 liters of liquid were added per hectare per treatment; control plots were sprayed with water.

To follow possible changes in the benomyl sensitivity of the natural population of *C. beticola* after 1972, commercial sugarbeet fields representative of different areas were chosen for sampling each year. Since crops usually are rotated, the fields were not the same each year; however, samples during each season were taken from the same fields from each area. At least 50 leaf spots were collected per field in each sampling and the pathogen was tested for benomyl sensitivity as described previously.

RESULTS

Experimental comparison of one sensitive and one resistant strain under field conditions.—The proportion of benomyl-resistant isolates recorded in the first sampling (26 June) of the 1973 experiment was somewhat different from that applied in the inoculum to each plot. Thus, plots that received only the sensitive or only the resistant strain yielded 1-5% of isolates of the other type. In plots which had received 10% of the resistant strain, the proportion of resistant isolates recovered was somewhat higher than 10% in the first sampling, before any fungicide had been applied. Apparently, therefore, despite precautions to avoid naturally occurring inoculum, the experimental field was not completely free of *C. beticola* inoculum before the experimental inoculation.

As shown in Fig. 1-S, benomyl exerted a very strong selection in favor of benomyl-resistant *C. beticola*. The mean percentage of leaf spots which yielded the resistant strain increased from 3.5 on 26 June to 91.5 on 1 August as a result of benomyl applications on 30 June and 13 July. Similarly, with the mixed inoculum, benomyl-resistant leaf spots increased from 19% to 87% between 26 June and 11 July after one benomyl spray (Fig. 1-SR). Disease control by benomyl was satisfactory only as long as most of the pathogen population was of the sensitive type. In the plots which received only resistant inoculum (Fig. 1-R), benomyl sprays only slightly reduced disease severity as compared to the unsprayed control. In the remaining cases (S and SR), the increase in percentage of resistant conidia was followed by a decrease in effectiveness of benomyl a few weeks later.

In contrast with the results for benomyl, the triphenyltin fungicide provided very good control of the disease until late in the season. On 14 September, for example, the mean percentage of spotted foliage in the treated plots was 11.0, 17.4, and 20.2 for S, R, and SR, respectively, whereas in the benomyl treatments the corresponding figures were 67.0%, 100%, and 99.5% for S, R, and SR, respectively. Alternating benomyl and triphenyltin was less effective than the use of triphenyltin for every spray, except where the level of the resistant population was very low at the onset of the epidemic (S). In this case, alternating benomyl and triphenyltin maintained good control of disease until late in the season. It must be noted, however, that even when the two fungicides were alternated, the percentage of benomyl-resistant leaf spots increased after each benomyl application and was 100% on 3 October in the plots which originally received only sensitive inoculum (Fig. 1-S). Obviously, disease severity would have increased if the availability of healthy foliage had been prolonged. The tank mix of the full rates of benomyl and triphenyltin was applied only in the case of the mixed original population of *C. beticola* (SR). This spray mixture kept disease severity to very low levels, but the difference between it and triphenyltin alone was not sufficient to justify the additional expense for benomyl (Fig. 1-SR).

No treatment was found to select against the benomyl-resistant strain of *C. beticola* (Fig. 1-R). The proportion of benomyl-resistant leaf spots increased even in the treatment with triphenyltin alone. This cannot be due to lower toxicity of triphenyltin for benomyl-resistant than benomyl-sensitive strains of *C. beticola*. In several in vitro experiments we have not observed a differential toxicity which is in agreement with the observations of Ruppel (9). Furthermore, the proportion of resistant conidia increased even in the unsprayed control, although at a much lower rate than in the treatments containing benomyl (Fig. 1-S and 1-SR). This was an unexpected finding. Since the conidia of *C. beticola* are not disseminated over long distances (4), it seems unlikely that outside inoculum had spread into the experimental plots between samplings in amounts sufficiently high to alter significantly the composition of the large population already present in each plot, particularly toward the end of the season. It seems more likely that the sensitive strain, which

TABLE 1. Incubation times of benomyl-sensitive (ATCC 24888) and benomyl-resistant (ATCC 24889) strains of *Cercospora beticola* on benomyl-free sugarbeet leaves

Experiment	Days required for the appearance of			
	50% of leaf spots		100% of leaf spots	
	ATCC 24888	ATCC 24889	ATCC 24888	ATCC 24889
1	17	12	24	22
2	15	12	26	22
3	15	12	26	23

happened to be used in the experiment, was less fit pathogenically than the particular resistant strain with which it was compared. This hypothesis is supported by the results of experiments in which the incubation time for each of the two strains was measured. Sugarbeet plants of cultivar Polybeta 13 grown in pots in a growth chamber were sprayed with a suspension of conidia and kept at 25 C. Relative humidity was kept at 100% for 4 days to assure infection and then decreased to 75%. As shown in Table 1, the benomyl-sensitive strain (ATCC 24888) took considerably longer to develop leaf spots than the resistant strain (ATCC 24889). Thus, it appears that when only these two strains are considered, the one resistant to benzimidazoles has a higher competitive ability.

