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Application of Image Analysis for Precise, Noninvasive Measurement of Plant Culture Growth

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Abstract–Accurately measuring the growth of solid cultures is important for evaluating regrowth following low temperature storage, cell screening on selective media, or the suitability of different media formulations. However, current procedures are inadequate to provide precise, quantitative data non-invasively. This paper describes the development of an image analysis technique to provide quantitative growth data for callus cultures. Various properties obtained from image analysis, such as area, perimeter, diameter, and integrated density value, were correlated with the observed mass of plant callus. Based on this data, a predictive linear model was developed. The image analysis method was used to estimate cell mass as a function of various media components and growth conditions. This work demonstrates that image analysis is a flexible and useful tool for monitoring cultures.

Key words: Image Analysis, Plant Cells, Callus Culture

INTRODUCTION

Accurately measuring cell growth is an important aspect of most plant cell culture experiments. For liquid cultures, growth can be monitored directly and quantitatively by taking a representative sample from the homogeneous mixture and measuring the dry weight, packed cell volume, or similar quantities. For solid cultures, directly monitoring growth is more problematic. Since solid cultures are not homogeneous, each sample must consist of an entire callus colony. This sampling method rapidly depletes the culture, necessitating a large number of colonies for each experimental condition to obtain a single replicate experiment. Furthermore, the results may not be accurate since the behavior and growth of individual colonies may vary substantially. Callus growth may also be monitored qualitatively (by visually rating increases in colony size, for example) or indirectly (by measuring changes in the total weight of the culture dish), but these methods can be subjective, vulnerable to bias, and incapable of detecting small changes.

The problem of monitoring solid cultures can be solved with the application of image analysis techniques. Image analysis techniques are suitable for non-invasive, real-time monitoring of cultures [Kinooka and Prenosil, 2000] and can rapidly and objectively monitor cell morphology [Chi et al., 1996] and behavior [Miyanaga et al., 2000]. This paper reports the application of an image analysis technique to the monitoring of solid plant cell cultures. Using this method, only a single culture plate is needed to obtain several repeated measurements for each experimental condition because the samples are not consumed over the course of the experiment. In this way, cultures can be monitored non-invasively and with a high degree of statistical confidence since the behavior of each colony can be observed over the course of the entire experiment.

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‡This paper is dedicated to Professor Hyun-Ku Rhee on the occasion of his retirement from Seoul National University.

MATERIALS AND METHODS

1. Culture Conditions

Callus cultures of *Nicotiana tabacum* cells were maintained on solid MS [Murishige and Skoog, 1962] media consisting of 4.3 g/L MS salt mixture, 30 g/L sucrose, 0.18 mg/mL KH_2PO_4, 0.1 mg/mL inositol, 1.0 μg/mL thiamine hydrochloride, and 0.2 μg/mL 2,4-dichlorophenoxyacetic acid (as a growth hormone), and 6 g/L agar. The pH was adjusted to 5.8 with 1 N KOH prior to autoclaving. Cultures were initiated by placing 9 colonies (approximately 50 mg each) on solidified media and maintained in sealed petri dishes at 30 °C.

Suspension cultures (for the comparison studies) were cultivated and in liquid MS media and sampled as previously described [Magnuson et al., 1996]. Cultures were initiated every 8 days by using an 8 percent inoculum and maintained on a gyratory shaker at 125 rpm and 30 °C.

2. Image Acquisition

Images for each experiment were obtained every two days with a Chemi Imager 440 low light imaging system (Alpha Innotech, San Leando, CA). Each plate was placed in the center of the trans-illuminating stage under the digital camera, which was mounted at a fixed height. After manually adjusting the contrast and brightness a singe grayscale image of the entire plate area was captured and saved to disk in a Tagged Image File Format (TIFF). The consistency of image size and quality was confirmed by recording the total plate area and the average grayscale intensity for each image.

