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Conditions for a High Plating Efficiency of Free Cell Suspensions of *Haplopappus gracilis* (Nutt) Gray

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Summary. Suspensions of *Haplopappus gracilis* cells, containing about 80% free cells, were obtained from log-phase cultures by filtration through 3 nylon sieves having decreasing mesh widths from 297, 210 and 88 µm. From the free cell suspensions, 75 to 90% of the cells developed into visible colonies when the plating procedure was divided into two steps: a) plating the cells at high concentration in soft agar on feeder agar; b) replating the resulting aggregations at appropriate concentrations on fresh feeder agar. From the results, it is inferred that, in the replating step, the volume of the inoculum is the deciding factor which influences the resulting plating efficiency.

Key words: Free-cell Suspension - *Haplopappus gracilis* - Plating Efficiency

Introduction

In several botanical subdisciplines (e.g. radiation-botany, plant breeding, plant pathology, plant genetics, etc.), an experimental system is needed which permits a quantitative evaluation of cellular responses to different treatments. Free-cell suspension cultures and isolated protoplasts appear a promising potential to supply this demand. (See for a comprehensive and up to date review Reinert and Bajaj 1977). A major drawback of free-cell suspension cultures is, however, the fact that in general the growth and division of single cells, when plated on agar-media in low and easily scorable numbers, is rather poor. Low plating efficiencies, at plating densities of 1000 cells per petri-dish or lower, have been reported by Bergmann (1960), Nagata and Takebe (1971), Logemann and Bergmann (1974), Engvild (1974), Vardi et al. (1975) and Raveh et al. (1973). Kao and Mickayluk (1975) achieved good growth of *Vicia* cells and protoplasts even at plating densities down to a few cells per ml. This suggests a high plating efficiency, but exact figures are not given. In this report, conditions are described which give a high plating efficiency (75-90%) of free cells of *Haplopappus gracilis*, plated at low densities.

Materials and Methods

Seeds of *Haplopappus gracilis* (Nutt) Gray were obtained from Dr. H. Smith, USA; Dr. R. Tanaka, Japan and Dr. E. Sparvoli, Italy. Cell-suspension cultures, strain '105 S III' (Eriksson, 1965) were obtained from Dr. H.J. Fritsch, W. Germany. Cellulase and pectinase were purchased from Sigma. All other chemicals used were of pa-grade.

Stem segments (1-5 cm) of mature plants were surface-sterilized by submersion in 5% $CaOCl_2$ for 5 min. They were then rinsed several times in sterilized, distilled water. After removing small lateral branches and leaves with forceps, the explants were placed on the surface of Gamborg's B5 medium (Gamborg et al. 1968) solidified with 1.5% agar. Callus resulting after three weeks incubation at 28°C was suspended in a few ml of B5 medium without agar and subsequently poured into an Erlenmeyer flask containing 40 ml of liquid B5 medium. The flasks were placed on a gyrotory shaker (200 rpm, amplitude 2.5 cm) at 28°C in continuous darkess. The culture was kept in logarithmic growth phase by transferring 8 ml of culture to 40 ml of new B5 medium every 7 days.

The weights of the cultures were determined by collecting the cells on pre-weighed, wet filter paper with a Buchner-funnel, and weighing them immediately. Dry weights were determined after incubating the collected cells at 80°C for 24 h. A longer incubation did not result in a further decrease of the dry weight of the sample.

In plating experiments, cell suspension were poured into petri dishes containing about 10 ml plating medium. Several plating media were used:

1. B5 agar. This is standard B5 medium (Gamborg et al. 1968) solidified with 1.5% agar.

2. Conditioned-agar: Cells were removed aseptically by filtration from suspensions near the end of the logarithmic growth phase. The resulting medium was heated to 40°C and added to an equal volume of B-5 medium containing 3% agar. The mixture was poured into petri dishes and allowed to solidify.

3. Feeder agar. Cell suspensions near the end of the logarithmic growth phase were distributed into petri dishes in portions of 1 ml. 10 ml of B5-agar at 40°C was added and the plates were incubated at 28°C

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in continuous darkness. After 4 days the plates were irradiated with 25 kR to kill all cells (Raveh et al. 1973).

4. Soft agar. This is standard B5 medium with 0.5% agar. It is made by mixing standard B5 medium with suspensions of free cells at 40°C in a volume ratio of 3 parts medium to 7 parts cell suspension. After cooling, the mixture is not completely solid but soft.

