Predicting Potential Fungicide Resistance in Fungal Populations by Using a Continuous Culturing Technique

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ABSTRACT

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A chemostat containing a continuously growing yeast population was used to study the development of resistance to pesticides after exposure to the fungicides, benomyl, CGA64251, or captan. The yeast population was dramatically reduced in response to each fungicide. Populations treated with benomyl recovered after approximately 2 days. The recovered population was resistant to benomyl and the individual resistant cells had a slower growth rate than the wild type, benomyl-sensitive cells. There was no recovery in populations treated with CGA64251 or captan.

Resistance of organisms to biocides has been a problem for over 30 yr. Bacterial resistance to medical drugs such as sulphonamides and antibiotics was reported in the 1940s; insects showing resistance to insecticides became a menace to agriculture during the 1950s; but plant pathogenic fungi resistant to fungicides generally were not a problem until the early 1970s. In fact, as late as 1967 it was generally believed that fungicide-tolerant strains of fungi were very rare (4). During the last decade, numerous cases of sudden failure of previously effective fungicides to control disease organisms in the field have been reported (2,3). The occurrence of resistance of the apple scab fungus *Venturia inaequalis* to dodine (Cyprex) in Nova Scotia is an example of this "sudden" and unexpected failure (14). In that province, dodine was used for scab control from 1961 until 1974 when several growers reported poor control with the chemical.

The sudden failure in fungicide effectiveness followed closely the introduction of single-site mode of action chemicals such as the benzimidazoles. Earlier, fungicides with multi-site modes of action such as the sulphurs, metal compounds, and carbamates did not seem to pose any pathogen resistance problems. The efficacy of the newer compounds is attributed to their systemic action and specificity, and that very minute quantities are required for inhibition. Their weakness has been their ability to select for resistant strains of plant pathogens when they are consistently used in the field.

Tolerant genotypes of fungi may be selected for in the laboratory simply by exposure of sensitive populations to a fungistat (8), or may be found in nature where no fungicidal selection pressure has been applied (9). It is likely that any population of a plant pathogen would contain within the gene pool some alleles for resistance to a fungicide. Whether these result from natural mutations or are chemically induced is not known (although some researchers indicate that the mutagenic properties of pesticides are not significant [12]), nor is it important. What is important, however, is how fast the pathogen population converts from sensitivity to resistance under normal field control programs, and past experience with benomyl shows that this phenomenon may be quite variable depending on the number of sprays applied per season, the concentration used, and the properties of the local pathogen population (1,5,15,16).

Population dynamics are quite different for different classes of fungi. *Monilinia* spp., for example, appear to be relatively stable in the field when benomyl is used for control whereas *Botrytis* spp. develop resistance quite rapidly.

Laboratory techniques used in the past 20 yr to screen for tolerance are generally based on plating the pathogen on the surface of agar, which has the fungicide suspended in it, and evaluating the ability of the pathogen to germinate and/or carry on normal growth in the presence of the chemical (13). Experience shows that this technique does not necessarily indicate the likelihood that resistance will develop in the field. In nature, there is no isolated infection cycle, but rather a series of recurring infection cycles within an infection period. The infection period is dictated by many parameters, and what happens within the infection period is influenced mostly by the intensity and duration of the selection pressure (ie, the concentration of the fungicide and the length of time of exposure). The use of a continuous culturing technique may offer some insight into the dynamics of the selection process, which is more applicable to the real field situation than the plating technique.

The use of yeast cells was chosen for these initial studies for the following reasons:

1. The chemostat system is more easily adapted to the study of single-celled organisms.
2. The yeast organism is taxonomically in the same class with many fungal pathogens and therefore may be expected to respond in a similar manner to fungicide treatment.

Continuous culturing of microorganisms was used originally as a source of microbes for industrial processes relying upon fermentation (7,10). Interest in continuous culture for microbiological research began in the 1940s and soon thereafter several types of culturing devices were described, such as the turbidostat, the chemostat, and the bactogen (reviewed by Kubitschek [6]). In addition to studies on mutation, selection, population dynamics, and enzyme induction, there have been applications to food and energy production. With recent advances and increasing interest in biotechnology, continuous culturing techniques are becoming even more commonplace. It appears that this technique may also prove invaluable in agricultural research with studies on the mechanism(s) of development of resistance in pathogenic populations.