3. Image Analysis Procedure

The resulting image files were analyzed with UTHSCSA Image Tool for Windows developed by Don Wilcox at the University of Texas Health Science Center at San Antonio. This program can be downloaded from http://ddsdx.uthscsa.edu/dig. Other software packages (such as LABVIEW with the IMAQ Vision image processing add-on, marketed by National Instruments Corp) may also work well. For analysis, each sample image was imported into Image Tool 2.0 (Fig. 1A). To make a clearer image, all background was removed

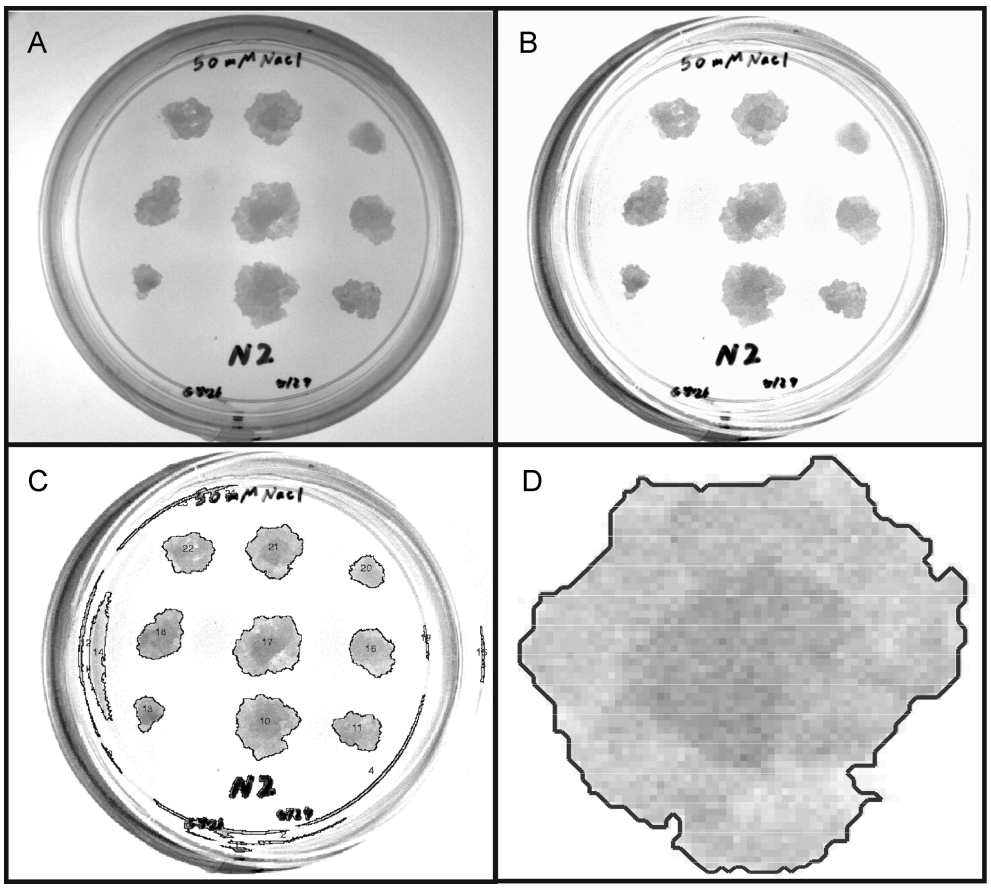

Fig. 1. Steps in the image analysis procedure: (A) original image, (B) processed image, (C) objects for analysis, and (D) an individual colony.

from the sample image with the Background Subtraction function (Fig. 1B) by using an empty plate of media as the reference image. Colonies of callus were identified as "objects" for analysis by using the Find Objects function (Fig. 1C). This function establishes a threshold intensity value to define a boundary around each colony or "object". The attributes of each colony, such as area (number of pixels in the object), perimeter (number of pixels on the boundary), feret diameter (an averaged diameter), and integrated density (number of pixels in the object multiplied by the mean intensity) (Fig. 1D) were measured by using the object analysis feature. This information was exported as a data file and organized for further analysis.

4. Calibration Experiment

To define a model for the relationship between image analysis data and the actual weight of callus, seven independent 10-inch plates were each inoculated with nine colonies of plant callus (for a total of 63 colonies). Every two days, images of these plates were taken and one colony from each plate was harvested with a sterile spatula. The harvested colony was weighed (to four decimal places) on a digital gram scale to determine the actual callus weight. With the Image Analysis technique as described above, the area, perimeter, feret diameter, integrated density, and other attributes were determined for each of the harvested samples.

5. Statistical Modeling

A linear regression analysis of the calibration data was performed by using SAS. To find the most significant linear model (with the highest F-value and R^2 value) regression analysis was performed for each predictor, by using callus weight as the dependent variable and each image analysis variable as an independent variable. Higher order regression analysis would also be easily accomplished. However, such analysis is not needed in cases where at least one linear model is highly significant and appropriate.