After plating an inoculum (1-5 ml) of the cell suspensions to be investigated, the petri dishes were sealed with parafilm and incubated at 28°C in continuous darkness. After an appropriate incubation time the number of visible colonies were counted. The plating efficiency (P.E.) is defined as the number of visible colonies per ml. $(\text{number plated units per ml})^{-1} \cdot 100\,\%$.

Results and Conclusions

Growth Characteristics of the Standard Suspension Culture

Standard conditions, as described in the previous chapter, result in a suspension culture that is continuously in logarithmic growth phase. The growth of such a suspension, expressed as increase in fresh and dry weight, is demonstrated in Fig.1. Logarithmic growth continues for 7 days. After this period, the culture gradually moves into a plateau-phase. It has not yet been investigated whether or not the cells in a plateau-phase-culture are arrested in a particular stage of the cell cycle.

The doubling time with respect to fresh and dry weight is approximately 3 days. Doubling time with respect to cell number could not be determined because cell counting is difficult and error-prone due to the occurrence of aggregations of cells that can not be fully macerated. The suspension is proportionly composed of 18% unicellular units and 82% multicellular aggregations. The number of cells in these aggregations range from two to several hundred. The ratio of free cells to multicellular units remains constant during logarithmic growth phase; it decreases when cultures go into plateau phase. All experiments which are described below have been carried out with one substrain that has retained its growth characteristics as described above for more than two years.

Production of Free-cell Suspensions

The occurrence of a high number of multicellular aggregations in the suspension cultures as described above,

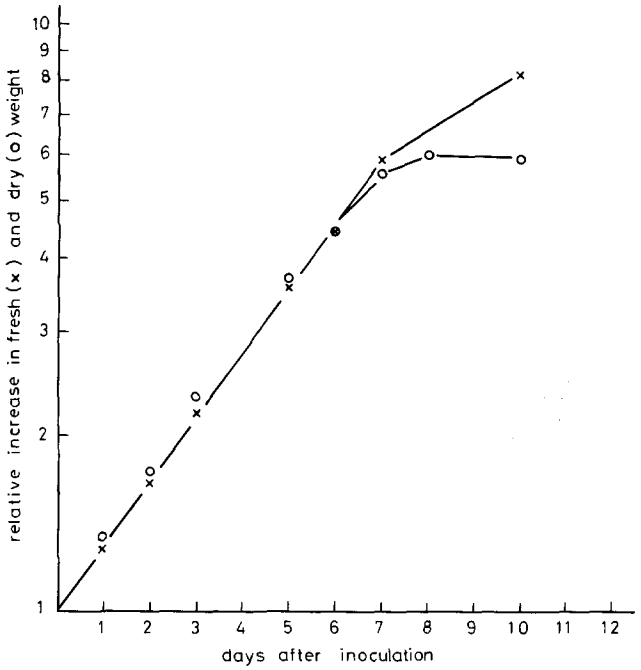

Fig.1. Increase of fresh and dry weight in exponentially growing suspension culture of *Haplopappus gracilis*. Erlenmeyer flasks containing 40 ml B-5 medium were inoculated with 10 ml exponentially growing suspension of *Haplopappus gracilis* cells (fresh weight 0.56 g, dry weight 0.058 g) and incubated. At intervals as indicated in the figure, three flasks were removed from the shaker and fresh and dry weights were measured

renders these cultures unsuited to a study of the plating efficiency of free cells. Therefore several procedures to increase the number of free cells have been explored.

A first suggestion was obtained from El Hinnawy's (1974) work: the application of chelating compounds with a high affinity for calcium would loosen the intercellular cement and, as a consequence, increase the number of free cells. The effect of EDTA (an example of a chelating agent) on suspension cultures of *Haplopappus* with respect to growth and number of free cells is shown in Fig.2. Growth rapidity is only slightly decreased at a concentration of 0.002% EDTA, but higher concentrations cause very anomalous and irreproducible growth. With respect to the number of free cells all concentrations tested resulted in only a slight increase. It is concluded from these experiments that addition of EDTA to the suspension cultures of *H. gracilis* is not a satisfactory procedure for the production of free cell suspensions. Other chelating compounds have not been tested because of their known deleterious effects on growth (El Hinnawy 1974).

