

Growth in Tissue Culture of Single-Cell Clones From Grape Stem & *Phylloxera* Gall^{1, 2}

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Introduction

Information about the behavior of clones of plant tissue of single-cell origin is valuable for our understanding of diseased and normal cell growth. Questions appeared about the variability of the cells in the insect-induced galls. Perhaps only a few diseased cells were responsible for the mass of abnormal, rapidly increasing cells, and the majority of the other cells provided a favorable nutritional and physical environment. A large number of single-cell cultures from the mother callus strains of both the normal grape stem tissue and gall tissue incited by *Phylloxera vastatrix* Planch, were available for study of cell variability (8). Increase in wet weight of the tissue culture resulted from the cell division and growth of the cellular mass, and indicated the degree of the activity of the tissues. Differences were observed in the fresh weight of the clones after 4 to 5 weeks of growth of both the normal and diseased clones when grown on basal mineral salts, 2.0 % sucrose agar medium, supplemented with coconut milk (CM) and additional growth promoting substances including alpha-naphthaleneacetic acid (NAA), calcium pantothenate, and 2,4-dichlorophenoxyacetic acid (2,4-D).

The purpose of this study was to clarify the behavior of the selected clones at different periods of their development, and to compare the growth characteristics of the clones with cell counts and dry weight.

Materials & Methods

Six single-cell clones, three from the gall tissue (GP4-21, GP4-8, & GP4-32) incited by *Phylloxera vastatrix* Planch and three from the normal grape stem tissue (GS6-24, GS6-81, & GS6-18), were studied. The criteria for such a selection were their color, texture, and growth rate.

Four seed tissue pieces were grown in each of 6-oz prescription bottles on 40 ml of a basal mineral salt, 2.0 % sucrose agar medium supplemented with CM (15.0 %), NAA (0.1 mg/liter), and calcium pantothenate (2.5 mg/liter) (6), spread on the flat narrow side of the bottles. All glassware was cleaned with potassium dichromate sulphuric acid cleaning solution, rinsed thoroughly with hot tap water, and finally with several changes of distilled water. Reagent grade chemicals were used. The medium was adjusted to pH 5.9 to 6.0 before autoclaving and remained unchanged afterward. In experimental bottles, six replications of each environment were used for every clone. Thus, with four tissue pieces in each bottle, 24 pieces were cultured for each experimental condition and every experiment was repeated three times. The bottles with cultures were kept in the dark at 26 C and at 55 % relative humidity which were optimum for growth.

Growth in terms of wet weight, dry weight, and cell count was measured at intervals of 5 days during growth periods of 10 to 40 days. The initial seed tissue piece selected from actively growing 2- to 3-week-old cultures weighed approximately 35 mg.

To determine wet weight, each clone of the tissue was removed from the culture bottle and weighed on a shadograph balance.

The clone pieces used for determining dry weights were dried overnight to constant weights at 65 C in aluminum pans and stored in desiccators. No reserve and excretory products were removed from the tissues. The dry weights of the tissue pieces were determined on a torsion balance.

Cell counts of the tissue were made adopting a modification of the methods suggested by Brown and Rickless (2), and by Gautheret (4). Nearly 50 mg of the tissue by fresh weight was fixed in 10 ml of aceto-alcohol (absolute alcohol & glacial acetic acid in the ratio 1:1) for 8 hours. The tissue was washed in distilled water and immersed in 5 ml of a solution containing 5.0 % chromic acid and 3.0 % hydrochloric acid in the ratio 1:1. The suspension was left at room temperature for 24 hours. The time for the acid treatment was not critical. The whole mass was shaken vigorously, and homogenized by passing it through a hypodermic syringe needle several times. The treatment yielded a turbid suspension without clumps visible to the naked eye.

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Cell counts were made with the Spencer Bright Line Haemocytometer. When maceration was apparently complete, a sample of the suspension was withdrawn with the pipette and introduced below the cover slip in a manner to prevent settling.

Cells were counted with a microscope having a 10 X objective and a 10 X eye piece. The total number of cells in the tissue piece was calculated. Six samples from each suspension were counted. Three suspensions were prepared and readings obtained for each harvest of a particular clone. Thus, each point on the curves in figures 9 and 10 is the average of the analysis of 18 samples. The method for determining cell numbers was relatively rapid and accurate. Replicate haemocytometer readings from the three suspensions counted for each point usually agreed within 5.0 %.