6. Kanamycin Toxicity

Nine callus colonies of untransformed tobacco callus were subcultured onto plates of solid MS media supplemented with 0, 12.5, 25, 37.5, 50, and 62.5 µg/mL kanamycin. Images of each plate were taken every 48 hours for a total of 22 days and analyzed by using the image analysis procedure to monitor growth.

7. Effect of Sodium Chloride on Growth

Tobacco cells were cultured on plates of solid MS media and in flasks of liquid MS media supplemented with an additional 0, 25, 50, 75, 100, and 125 mM of NaCl. For solid cultures, nine colonies of tobacco callus were subcultured onto one plate at each salt concentration. As before, images were taken every 48 hours and the callus weight determined by using the regression model. For liquid cultures, cell growth was measured directly [Mills and Lee, 1996] for duplicate flasks at each salt concentration.

8. Low Temperature Storage and Growth

Nine colonies of untransformed tobacco callus were subcultured onto plates of solid MS media. After the colonies were allowed to

double in size, the plates were transferred to refrigerator storage (4 °C) for 4, 8, 12, 16, or 20 days. Following storage, the colonies were cultivated in a temperature controlled growth room at 30 °C. Images were taken every 48 hours during storage and cultivation.

9. Effect of Sugar Concentration on Growth Rate

Tobacco cells were cultured on plates of solid MS media and flasks of liquid MS media supplemented with 10, 20, 30, 40, 50, and 60 g/L of either sucrose or fructose. For solid cultures, nine colonies of tobacco callus were subcultured onto one plate at each salt concentration. As before, the callus weight was determined from image analysis date by using the regression model. For liquid cultures, cell growth was measured directly for duplicate flasks at each sugar concentration.

RESULTS AND DISCUSSION

1. Calibration Experiment and Statistical Modeling

First, it was necessary to define a statistical relationship between image analysis data and the actual weight of callus. To accomplish this, the growth of 63 callus colonies was monitored on seven culture plates by using image analysis and direct measurements as described in Materials and Methods. As a result, the area, perimeter, feret diameter, integrated density, and callus weight were determined for each of the harvested samples.

To compare these image analysis variables as predictors for callus growth, a multiple linear regression analysis was performed. This analysis, including parameters for each model, is summarized in Table 1. The most significant linear model (with the highest F-value and R^2 value) used integrated density as a predictor for callus weight. This was not surprising since the integrated density represents an estimate of area (the 2-dimensional area multiplied by the intensity factor). Results using area as a predictor were also very good. This result was somewhat surprising, since the height of the colony was not constant. Using the perimeter or feret diameter was also acceptable, but these parameters could not accurately predict the weight of small colonies (data not shown). A calibration curve (Fig. 2) correlating callus weight and integrated density was constructed from this result. One might expect some limitation in prediction accuracy might be expected for extremely small or large colonies. However, the observed percent error (for predicted weight versus observed weight) was nearly constant for the experimental conditions tested. Therefore, it was possible, on the basis of this calibration curve, to determine the growth of plant callus cultures solely from Image Analysis data.

Table 1. Linear regression results correlating callus weight with image analysis data

Predictor	R^2	Sum of squares	F-Value
Area	0.96	2.190	1393
Perimeter	0.83	1.895	292
Feret diameter	0.79	1.797	221
Integrated density	0.97	2.213	1863

2. Kanamycin Toxicity

The new image analysis method was applied to monitor the effectiveness of different concentrations of kanamycin (a common selection resistance gene) at killing non-resistant tobacco callus. As described in Materials and Methods, colonies of untransformed tobacco callus were cultured on MS media supplemented with 0, 12.5, 25, 37.5, 50, and 62.5 μg/mL kanamycin and monitored by using image analysis. Following image analysis, the integrated density values for each colony were averaged and converted to callus weight by using the regression model. As shown in Fig. 3, cell growth was normal with no kanamycin (0 μg/mL), significantly reduced with 12.5 μg/mL kanamycin, and completely inhibited with 25 μg/mL kanamycin or higher.

