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A Model of Mutant Selection in Plant Suspension Cultures

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Summary

In plant cell suspension cultures, the fastest growing cell type will eventually dominate the culture. This selection process is often proposed as the mechanism by which cultures become habituated to stressful conditions. Equations which model the selection hypothesis predict a sinusoidal transition from sensitive to tolerant cells. Culture growth during the habituation process as predicted by this model closely matches the habituation of tobacco cells to NaCl as described by Nabors, Daniels, Nadolny, and Brown (1975). The equations can also be used to estimate a theoretical mutation rate from experimental results.

Key words: Model, mutant, selection, tissue culture, tolerance.

Introduction

Numerous studies have shown that cultured plant cells, placed in a stressful environment, gradually become habituated to the stress (see review by Maliga, 1978). It is difficult to determine whether the habituated cells are normal cells which have adapted to the stress or whether there has been selection for mutant cells. In some cases, genetic studies of plants regenerated from habituated cells have confirmed the latter hypothesis (see review by Maliga, 1980). We present here a model that was developed to predict the growth characteristics of suspension cultures during the habituation process. This model is based on the hypothesis that the mutant is selected. All wild type cells survive in the stressful media, but grow slowly. This is representative of cases where tolerance is a quantitative rather than qualitative trait and is likely controlled by more than one gene. Mutant cells are very rare initially but they grow and divide more rapidly in the stressful media, and eventually dominate the culture (Nabors 1976, Street 1976, Bressan, Hasegawa, and Handa 1981, Chaleff 1983, Meins 1983).

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Abbreviation: EMS-ethyl methane sulfonate.

Construction of the model

The model assumes that normal culture growth occurs at a constant, exponential rate defined (Radford, 1967) by

(1) $$W = W_0 e^{rt}$$

where W is indication of culture size, i.e. fresh weight, dry weight, packed cell volume, or cell number,
W_0 is the initial value of W,
e is the base of the natural logarithm, approximately 2.71828,
r is the relative growth rate,
t is elapsed time.

The relative growth rate of a culture can be determined by

(2) $$r = \frac{\ln W - \ln W_0}{t}.$$

where ln is the natural logarithm.

The assumption of exponential growth is reasonable if the inoculum density is sufficient to avoid inducing a lag phase and if the culture is subcultured frequently to ensure that culture size is kept well below the density at stationary phase (Wilson, King and Street, 1971).

The growth of serial subcultures during the habituation process can be modeled by assuming that a culture of sensitive, wild type cells and a culture of tolerant, mutant cells, with different values for r and W_0, are growing concurrently in the culture flask. The number of cells in the culture at any given time is

(3) $$W = S + T = e^{At} + ne^{Bt}$$

where S is the number of sensitive, wild type cells,
T is the number of tolerant, mutant cells,
A is the relative growth rate of the sensitive cells in the selection media,
B is the relative growth rate of the tolerant cells in the selection media,
n is the number of tolerant cells per sensitive cell in the culture at the start of selection (i.e. the mutant frequency).

The proportion of tolerant cells in the culture (P) is

(4) $$P = \frac{T}{S+T} = \frac{ne^{Bt}}{e^{At} + ne^{Bt}}$$

This equation is the mathematical description of the model. It defines most of the components of the system and the way that they change in time.

Equations derived from the model

A set of parametric equations can be derived from (4) which describe the mutant selection process. The mutant frequency in the culture prior to selection is

(5) $$n = \frac{e^{(A-B)t}}{\frac{1}{P} - 1}$$

The proportion of cells (P) which are tolerant at any given time is

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(6) $$P = \left[\frac{e^{(A-B)t}}{n} + 1\right]^{-1}$$

The time (t) required to obtain a culture with a given fraction of tolerant cells (P) is

(7) $$t = \frac{\ln(n(\frac{1}{P} - 1))}{A - B}$$

The current culture growth rate (CCGR) at any time during the habituation process is determined by

(8) $$CCGR = A + \frac{B - A}{\frac{e^{(A-B)t}}{n} + 1}$$

Several other assumptions of the model may affect the conclusions drawn from it:

A) Tolerant cells do not affect the growth rate of sensitive cells and vice versa. Suspension cultures are composed of clusters of cells. Cross-feeding is common and may influence the growth rates of the different cell types. This effect may change the rate of the selection process or lead to stable or cyclical mixtures of cells (Meins, 1983).