MATERIALS AND METHODS

Growth of yeast populations. Cultures of yeast (Fleischmann's commercial baker's yeast) were grown in chemostats similar to

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those of Novick and Szilard (11). The selection of a yeast that is sensitive to the particular fungicides to be compared is critical. Of the several yeasts screened, only two were sensitive to benomyl, Fleischmann's dated 2 September 1969 and Fleischmann's dated pre-1974.

The water column flow regulator described by Novick and Szilard was replaced with a peristaltic pump system. A nonbuffered system of nutrient broth (NB) was used (0.5%) supplemented with 0.05% glucose, streptomycin (40 μg/ml), and penicillin (20 units per milliliter). The concentration of glucose required to sustain a population of approximately 10^7 cells per milliliter was found in subsequent experiments to vary with the yeast source and had to be determined beforehand. Nutrient was supplied at the rate of 3 ml/hr, which is equal to the overflow rate. Samples for determining cell numbers were withdrawn directly from the fermentation vessel.

The nutrient flow rate determines the population size stability. If the nutrient flow rate is too great, a "washout" occurs and the population never builds up. If w = dilution rate (fractional rate of replacement flow), W = rate of flow, V_0 = culture volume, then $w = W/V_0$ (units = time). Now, if N = the number of cells, then, for nondividing cells, the rate of decrease of cells is given by $dN/dt = -wN$. So at time t, $N = N_0e^{-wt}$, the time (τ) it takes to replace V_0 is given by $\tau = V_0/W = 1/w$. Since N is constant then the culture would double in time τ, but in a practical sense some cells wash out before they divide so the remaining must divide in less than τ to keep N constant. Since $N/N_0 = 2 = e^{-wt}$, then T (cell generation time) = ln $2/w$. However, $1/w = \tau$, and, therefore, $T = \ln 2(\tau) = 0.693\tau$ *at steady state.*

Stock culture. NB (0.5%) supplemented with 1% glucose and streptomycin-penicillin was seeded with yeast and incubated at 30 C. This culture was routinely transferred to 5 ml of NB (0.5%) supplemented with the lower glucose concentration (0.05%) and allowed to grow overnight at 30 C and then added to the chemostat. The population was stabilized at approximately 10^6 cells per milliliter, 24 hr after being placed in the chemostat. The glucose concentration necessary to limit maximum growth to approximately 10^6 cells was found to vary considerably for various sources of yeast.

Fungicides. The three fungicides compared were: methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (benomyl; Dupont of Canada, 50% WP Com); *N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide) (captan; Stauffer Chemical Co., 50% WP Com), and (*t*)-*cis*/*trans*-1-[2-(2,4-dichlorophenyl)-4-ethyl-1,3-dioxolan-2yl-methyl] 1H-1,2,4-triazole (CGA64251; Ciba Geigy, 10% WP, experimental), and their concentrations (active ingredients) in the chemostat were: 20 μg/ml, 10 μg/ml, and 20 μg/ml, respectively. These concentrations were selected on the basis of their toxicity to our yeast population and may vary for yeasts from different sources.

Evaluation of resistance within the population. The total number of cells in the chemostat was monitored twice daily by making appropriate dilutions and plating the cells on potato-dextrose agar (PDA). All cell concentrations given are the average counts of four plates. At the same time, PDA plates with fungicide suspended in the agar were used to determine the percentage of resistant cells in the total population. Plates were counted 48 hr after plating. Screening for benomyl resistance was carried out at 250 μg/ml, for captan resistance at 16 μg/ml and 0.5 μg/ml, and for CGA64251 at 10 μg/ml. The choice of fungicide concentration in the PDA plates used for screening for resistance was determined by the tolerance of the population in question.