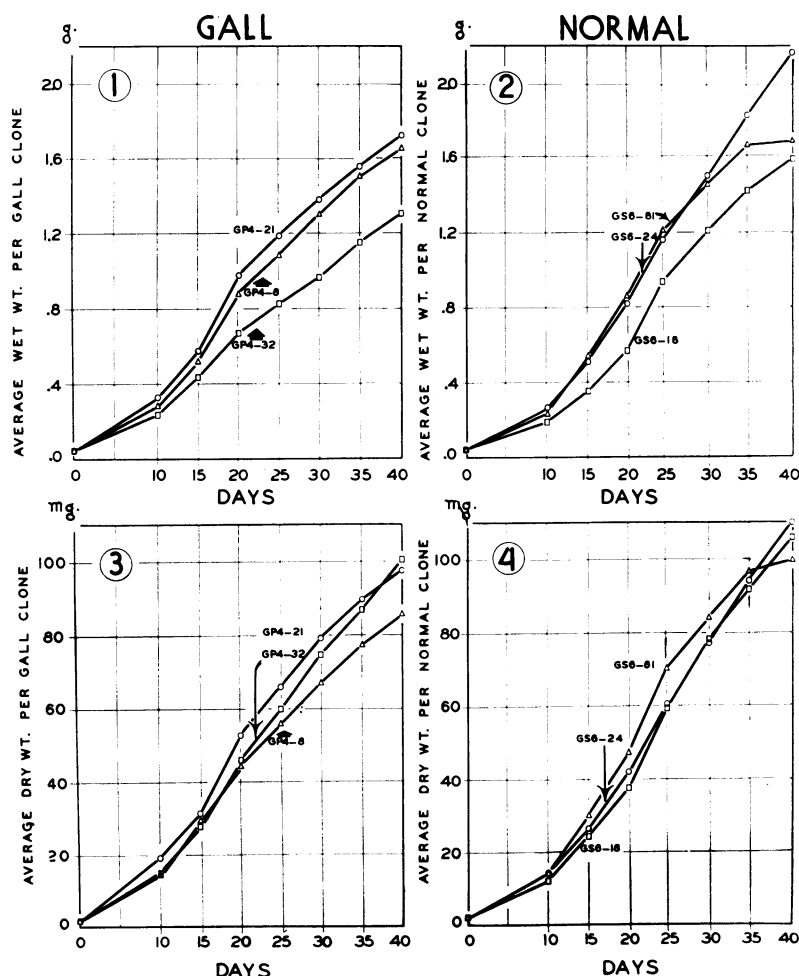
Dimensions of the cells of the clones were determined, and photomicrographs (fig 11-16) made after the following treatment. The cell suspension in the chromic-hydrochloric acid solution was filtered, the cell mass left on the filter paper was washed with distilled water several times, and the cells removed

to 5 ml of 1.0 % aqueous solution of crystal violet. Cells were stained in the solution for 15 minutes and afterwards centrifuged at 250 rpm for 10 minutes. The supernatant was decanted off and cells suspended in distilled water. Microculture technique used earlier in the laboratory by Jones et al. (9) was used for making stained cell preparations.

Experimental Results

The means of the individual harvests in terms of wet weights and dry weights of the six single-cell clones of the gall and normal tissues were obtained and plotted in figures 1 to 4. Tobacco tissue culture (3) for the first 5 or 6 weeks indicated constant exponential growth. Caplin used the formula: $\text{Log}_e (W_1/W_0) = rt$ as originally devised by Blackman (1). W_1 and W_0 were the final and initial weights of the tissue, e the base of the natural logarithms, r the rate of increase, and t the time. The rate of increase was expressed as percentage increase per day. This formula was used to calculate the growth rate of the six single-cell clones in terms of percentage increase

Fig. 1-4. Average wet and dry weights of the single-cell clones of *Phylloxera* gall (fast, GP4-21; medium, GP4-8, & slow growing GP4-32) and normal grape stem callus (fast, GS6-24; medium, GS6-81, & slow growing GS6-18) through different periods of growth ranging from 10 to 40 days. Each point on the curves represents the average wet (1 & 2) or dry (3 & 4) weight of 24 tissue pieces from each of the three experiments conducted at different times.



in both the wet and dry weight per day. The data appear graphically in figures 5 to 8. The results of the cell counts of the clones appear in figures 9 and 10.