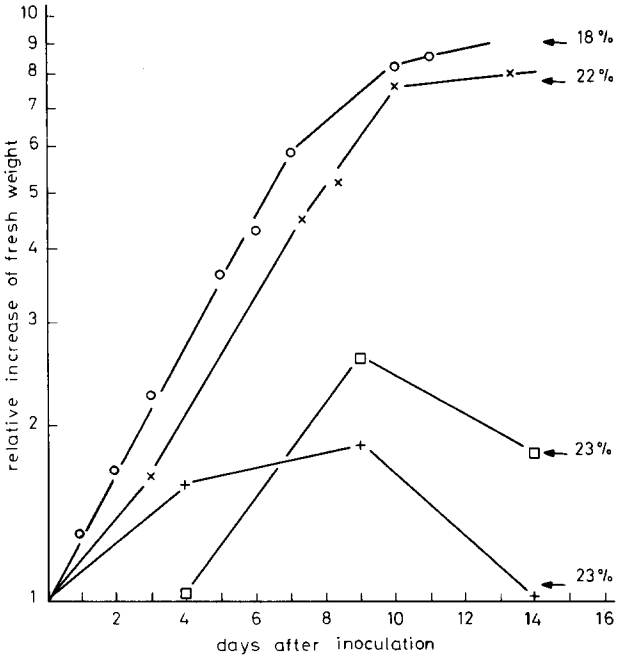

Fig.2. Effect of EDTA on the growth and free cell content of a suspension culture of *Haplopappus gracilis*. Experimental procedure is as described for Fig.1 except that at the time of inoculation, EDTA was added in concentrations as indicated. The percentage of free cells was determined at the 7th day after inoculation
○ —— ○ untreated control
× —— × 0.002% EDTA
□ —— □ 0.004% EDTA
\+ —— + 0.008% EDTA

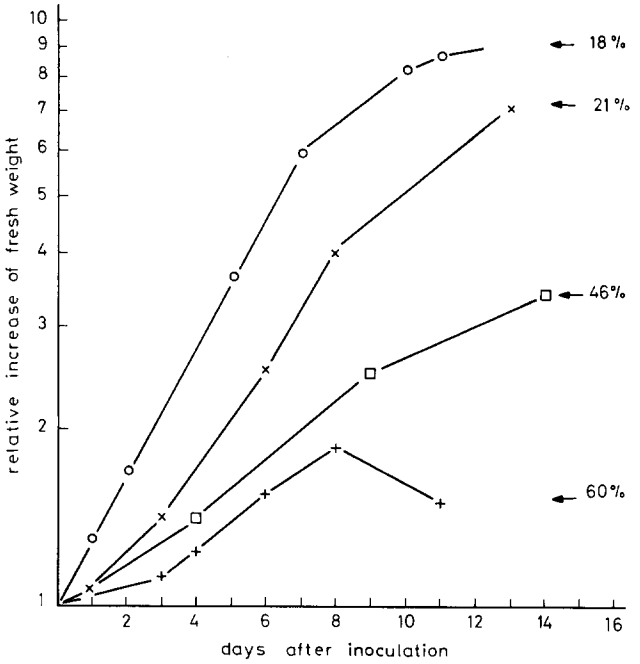

Fig.3. The effect of cellulase + pectinase on the growth and free cell content of suspension culture of *Haplopappus gracilis*. Experimental procedure as described for Fig.1 except that at the time of inoculation, cellulase and pectinase were added in the concentrations indicated. The percentage of free cells was determined at the 7th day after inoculation
○ —— ○ untreated control
× —— × 0.025% cellulase + 0.0125% pectinase
□ —— □ 0.05% cellulase + 0.025% pectinase
\+ —— + 0.10% cellulase + 0.05% pectinase

In a second attempt, the effect of cellulase and pectinase on the number of free cells was studied. The attempt to use these enzymes to produce high numbers of single-cells in suspension cultures was based on the fact that they are successfully employed in methods for the release of viable protoplasts from plant material. Fig.3 shows the result of a representative experiment of the effect on growth and number of free cells of the simultaneous addition of cellulase and pectinase to a logarithmically growing culture: low concentrations, up to 0.025% + 0.0125% have only a minor and perhaps acceptable effect on growth characteristics; on the other hand, the number of free cells in the suspension is not substantially increased. Higher concentrations, while resulting in high numbers of free cells, greatly impair the growth of the suspension cultures. This anomalous growth may be explained by the enzymatic weakening of the cell wall, resulting in cells that are osmotically incompatible with the standard growth medium.

