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Analysis of Distributions of Mutants in Clones of Plant-cell Aggregates

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Summary. The organization of plant cells (or any other genetic elements, such as organelles) into aggregates modifies the expected distribution of mutants in clones. The reason for the modification, and its effect on the use of the Luria-Delbrück fluctuation test, are discussed. The Luria-Delbrück test was used to show that the trait for chlorate resistance in cultured rose-cell aggregates appeared spontaneously and in the absence of chlorate ion.

Key words: Luria-Delbrück – *Rosa* – Chlorate resistance – Mutation rates – Cell culture

Introduction

Phenotypic variants in cultured plant cells may represent classical genetic mutations (Maliga 1978) as well as "epigenetic" or "physiological" changes (Meins and Binns 1977; Widholm 1977). It is difficult in any particular case to discern the nature of a phenotypic change without regenerating plants from the affected cells and performing standard mating experiments. However, with cultured cells of some species (e.g., rose and soybean) regeneration is not yet possible and other tests must be used.

One useful test, the fluctuation test, has been generally ignored by plant tissue culture geneticists. Luria and Delbrück (1943) introduced this test to distinguish between induced and spontaneous viral resistance in bacteria. They compared the pattern of appearance of phenotypic variants in separate clones to the pattern expected from non-hereditary causes (induction) and to that expected from hereditary events (spontaneous mutation). In the first case, the distribution was expected to follow a binomial (under limiting conditions, a Poisson) distribution. In the second case, the distribution would depart from the Poisson distribution, with significant numbers of clones, those with early mutations, having disproportionately high numbers of variants.

The data below show an application of the fluctuation test to chlorate resistance in suspension cultured rose cells. I demonstrate that the multicellular nature of the aggregates in the rose-cell suspensions modifies the expected distribution of variants appearing by mutational events but does not prevent the test from detecting such events.

Materials and Methods

Conditions for the growth and plating of a standard culture of *Rosa damascena* Mill. var. 'Gloire de Guillan' have been described by Murphy et al. (1979). "Clonal" populations of rose cell suspensions were prepared by plating several samples each containing approximately 100 aggregates of 60 to 350 cells each. Twenty to forty isolated aggregates per plate were identified by microscope and marked. After 2 to 3 weeks, 60% of the marked aggregates had grown into large, friable colonies, which contained an average of 3×10^5 cells each. Individual colonies were gently macerated and transferred to 25 ml of liquid medium. The flasks containing the colonies were incubated 7 to 14 days until they formed stationary phase suspensions of small aggregates. – The concentrations of cells and aggregates were measured by counting them in a cytometer under a microscope. To count cells, suspensions were first treated with chromic acid. The number of cells per aggregate was calculated by dividing the concentration of cells by the concentration of aggregates. The concentration of colony-forming units was measured by plating samples on agar medium without $NaClO_3$. The concentration of chlorate-resistant colony-forming units was estimated by plating samples on agar medium containing 56 mM $NaClO_3$; putative resistant colonies were retested on agar containing 67 mM $NaClO_3$. Details of the test for chlorate resistance, including demonstrations of the stability of chlorate resistance in the colonies that are selected, have been described by Murphy and Imbrie (1981).

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Results

The Effect of Aggregate Size on the Distribution of Numbers of Resistant Variants

A fundamental difference between rose cell cultures and the bacterial cultures studied by Luria and Delbrück (1943) involves the state of aggregation of the cells. The bacterial cells were individuals; each was a colony forming unit. The numbers of resistant and sensitive cells could be determined by the numbers of resistant and sensitive colonies. The rose cells were plated as aggregates of 60–350 cells; the aggregate was the colony forming unit. A resistant aggregate might have one or more resistant cells. This aggregation tends to distort experimental estimates of the number of resistant cells, of the fraction of cells that were resistant, and of the distribution of resistant cells in a sample.