3. Salt Effect

It is desirable to use the observed behavior of solid cultures to predict the optimum growth conditions for plant suspension cultures. To test this possibility, tobacco cells were cultured on plates of solid MS media and in flasks of liquid MS media supplemented with an additional 0, 25, 50, 75, 100, and 125 mM of NaCl. Fig. 4 shows the effect of additional salt cell growth behavior for the solid (A) and liquid (B) cultures. For both solid and liquid cultures, the rate of growth decreased linearly with increasing salt concentration. Although the overall growth rate differs substantially between solid cultures and liquid cultures, the relative change in growth rate was very similar for the solid and liquid cultures. In fact, graphing the average specific growth rate versus salt concentration for suspension and callus growth (Fig. 5) gives straight lines with statisti-

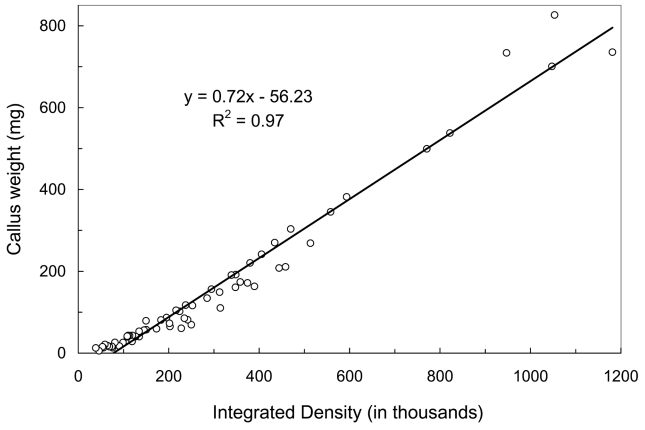

Fig. 2. The linear relationship between callus weight and the integrated density (IDV) obtained by image analysis.

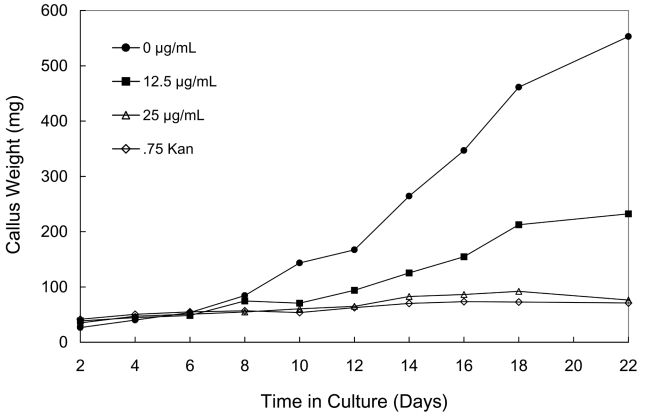

Fig. 3. Growth inhibition and cell death from kanamycin toxicity as monitored using image analysis.

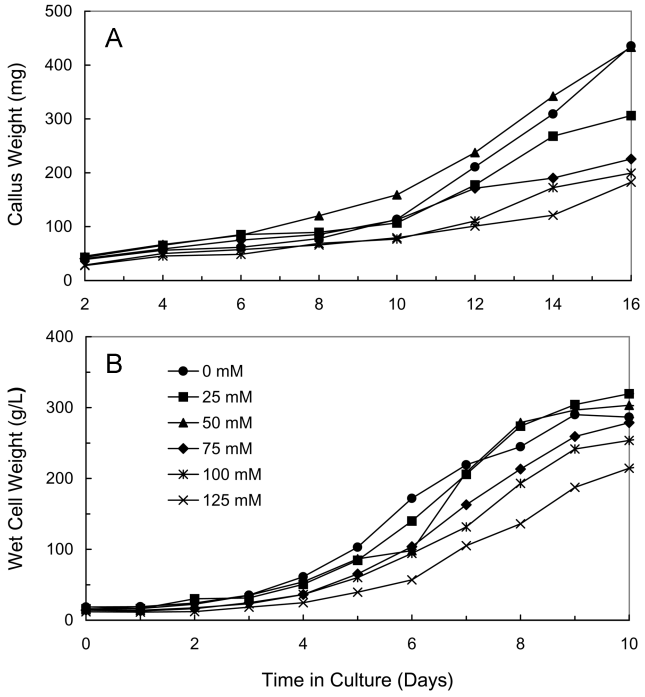

Fig. 4. Salt effect on (A) solid cultures monitored by image analysis and (B) liquid cultures monitored by direct measurement.

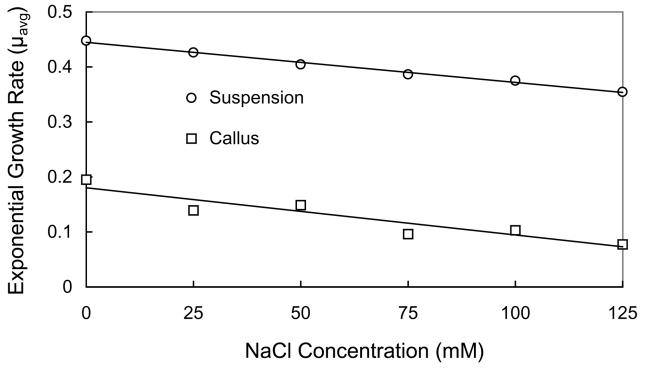

Fig. 5. The linear relationship between salt concentration and growth inhibition for solid and liquid cultures.

cally indistinguishable slopes. This result suggests that information obtained for simple solid culture experiments can be used to determine culture conditions for larger suspension cultures.