RESULTS

The limiting and optimal dextrose concentrations were determined for this system (Fig. 1). The concentration 0.05% dextrose was chosen because it supported approximately 10^6 cells per milliliter, which was an ideal total population for these experiments. The system also responded quickly to changes in the rate of nutrient flow. As shown in Fig. 2, when the nutrient exchange rate was increased after the population had stabilized, the population dropped approximately 100-fold and when the nutrient

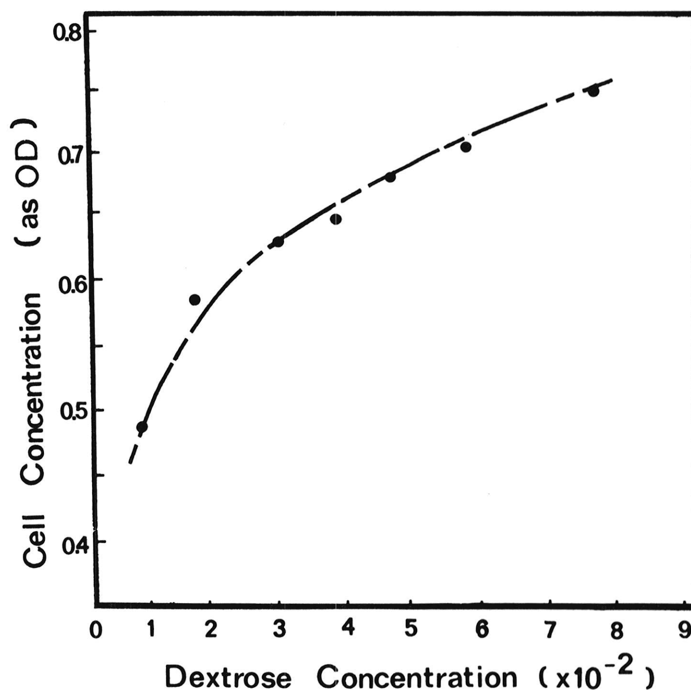


Fig. 1. The number of cells (total population) supported by various concentrations of dextrose in 0.5% nutrient broth.

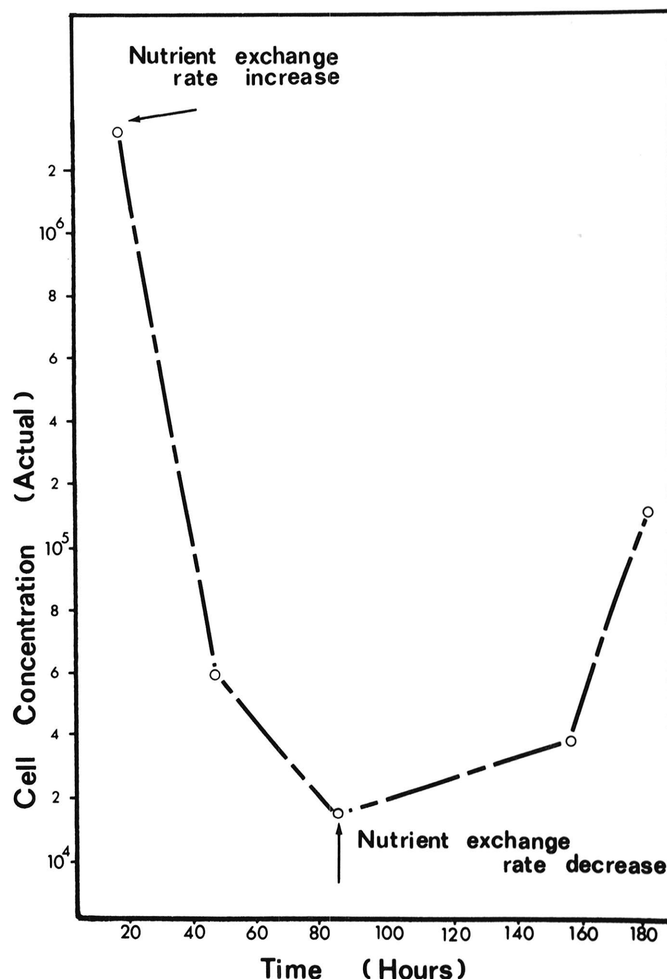


Fig. 2. The response of a stable population of yeast (10^6 cells per milliliter) to changes in the nutrient flow in a chemostat. A stable population was maintained at a flow rate of 3.6 ml/hr. When the flow rate was increased to 10 ml/hr at 20 hr, the population declined due to elution from the chamber. At 80 hr, the flow rate was returned to 3.6 ml/hr and the population began to recover.