The reason for the aggregate effect can be seen in Fig. 1. Consider an aggregate of n cells, one of which is a resistant mutant. Assume that in each generation all cells divide, then the aggregate splits into two aggregates of equal size. If at each split the two mutant progeny cells are divided, then the number of aggregates that contain a mutant cell increases as fast as the number of mutant cells (curves A and D). This is what occurs with bacteria that are in "aggregates" of n = 1. If the mutant cells are not divided until they make up an entire aggregate, there is a delay of $\log_2$ (n) generations before the number of aggregates with mutant cells starts to increase (curves C and F). If the division of the

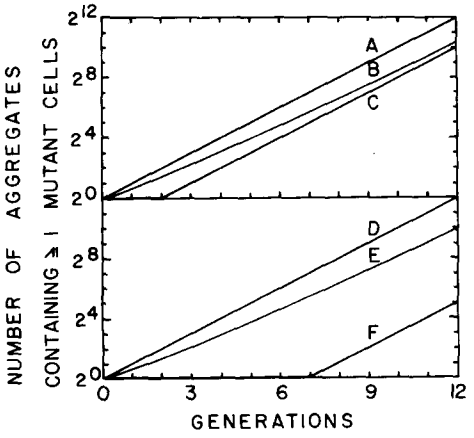

Fig. 1. Effect of aggregation on the number of colony-forming units containing mutant cells, following a mutation of a single cell. Ordinate: number of aggregates (colony-forming units) containing ≧ 1 mutant cell. Abscissa: number of generations of growth. The upper curves assume that one aggregate of size n = 4 contained one mutant cell at generation 0. The lower curves assume that one aggregate of size n = 128 contained one mutant cell at generation 0. The numbers of mutant cells, non-mutant cells, and aggregates all doubled once each generation. For conditions yielding curves A to F, see text

mutant and non-mutant cells occurs randomly, the number of aggregates with one or more mutant cells (curves B and E) lies between the values given by the first two curves, but approaches the second curve as a limit (see Appendix). In the cases represented by curves B, C, E, and F, aggregates that are initially mixtures of mutant and wild type cells grow into populations of aggregates that are all mutant or all wild type.

Two observations help us assess the situation in a real plant-cell suspension. The first involves chromic acid digestion of cell aggregates. This treatment seldom separates aggregates into individual cells; rather, it leaves small aggregates (n≧2) unless it is continued so long that the cells themselves break up. This suggests that cell plates are resistant to separation for a few generations, so clones within an aggregate would tend to stay together. The second observation involves division patterns seen in aggregates (Street 1977). Active regions of division often occur near the surface of aggregates, suggesting that new aggregates are formed from "clones" within the aggregate rather than from a random assortment of cells in the aggregate. Both sets of observations mean that the real situation probably falls between curves B and C (or E and F) in Fig. 1. This means that the number of aggregates that contain mutants will tend to become 1/n the number of mutant cells. Of direct importance to fluctuation testing, the ratio of the number of aggregates containing mutants to the number of mutant cells decreases as a clone that contains mutants grows. This would reduce the size of the "tail" of the distribution of mutants within samples and could make it difficult to distinguish the distributions expected from mutational and non-mutational events.

Figure 2 illustrates the potential magnitude of the aggregation effect in two ways. When mutation rate is held constant, aggregation lowers the mean number of mutant-containing aggregates in a clone (Fig. 2a). The effect is striking for even a low degree of aggregation (n = 4). When mutation rate is varied so that the mean number of mutant-containing aggregates remains constant, aggregation changes the probability distribution for the number of such aggregates appearing in a large number of samples, as well as the ratio of the variance to the mean (Fig. 2b). A high degree of aggregation (n = 128) makes the probability distribution indistinguishable from a Poisson distribution with the same mean.

While aggregation may limit the use of the fluctuation test as a means of distinguishing between mutational changes in aggregates, it need not preclude its use. The actual pattern of aggregate growth and division and the distribution of aggregate sizes are important and unknown factors. The assumptions used in Fig. 2b (n = 128) represent a worst-case model. The