4. Low Temperature Growth

Solid cultures may be maintained at a reduced temperature (4 °C) for extended culturing of important cell lines. It is believed that low temperature storage can be used for long-term storage because cell growth is reduced or halted. However, cell behavior during storage may only be evaluated qualitatively without the aid of image analysis. Using the image analysis technique it is possible to precisely monitor the growth behavior of solid cultures during low temperature storage and recovery. To determine the growth behavior during low temperature storage and recovery, tobacco callus was subcultured onto plates of solid MS media and monitored as described in Materials and Methods. Fig. 6 shows the behavior of these cultures during storage (open symbols) and recovery (closed symbols).

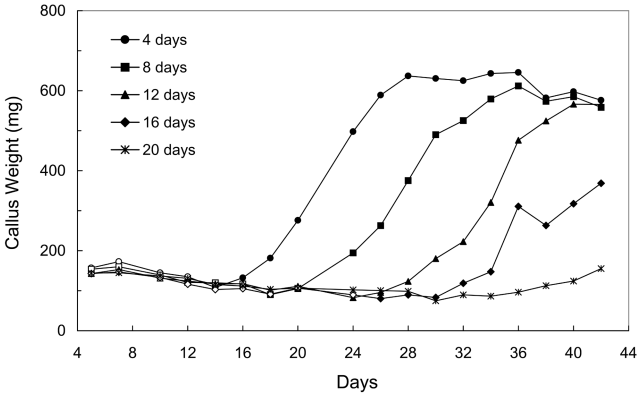

Fig. 6. Growth and recovery of plant callus cultures following low temperature storage.

Table 2. Growth performance of callus as influenced by shrinkage during storage

Sample	Storage duration (days)	Recovery time[1] (days)	Regrowth time[2] (days)	Shrinkage (%)
1	4	6	10	25.3
2	8	6	10	35.4
3	12	8	12	37.2
4	16	10	12	39.7
5	20	14	>24	45.4

[1]Recovery was defined as the amount of time elapsed before growth could be detected.
[2]Regrowth was defined as the amount of time required for the colony size to double.

Cell growth and recovery was hindered for cultures that endured longer storage periods. As summarized in Table 2, this decrease in growth potential was coincident with shrinkage during storage. Based on an examination of the colonies following storage, the observed shrinkage was due to water loss by the callus. Therefore, it is likely that adding moisture to the culture plates could lengthen the storage time. This experiment illustrates the discovery of previously unnoticed changes during cold storage by using the image analysis technique.

5. Effect of Sugar Concentration on Growth Rate

To determine the effect of sugar type and concentration on growth performance, tobacco cells were cultured on plates of solid and liquid media supplemented with 10, 20, 30, 40, 50, and 60 g/L of either sucrose or fructose. Fig. 7 shows the growth performance for the solid (A) and liquid (B) cultures supplemented with sucrose. Fig. 8 shows the growth performance for the solid (A) and liquid (B) cultures supplemented with fructose. The kinetic growth data for these cultures is summarized in Table 3. Although the overall growth rate differs substantially between solid cultures and liquid cultures, the optimum sugar concentrations were similar for both culture types. For cultures supplemented with sucrose, the highest specific growth rate was observed at 20 g/L while the highest cell density was observed at 40 g/L. For cultures supplemented with fructose, the highest specific growth rate and the highest cell concentration were observed at 20 g/L. Also, cell growth was severely inhibited at the high-