flow rate was decreased to the original level the population began to increase again. The chosen nutrient flow rate was very critical for maintenance of a given population.

Although the system was sensitive to changes in the nutrient flow rate, the control populations, when the experimentally defined conditions were held constant, remained relatively stable (Fig. 3, curves 1, 2, and 3).

The three fungicides tested were added via the nutrient supply. A finite time was required for the concentration to build up in the chemostat, usually resulting in a lag period before the population showed a dramatic decline. Figure 3 (curves 4 and 5) shows graphically the population collapse for two benomyl experiments. In each of these cases, there was no sign of a recovery of the population, as would be expected if the original population contained a significant number of resistant organisms, even though the experiment was continued for more than 100 hr.

Figure 4 compares the responses of yeast populations to the three fungicides, benomyl, captan, and CGA64251, and in each of these cases the population collapsed in response to their fungicidal properties, as was the case in earlier experiments (Fig. 3). However, when the nutrient exchange rate was reduced to 0.5 ml/hr in the benomyl-treated vessel, the population began to recover (Fig. 4A). The number of cells resistant to benomyl at each sampling time is shown in Fig. 4B. Initially no resistant cells were detected, but after the introduction of benomyl to the system, the number of benomyl resistant cells increased to approximately 10^3 cells over the next 24 hr. A comparison of Figs. 4A and B shows that by this time (52 hr), the total population was composed of 30% resistant cells. Comparing the same two curves at 72 hr, it is apparent that the entire population was resistant. In the earlier experiments (Fig. 3), it was noted that the total population continued to decrease until no cells were detectable; however, in the benomyl experiment shown in Fig. 4A the population recovered after the nutrient exchange rate was reduced (72 hr). Thus, it appears that the resistant cells had a slower growth rate than the "normal" or benomyl-sensitive cells that comprised most of the original population. This may illustrate a very basic field principle. Ogawa et al (12) suggested that resistance is intrinsic in the population gene pool rather than being