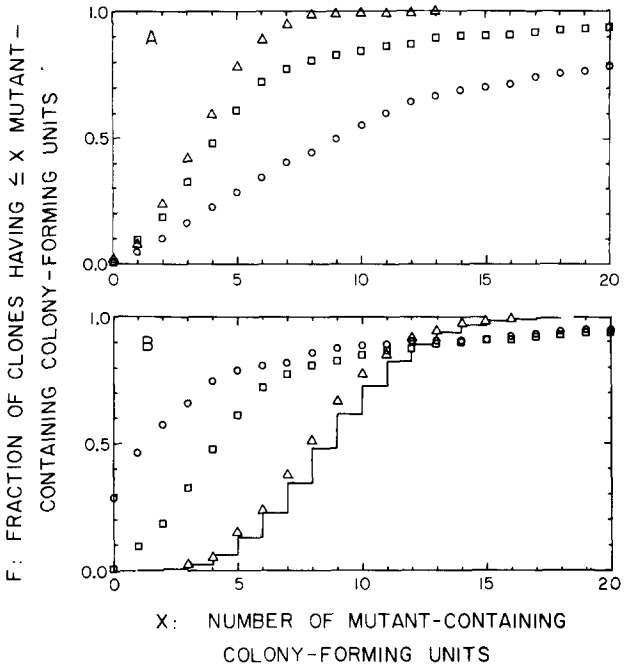

Fig. 2 A and B. Effect of aggregation on the frequency distribution of mutant colony-forming units in clonal growth. The data shown (○, □, △) are simulations performed by computer (Fortran IV program available on request). Each curve gives the fraction of clones that had ≦ X mutant – containing aggregates at the end of the growth period. Each curve was calculated from 500 replications of clonal growth, 25 generations each, in which mutant cells were assumed not to separate until they made up an entire aggregate (curves C and F of Fig. 1). **A** ○: cells/aggregate (C/A) = 1, mutation rate (**A**) = 6×10^8, mean number of mutant-containing aggregates per clone (m) = 22.7, variance/mean (var/m) = 186. □: C/A = 4, a = 6×10^8, m = 8.9, var/m = 40. △: C/A = 128, a = 6×10^8, m = 4.1, var/m = 1.0. **B** ○: C/A = 1, a = 2×10^8, m = 8.8, var/m = 205. □: C/A = 4, a = 6×10^8, m = 8.9, var/m = 40. △: C/A = 128, a = 13×10^8, m = 8.8, var/m = 4.5. ——: cumulative Poisson distribution, mean = 8.8

observations below demonstrate a successful case in which the fluctuation test detected mutational events in cell aggregates.

Distribution of Chlorate Resistance in Rose-Cell Cultures

The first part of the experiment was a control to show the effects of plating and scoring procedures on the distribution of resistant colonies. Thirty-six 1-ml samples of the standard rose culture were plated on medium containing $NaClO_3$. Each sample contained an average 946 aggregates with 113 cells per aggregate. Plating efficiency of the aggregates measured without $NaClO_3$ was 55%. The samples each formed an average of 8.89 colonies that were judged to be resistant to $NaClO_3$. The cumulative distribution of the numbers of chlorate-resistant colonies per sample is shown in Fig. 3a. A cumulative Poisson distribution with the same mean is also shown. The relationship between the observed distribution and the Poisson distribution was tested using the Kolmogorov-Smirnov test for goodness of fit (Massey 1951). The test uses the statistic D = max | F (observed) – F (expected) | and is nonparametric. In this case, the test indicated no significant difference ($P > 0.15$) between the observed distribution and the Poisson distribution. An independent repetition of this part of the experiment gave the same result (data not shown).

The second part of the experiment showed the effect of "clonal" growth, that is, growth from a single aggregate, and mutation on the distribution of variants.