Variation in the growth patterns of all the six clones was distinct in their growth rates under all the three experimental conditions. Characteristic curves developed for each clone. The results of the behavior of the clones under different experimental methods may be summarized as follows:

► **Wet Weight Determinations:** The pattern of growth of all the three *Phylloxera*-gall clones (GP4-21, GP4-8, & GP4-32) remained specific through all the growth periods. Each clone developed its characteristic curve in accordance with its rate of growth. The clones were grouped as fast-, medium-, and slow-growing. GP4-21, a fast-growing clone, was whitish-grey, translucent, and soft. GP4-8, a medium-growing clone, was whitish-yellow, translucent, and soft. The slow-growing GP4-32 was lemon-yellow, translucent, and firm.

The maximum increase in the total wet weights of all the three clones took place in 15 to 20 days of

growth. After 20 days, the growth slowed gradually but the clones continued to grow at the reduced rate even on the 40th day. However, when the growth rates were calculated in terms of percentage increase per day, the maximum rate of increase in the wet weights was obtained by all the three clones during the first 10 days of growth. Then a gradual decline appeared in the rate of increase of the wet weights.

Among the normal tissue clones, the behavior of GS6-24, (a fast-growing, whitish-yellow, translucent, & soft clone) was very interesting. This clone grew slower than GS6-81 (a medium-growing, whitish-grey, translucent, & soft-textured normal clone) until the 25th day of growth, but between 25 to 30 days it grew faster than GS6-81. The decline in the growth rate of GS6-24 was correspondingly gradual when compared with that of GS6-81 after 30 days of growth. The firm, yellow-colored, GS6-18 showed no change in its behavior. Through all periods of growth it remained a slow-growing clone. All the three normal clones obtained optimum increase in the wet weights in 20 to 25 days of growth. The percentage increase of growth per day followed the same