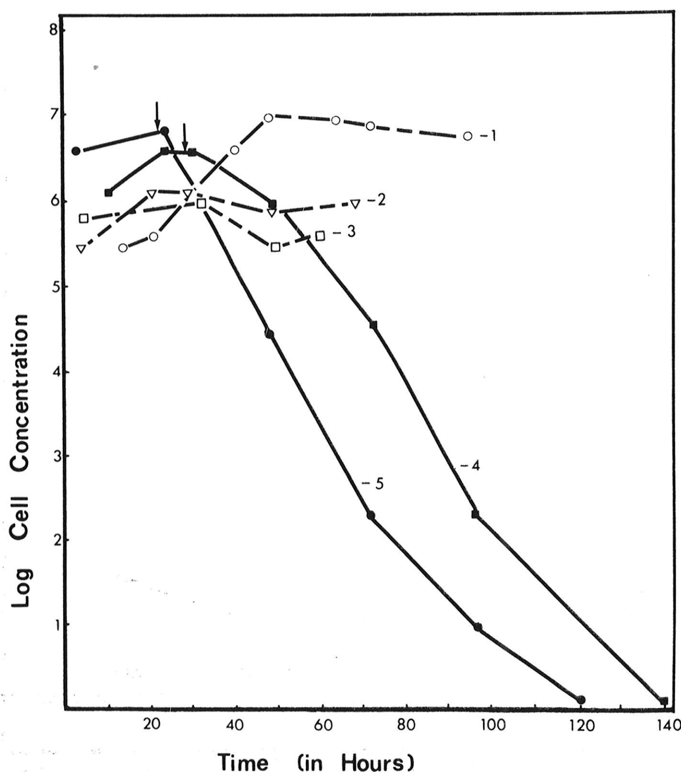
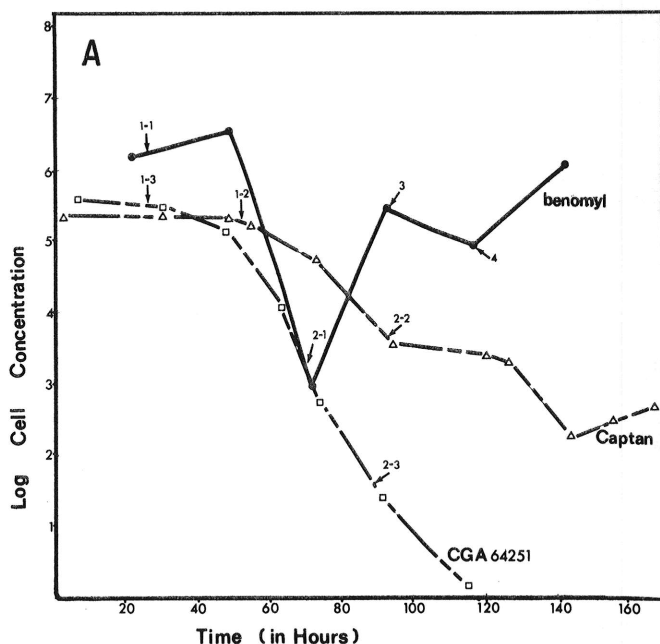


Fig. 3. The relative stability of the population of yeast cells at a constant flow rate in a chemostat is illustrated by curves 1, 2, and 3. These curves are stable 24–48 hr after inoculation. Zero on the time scale indicates initiation of population monitoring. This is approximately 24 hr after inoculation. Variation between curves 1, 2, and 3 reflect small differences in flow rate. Curves 4 and 5 show the population collapse resulting from the addition of benomyl. The time of benomyl addition is indicated by the arrows on curves 4 and 5.