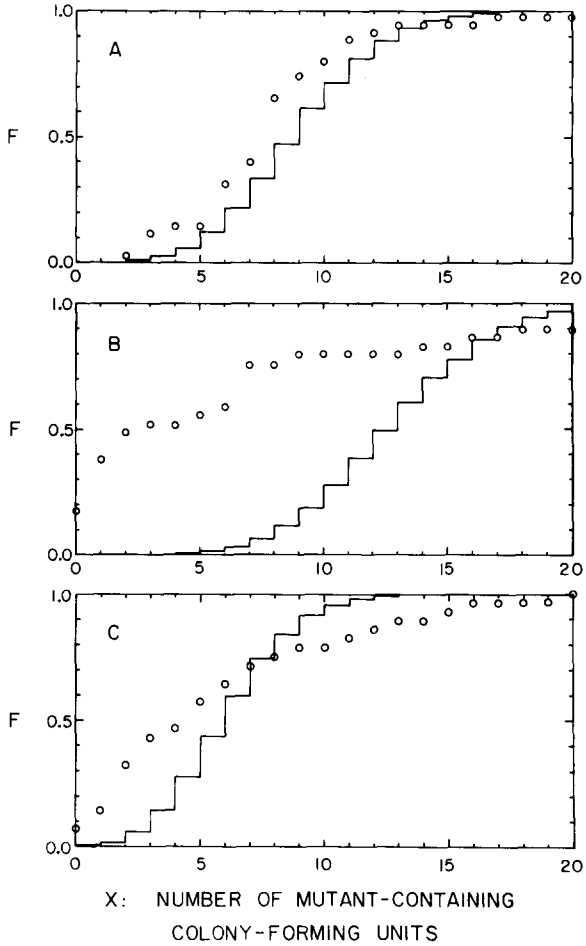

Fig. 3 A–C. Frequency distribution of $NaClO_3$-resistant colonies in rose-cell cultures. **A** Samples from a long-term suspension culture. ○: Cumulative fraction of plates containing ≦ X resistant colonies (mean = 8.89). ——: cumulative Poisson distribution (mean = 8.89). **B, C** Samples from cloned cultures. ○: cumulative fraction of samples (3 plates per clone) containing ≦ X resistant colonies. ——: cumulative Poisson distribution. In **B** mean = 12.7; in **C** mean = 6.07

Twenty-nine isolated colonies were incubated in liquid medium to form 29 clonal suspensions of small aggregates. The suspensions contained, on the average, 4.22×10^4 ($\pm 1.70 \times 10^4$) aggregates per ml with 129 (± 53) cells per aggregate. The average total growth corresponded to 26.7 (± 0.34) generations. From each suspension three 1 ml samples were plated on agar medium containing $NaClO_3$. Plating efficiency in the absence of chlorate averaged 0.35 (± 0.05). For each clone, the numbers of chlorate-resistant colonies in the three 1-ml samples were summed. Figure 3b shows the distribution of these sums for the 29 clones. The Kolmogorov-Smirnov test indicated a highly significant difference ($P < 0.01$) between the cumulative distribution function for the observed number of resistant colonies and a Poisson distribution with the same mean, 12.7. In fact, there was a highly significant difference between the observed distribution and every possible Poisson distribution. Relative to the Poisson distribution, the observed distribution had many more clones with few variants and many clones with many variants, the situation expected when hereditary mutants accumulate randomly. An independent repetition of this experiment using a new set of aggregate clones gave similar but less pronounced results (Fig. 3c): the observed distribution of mutants was significantly different ($P < 0.05$) from a Poisson distribution with the same mean, but not significantly different from every Poisson distribution. This repetition may demonstrate the results of a greater degree of aggregation, since the average number of cells per aggregate was somewhat greater than in the experiment described above (164 ± 76).

The mutation rate can be calculated by applying the formula of Luria and Delbrück (1943) and taking into account the possible effects of aggregation. The mutation rate per cell (or per generation), a, is obtained by a numerical solution of the equation $r = a N_t \ln (C\, a N_t)$, where r = the likely average number of mutant cells per sample in a limited number of samples, C = the number of samples, and N_t = the total number of cells per sample. As discussed above, the number of mutant cells in a culture will fall somewhere between the number of resistant aggregates and n times the number of resistant aggregates (n = cells per aggregate). However, assuming random division of aggregates, curve E in Fig. 1 suggests that even with large aggregates a reasonable estimate of the number of mutant cells would be no more than 2 to 4 times the number of resistant aggregates, since most of the mutants would have appeared late in the growth period, certainly no more than 12 generations before the time the cultures were tested. With this assumption, the data in Fig. 3b obtained from the 29 samples of clonal suspensions described above, corrected for sample size and for plating efficiency, give estimates of r of 278 (r = number of resistant aggregates) to 1,113 (r = 4 times the number of resistant aggregates). These estimates yield values for a of 3.6×10^{-7} to 12×10^{-7}. These values are higher than that calculated by the more familiar formula, $a = -\ln (F_0)/N_t$, where F_0 is the fraction of clone samples that had no mutants. This calculation yields $a = 1.2 \times 10^{-7}$.

Discussion

The aggregation of plant cells changes the distribution of variants expected from random mutations. Either random cell division patterns or cell division in which siblings do not separate immediately will tend to decrease the number of clones that show very high numbers of resistant variants. A major effect will be to lower the variance of the distribution, possibly enough to erase the distinction between this distribution and a Poisson distribution. The effects of aggregation are similar to those of delayed phenotypic expression, as described by Crump and Hoel (1974).

