Carbohydrate-limited Growth Kinetics of Tobacco (*Nicotiana rustica* L.) Callus[1]

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ABSTRACT

Logistic curves were fitted to sigmoidal growth data obtained from tobacco (*Nicotiana rustica* L.) callus grown on media prepared with 0.1, 0.03, 0.01, and 0.003 M sucrose. Analysis of the growth curves indicated that final yields and specific growth rates were influenced by the initial sucrose concentration. Growth yields from the four treatments were similar (0.61 ± 0.04 gram dry tissue per gram sucrose supplied). Initial specific growth rates exhibited a Michaelis-Menten dependency on initial sucrose concentration such that the $V_{max} = 0.18$ g g^{-1} day^{-1} and $Km = 0.0037$ M sucrose.

Plant tissues cultured *in vitro* commonly derive carbon and energy from glucose or sucrose supplied in the medium (17). Upper *et al.* (16) established that the quantity of sucrose supplied in Linsmaier and Skoog's (10) medium determined the maximum obtainable yield of tobacco callus tissue, but they and others have been unable to detect any clear quantitative dependency of growth rate on sucrose concentration. The nature of this response function is relevant to distinguishing the nature and proportion of the control that substrate level exerts over growth and morphogenesis.

Reports of the activities of sucrose-degrading enzymes in plant tissue cultures (3, 14, 15), for example, vary in the degree of correspondence observed between enzyme activity and growth rate. Growth rate may be limited by substrate concentration as well as by enzyme concentration, and a simple correspondence between enzyme activity and growth is unlikely to exist throughout a culture period during which the carbohydrate supply in the medium may become completely exhausted (16).

The kinetics of growth in relation to carbohydrate concentration are also pertinent to the construction of explanatory growth models designed to predict whole plant and crop growth on the basis of hypotheses and data derived from studies at lower levels of plant organization. Integrative models such as ELCROS (1, 4), SUBGRO II (5, 6), and SUBGOL I (9) contain functions that regulate growth according to the concentration of "reserves" (carbohydrate) as one means of simulating metabolic control.

This paper reports an attempt to evaluate the dependency of growth rate at the tissue (callus) level on carbohydrate supply with a curve fitting and analysis approach.

MATERIALS AND METHODS

Tobacco callus cultures grown on Linsmaier and Skoog's medium (10) were used following preliminary tests with a large number of cultivars of four species on four basic media with a range of hormonal concentrations. The Linsmaier and Skoog medium was developed with a view towards optimizing medium requirements for growth (10, 11) and, with tobacco, gave us the highest growth rates of relatively undifferentiated callus.

The callus was generated from the culture of internodal pith explants from greenhouse-grown tobacco plants (*Nicotiana rustica* L.). After several subcultures on Linsmaier and Skoog's medium (10) in darkness at 27 C, three vigorously growing pieces were dissected for inoculum.

Ninety-six, 125-ml Erlenmeyer flasks containing 40 ml of Linsmaier and Skoog's medium were prepared for inoculation with four treatment sucrose concentrations (0.1, 0.03, 0.01, and 0.003 M). Cytokinin was added at a nonlimiting concentration (0.93 μM kinetin). All flasks were inoculated with four evenly spaced pieces of tobacco callus (3 mm^3 per piece). The callus was firm to cut but disintegrated easily. Loose tissue was not used as inoculum as the size could not be estimated. The flasks were incubated in darkness at 27 C.

Fresh and dry weight measurements were made of 20 pieces of inoculum (collectively) and of the callus from six randomly selected flasks for each treatment on four sampling dates: 7, 28, 43, and 55 days after inoculation. Dry weights were determined after drying for 24 hr in a Thermo-Vac RD-6 freeze-dryer.

