Planta 149, 7–18 (1980)

Planta

Nutritional Requirements of Protoplast-derived, Haploid Tobacco Cells Grown at Low Cell Densities in Liquid Medium

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Abstract. Preliminary attempts to define a completely synthetic medium able to support divisions of haploid tobacco mesophyll protoplasts at low initial densities have failed. High protoplast concentrations together with large amounts of naphtaleneacetic acid in the medium (3 mg l^{-1} NAA) were required for maximal induction of protoplast division. However, cell suspensions derived from haploid protoplasts after four days of preculture at high initial cell densities could be diluted to densities as low as 1–4 cells ml^{-1}, provided the concentration of NAA in the medium was lowered to below 0.3 mg l^{-1}. The optimal NAA supply for low cell density growth was affected by the nature of the nitrogen source.

A simple minimal medium which supports the growth of these haploid cells with a plating efficiency of 30–40%, independent of the cell density in the range of 1–4 to $3 \cdot 10^4$ cells ml^{-1}, has been established. In this medium inositol was the only vitamin stringently required for growth.

Growth of cells at low densities was also possible in a medium initially containing 3 mg l^{-1} NAA, provided it was conditioned by the growth of protoplasts at high densities. Preliminary experiments with [^{14}C]NAA showed that the amount of free NAA remaining in the medium after preincubation at high densities was drastically reduced. Simultaneously, NAA conjugates accumulated in the medium. The implications of these results are discussed.

Key words: Auxin metabolism – Cell suspensions (low density) – Medium conditioning – *Nicotiana* – Nitrogen metabolism – Protoplasts – Vitamin requirements.

Abbreviations: BA = 6-benzyladenine; EDTA = ethylene diaminetetraacetic acid; NAA = naphtaleneacetic acid

Introduction

Mesophyll protoplasts, e.g., from haploid plants of tobacco (*Nicotiana tabacum*), should provide a suitable material for the in vitro selection of various "biochemical" mutants (Carlson 1973; Bourgin 1978). However, as for other systems of plant cells cultured in vitro, relatively high protoplast densities are required to allow colony formation (e.g., Nagata and Takebe 1971). This requirement presents several drawbacks for the extension to higher plant protoplasts of the genetic methodology developed for microorganisms or animal cells cultured in vitro.

(i) Determination of the actual toxicity of mutagenic agents and of selective drugs might be considerably biased under conditions involving high lethalities, because the few remaining living cells would not divide.

(ii) Cross-feeding effects could hamper the recovery of mutant clones in selection procedures. For instance, drug-susceptible wild-type cells when killed by the selective agent might induce a secondary poisening of drug-resistant cells (Fujimoto et al. 1971).

Techniques involving the use of feeder layers to support growth of cells at low density through conditioning effects could partially overcome these problems (Raveh et al. 1973; Weber and Lark 1979). This type of procedure should be useful for quantifying the effect of mutagenic treatments. However, the use of a defined medium allowing the proliferation of cells or protoplasts at low cell densities would be, at least theoretically, a preferable alternative to the in vitro selection of mutant cells, since it could be employed more easily and possibly used for selecting a wider range of mutant types. Recent studies on the growth requirements of cells cultured at low population densities, either in agar-solidified medium (Lo-