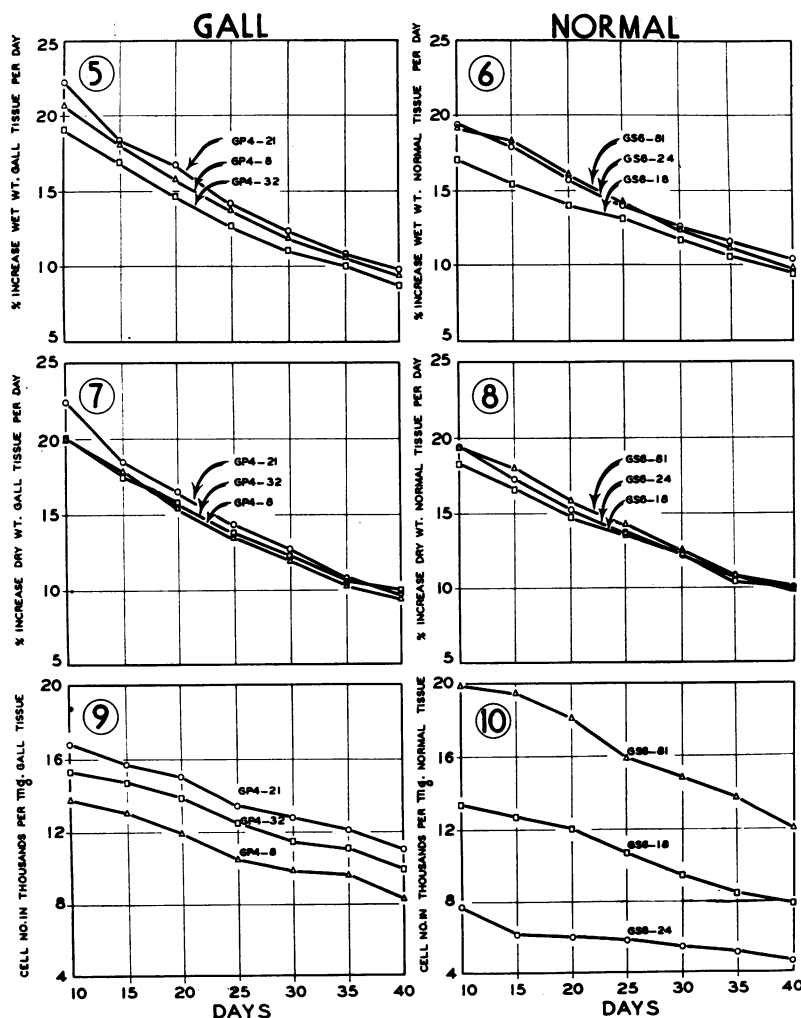


Fig. 5-10. The rate of increase of wet (5 & 6) and dry (7 & 8) weights calculated in terms of percentage increase applying the formula $\text{Log}_e (W_1/W_0) = rt$, and the result of cell counts (9 & 10) in terms of number of cells per mg of the wet weight of the callus tissue of single-cell clones as given in figures 1 to 4 of *Phylloxera* gall and normal grape stem from 10 to 40 days of growth period. Each point on the curves in figures 5 to 8 is based on the average of 72 samples, and in figures 9 to 10 the average of analysis of 18 samples.

pattern as that of the gall clones described above (fig 6).

► **Dry Weight Determinations:** The growth patterns displayed by the gall clones GP4-21 and GP4-8 on a dry weight basis were identical to those obtained under the wet weight method. GP4-32, a slow-growing clone, however, showed a considerable degree of fluctuation. During the first 15 days of growth the dry weights equalled those of GP4-8, but later started leading and after 40 days weighed the most. The decline in its weight was very gradual.

Dry weights of the normal clones GS6-24, GS6-81, and GS6-18 followed more or less the corresponding growth pattern obtained with wet weights. Only the weights of GS6-18, a slow-growing clone, failed to show similarity in the decline of its growth after 15 days.

The dry weight relationship of the clones with the corresponding wet weights was calculated in terms of percentage dry weight of the wet weight. The range of variation in the GP4-21, a fast-growing gall clone, was from 5.3 to 5.8 %; in the medium-growing gall clone GP4-8 from 5.2 to 5.4 %; and in the slow-growing GP4-32, from 6.3 to 7.7 %. Among the normal clones, in the fast-growing GS6-24, the range was from 5.1 to 5.7 %; in the medium-growing GS6-81, from 5.1 to 5.9 %, and in the slow-

growing GS6-18, from 6.3 to 8.4 %. The results indicated that with the exception of GP4-32 and GS6-18, both slow-growing clones of gall and normal tissues, respectively, the range of difference in the dry and wet weight methods was nominal. However, the slow-growing clones showed comparatively more pronounced differences in the results of the two methods. Also, the weights of the dried clones were reduced to a degree that the curves ran close to one another making it difficult to differentiate them on the basis of their growth rates.

► **Cell Counts:** In all the six clones of gall and normal origin, the average number of cells per milligram of fresh weight recorded the highest count on the 10th day. Subsequent readings revealed a gradual decrease to the minimum after 40 days. A shift, in the respective positions of GP4-32 and GS6-24 obtained in the dry and wet weight determinations, was observed (fig 5-10). The slow-growing gall clone GP4-32 gave cell counts to place it in an intermediate position in the three gall clones. Among the normal clones, the fast-growing GS6-24 gave the smallest number of cells per milligram occupying the third position.

Differences in the cell sizes of various clones were observed during counts of cells on the haemocytometer slide. The sizes of 100 cells of each clone