B) Phenotypic expression is rapid. In cells that result from new mutations, the expression of some types of mutations (such as those related to membrane bound enzymes) may be delayed for several generations (Hayes, 1968). The assumption is realistic because the model is based on multiplication of existing mutant cells, not new mutations.

C) The mutation for tolerance is a single change in the cell's genotype. Alternatively, a second mutation may further increase the tolerance of a cell which has the initial mutation. Such a change would be more likely near the end of the selection process, when the initial mutant is more frequent. Or, there may be several mutations which have similar phenotypes (Maliga, 1980). Regardless of the nature of the mutation(s), the proportion of the cell type with the fastest growth rate will increase until that cell type is dominant or until another cell type arises with a faster growth rate.

D) The forward mutation rate is significant only when determining the equilibrium mutant frequency before selection (see discussion of mutant frequency versus mutation rate below). The importance of each new mutation rapidly decreases as the proportion of mutant cells increases. Likewise the backward mutation rate is significant only when determining the equilibrium frequency of wild type cells at the end of the selection process. If these mutation rates are not substantially less than one, then the assumption is not justified.

Use of the model and comparison with experimental results

The culture growth characteristics predicted by the model can be compared to experimental results by extracting estimates of three parameters (the duration of the selection process, and the growth rates of tolerant and sensitive cells in stressful media) and using those values in the equations presented above to predict culture growth. The experimental data is from a study by Nabors et al. (1975), in which Samsun tobacco (*Nicotiana tabacum* L.) cells were serially subcultured in 27 mM NaCl after treatment with a mutagen, 0.15 % EMS for 60 minutes (Figure 1). Culture

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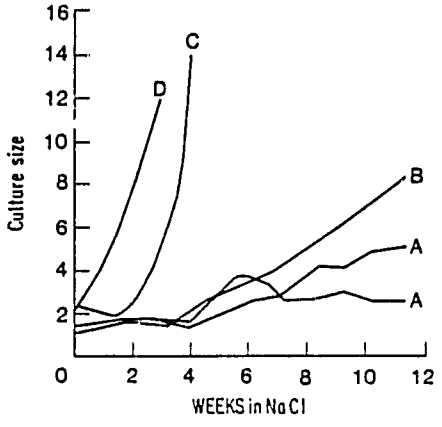

Fig. 1: Progressive habituation of mutagen treated tobacco suspension cultures grown in 27 mM NaCl (from Nabors, Daniels, Nadolny, and Brown, 1975).
A: non-mutated culture. – B: culture treated with 0.15% EMS for 60 min. – C: subculture of B culture (5 way division). – D: subculture of C culture (5 way division).

size was measured as settled height in a side arm flask and has been shown to be proportional to cell number. The cells were then serially transferred through a series of media with progressively higher NaCl concentrations. It is hypothesized that each step is associated with mutation(s) that make habituation possible and that the mutagen only enhanced the natural mutation rate. Plants regenerated from these cells were selfed for two generations and the progeny were shown to have increased salt tolerance (Nabors et al., 1980). Thus, this selection experiment, of which Nabors et al. (1975) was the first step, produced tolerant plants which were genetically different. In this paper only the first step (to 27 mM NaCl) will be discussed.

Using data estimated from Figure 1, growth rates of the salt tolerant cells (represented by the average of the non-mutated A cultures) and salt tolerant cells (represented by the product of the habituation process, culture D) were calculated using (2):

$$A = (\ln 3.75 - \ln 1.5)/77 \text{ days} = 11.9 \times 10^{-3}/\text{day}$$

$$B = (\ln 12 - \ln 2.3)/18 \text{ days} = 91.8 \times 10^{-3}/\text{day}$$

The duration of the experiment (t) was 18 weeks. The proportion of salt tolerant cells in the culture (P) at the end of the process is assumed to be 0.99. With these values, the theoretical initial mutant frequency necessary to explain these results can be calculated with (5): $n = 4.2 \times 10^{-3}$/day.

The proportion of tolerant cells in the culture at various times during the selection process was determined with (6) (Figure 2). The transition of cell types in the culture, from predominantly sensitive to predominantly tolerant, is clearly sinusoidal. The current culture growth rate at various times during the selection process (Figure 3), calculated with (8), reflects this transition.