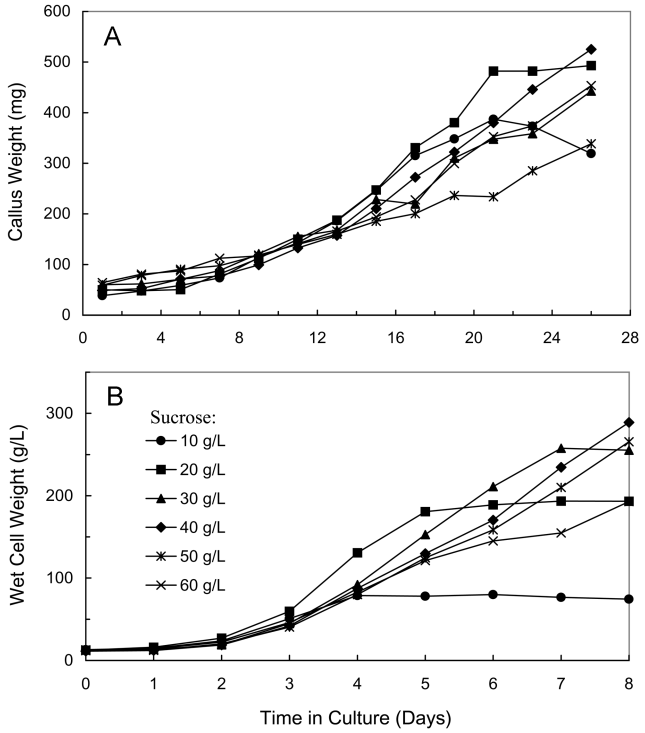

Fig. 7. Growth behavior of (A) solid and (B) liquid plant cell cultures at various sucrose concentrations.

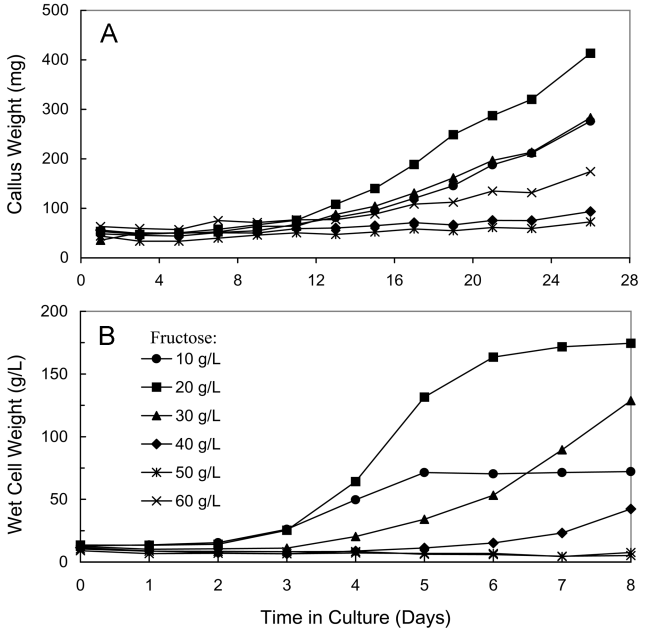

Fig. 8. Growth behavior of (A) solid and (B) liquid plant cell cultures at various fructose concentrations.

est (50 and 60 g/L) fructose concentrations. These results also suggest that information obtained for simple solid culture experiments can be used to predict growth conditions for suspension cultures.

CONCLUSIONS

The results of this paper indicate that the growth of solid cultures can be monitored effectively and non-invasively by using image analysis. The actual weight of a given callus sample can be linearly correlated with the integrated density or area (obtained from image analysis) with a high degree of confidence. The image analysis technique was useful for monitoring cell growth for a variety of experiments, including kanamycin toxicity studies, and media optimization studies. Image analysis was particularly well suited for monitoring the slow growth of cells during low temperature preservation revealing that moisture loss may be the major cause of hindered cell growth and recovery following storage. Furthermore, the comparison of callus and suspension culture behavior indicates that the observed behavior of solid cultures can be used to predict the optimum growth conditions for suspension cultures. Together these findings show that image analysis is a flexible and statistically powerful tool for monitoring plant cell cultures.

Table 3. Growth attributes of plant callus and suspensions at various sugar concentrations

Substrate	Conc. (g/L)	μ_{exp} (day^{-1})		Max. cell weight	
		Suspension	Callus	Suspension (g/L)	Callus (mg)
Sucrose	10	0.54	0.088	77	370
	20	0.66*	0.11*	190	490
	30	0.35	0.069	260	440
	40	0.27	0.083	290*	530*
	50	0.29	0.057	270	340
	60	0.37	0.073	190	450
Fructose	10	0.46	0.099	72	280
	20	0.68*	0.12*	175*	410*
	30	0.45	0.10	130	280
	40	0.39	0.048	42	94
	50	0.029	0.040	7.6	73
	60	0.081	0.061	5.2	170

*Indicates the highest observed value for the corresponding culture and sugar type.

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