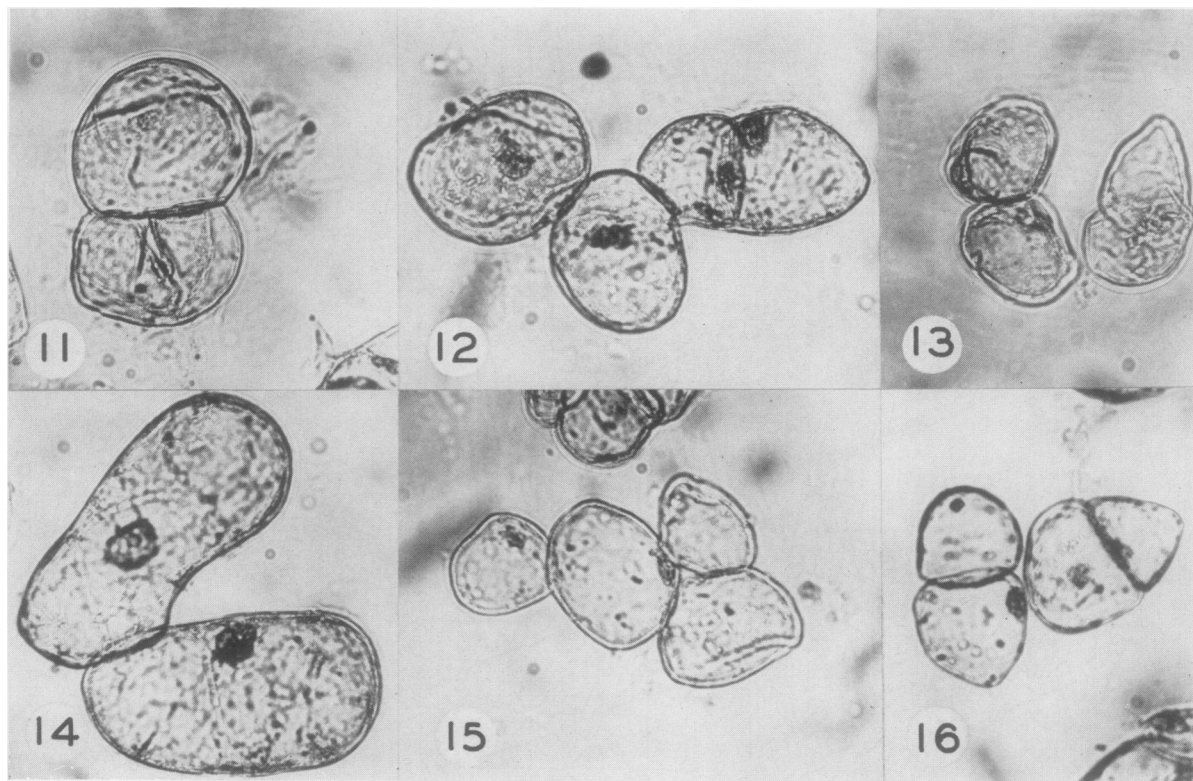


Fig. 11-16. Photomicrographs of the stained representative cells of 20-day-old cultures of single-cell clones of *Phylloxera* gall (11, GP4-21; 12, GP4-8; 13, GP4-32) and normal grape stem callus (14, GS6-24; 15, GS6-81; & 16, GS6-18) showing differences in cell sizes. ($\times 1,400$)

were, therefore, measured after 20 days of growth. The cell sizes of GP4-32 and GS6-24 were remarkably different from the cell sizes of the other clones. The mean sizes of cells of the various clones measured as follows: GP4-21, a fast-growing gall clone, $59 \times 46 \mu$ ($43-82 \times 35-62 \mu$); GP4-8, a medium-growing gall clone, $59 \times 46 \mu$ ($46-73 \times 38-62 \mu$); GP4-32, a slow-growing gall clone, $54 \times 40 \mu$ ($40-67 \times 32-57 \mu$); GS6-24, a fast-growing normal clone $86 \times 67 \mu$ ($59-135 \times 54-94 \mu$); GS6-81, a medium-growing normal clone, $58 \times 44 \mu$ ($43-74 \times 32-67 \mu$), and GS6-18, a slow-growing normal clone, $58 \times 44 \mu$ ($43-76 \times 32-65 \mu$).

Discussion

A number of methods have been described for the evaluation of the growth of the plant tissue cultures (4). Some of the important ones are cell and nuclei counts, fresh and dry weights, tissue areas and volumes, as well as determinations of protein nitrogen or nucleic acid contents. In the present investigations wet weights, dry weights and cell counts were made for comparing the growth of six single-cell clones from grape stem and *Phylloxera* gall tissues. All the three methods gave comparable results. The 10th day readings showed the maximum rate of growth indicated by the highest percentage increase in wet and dry weight per day and largest number of cells per milligram of fresh weight of the tissue. This was followed by a gradual decrease to a minimum after 40 days. Different clones varied in their growth rates which were manifested in the formation of their specific curves, and their being grouped into fast-, medium-, and slow-growing clones. The individual clones generally maintained the steady pattern of growth. However, GS6-24, a fast-growing normal clone, came a close second to GS6-81, a medium-growing normal clone, up to 25 days, but between 25 to 30 days its curve crossed that of the GS6-81 and ultimately led up to 40 days when the last readings were made. This might be explained since A. the growth curve of GS6-24 did not show a normal gradual decline after 25 days of growth, and B. the growth curve of GS6-81, comparatively, dropped rapidly, making a substantial difference in their wet weights in the final count.