In order to overcome the presumed osmotic incompatibility of the growth medium, sorbitol, an osmotic agent, was added to counteract the negative effects of cellulase and pectinase. The effect of sorbitol alone is demonstrated in Fig.4. Up to a concentration of 4% the growth characteristics with respect to the rate of increase in fresh weight and the number of free cells do not deviate from the untreated control. However, the appearance of a lag-period which lasts one day, indicates that the cells have to adapt to the new osmotic conditions of the medium.

The combined effect of sorbitol and cell wall degrading enzymes is demonstrated in Fig.5. Sorbitol, in concentrations from 0.5 to 2.0% and in combination with 0.05% cellulase + 0.025% pectinase, causes the same high percentage of free cells in the suspension as

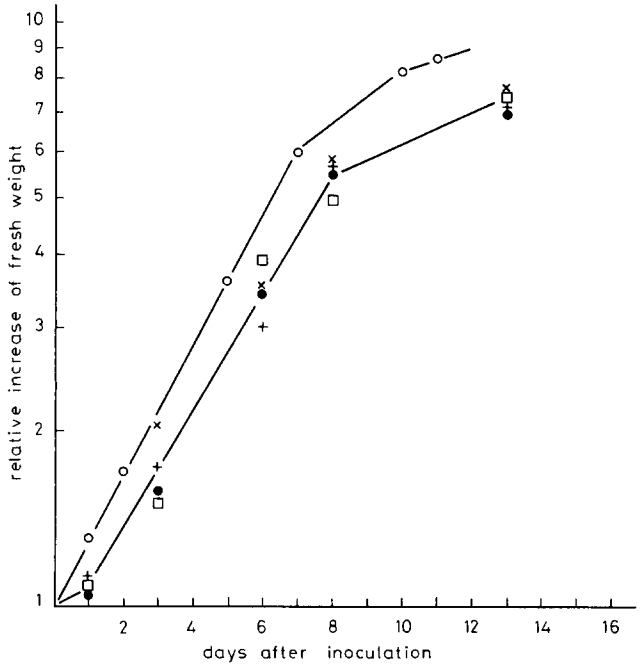

Fig.4. The effect of sorbitol on the growth and free cell content of the suspension culture of *Haplopappus gracilis*. Experimental procedure as described for Fig.1 except that at the time of inoculation sorbitol was added in concentrations as indicated
○——○ untreated control
×——× 0,5% Sorbitol
□——□ 1,0% Sorbitol
+——+ 2,0% Sorbitol
●——● 4,0% Sorbitol

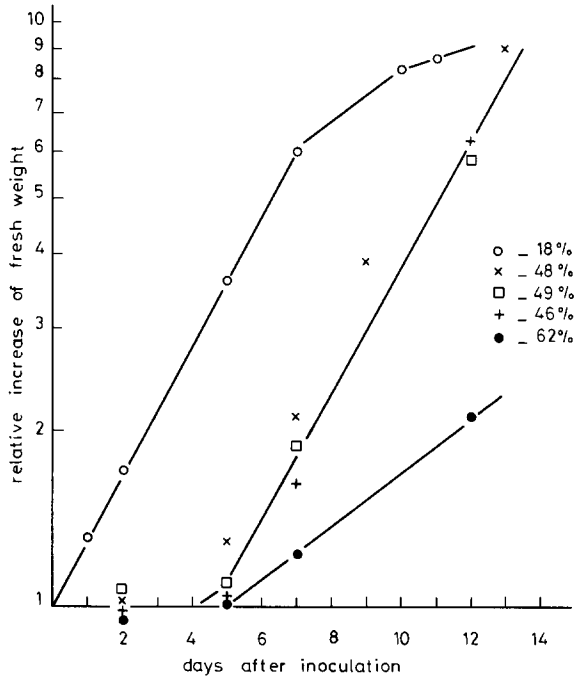

Fig.5. The combined effect of sorbitol and cell wall degrading enzymes on the growth and free cell-content of suspension culture of *Haplopappus gracilis*. Experimental procedure as described for Fig.1 except that at the time of inoculation, cellulase and pectinase were added in a concentration of 0.05% and 0.025% respectively, and sorbitol to concentrations as indicated. The percentage of free cells was determined at the 7th day after inoculation
○——○ untreated control
×——× 0.05% cellulase + 0.025 pectinase + 0.5% sorbitol
□——□ 0.05% cellulase + 0.025 pectinase + 1.0% sorbitol
+——+ 0.05% cellulase + 0.025 pectinase + 2.0% sorbitol
●——● 0.05% cellulase + 0.025 pectinase + 4.0% sorbitol

does 0.05% cellulase + 0.025% pectinase. The rate of increase of fresh weight does not deviate from the untreated control, but a very long lag period of about four days preceeds the logarithmic growth phase. At a higher concentration of sorbitol, the percentage of free cells is even higher than at low concentrations, but growth is much slower after an even longer lag-period.