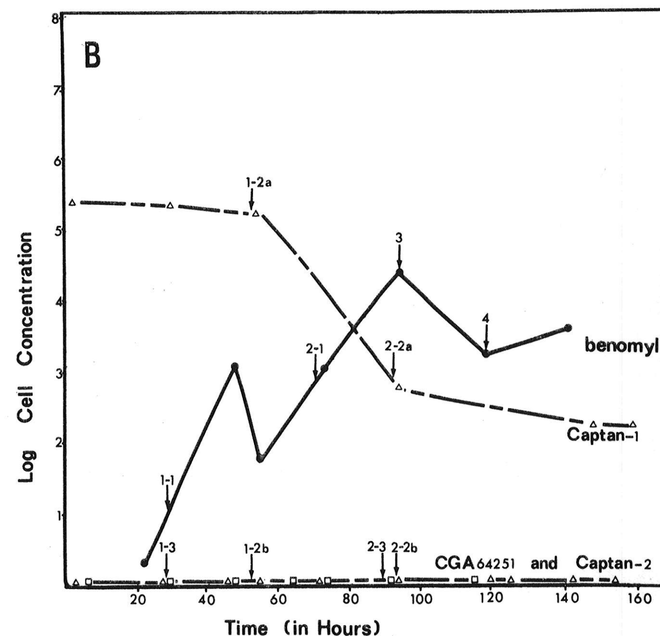


Fig. 4. A, A comparison of the response of a stable population of yeast cells in a chemostat to the addition of fungicides to the system. Changes to the system are indicated on the curves. 1-1, 1-2, and 1-3 indicate time of addition of the respective fungicides. 2-1, 2-2, and 2-3 indicate the time at which the flow rate was changed. For benomyl, the rate changed from 3.6 ml/hr to 1 ml/hr. For CGA64251 and captan, the flow rate was changed from 3.6 ml/hr to 1 ml/hr then to 0 ml/hr. For benomyl, arrow 3 indicates a return to a flow rate of 3.6 ml/hr and arrow 4 indicates removal of benomyl from the system. **B,** The number of fungicide-resistant cells as determined by plating on agar containing the various fungicides. The number shown on the curves correspond to the number in Fig. 4A. For captan, an additional "a" or "b" is used to distinguish between the two different fungicide levels used for screening for resistance. The benomyl resistant strains were selected from agar plates containing 250 μg (a.i.) benomyl per milliliter. The captan resistance was screened for on agar containing 16 μg (a.i.) of captan (captan-2) and 0.5 μg (a.i.) captan (captan-1) per milliliter, and CGA resistance on agar containing 10 μg (a.i.) of CGA64251 per milliliter. The CGA64251 and captan-2 curves represent zero resistant cells.

introduced as a result of the mutagenic properties of the chemical used as a fungicide. This certainly is supported by the isolation of chemical-resistant strains from untreated populations, and if it is generally true, the fact that the resistant strains normally remain as an insignificant part of the total population may be explained by their slower growth rate. They are less fit under normal field conditions and only have an advantage over the faster growing, sensitive strains when the population is subjected to a selection pressure, which is the case when a chemical spray program is used.

In the benomyl test the increase in population, caused by reduction of the nutrient flow rate (arrow 2, Fig. 4A), was reversed by a return to the original flow rate (arrow 3, Fig. 4A). This decrease in population was again reversed by removal of benomyl from the system (arrow 4, Fig. 4A) (without any change in the nutrient exchange rate). Comparing Fig. 4A and B, the total population increased 10-fold from 116 to 142 hr, while the number of resistant cells increased by only one-third over the same time span. This confirms the survival advantage of the sensitive strains under the conditions of the experiment.

The population responded very differently to either captan or CGA64251 than to benomyl. The population collapsed, as expected, but failed to respond to a major change in the nutrient exchange rate over a time period similar to that used in the benomyl experiment and no resistant strains were detected. Response to the captan treatment differed slightly from the CGA64251 treatment in that the population did not completely collapse but was maintained at a low level (4×10^2 cells per milliliter at 160 hr). The population showed low level resistance throughout the treatment when tested on plates containing 0.5 μg of captan and some initial resistance to captan on 8 μg/ml plates, which disappeared with time. There were no cells from the fungicide-treated population resistant to 16 μg/ml captan.

DISCUSSION

There are a number of uncertainties related to the initiation and buildup of fungicide resistance in a fungal population in the field that are as yet unresolved. Tank mixing of chemicals has been generally accepted as a technique for avoiding the buildup of resistant strains in the field, yet a question remains as to whether alternating chemicals in a spray program might not be as good or better. It is difficult to resolve these questions, however, because the time frame for field observation is long and because the natural host-pathogen system in an orchard is extremely complex. Also, much of the field data available from commercial applications are, for numerous reasons, scientifically unreliable, and it is extremely difficult to focus on individual parameters in an effort to establish the basic principles of resistance buildup. This continuous culturing technique indicates, however, that the response of a fungal population to fungicide selection pressure can be monitored under controlled conditions. This introduces the potential for evaluating new chemicals such as CGA64251, and comparing them against established ones such as benomyl, and captan for which field responses are well known. In this system, many of the environmental parameters, such as flow rate, nutrient concentration, and fungicide concentration can be closely controlled. Under such conditions, the population dynamics are mathematically defined.

Many factors contribute to the frequency and survival of resistant mutants in the field. Virulence, reproduction rate, and speed of colonization are three important factors. The influence of the reproduction rate was illustrated by the fact that the benomyl resistant strains grew more slowly than the sensitive strains and were unable to become established in an environment that was acceptable to the sensitive strain. This example clearly supports the argument by Wolfe (17), that "The theory of genetic homostasis indicates that, although particular individuals with a characteristic such as fungicide tolerance may be selected, the whole population does not easily shift in this direction since there are many characteristics in the population which are held in a complex balance in the existing environmental situation." He also notes, "Thus, if the new selection pressure is relaxed or incomplete, the population may tend to shift back to its previous state of balanced adaptation." This was observed during the benomyl experiments (Fig. 4A and B).

