Determination and Significance of the Mutation Rate of *Colletotrichum coffeanum* From Benomyl Sensitivity to Benomyl Tolerance

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ABSTRACT

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The fluctuation test of Luria and Delbrück applied to spore suspensions of *Colletotrichum coffeanum* made from sporulating lesions on green coffee berries suffering from coffee berry disease (CBD), showed that conidia tolerant to benomyl arose in a coffee field as a result of preadaptive, spontaneous mutations. The mutation rate from benomyl sensitivity to benomyl tolerance was 2.4×10^{-6} per spore per division. Selection for tolerant mutants due to repeated spray applications of benzimidazole fungicides was simulated by using a mathematical model constructed by Kable and Jeffery. At good spray coverage, the 29 to 34 theoretical spray applications required for the tolerant subpopulation frequency to increase from 0.000042% ($2.4 \times 10^{-6} \times 100$) to 95%, compared well with published data from several benzimidazole fungicide trials on CBD control. The prospect of delaying the buildup of tolerant mutants to unacceptable levels by using these potent fungicides in mixtures with other fungicides to which no tolerance has developed is discussed.

Additional key words: asexual reproduction, sectors.

Colletotrichum coffeanum is the causal fungus of coffee berry disease (CBD) on *Coffea arabica* L. Recently, strains of this pathogen tolerant to benzimidazole fungicides (benomyl, carbendazim, and cypendazole) appeared in Kenya. Their distribution within growing areas of Kenya, their degree of tolerance to the fungicides, and their stability and fitness (reproductive and pathogenic ability relative to the normal sensitive strain) in vivo and in vitro have been well elucidated (2,5,8).

The origin of tolerant strains was not clarified, although mutation from sensitivity to tolerance was suggested as a possibility (2,8). In the absence of knowledge of sexual or parasexual stages of the fungus (9), resolution of this point seemed possible by applying the fluctuation test of Luria and Delbrück (7).

Benomyl tolerance was investigated because this fungicide

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selects strains tolerant to the other benzimidazole fungicides (2,8) and also because it is sometimes used for CBD control in combination with other broad-spectrum protective fungicides to which no tolerance exists. To elucidate the effect of such combinations on the tolerant subpopulation frequency, and the significance of the calculated mutation rate, a theoretical mathematical model constructed by Kable and Jeffery (6) was used. The model simulates the process of selection that occurs when a pathogen population containing a component tolerant to a fungicide is repeatedly exposed to the fungicide mixed with a second fungicide to which no tolerance exists.

MATERIALS AND METHODS

All collections of green diseased or healthy berries were made from a coffee plot (cultivar SL 28) established in 1975 at the National Agricultural Laboratories, Nairobi. The plot was isolated, with no other coffee growing within 10 km of it. The trees were not sprayed with any of the anti-CBD fungicides from the time seedlings were transplanted. However, the seedlings were sprayed with captafol and copper in the nursery.

Diseased berries were washed well in tap water containing a little teepol. After they had dried, the berries were placed on damp cellulose wadding (with the lesions facing upward) in a covered dish for 48–72 hr at 24 C to promote sporulation. To determine the effect of benomyl on sporulation, 40 similarly incubated diseased berries were lightly sprayed with a suspension containing 250 μg of benomyl per milliliter.

Spores or colony forming units (cfu) are herein defined as tolerant or sensitive, based on ability to grow on malt extract agar (MEA) (Oxoid Ltd.) containing 250 μg (a.i.) of benomyl per milliliter according to established procedures (2,5) from the 50% wettable powder (WP) formulation of Benlate (E. I. du Pont de Nemours & Co., Wilmington, DE 19898).

Spore suspensions were made as follows. A single naturally diseased or laboratory inoculated green coffee berry was selected. The berry bearing only one actively sporulating anthracnose lesion approximately 4–6 mm long was placed in a small, sterile, screw-capped bottle containing 1, 3, or 9 ml of sterile distilled water and 0.001% teepol plus 50 μg of streptomycin per milliliter. Spores were dispersed by vigorously shaking the bottle for 20 min in a reciprocal elbow shaker. The berry was then removed, rinsed with 1 ml of sterile distilled water, which was added to the suspension. Microscopic examination of washed lesions showed that almost all spores were removed by this method. The number of spores per milliliter in suspension was determined with a haemocytometer.