Generally, the pattern of growth of the clones displayed by dried weight estimation was identical to the one obtained through the wet weight method. The results confirmed the observations recorded earlier by Hildebrandt and Riker (7) in the case of sunflower and tobacco tissues *in vitro*, and recently by Lance (10) who compared the fresh and dry weight, and nitrogen content of the tissue cultures and found wet and dry weights gave parallel results. Only in the case of GP4-32, a slow-growing *Phylloxera*-gall clone, was a deviation observed in its behavior in terms of dry weight estimation. Up to 15 days, growth of GP4-32 resembled that of GP4-8,

a medium-growing gall clone, but between 15 to 20 days its curve crossed that of GP4-8, and later occupied a middle position between GP4-21 and GP4-8, fast-, and medium-growing gall clones. White (11) has considered increase in the living material in the tissues as the criterion for its growth as distinct from non-living secretions. Accordingly, if dry weight samples for growth determination were taken when the cultures were in an actively growing state, it might give a fairly accurate indication of plant activity. At the differentiating stage in development, there may be a rapid increase in dry weight along with an actual decrease in living material. Gautheret (4) also thought that the determination of dry weight had the distortion of including reserve materials. Goris (5) earlier suggested that if the reserve and secretion products were estimated and deducted from the total increase in dry weight of the tissues, the corrected values fairly represented the increase in the living materials. Possibly, the abnormal rise in the dry weights of GP4-32 might be explained by an excessive discharge of some such materials, but this was not determined.

The method of cell and nuclei counts has been considered useful to measure growth (4). High and low rates of cell counts per milligram of the fresh tissue as obtained in the case of GP4-32, a slow-growing gall clone, and GS6-24, a fast-growing normal clone, respectively, which altered their respective positions, could be explained on the basis of the size of the cells. For example, the cells of GS6-24 were comparatively larger than those of GS6-81 and GS6-18. As a result, a lesser number of cells per milligram of tissue by fresh weight were counted, giving GS6-24 third position among normal clones. Its fast growth rate measured in terms of wet and dry weights may have been due to enlargement of the cells rather than to rapid multiplication, or due to both. The cells of GP4-32 on the other hand, were relatively smaller and consequently a larger number of cells were found per milligram of the fresh weight of the tissue, which placed it second among the gall clones. This again may not be due to rapid multiplication of the cells but to a smaller size of the cells. On the basis of cell counts (fig 9, 10), relatively large differences appeared between the three normal clones and only small differences between the three gall clones. The reason for this is obscure. However, one can speculate that the normal clones were derived from cells which had reached varying degrees of differentiation while the gall clones came from cells which had lost such differentiation. It may be useful to examine critically the multiplication and enlargement of the cells at different periods of tissue growth and more accurately to describe growth variability of the clones. More information about variability and growth specificity of different clones of gall and normal origin may be important in clarifying the basic problem about the nature and pattern of diseased growth in plants.

Summary

Six single-cell clones, three (GS6-24, GS6-81, & GS6-18) from the grape stem callus and three (GP4-21, GP4-8, & GP4-32) from the gall tissue incited by *Phylloxera vastatrix* Planch were grown on a basal mineral salts, 2% agar medium supplemented with coconut milk (15.0%), alpha-naphthaleneacetic acid (0.1 mg/liter), and calcium pantothenate (2.5 mg/liter). Growth of the clones was measured as wet weight, dry weight, and cell count at intervals of 5 days during growth periods of 10 to 40 days. The clones were differentiated into three groups: fast-, medium-, and slow-growing. They also varied in their color and texture.

Results of all the methods used were comparable. The first readings taken on the 10th day recorded highest percentage increase in wet and dry weight per day, and the largest number of cells per milligram of the fresh weight of the tissues. This was followed by gradual decrease, attaining minimum after 40 days.

In general, fast-, medium-, and slow-growing clones of both gall and normal origin maintained uniformity in their specific growth pattern through all the growth periods tested. However, GS6-24, a fast-growing normal clone, was medium in growth during a 15- to 20-day period. Among the gall tissue clones, GP4-32 (a slow-growing clone according to wet weight estimation) made a shift to a medium growth rate when comparisons were made in terms of dry weights and cell counts. The GS6-24 (a fast-growing clone) also showed the fewest number of cells by the cell count method, compared with medium-, and slow-growing normal clones. More striking differences were observed among the normal clones than in the gall clones on the basis of cell counts. Cells of all the six clones were measured and differences in their cell sizes were recorded. Most notable were the sizes of cells of GS6-24 which were largest and those of GP4-32 which were smallest.

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