RESULTS

The growth of tobacco callus for the four sucrose supply treatments is illustrated in Figure 1. Yields followed a sigmoidal pattern with time and weighted logistic growth curves were fitted to the data by an iterative program that minimized the residual variance of the data points from the curve. The form of the logistic was:

$$W = \frac{A}{1 + B \cdot e^{-C \cdot t}} + d$$

where W = callus yield, t = days of growth, A, B, and C are constants, and d = unaccounted variation. The maximum rate of growth, and the time (t) when this occurred, were given by (ln B)/C and $AC/4$, respectively. A is the asymptotic estimate of maximum yield.

The relationships between the maximum and specific growth rates (as estimated from the fitted logistics) and initial sucrose concentrations are illustrated in Figures 2 and 3. The growth yields (Y), calculated from A, and the initial sucrose content of the media are presented in Table I.

The effect of sucrose concentration on specific growth rate was assessed from initial growth data as sucrose depletion and evapo-
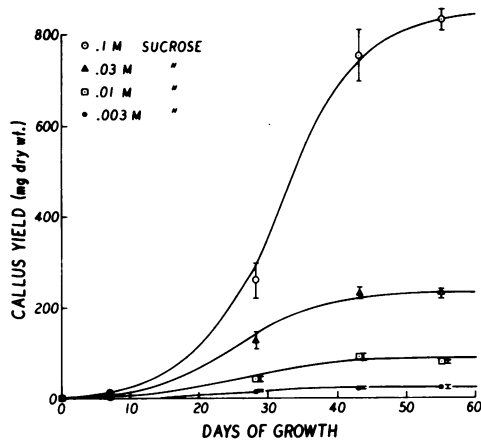
FIG. 1. Growth of tobacco callus on four media varying in initial sucrose concentration. Logistic curves have been fitted to the data. Vertical bars indicate standard deviations from sample means.
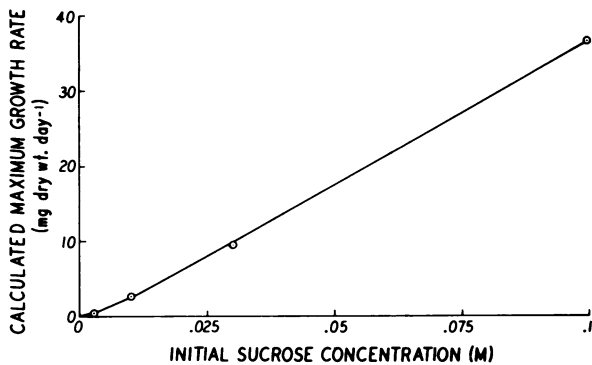

FIG. 2. Maximum growth rate estimates (calculated as the maximum slope of the logistics) of tobacco callus for four initial media sucrose concentrations.
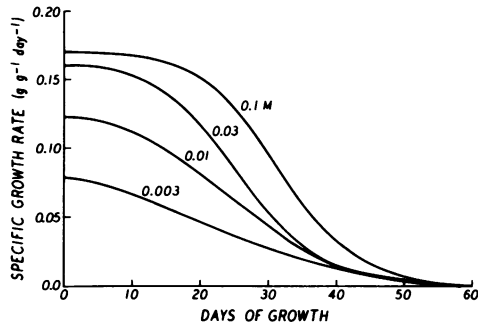

FIG. 3. Specific growth rates (calculated from fitted logistics) of tobacco callus with four initial media sucrose concentrations.

ration were minimal during the 1st week of growth and good estimates of initial specific growth rates could be obtained from Figure 3. These data (Fig. 4) indicate that the kinetics of tobacco callus specific growth rate followed a Michaelis-Menten dependency on sucrose concentration. The curve in Figure 4 was generated from a linear regression, least squares fit to a Lineweaver-Burk plot of the data (Fig. 5). The V_{max} estimate for specific growth rate (0.18 g g^{-1} day^{-1} or 0.26 doublings day^{-1}) agrees well with previous estimates of maximum tobacco callus growth rates on Linsmaier and Skoog's medium (7, 8, 16).