TABLE 2. Fitness of benomyl-resistant strains in natural populations of *Cercospora beticola* in northern Greece

Area and sampling date	Mean percentage of benomyl-resistant leaf spot		
Area A[a]	1973	1974	1975
10 June	...	93.0	91.2
25 June	91.7	96.5	93.0
10 July	93.4	94.5	...
25 July	97.3	92.6	93.0
10 August	95.8	95.3	...
25 August	94.1	97.4	96.8
10 September	94.3	93.8	89.6
Area B	1974[b]		1975[c]
	Field sprayed twice with triphenyltin	Nonsprayed field	Nonsprayed
15 July	...	...	20.1
8 August	13.1	13.0	...
23 August	19.8	18.5	...
9 September	19.7	18.9	...
22 October	15.0	18.0	24.3

[a]Regularly sprayed with triphenyltin. At least 50 leaf spot lesions from each of 30 fields were tested per sampling.

[b]At least 50 leaf spot lesions from each of one sprayed and one nonsprayed field were tested per sampling.

[c]At least 50 leaf spot lesions from each of seven fields were tested per sampling.

Fitness of sensitive and resistant strains in mixed natural populations.—Differences in fitness between benomyl-sensitive and benomyl-resistant portions of natural populations of *C. beticola* were sought in a number of sugarbeet-growing areas of northern Greece; commercial fields either unsprayed or regularly sprayed with triphenyltin were sampled. This search has provided no evidence that the frequency of benomyl-resistant strains declines after benzimidazole-type fungicides were discontinued. Two examples are given in Table 2 to demonstrate this point.

In area A of the Platy district, the frequency of benomyl-resistant strains was very high at the end of 1972 and, because conditions are very favorable for leaf spot, triphenyltin was applied six to eight times per year in 1973-1975. In this area, 30 representative fields were sampled, each several times each growing season, as described above. As shown in Table 2, although benomyl-sensitive strains are present in the area, their frequency did not increase during the 3 years when benzimidazole fungicides were not used.

In area B of the Larissa district, conditions are rather unfavorable for leaf-spot development and the frequency of benomyl-resistant strains in this area never reached high levels. Only twice in 1974 was it necessary to spray some of the fields in this area with triphenyltin. One sprayed and one nonsprayed field were sampled four times during 1974. Fields of area B were not sprayed in 1975. Seven such fields were chosen and sampled at the beginning and at the end of the 1975 growing season. In both years, we found that the ratio of benomyl-sensitive to -resistant leaf spots tended to remain constant (Table 2).

It is noteworthy that the frequency in which benomyl-sensitive strains of *C. beticola* were isolated in northern Greece has not increased despite the repeated introduction of benomyl-sensitive inoculum on seed. All sugarbeet seed used in Greece is imported each year, mostly from Central Europe. As a rule, strains of *C. beticola* imported in this way are benomyl-sensitive. This is indicated by the lower frequencies in which benomyl-resistant leaf spots are found on young seedlings, for which infection from seed-borne inoculum is more important than on older plants. The example in Table 3 was taken from a 1975 experiment in which a number of sugarbeet cultivars were compared in area A of Table 2.

TABLE 3. Apparent effect of benomyl-sensitive seed-borne *Cercospora beticola* on the frequency of benomyl-resistant sugarbeet leaf spot early in the season

Cultivar tested	Country of seed origin	Mean[a] benomyl-resistant leaf spot (%)	
		5 May 1975[b]	5 June 1975[c]
Kawepoly	W. Germany	54.8	97.8
Maribo Monova CR	Denmark	50.2	91.6
K.W.S.	W. Germany	59.2	89.4
Kawecercopoly	W. Germany	51.4	94.8
Hil; Mono 2419	Sweden	67.4	92.4
Monofort	The Netherlands	59.9	97.3
G.W. Mono Hy E_1	U.S.A.	70.2	94.7
KWS 37438	Italy	53.2	89.6

[a]At least 50 leaf spot lesions from each cultivar were tested per sampling.

[b]On 5 May 1975 disease incidence was very low and spots could be found mostly on the cotyledons of seedlings.

[c]On 5 June 1975 the mean percentage of spotted leaf area had increased to 15.8. The plots had received no fungicide applications.

In all cases, the frequency of benomyl-resistant leaf spots increased considerably between 5 May and 5 June 1975 in the absence of any selection for resistant strains due to the use of benzimidazoles (Table 3).