During the long lag phases, inherent to this procedure, a considerable decrease in fresh weight is often observed. This indicates that some cells lyse during that period. Since it cannot be excluded that this process of lysing involves some selection of cells, this method is not suited to our purposes in spite of the high number of free cells produced.

The chemical treatments have consequently been rejected and a simple filtration technique of the untreated suspension culture was adopted in their place to separate free cells from the multicellular aggregations. Suspension cultures near the end of logarithmic growth phase were aseptically filtered through a combination of nylon sieves having a mesh width of 297, 210 and 88 µm successively. The resulting suspension contains about 2.5×10^3 viable units per ml. Of these units, 70 to 80%, depending on the quality of the original suspension, are unicellular, the rest is bicellular and a negligible (< 1%) part is multicellular. Most of the bicellular units are probably cells that have not fully completed cell division. In view of the high proportion of free cells in the filtrate and the fact that no physiological selection for special cells occurs, this procedure meets the requirements for a study of the plating efficiency of free cells.

Plating Efficiency of Free Cells

The simplest experimental procedure with which to study the plating efficiency of the cells is to spread a small volume of cell suspension over solid growth medium in a petri dish, incubate the plates at the appropriate conditions and observe the development of the plated cells. In Table 1, the results of several experiments based on this concept are presented. In these experiments, cellular development was studied by the microscopical observation, three days after the plating, of the number of single cells that had undergone at least one division, and secondly, six weeks after plating, the number of visible colonies was counted.

None of the plating conditions presented in Table 1 proved to be satisfactory since the highest plating efficiency that was reached was only 10%. However the following observations are of importance.

a. The quality of the solid growth medium influences to a great extent the development of colonies. Feeder agar allows 10% of the plated units to develop into colonies, whereas none or very few units do so when plated on B5 or conditioned agar.

b. At least some cells develop into visible colonies when the inoculum is mixed into the plating medium. It seems, therefore, that the physical properties of the medium also influence the development of free cells to colonies.

c. In all the experiments presented in Table 1, a rather high proportion of the free cells plated had divided at least once by the second day after plating. Further microscopic observation revealed that in experiment 4 nearly all the free cells had divided and that division ceased after the resulting colonies reached the number of 30-60 cells. Most likely at this stage of development some shortage of nutrients or inefficiency of gas exchange occurs which causes the colonies to stop further growth.

Using these observations, a new experimental concept was developed: free cells were suspended in soft agar (b) and plated on feeder agar (a). After the developing colonies had reached the stage of 30-60 cells (3 weeks), the soft agar was diluted with liquid (40°C) soft agar to an appropriate concentration of colonies and the resulting suspension was replated on fresh feeder agar. By this last step the presumed blockage in development, probably caused by nutrient shortage and/or inefficient gas exchange, is circumvented. Visible colonies were counted 2 weeks after the replating. In Table 2 the results of a number of experiments, based on this concept are presented.

In experiment 1, where the procedure is in full accordance with the new concept, a rather high plating efficiency is achieved. From the results of these several similar experiments (exp 1 a-f), it can be concluded that there is some variation in the plating efficiency. Deviations from the procedure employed in exp 1, with respect to the quality of solid media (exp 2 + 3), and technique of first plating (exp 4) result in consistently lower plating efficiencies.

The cause of the experimental variation of the plating efficiency, as observed in the experiments 1 a-f, may be found in the observation that the greater the volume of the inoculum, the lower the plating efficiency. Consequently, the effect of the volume of inoculum on plating efficiency has been investigated. From the result of this experiment, shown in Fig.6, it is concluded that the volume of inoculum in the replating step is the deciding factor which influences the resulting plating efficiency. It is improbable that

Table 1. The effect of plating conditions on the plating efficiency of *Haplopappus gracilis* cells