The number of tolerant cfu in spore suspensions was determined by seeding agar plates. The spore suspension from each lesion was poured into two or three petri plates (about 1–3 ml per plate). The inside of the bottle was rinsed with 1 ml of sterile distilled water, which was added to the last petri plate. Fifteen milliliters of cool molten MEA containing 250 μg of benomyl and 100 μg of streptomycin per milliliter was added to each plate. After gentle agitation, the solidified seeded agar plates were incubated at 24 C for 7 days after which the colonies that developed in each plate were counted. To determine whether the plating technique caused unusual variation in the number of colonies, the numbers of tolerant cfu in 1-ml aliquots from four or eight pooled 10-ml spore suspensions were similarly determined. Total cfu (tolerant + sensitive) in suspensions were determined on MEA containing 100 μg of streptomycin only per milliliter, by using 0.5-cc, 1-cc, or 2-cc

TABLE 1. Variation in recoveries of spores of *Colletotrichum coffeanum* from single infected coffee berries[a]

Set[b] no.	Suspension number[c] 1	2	3	4	5	6	7	8	9	10	Mean	Variance
1	6.4	6.4	5.0	3.5	9.5	7.0	4.4	3.2	1.6	12.5	5.95	10.30
2	8.4	9.6	7.6	2.4	2.4	6.4	5.2	2.4	5.6	3.2	5.32	7.20
3	1.4	2.3	0.4	2.7	0.6	0.7	1.9	2.2	1.2	1.5	1.49	0.61
4	9.6	11.2	9.6	8.0	16.0	9.6	11.2	17.6	21.2	17.6	13.20	19.32

[a] Each selected berry bore a single lesion 4–6 mm long.
[b] Four sets of 10 berries each.
[c] Each suspension was prepared from a single berry and each value represents the number of spores divided by 10^3.

TABLE 2. The number of tolerant *Colletotrichum coffeanum* spores in a series of eight similar parallel spore suspensions prepared from single infected coffee berries

	Set number[a] 1	2	3	4	5	6	7	8
Number of cultures	10	10	10	10	10	5	10	10
Volume of culture (ml)	4	10	4	4	4	2	4	4
Volume plated (ml)	4	10	4	4	4	2	4	4
Suspension number[b]								
1	0	46	95	0	32	9	205	3
2	0	4	0	14	3	24	154	67
3	0	3	1	0	2	2	2	3
4	0	6	0	45	4	7	0	0
5	11	20	3	0	15	9	0	9
6	0	3	1	0	2	···	26	25
7	0	4	82	178	7	···	16	0
8	3	12	0	9	3	···	0	16
9	5	8	3	1	4	···	0	0
10	7	5	29	0	4	···	0	5
Average number of tolerant spores per culture	2.60	11.1	21.6	24.70	7.60	10.20	40.30	12.80
Variance	11.70	178.0	134.0	3,098.00	88.00	68.00	5,604.00	428.00
Total spores per culture	8.00×10^5	5.12×10^5	5.40×10^6	2.97×10^6	1.90×10^6	1.29×10^6	5.30×10^6	3.20×10^6
Mutation rate	1.40×10^{-6}	6.20×10^{-6}	0.99×10^{-6}	1.99×10^{-6}	1.26×10^{-6}	2.30×10^{-6}	1.68×10^{-6}	1.11×10^{-6}
(Standard deviation)/mean								
Exper.[c]	1.32	1.20	1.71	2.25	1.24	0.81	1.85	1.60
Calcu.	1.32	0.91	0.79	0.76	0.98	0.76	0.70	0.88

[a] Each set represents 10 coffee berries with single lesions 4–6 mm long.
[b] Spore suspensions were prepared from individual berries. Sets 1–6 were prepared from naturally field-infected berries. Sets 7 and 8 were prepared from berries artificially inoculated in the laboratory with spores from a single benomyl-sensitive colony.
[c] Exper. = experimented ratio; Calcu. = calculated ratio.

aliquots from 10^{-4} dilutions. The color and other characteristics of colonies were carefully assessed after 15 days of incubation according to the descriptions of Gibbs (3).

Pathogenicity of 20 tolerant isolates was determined by the method of Gibbs (3). Spore suspensions for inocula were prepared from second culture generation of tolerant isolates. Forty berries per isolate were inoculated with 0.01 ml of suspension containing about 10^6 spores per milliliter. Two sets of berries, one inoculated with a sensitive isolate and the other with distilled water, were included as controls.