DISCUSSION

Fitting a logistic. The logistic function appears to be appropriate on physiological grounds to describe our data on the time course of tobacco callus yield. The derivation of the logistic is based on the dual assumptions that growth is inherently exponential and that the maximum growth obtainable is eventually limited by a shortage of environmental resources (13). Evidence supporting the validity of both assumptions for tobacco callus growth can be found in the literature.

Exponential tobacco callus growth was observed by Caplin (2) even at a relatively low specific growth rate (0.05 − 0.06) g g^{-1} day^{-1}). Exponential callus growth may be observed in terms of fresh weight, dry weight, and cell number, suggesting that individual cellular synthetic activity is constant: the exponential character being maintained by the increasing number of cells (18). The specific growth rate of exponentially growing tobacco callus can be controlled (on Linsmaier and Skoog's medium) by the cytokinin concentration (7) and exhibits some sensitivity to gibberellic acid (8).

The exponential growth phase is sometimes preceded by a short lag phase of 1 to 3 days following transfer (7). The occurrence of a lag would affect fitting of the logistic function and our sampling dates are spaced too widely to detect such a lag visually from Figure 1. However, the introduction of lags of 1, 2, or 3 days in the fitting program increased the residual variance term giving a poorer fit to the data. The discrepancy with 1 day

Table I. *Growth Yields (Y) for Tobacco Callus Growth with Different Initial Sucrose Concentrations in Medium*

Y = maximum estimated dry weight callus/weight of sucrose supplied.

	Initial Sucrose Concentration (M)				Mean
	0.1	0.03	0.01	0.003	
Y	0.62	0.56	0.65	0.62	0.61 [1]

[1] ± 0.04 as standard deviation for combined treatments.
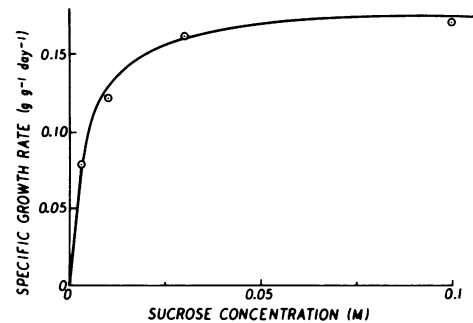

FIG. 4. Kinetics of tobacco callus specific growth rate as influenced by medium sucrose concentration. Data points represent estimates of initial specific growth rates obtained from Fig. 3.
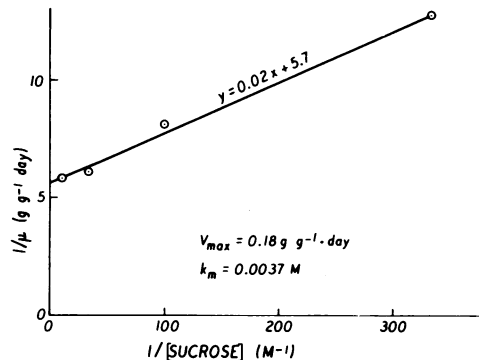

FIG. 5. Lineweaver-Burk plot of the data presented in Fig. 4. The straight line was fitted by regression analysis. μ = specific growth rate, [sucrose] = sucrose concentration.

was small and with 2 or 3 days, quite large. A 1-day lag changed our estimates of initial specific growth rates (Figs. 3 and 4) by 2 to 3% without changing the interpretation. Because of the poorer fit, the differences in estimates of later specific growth rates were larger. We conclude that a lag time, if it occurred, was smaller than 1 day, or, was compensated before the observation on day 7.

The constant value of the growth yield estimate (Table I) indicates that in each treatment the maximum callus yield was determined by the quantity of sucrose supplied. A similar result was obtained by Upper *et al.* (16) who also demonstrated that the time at which the maximum obtainable dry weight was reached coincided with the time at which the supply of carbohydrate in the medium was exhausted. Growth yield estimates (0.60–0.65) for tobacco callus grown on Linsmaier and Skoog's (10) medium (0.088 M sucrose) published by Upper *et al.* (16) agree closely with those in Table I (0.61 ± 0.04). These data support the relevance of the logistic as an appropriate model of tobacco callus growth, during which inherently exponential growth is limited by the supply of carbohydrate from the environment.