DISCUSSION

In locations where the use of a fungicide must be discontinued because of a high frequency of resistant forms of the target fungus, it is important to determine whether the frequency will decrease when the selection pressure is relaxed. This is because alternative control measures may not always be available and because the effectiveness of alternative methods of control, no matter whether chemicals or host resistance genes, may break down because of pathogen variability (5). The frequency of resistant strains will decrease if such strains are not fitted for survival and competition in the absence of the selective agent. In much of the available literature this problem has been investigated in the laboratory and greenhouse, but field comparisons of particular resistant to particular sensitive strains have been made less often. In the case of benomyl-resistance of *C. beticola,* inoculations of sugarbeets with a mixture of equal numbers of conidia from a sensitive and a resistant strain by Ruppel (9) yielded leaf spots from which the sensitive strain was isolated at a much higher frequency. In our 1973 field experiment, however, the resistant strain, ATCC 24889, was isolated more frequently (Fig. 1, Table 1).

It is doubtful, however, whether differences in fitness demonstrated by the study of a limited number of strains, are of real value in predicting the fate of fungicide-resistant fractions of natural populations. Even though the compared strains were taken from natural populations they did not necessarily represent the most competitive members. Furthermore, changes in virulence (1) or other fitness-determining characters may take place even with short maintenance in culture. The two strains of *C. beticola* used in our 1973 field experiment, for example, had been isolated the previous year and maintained by transferring on PDA slants. It is possible that the lower fitness of the benomyl-sensitive strain, ATCC 24888, was the result of such a change in culture. On the other hand, when a natural population is studied as a whole, the most competitive members of both its fungicide-resistant and -sensitive fractions will be considered. Thus, our study showed that in the case of *C. beticola* in Greece, benomyl-resistant strains of high adaptability have evolved in nonsprayed and triphenyltin-treated crops (Table 2). The maintenance of high frequencies of resistant strains 3 years after the cessation of benomyl usage cannot be due to fungicide residues, particularly since crop rotation is the rule. In addition, benomyl-resistant strains were found at low but constant frequencies even in areas where benomyl was never used.

In addition to demonstrating high adaptability of benomyl-resistant strains of *C. beticola,* our results provide an explanation for the suddenness with which the problem appeared in 1972 (7). Although the two strains compared in the 1973 field experiment apparently were not equally competitive, the selection pressure in favor of the resistant strain exerted by benomyl sprays was very clearly demonstrated. It was also shown that the effectiveness of benomyl for disease control is greatly reduced as soon as the resistant strains attain a high frequency in the population (Fig. 1). In part S of the experiment, for example, with virtually undetectable amounts of benomyl-resistant *C. beticola* at the end of June, benomyl became almost ineffective by the end of August so that the incidence of diseased foliage increased from 17 to 72% within 2 weeks. Because of this fast breakdown of effectiveness and the high fitness for survival of the benomyl-resistant strains in northern Greece the use of benzimidazoles cannot be recommended even in areas where resistant strains presently exist in very low frequencies.

LITERATURE CITED

1. DAY, P. R. 1974. Genetics of host-parasite interaction. W. H. Freeman, San Francisco. 238 p.
2. DEKKER, J. 1976. Acquired resistance to fungicides. Annu. Rev. Phytopathol. 14:
3. FLETCHER, J. T. 1975. Tolerance of fungal pathogens to systemic fungicides in relation to disease control. Pages 751-762 *in* Proc. 8th Br. Insectic. Fungic. Conf., Brighton, 17-20 November, 1975. 1069 p.
4. FRANDSEN, N. O. 1956. Untersuchungen über Cercospora beticola. V. konidienproduction. Zucker 9:51-53.
5. GEORGOPOULOS, S. G. 1971. Microbial genetics and the control of pathogens in agricultural industries. Pages 129-133 *in* Radiation and radioisotopes for industrial microorganisms. International Atomic Energy Agency, Vienna. 388 p.
6. GEORGOPOULOS, S. G. 1976. Development of fungal resistance to fungicides. Pages *in* H. D. Sisler and M. R. Siegel, eds. Antifungal compounds, Vol. II. Marcel Dekker, New York. (In press).
7. GEORGOPOULOS, S. G., and C. DOVAS. 1973. A serious outbreak of strains of Cercospora beticola resistant to benzimidazole fungicides in northern Greece. Plant Dis. Rep. 57:321-324.
8. GREENAWAY, W., and F. R. WHATLEY. 1975. Resistance to systemic fungicides—causes and cures. Curr. Advan. Plant Sci. 7:335-347.
9. RUPPEL, E. G. 1975. Biology of benomyl-tolerant strains of Cercospora beticola from sugar beet. Phytopathology 65:785-789.
10. YONG JOON, L. A. 1963. Conidial production of Cercospora beticola Sacc. on tomato juice agar. Korean J. Bot. 6:8-10.