Exp. No.	Plating condition	Volume of inoculum	Cell density $\times 10^3$ per ml inoculum	% free cells divided	P.E.
1	Inoculum on top of B-5 agar	1 ml	6.1	60	0
2	Inoculum on top of conditioned agar	1 ml	6.1	56	0
3	Inoculum mixed in conditioned agar	10 ml	1.2	61	1%
4	Inoculum on top of feeder agar	1 ml	3.4	81	10%

Table 2. The effect of plating conditions in the replating procedure on the plating efficiency of *Haplopappus gracilis* cells

Exp. No.	Plating medium	First plating		Replating			P.E.
		vol. of inoculum	cell density $\times 10^3$ per ml	plating medium	vol. of inoculum	cell density $\times 10^3$ per ml	
1	Feeder agar	exp. a 10 ml	0.2	feeder agar	2 ml	0.02	60 %
		b 10 ml	0.05		4 ml	0.01	9 %
		c 10 ml	1.7		1 ml	0.35	89 %
		d 10 ml	1.6		1 ml	0.32	78 %
		e 10 ml	1.9		1 ml	0.39	76 %
		f 10 ml	5.3		1 ml	0.53	80 %
2	B-5 agar	10 ml	9.1	feeder agar	1 ml	0.9	63 %
3	B-5 agar	10 ml	3.2	B-5 agar	1 ml	3.2	54 %
4	B-5 liquid medium	3 ml	16.0	B-5 agar	1 ml	2.0	29 %

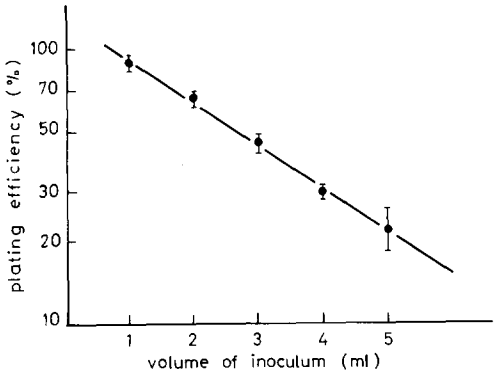

Fig. 6. The effect of the volume of inoculum in the replating step on plating efficiency of *Haplopappus gracilis* cells. After the first plating step, the soft agar containing small aggregations was diluted and divided in protions of 1 ml, each containing 275 units. Soft agar was added to give final volumes of 1, 2, 3, 4 and 5 ml and the resulting inocula were replated. After two weeks the visible colonies were counted. The standard error is indicated by the bars

the shortage of nutrients is the reason that the remaining aggregations have not grown to visible colonies since they are replated in fresh feeder-medium. However, inefficient gas exchange may still occur with those aggregations which are situated at the bottom of the medium-layer of the inoculum.

Discussion

It is clear from the results presented in Tables 1 and 2 that the procedure developed in this investigation leads to a high plating efficiency of free plant cells. At low plating densities (< 1000 cells per petri dish), about 80 % of the plated free cells develop into a visible colony.

Extrapolation of the relationship between the volume of the inoculum used in the replating step and the plating efficiency indicates that at a volume of 0.75 ml the plating efficiency would be 100 %. In this investigation petri dishes were used which have a diameter of 90 mm. Consequently a volume of 0.75 ml corresponds to a depth of 0.12 mm which is the average diameter of the aggregations at the time of replating. Due to surface tension problems it is not possible to spread 0.75 ml of inoculum over the total surface of the feeder agar in the petri dish and it has not yet been possible to achieve layers of inocula shallower than 0.15 ml, the depth achieved when 1 ml of inoculum is completely spread over the petri dish. This means that about 20 % of the replated aggregations will be covered with a layer of soft agar which probably prevents optimal gas exchange and inhibits development into visible colonies.

The result showing that the volume of the inoculum used in the replating step determines the plating efficiency indicates that the cells that do not develop into a visible colony do not represent a special group of the population. Therefore, this new procedure is ideally suited for a study of the cellular effects of radiation, environmental pollutants, mutagenic agents, viruses, pathogenic microorganisms, etc.

Inherent to the procedure adopted for the production of free cell suspensions is a considerable loss of cell material. This is due to the low number of free cells in the untreated suspension cultures. It may be possible, however, that variation in the physiological conditions during the treatments with chelating agents

and cell wall degrading enzymes will eventually lead to cell suspensions which have high numbers of free cells and growth characteristics comparable to the untreated cultures. These problems are currently being studied, but in the mean time the replating technique as described here is being successfully used in our laboratory to study the cellular effects of ionizing radiation on free plant cells.

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