To facilitate operations with the mathematical model of Kable and Jeffery (6), a program for a desk-top computer (Compucorp 445) was written by the second author. To minimize bias in the results, the main variables efficacy (F) and spray coverage (E) were each assigned a range of values. F_{1A} and F_{2A} indicate efficacy of benzimidazole (fungicide A) against tolerant (subpopulation 1) and sensitive (subpopulation 2) strains of *C. coffeanum*, respectively.

Since it was shown that the protective property of anti-CBD fungicides is by far more important than their sporulation inhibiting property (3,4,10), efficacy in this paper is measured in terms of protective effect. F_{1A} and F_{2A} values were obtained from published dose-response histograms for benomyl against tolerant and sensitive strains, respectively. At the recommended field concentration of benomyl (500 μg/ml), F_{1A} values were 0.20 at 15 C and 0.30 at 20 C (5). F_{2A} values were 0.595 at 15 C and 0.695 at 20 C (5). F_B values were obtained from similar unpublished data for captafol 80% WP (Chevron Chemical Co., San Francisco, CA 94104), at the recommended field concentration of 3,200 μg/ml of active ingredient. F_B values were 0.75 at 15 C and 0.52 at 20 C (*personal communication*, Javed, Coffee Pathologist, Coffee Research Station, P.O. Box 4, Ruiru, Kenya).

Calculations were made with a starting frequency of the tolerant subpopulation equal to the experimentally determined mutation rate from benomyl sensitivity to benomyl tolerance.

RESULTS

A culture of *C. coffeanum* was a suspension containing the population of spores formed in a single anthracnose lesion (4–6 mm long) after 48–72 hr of incubation at 24 C. It was found that such young lesions on green berries were not colonized by saprophytic forms of *Colletotrichum* described by Gibbs (3).

Detection of tolerant strains of the pathogen among conidia from diseased berries. Tolerant colonies were detected in some of these suspensions or in aliquots of pooled suspensions. Their number formed a very small fraction of the 10^5 or 10^6 spores per milliliter that had been plated. Therefore, it was unlikely that the diseased berries had been colonized by conidia of tolerant strains of the pathogen. This was confirmed in two experiments in which none of the diseased berries sprayed with 250 μg/ml benomyl showed signs of sporulation as compared to the unsprayed diseased berries.

Characteristics of the tolerant isolates. Inoculations of berries with each of 20 tolerant isolates caused between 12.5 and 25% infection, which was similar to the proportion infected from inoculations with a normal, sensitive CBD isolate.

In agar plates the white mycelium of each of the 20 isolates that appeared in 4–7 days turned grey to dark grey in about 12–15 days. Acervuli were not observed even after several weeks of incubation, but conidia were present on tips of solitary hyphae. These tolerant isolates thus conformed to CBD strains described by Gibbs (3). Not all isolates were tested for pathogenicity, but since their colony characteristics were very similar to the 20 isolates studied, they were considered typical *C. coffeanum*. Sectoring was not observed in any of the colonies.

Viability of spores in suspensions. The use of teepol minimized clumping. The spore concentrations in three suspensions counted in the haemocytometer were 1.5×10^6, 4.4×10^5, and 4.5×10^5 spores per milliliter, respectively. The number of cfu determined from colony counts on agar for the same three suspensions were 1.2×10^6, 2.7×10^5, and 4.2×10^5 cfu per milliliter, respectively. Therefore, it could safely be assumed that loss in viability was minimal and that a cfu represented a single viable spore.

Variation in the sporulating capacity of diseased berries. Table 1 shows the variation in total number of spores (tolerant and sensitive) within four sets of parallel suspensions made with diseased berries obtained from the plot where infection and lesion development occurred under normal uncontrolled field conditions. The variation within each set was clearly within the range of normal sampling error.

The fluctuation test of Luria and Delbrück and calculation of the mutation rate. The occurrence of tolerance being a rare event, distribution of the number of such occurrences follows a Poisson series in which the variance is equal to the mean. According to the mutation hypothesis of Luria and Delbrück (7) tolerant cells that arise among cultures (or single sporulating lesions) before exposure to benomyl would generate sectors of various sizes containing clones of tolerant spores, depending upon the time of mutation. The variance/mean ratio would then be significantly >1. Induction of tolerance by benomyl would generate sectors of approximately equal sizes and the variance/mean ratio would then be close to $\cong 1$.