Any isolated systems such as the one described here will suffer from limitations when an attempt is made to extrapolate the data to field situations. An obvious example is the inability to account for the relative availability of various chemicals at the host-pathogen interface (ie, the relative availability of systemic and nonsystemic chemicals at the disease site). However, comparison between chemicals still indicates the relative likelihood of a resistant population developing under defined conditions and thus gives an indication of the potential for a resistance problem in the field.

As early as 1971, Wolfe was advocating routine monitoring of resistant strains to detect, in the early stages, what potential risk for resistance buildup existed. Ogawa (12) stated, "The relationship between genetic studies in the laboratory and resistance in the field has not been established." Effective preventive monitoring is still a pressing need, but the continuous culturing technique offers an opportunity to determine the potential for a chemical generating and/or selecting for resistance within a population prior to extensive field testing and thus, moves somewhat in the direction of solving these experimental problems.

LITERATURE CITED

1. Berger, R. D. 1973. Disease progress of *Cercospora appi* resistant to benomyl. Plant Dis. Rep. 57:837-840.
2. Dekker, J. 1976. Acquired resistance to fungicides. Annu. Rev. Phytopathol. 14:405-428.
3. Dekker, J. 1977. The fungicide-resistance problem. Neth. J. Plant Pathol. 83 (Suppl. 1):159-167.
4. Georgopoulos, S. G., and Zaracovitis, C. 1967. Tolerance of fungi to organic fungicides. Annu. Rev. Phytopathol. 5:109-130.
5. Jones, A. L., and Ehret, G. R. 1976. Isolation and characterization of benomyl-tolerant strains of *Monilinia fructicola*. Plant Dis. Rep. 60:765-769.
6. Kubitschek, H. E. 1970. Introduction to research with continuous cultures. Prentice-Hall, Englewood Cliffs, NJ. 195 pp.
7. Malek, I. 1958. The physiologic state of microorganisms during continuous culture. Pages 11-28 in: Continuous Cultivation of Microorganisms. I. Malek, ed. Publishing House of the Czech. Acad. Sciences, Prague.
8. McPhee, W. J. 1980. Some characteristics of *Alternaria alternata* strains resistant to iprodione. Plant Dis. 64:847-849.
9. Meyer, J. A., Pourtois, A., Lumande, K., and Maraite, H. 1977. Tolerance to benomyl of *Botrytis cinerea* and *Fusarium oxysporum*. Neth. J. Plant Pathol. (Suppl. 1) 83:215-227.
10. Novick, A. 1955. Growth of bacteria. Annu. Rev. Microbiol. 9:97-110.
11. Novick, A., and Szilard, L. 1950. Description of the Chemostat. Science 112:715-716.
12. Ogawa, J. M., Gilpatrick, J. D., and Chiarappa, L. 1977. Review of plant pathogens resistant to fungicides and bactericides. FAO Plant Protection Bull. 25:97-111.
13. Partridge, A. D., and Rich, A. E. 1962. Induced tolerance to fungicides in three species of fungi. Phytopathology 52:1000-1004.
14. Ross, R. G., and Newbery, R. J. 1977. Tolerance of *Venturia inaequalis* to dodine in Nova Scotia. Can. Plant Dis. Surv. 57:57-60.
15. Szkolnik, M., and Gilpatrick, J. D. 1977. Tolerance of *Monilinia fructicola* to benomyl in western New York state orchards. Plant Dis. Rep. 61:654-657.
16. deWaard, M. A., and Gieskes, S. A. 1977. Characterization of fenarimol-resistant mutants of *Aspergillus nidulans*. Neth. J. Plant Pathol. 83 (Suppl. 1):177-188.
17. Wolfe, M. S. 1971. Fungicide and the fungus population problem. Proc. 6th Insectic. Fungic. Conf., Plant Breeding Institute, Cambridge, England.