Growth Kinetics. The maximum absolute tobacco callus growth rate increased with sucrose supply (Fig. 2) owing to the greater amount of growing tissue generated and higher specific growth rates (Fig. 3). The dependency of specific growth rate on sucrose concentration indicated in Figure 3 is at variance with the conclusions of Upper *et al.* (16) who were unable to detect a similar response from log plots of short term tobacco callus growth data. Inspection of Figure 4 indicates that the sucrose concentration recommended by Murashige and Skoog (11) and retained by Linsmaier and Skoog (10) (0.088 M) should result initially in near maximum growth rates (0.17 g g^{-1} day^{-1} or 0.25 doublings day^{-1}). Where medium sucrose concentration is limiting, the estimated Michaelis constant is sufficiently low (0.0037 M) that substantial reductions in the medium sucrose concentration below 0.088 M would be predicted to result in relatively small reductions in growth rate.

Upper *et al.* (16) examined tobacco callus growth responses to Linsmaier and Skoog's medium containing 0.029, 0.0088, and 0.0029 M sucrose. While the lowest concentration (close to the above estimated Km) did result in slower tobacco callus growth, the remaining two treatments resulted in growth rates similar to those obtained with 0.088 M sucrose (0.24 doublings day^{-1}). Growth rates predicted by the relationship in Figure 4 were 0.17, 0.16, 0.13, and 0.08 g g^{-1} day^{-1} (0.25, 0.23, 0.18, and 0.11 doublings day^{-1}) at sucrose concentrations of 0.088, 0.029, 0.0088 and 0.0029 M, respectively, *i.e.* the kinetics of the system are such that relatively minor differences in specific growth rate may be expected over much of the range considered by Upper *et al.* (16) and their data are not entirely incompatible with the relationship expressed in Figure 4.

The maximum tobacco callus growth rates (0.26 g g^{-1} day^{-1}) observed after one weight doubling in response to the addition of 2 μM gibberellic acid to Linsmaier and Skoog's medium (8) are well in excess of the V_{max} (0.18 g g^{-1} day^{-1}) estimated above for the gibberellic acid-free system. This suggests that the gibberellic acid influence may be exerted at least partially by an increase in the size of the enzymic component associated with carbohydrate metabolism in newly formed tissues.

Application of Growth Kinetics to Logistic. The logistic equation may be modified to test the extent to which observed tobacco callus growth patterns may be simply related to carbohydrate growth kinetics. The differential form of the Verhulst-Pearl logistic equation

$$\frac{dW}{dt} = W \cdot f(W)$$

assumes that $f(W)$ is linear

$$f(W) = a - b \cdot W \qquad (a, b > 0)$$

giving

$$\frac{dW}{dt} = W(a - b \cdot W) \quad (13)$$

where, for this discussion, W is callus dry weight and dW/dt is callus growth rate. The above data suggest, however, that simply in terms of carbohydrate kinetics, the specific growth rate for tobacco callus may be expressed as

$$\frac{1}{W} \cdot \frac{dw}{dt} = \frac{V_{max} \cdot [S]}{K m + [S]}$$

so that

$$\frac{dW}{dt} = W \cdot \frac{V_{max} \cdot [S]}{K m + [S]} \quad (1)$$

where $[S]$ is the sucrose concentration in the medium.