Aggregation effects may explain why workers studying organellar genetics have observed low variance/mean ratios in fluctuation tests (Sager 1962; Gillham and Levine 1962; Dujon et al. 1976). If there are multiple copies of mitochondrial and chloroplast genes within the cells investigated (Gillham 1974), the cells could be considered to be aggregates of organelles, and the arguments above concerning the effect of aggregation on the "tail" of the mutant distribution (Figs. 1, 2) would apply. It would thus be unnecessary to invoke "induction" or "intracellular selection" to explain the low variance/mean ratios.

Fluctuation analysis for $NaClO_3$ resistance in rose cells showed a distribution of variants among clones which differed significantly from that expected assuming non-mutational, non-hereditary resistance. The difference could not be attributed to idiosyncrasies of the plating assay; instead, the observed distribution followed the distribution expected assuming that resistance came from mutations that occurred randomly during "clonal" growth. Previous experiments (Murphy and Imbrie 1981) established that chlorate-resistant rose-cell strains selected on plates containing $NaClO_3$ retained their resistance for many generations in the absence of chlorate. The fluctuation analysis adds the information that resistance can appear in the absence of chlorate.

The advantage of the fluctuation-test protocol includes information regarding mutation rate. This is especially important when one deals with mutants that are not selected against during normal growth, such as the chlorate-resistant rose cells, because with such mu-

tants the fraction of mutant cells in a long term culture does not indicate the forward mutation rate. Two methods for the calculation of mutation rate, one based on the number of mutants per clone and the other on the fraction of clones with no mutants, gave different values. This was apparently not a function of aggregate size. Luria and Delbrück (1943) also observed this effect with bacteria. They pointed out that it reflects a bias in calculations performed by the first method and on an "excess of early mutations" relative to the assumptions involved in the derivation of the equation for r.

The data from a fluctuation test may also help one select mutants in cases where the resistant colonies represent a mixture of mutants and nonmutational variants. Clones that show many resistant colonies are ones that have experienced early, stable mutations; selection of resistant colonies from these clones should yield useful mutants with high probability.

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Appendix

Calculation of the Distribution of Mutant Cells Among Progeny Aggregates

Assume a population of aggregates of uniform size, each aggregate containing "mutant" and "non-mutant" cells. Assume further that once each generation every cell in each aggregate divides, then the aggregate itself splits into two equal pieces randomly such that all combinations of progeny cells in the progeny aggregates are equally likely. Let n = aggregate size, y, t = number of mutant cells in an aggregate at generation t, $Q_{y,t}$ = the fraction of aggregates with y mutant cells at generation t, and EQ = the expected value of that fraction. Given values of $Q_{y,t-1}$ or $EQ_{y,t-1}$, one can calculate values of $EQ_{y,t}$ using the hypergeometric distribution.

$$EQ_{y,t} = \sum_{y,t-1=0}^{n} \left[\frac{\binom{2(y,t-1)}{y,t} \binom{2n-2(y,t-1)}{n-(y,t)}}{\binom{2n}{n}} \right] [Q_{y,t-1}].$$

The curves in Fig. 1 were calculated by assuming that at generation 0, the value of $Q_{1,0} = 1.0$ and the values of $Q_{y,0} = 0.0$ for all values of $y \neq 1$. Table 1 gives $EQ_{y,t}$ for $n = 4$ and shows how, with random division, aggregates tend to become homogeneously mutant and wild-type as the growth of the culture proceeds. The table also show that $EQ_{y,t}$ tends toward 1/n as t approaches infinity.

Table 1. Values of $EQ_{y,t}$ for $n = 4$ and $Q_{y,0} = 1.0$

Generation	y = number of mutant cells				
(t)	0	1	2	3	4
0	0.000	1.000	0.000	0.000	0.000
1	0.214	0.571	0.214	0.000	0.000
2	0.339	0.375	0.232	0.048	0.003
3	0.423	0.267	0.210	0.081	0.016
4	0.483	0.201	0.183	0.094	0.037
5	0.529	0.156	0.157	0.095	0.060
10	0.654	0.060	0.072	0.056	0.156
15	0.706	0.027	0.033	0.026	0.206
20	0.729	0.012	0.015	0.012	0.229
25	0.740	0.005	0.007	0.005	0.240

Dr. Stephen Portnoy, Dept. of Mathematics, University of Illinois at Urbana-Champaign, has derived an equation for the variance and standard deviation of $Q_{y,t}$. His computations for $n = 4$ and $n = 10$ indicate that the standard deviation of $Q_{y,t}$ quickly (in 15 generations or less) becomes less than the expected value of $Q_{y,t}$ for all values of y (personal communication).

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