0032-0935/80/0149/0007/$02.40

Table 1. Cell and protoplast culture media

Compound	Protoplast culture medium T_0		Cell culture medium A		Cell culture minimal medium M	
	mg l^{-1}	mM	mg l^{-1}	mM	mg l^{-1}	mM
Macronutrients						
NH_4NO_3	825	10.3	800	10	400	5
KNO_3	950	9.4	1,010	10	–	–
$CaCl_2 \cdot 2\,H_2O$	220	1.5	440	3	293	2
$MgSO_4 \cdot 7\,H_2O$	185	0.75	738	3	246	1
KH_2PO_4	85	0.62	136	1	68	0.5
	mg l^{-1}	µM	mg l^{-1}	µM	mg l^{-1}	µM
Micronutrients						
$FeSO_4 \cdot 7\,H_2O$	27	100	27	100	27	100
Na_2EDTA	37	100	37	100	37	100
H_3BO_3	1	16	3	49	6.2	100
$MnSO_4 \cdot H_2O$	0.1	0.6	0.3	1.8	0.17	1
$ZnSO_4 \cdot 7\,H_2O$	1	3.5	3	10.4	0.28	1
$CoCl_2 \cdot 6\,H_2O$	–	–	0.01	0.04	0.024	0.1
$CuSO_4 \cdot 5\,H_2O$	0.03	0.12	0.09	0.36	0.025	0.1
$Na_2MoO_4 \cdot 2\,H_2O$	–	–	0.1	0.41	0.024	0.1
$AlCl_3$	0.03	0.22	0.09	0.66	–	–
$NiCl_2 \cdot 6\,H_2O$	0.03	0.13	0.09	0.40	–	–
KI	0.01	0.06	0.03	0.18	–	–
	mg l^{-1}	µM	mg l^{-1}	µM	mg l^{-1}	µM
Organic substances						
Inositol	100	555	100	555	180	1,000
Thiamine·HCl	1	3	1	3	–	–
Pyridoxine·HCl	1	5	1	5	0.5	2.5
Nicotinic acid	1	8	1	8	–	–
Ca pantothenate	1	2	1	2	–	–
Biotine	0.01	0.04	0.01	0.04	–	–
NAA	3	16.1	0.1	0.53	0.1	0.53
BA	1	4.4	1	4.4	1	4.4
Sucrose	$2 \cdot 10^4$	0.058 M	$2 \cdot 10^4$	0.058 M	$2 \cdot 10^4$	0.058 M
Mannitol	$8 \cdot 10^4$	0.44 M	$8 \cdot 10^4$	0.44 M	$8 \cdot 10^4$	0.44 M
Tween 80	8	–	–	–	–	–
pH of the medium	5.6		5.6		6.2	

Culture media were brought to pH 5.5–5.7 with sodium hydroxyde after addition of all ingredients and then sterilized by autoclaving for 20 min at 110° C. Media T_0 and A contained the vitamin mixture of Morel and Wetmore (1951). Medium A was often supplemented with 1 mM glutamine aseptically added after autoclaving (Medium AG)

gemann and Bergmann 1974) or in liquid medium (Kao and Michayluk 1975), did not lead to the characterization of chemically defined media able to support growth with satisfactory plating efficiencies of cells cultured at very low population densities (such as 1–4 cells ml^{-1}). As attempts to develop a defined medium supporting the growth of haploid tobacco mesophyll protoplasts at low cell densities have failed, the present paper describes an alternative procedure which allows the growth at very low densities in a defined medium of cells derived from protoplasts after their preculture at high population densities. A preliminary study of the mechanism involved in the conditioning process of the culture medium by these high density populations of protoplasts is also reported.

Materials and Methods

Plant Material. Nicotiana tabacum (c.v. Xanthi) haploid plants were derived from a single clone obtained by anther culture. These plants were grown under semi-controlled light and feeding conditions, as described previously (Chupeau et al. 1974).

Media. Two types of media, differing mainly in their NAA content, were used in these studies. The composition of these media is given in Table 1. Medium T_0, containing 3 mg l^{-1} NAA, was used