The sucrose concentration changes during growth as a result of depletion by the growing tissue and evaporation of H_2O from the medium. As diffusive resistances may also establish gradients in $[S]$ into the medium from the site of absorption by the tissue, calculation of the effective $[S]$ is beyond us. As an approximation, $[S]$ may be substituted for in terms of W. Assuming that changes in medium volume are small relative to sucrose depletion, equation 1 becomes

$$\frac{dW}{dt} = W \cdot \frac{V_{max} \cdot S}{K m \cdot v + S}$$

where v is the volume of the medium and S is the amount of sucrose it contains. As the initial amount of sucrose and the growth yield (Y) are known

$$\frac{dW}{dt} = \frac{W \cdot V_{max} \cdot (W_{max} - W)}{K m \cdot v \cdot Y + (W_{max} - W)} \quad (2)$$

and $f(W)$ takes the form of a rectangular hyperbola. W_{max} is the maximum callus yield calculated from the initial amount of sucrose and Y. The validity of equation 2 as a model of tobacco callus growth depends primarily on the accuracy with which $W_{max} - W$ approximates the effective sucrose availability for growth.

Equation 2 was solved iteratively for the four treatments in Figure 1 using a 5-min integration interval on a PDP 11/45 computer. A comparison between the specific growth rates from the logistic fitted to the tobacco callus data and those calculated using the modified logistic is made in Figure 6. The modified logistic predicted initial specific growth rates close to those observed and growth rates fell to zero when the medium sucrose was exhausted (or $W = W_{max}$). Good agreement between the
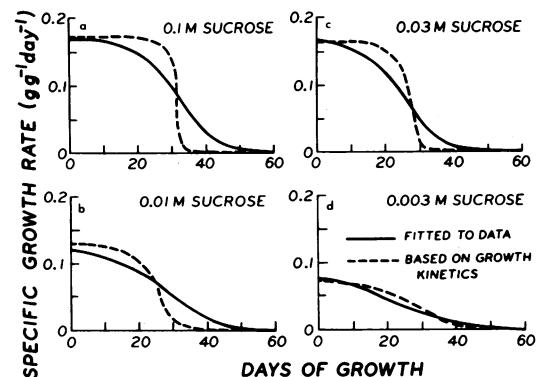

FIG. 6. Comparison between specific growth rates fitted to growth data and specific growth rates calculated from a model based on carbohydrate growth kinetics for tobacco callus.

fitted curve and the modified curve throughout the growth period was obtained only at the lowest sucrose concentration. The modified logistic tended to overestimate the period for which the specific growth rate determined by the initial sucrose concentration could be maintained, the discrepancy increasing with initial sucrose concentration.

The degree of agreement between the curves compared in Figure 6 tests the hypothesis that tobacco callus growth rates observed on Linsmaier and Skoog's medium can be explained simply in terms of the carbohydrate growth kinetics, assuming that all carbohydrate in the medium is freely available for growth. It appears likely that the modified logistic overestimates the sucrose availability during the most rapid growth phase and that significant sucrose gradients exist from the tissue into the medium. If so, the magnitude of such gradients would increase with absolute callus growth rates and therefore initial sucrose concentration (Fig. 2) explaining the increasing divergence with initial sucrose concentration between the curves compared in Figure 6. Apparently, with 0.003 M sucrose as the initial concentration, absolute growth rates were never high enough to maintain a significant sucrose gradient in the medium, with the result that observed changes in specific growth rate with time could be predicted from carbohydrate growth kinetics and the growth yield.

If the existence of sucrose gradients in the medium of rapidly growing tobacco callus cultures implied by our results and calculations is real, observed growth patterns may vary with geometrical relationships between growing tissue and medium (e.g. shape of culture vessel and the spacing of inoculum) as well as with sucrose concentration. A culture system that minimizes gradients (e.g. suspension culture) would be predicted by the modified logistic to produce "J-shaped" (12) growth curves with 0.1 M sucrose since a high specific growth rate would be maintained for almost the entire growth period (broken line, Fig. 6a). At lower initial sucrose concentrations (0.01, 0.0037 M) the smaller mass of tissue accumulated before sucrose concentration becomes limiting results in a slower rate of sucrose exhaustion (growth rate decline, Fig. 6, b and d) and a sigmoid pattern of growth.

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