The ratios in Table 2 were all significantly >1. Variation in sets 7 and 8 was similar to that in sets 1–6 (Table 2). The former two sets were made with diseased berries that were inoculated in the laboratory with spores harvested from a single agar colony of the normal, sensitive strain of the pathogen. The similarity between variances and means of the number of tolerant spores in 1-ml aliquots from pooled 10-ml spore suspensions (Table 3) showed that the plating technique introduced only normal sampling error in this result.

In defining the mutation rate for a growing bacterial culture, Luria and Delbrück (7) represented time in units of the average division time T of a bacterial cell, divided by $\log_e 2$ (ie, bT divided by $\log_e 2 = 1$, in which T = the average time taken by a single bacterial cell to divide, and b = the division rate). Bacterial cell divisions are analogous to nuclear divisions in fungal mycelia and conidiophores. A mutant conidiophore would produce sectors of mutant conidia. Therefore, in this paper the mutation rate is defined as the chance of mutation per division.

The mutation rate from benomyl sensitivity to benomyl tolerance was obtained from the equation $r = aN_t \log_e(N_t\, Ca)$, in which r = the average number of mutants in a limited number of cultures; N_t = the total number of spores per culture; C = the number of cultures; and a = the chance of mutation per division (7). The mean of eight estimates of 'a' (Table 2) was 2.15×10^{-6} per spore per division, while the mean of sets 1 to 6 (field infected berries only, Table 2) was 2.4×10^{-6} per spore per generation.

To test the fit of the experimental results to theory, the experimental ratio of standard deviation per mean was compared with the theoretical ratio obtained by equation 12 of Luria and Delbrück (7) (Table 2).

Simulation of the process of selection of tolerant spores using the model of Kable and Jeffery. Results of two simulations with F_{1A} = 0.20, 0.25, 0.30, and 0.40 and F_{2A} = 0.55, 0.60, 0.65, and 0.70 are shown in Tables 4 and 5. The first simulation examines the effect on selection of benzimidazole alone ($F_B = 0.0$) and in mixture with another anti-CBD fungicide (F_B = 0.50, 0.59, 0.70, and 0.80) at good spray coverage ($E = 0.05$ escapes). The second simulation examines the effect of different spray covers (E = 0.0, 0.05, 0.10, 0.30, and 0.50 escapes) since this variable was shown to have the

TABLE 3. Distribution of numbers of benomyl-tolerant *Colletotrichum coffeanum* colonies from spore suspensions prepared from single infected coffee berries

Suspension		Number of tolerant colonies							
No.	Vol. (ml)	0	1	2	3	4	5	Mean	Variance
1	10	···	···	3[a]	2	4	1	3.3	1.12
2	80	60	18	2	···	···	···	0.28	0.22
3	40	36	3	1	···	···	···	0.13	0.17

[a]Number of 1-ml aliquots having 0, 1, 2, 3, 4, or 5 tolerant colonies. Mean = mean number of colonies.

largest effect on selection (6). In the second simulation, F_B was held constant at 0.59 because the average efficacy of captafol 80% WP, the most recommended anti-CBD fungicide, is close to this value. The theoretical number of sprays required for tolerance to increase from 0.000042 to 95% was calculated.

At good spray coverage, 29–34 theoretical spray applications would be required with the benzimidazole alone (Table 4). In a mixture with a second fungicide to which no tolerance develops, 29–42 challenges would be required at good-to-fair spray coverage ($E = 0.0$, 0.05, and 0.10; Tables 4 and 5). However, in a mixture at poor spray coverage ($E = 0.30$ and 0.50), 82–170 challenges would be required (Table 5).

If the benzimidazole was used alternately with the other anti-CBD fungicides, 58–68 spray challenges would be required. As explained by Kable and Jeffery (6), these figures were obtained by doubling the number of challenges with exclusive use of benzimidazole ($F_B = 0.0$; Table 4).

DISCUSSION

The abnormally high variance of the distribution of tolerant spores in a series of parallel spore suspensions can be explained by the occurrence of preadaptive, random, spontaneous mutations that occur at any moment in time during the growth of fungal colonies resulting in fungicide-tolerant, sporulating sectors that vary in size from colony to colony depending upon the time of mutation. Consequently the proportions of tolerant spores varied greatly from suspension to suspension. This explanation is tenable because the abnormally high variance could not be accounted for by: colonization of some diseased berries by tolerant strains of the pathogen; variations in the sporulating capacity of lesions on diseased berries; method of preparation of spore suspensions or amount of suspending volume; or the source of infected berries (field infected or laboratory infected).