A computer program was written to simulate the actual conditions used in Nabors et al. (1975) experiment. Culture size was determined at weekly intervals using (3). Culture size was set to a relative value of 1 at the start of the experiment and at inoculation of each subculture. Each culture was subcultured when the relative size of the culture rose above nine except for a premature subculturing after 11 weeks. The

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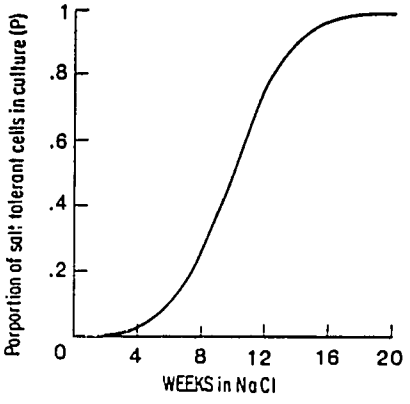

Fig. 2

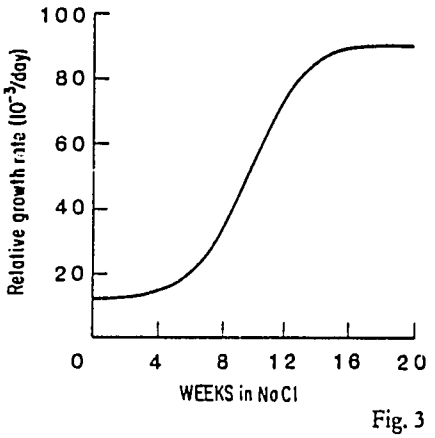

Fig. 3

Fig. 2: Fraction of cells which are salt tolerant in a culture as predicted by a mutant selection model of the habituation process (see text for parameters). Because the salt tolerant cells divide more rapidly, they eventually become more prevalent than the salt sensitive cells.

Fig. 3: Current culture growth rate during habituation process as predicted by a mutant selection model. Culture growth rate reflects the transition from salt sensitive to salt tolerant cells.

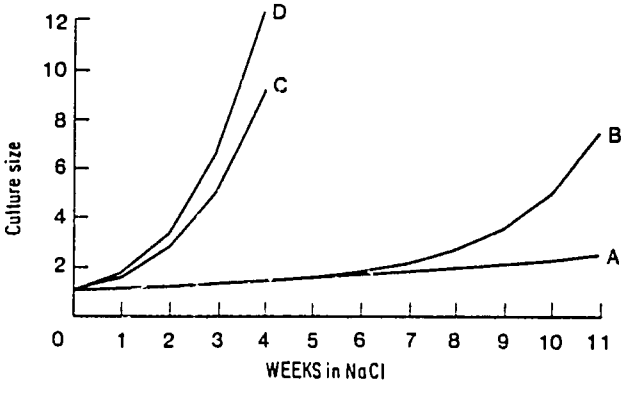

Fig. 4: Growth of serial subcultures in selection media as predicted by a mutant selection model of the habituation process.
Cultures: A: Culture with mutant frequency of 10^{-6} or less. – B: First passage of mutagen treated culture. – C: Subculture of B culture. – D: Subculture of C culture.
Parameters (see text): r of sensitive cells (A) = 11.9×10^{-3}/day; r of tolerant cells (B) = 91.8×10^{-3}/day; mutant frequency (n) = 4.2×10^{-3}.

predicted results (Figure 4) closely match the actual results of the experiment (Figure 1).

The period in which the current culture growth rate is rapidly increasing (weeks 5 through 15) is preceeded by a period of slow growth that might erroneously be

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termed a «lag phase». However, even in this phase, growth of both the sensitive and tolerant portions of the culture is exponential.

The model explains the growth curves which are characteristic of the habituation process: 1) the initial phase of slow growth, 2) the progressively faster growth of the serial subcultures, and 3) the eventual limit to the increasing culture growth rate.

Nabors et al. (1975) experimental results provide further evidence for the selection model. The growth of the mutagen treated culture (B in Figure 1) was clearly more rapid than the non-mutagen treated culture (A). This indicates that a higher mutation rate increased the rate of habituation. Thus, mutations were an integral part of the process. If the cells were merely adapting, the mutagen treatment would have had no effect. EMS may have had other, more direct, effects on the cells, but it seems unlikely that such effects would persist long after exposure to EMS.