Luria and Delbrück (7) showed that the peculiar "clonal distribution" of mutants would occur whatever the magnitude of the mutation rate, provided that the initial number of dividing cells in a culture is so small that the number of mutations that take place during the first division cycle is very small. Our cultures were sporulating lesions presumably initiated by single spores. The reasonably close agreement between experimental and theoretical standard deviations/means ratios suggested that Luria and Delbrück's (7) condition was met.

The results showed that tolerant spores arose in the coffee plot by spontaneous mutations that occurred at a rate of 2.4×10^{-6} per division. This rate, and the fact that the mutants have no fitness advantage over the parent (2,5,8) explain the low frequency of benzimidazole-tolerant mutants in coffee fields devoid of selection pressure for the mutants. Under intense selection pressure that was created, in two spray trials, by 10 spray applications of benzimidazole fungicides per year at intervals of 4 wk, tolerant mutants nearly replaced the sensitive parents in 2–3 yr or 20–30 sprays (2).

At good spray coverage, the 29–34 benzimidazole spray applications theoretically calculated for the pathogen population to become 95% tolerant, compares favorably with the above field observation. In making this comparison and in drawing inferences from results obtained with the model of Kable and Jeffery (6), it must be pointed out that one underlying condition of the model, that efficacy must remain constant from challenge to challenge, may not be completely met. In the absence of concrete information on the change in efficacy due to fungicide deposits, it was assumed that this condition was partially fulfilled because the spray interval of 4 wk was much longer than the incubation period of *C. coffeanum* (1). All the other underlying conditions of the model were met by the CBD system: the pathogen reproduces asexually (9); the disease system is closed in that inoculum rarely, if at all, comes from without (11); and the tolerant mutant is as fit as the sensitive parent (2,4,8) making it unlikely for the subpopulation ratios (tolerant/sensitive) to change significantly between sprays.

The CBD system furnishes a good example of the entry into a pathogen's field population of preadaptive mutants tolerant to a fungicide having a specific mode of action, presumably involving one or only a few gene sites. The mutants are invariably selected whether the fungicide is used alone, in mixtures with other fungicides to which no tolerance exists, alternately with other fungicides, or in a planned sequence with other fungicides. These strategies for disease control only reduce the rate of selection, thus minimizing the likelihood of sudden and complete breakdown of control. The theoretical results indicate that at good spray coverage, a limited number of alternate or sequential applications of benzimidazoles for CBD control could be the preferred strategy. There is, however, an obvious risk of high disease incidence after only one application, should the initial frequency of the tolerant subpopulation be unacceptably high and the disease pressure intense. This risk can be evaluated by the development of an efficient monitoring method for reliably determining the tolerant subpopulation frequency. For reasons given by Kable and Jeffery (6), the unacceptable frequency should be set at 1%.

TABLE 4. The theoretical number of sprays of a fungicide mixture at good spray coverage ($E = 0.05$ escapes) required for a pathogen population to attain 95% tolerance

Efficacy[a]		F_B = efficacy of second fungicide against both subpopulations				
F_{1A}	F_{2A}	0.0	0.50	0.59	0.70	0.80
0.20	0.55	34	37	38	40	45
0.25	0.60	31	34	35	38	42
0.30	0.65	29	31	33	35	40
0.40	0.70	29	32	34	37	42

[a] F_{1A} = efficacy of benomyl against the tolerant subpopulation. F_{2A} = efficacy of benomyl against the sensitive subpopulation. The starting frequency of the tolerant subpopulation was 1 in 2.4×10^6 spores.

TABLE 5. The effect of spray coverage (E) on the theoretical number of sprays of a fungicide mixture required for a pathogen population to attain 95% tolerance

Efficacy[a]		E = proportion of spores escaping fungicide contact				
F_{1A}[a]	F_{2A}	0.0	0.05	0.10	0.30	0.50
0.20	0.55	31	38	45	84	155
0.25	0.60	29	35	43	82	152
0.30	0.65	26	33	40	79	150
0.40	0.70	26	34	42	88	170

[a] F_{1A} = efficacy representing benomyl against the tolerant subpopulation. F_{2A} = efficacy representing benomyl against the sensitive subpopulation. The starting frequency of the tolerant subpopulation was 1 in 2.4×10^6 spores and the efficacy of the second fungicide (F_B) to which no tolerance develops was kept constant at 0.59.

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