The relationship of mutant frequency and mutation rate

If the growth rate of the mutant cells in non-stress media is greater than the growth rate of the wild type cells, the mutants will have become the dominant cell type even before transfer to the stressful media. If their growth rate is less (specifically, if $B'-(1-2m)A'<0$, where m is the mutation rate, and A' and B' are the growth rates of the wild type and the mutant cells, respectively, in non-stress media) then according to Northrup and Kunitz (1957) the frequency of mutants will approach an equilibrium of

(9) $$n = \frac{2mA'}{A'-B'}$$

This equilibrium will be approached with a half time of $t_{0.5} = \ln 2/(A'-B')$. Under these conditions the frequency of mutants present in a culture prior to selection may be substantially higher than the mutation rate, depending on the growth rates of the two cell types in the control media. Assuming that equilibrium has been reached (this assumption is more appropriate if the cell line has not been cloned recently), the mutation rate is

(10) $$m = \frac{(A'-B')n}{2A'}$$

Direct estimation of m in the tobacco example is not possible because the growth rate of the tolerant cells in the absence of salt (B') was not determined. However, the reciprocal relationship of m and B' in (10) can be demonstrated by considering the range of possible values of B. For example, if the tolerant cells do not grow at all in the absence of salt ($B'=0$) then $m = n/2 = 2.1\times10^{-3}$. This is the extreme case in which an extremely high mutation rate is necessary to explain the mutant frequency. Alternatively, if B' is larger, m will be smaller: if $B'=.9A'$ then $m = 2.1\times10^{-4}$; if $B'=.99A'$ then $m = 2.1\times10^{-5}$; etc. As mentioned above, if B' is greater than A', the salt tolerant cells will become the predominant cell type even without transfer to a saline medium.

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Typical mutation rates in plant cell cultures range from 10^{-5} to 10^{-8} (Maliga, 1978), which may be enhanced 1 to 140 fold by mutagens (Sung, 1976). These values are compatable with most of the range of m calculated above. Calculation of m with (10) is clearly imprecise if B′ is approximately equal to A′. More direct methods of determining mutation rates in cultured plant cells are discussed by Meins (1983).

Discussion

Other situations where the model may apply

Selection with calli should be quite similar. Tolerant cells may appear as actively growing regions on an otherwise slow growing callus (Maliga, Marton, Sz. Breznovits, 1973). This allows segregation of the cell types when subculturing. Alternatively, the tolerant cells may act as feeder cells to the sensitive cells, leading to the formation of chimeric cultures.

The model may also apply to culture initiation. Cultures frequently display a prolonged «adaptation» period after initiation during which successive subcultures grow progressively faster. After a long period, the culture's growth rate stabilizes (Gamborg and Shyluk, 1981). The resulting cells, unavoidably selected for rapid growth in culture, may have accumulated mutations (most notably, changes in chromosome number) which will affect plants regenerated from them (Street, 1976).

Another application of the model is with tests of the stability of tolerance. Cells selected for salt tolerance are often grown in the absence of salt for a few passages and then returned to saline media to test the stability of the salt tolerant phenotype. Such experiments are the reverse of selection for salt tolerance (Bressan et al., 1981). If, as was assumed, the growth rate of a few salt sensitive cells (which are maintained at a low equilibrium frequency by back mutation in the selected culture in stressful media) is substantially higher than the growth rate of the salt tolerant cells when grown in the absence of salt, there will be selection for salt sensitive cells. In this case, the result will be a culture very similar to the one before selection: predominantly wild type cells with mutants maintained at a low frequency by mutation. Thus, phenotypic instability after long periods in non-selective media should not be construed as proof that the tolerant cells are not true mutants.

Validation

The fact the model closely matches the experimental results of Nabors et al. (1975) is evidence in support of, but not proof of, the validity of the selection model. Further support could come from: 1) comparison of the model with results from similar experiments, 2) genetic analysis of plants regenerated from such studies, 3) cytogenetic studies of cells before and after habituation, or 4) changes in protein banding patterns. It should also be possible to use suspension cultures to select for resistant lines in cases where the mutation rate has already been determined by other means, and compare actual culture growth with culture growth as predicted by the model.

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