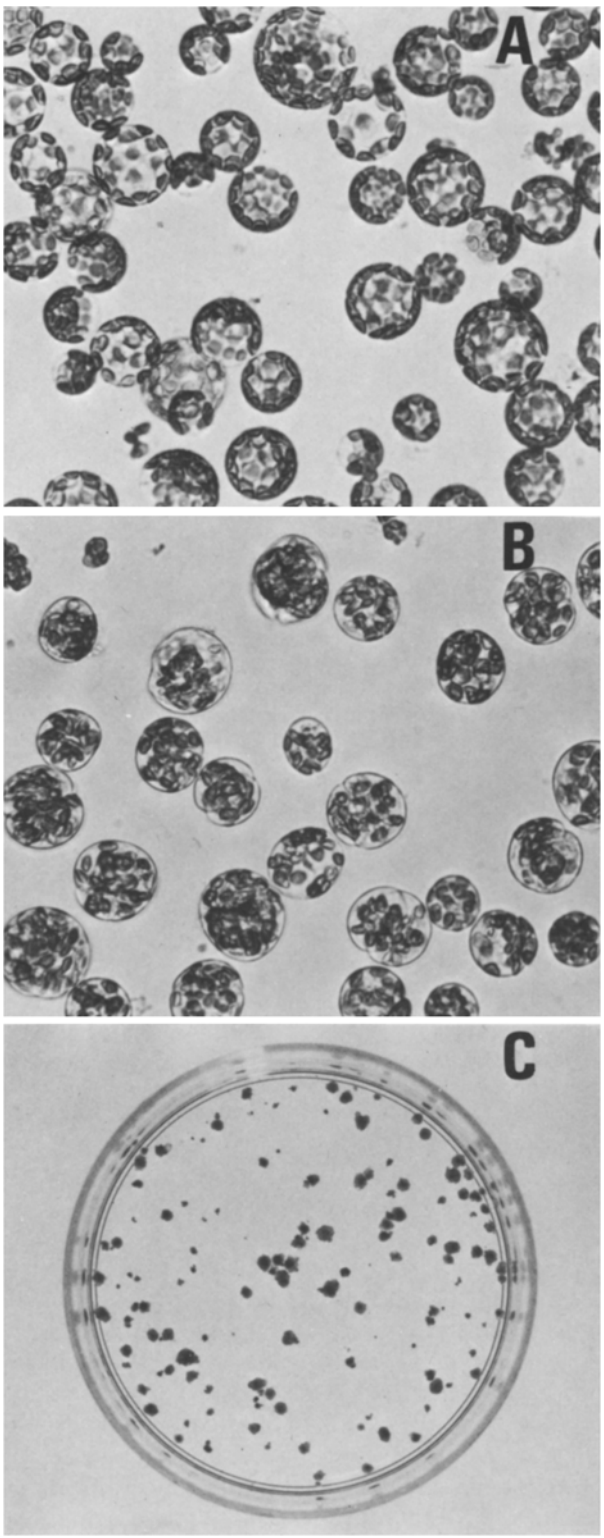

Fig. 1 A–C. Growth at low cell densities of protoplast-derived cells. **A** Protoplasts at the time of isolation, before plating in medium T_0. **B** Protoplast-derived cells after four days of incubation in medium T_0 and at the time of their transfer to medium AG. **C** Culture incubated for one month after plating of 400 protoplast-derived cells in 4 ml of medium AG

for protoplast isolation and plating as described previously (Bourgin et al. 1979). Medium A, containing 0.1 mg l^{-1} NAA, was used to grow protoplast-derived cells at low densities. The macrosalt composition of this medium was derived from that of medium T_0 where calcium chloride and magnesium sulfate concentrations were increased to 3 mM each. Generally, medium A was supplemented with 1 mM filter-sterilized glutamine, aseptically added after heat sterilization of the medium (medium AG). In experiments where the nitrogen source had to be varied, a basal medium identical to medium A but lacking ammonium and potassium nitrates was used (medium N^-).

Protoplast Isolation. The procedure for protoplast isolation has been described in detail by Chupeau et al. (1974). Briefly, sterilized leaves were peeled and incubated overnight in a T_0 medium lacking sucrose and containing 0.02% Macerozyme R10, 0.1% Cellulase Onozuka R10, and 0.05% Driselase. The liberated protoplasts were washed twice by low-speed centrifugation in the W 0.6 macrosalts solution of Meyer and Abel (1975).

Protoplast Culture. Washed protoplasts were resuspended in medium T_0 with the standard dilution of $7 \cdot 10^4$ protoplasts ml^{-1} (Fig. 1 A). They could be kept at 4° C for 1 week before plating without significant losses of viability. Protoplast cultures were incubated in Petri dishes at 25° C in the dark for four days and then transferred under fluorescent lamps (2,500 lx, 16 h per day). The first protoplast divisions occurred two days after the beginning of incubations.

Plating of Protoplast-derived Cells at Low Densities. Protoplast cultures were used for low-density growth experiments when on the average 30–60% of protoplasts had divided once. This was usually the case on the fourth day of incubation, provided that relatively young leaves (5–8 cm wide) had been used for the preparation of the protoplasts (Fig. 1 B). A low but significant proportion of colonies at the four-cell stage was also observed (up to 15% of the plated protoplasts in some experiments). These cells, obtained after a four-day incubation of protoplasts, were used for growth experiments at low cell concentrations and will be hereafter referred to as "protoplast-derived cells". Cells were then collected by low-speed centrifugation and washed once in medium A. Cell concentration was usually decreased from 10^5 to 10^2 cells ml^{-1} by serial ten-fold dilution in medium A, and the resulting suspensions were plated in 5.5 cm Petri dishes (4 ml/dish). The dishes were then incubated in standard light conditions at 25° C in tightly closed, transparent plastic boxes.

Determination of Plating Efficiencies. Plating efficiencies were expressed as the percentage of protoplasts or protoplast-derived cells growing to colonies. When plating efficiencies were estimated in high density growth conditions the amount of colonies per ml of culture was counted with an Agasse-Lafont hematocytometer on two-week-old cultures. In medium T_0, plating efficiencies of protoplasts were in the range of 60–90%, provided young leaves had been used. When plating efficiencies were estimated at low cellular densities (1 to 300 cells ml^{-1}), visible colonies were directly scored after three weeks of incubation (Fig. 1 C). In medium AG, the plating efficiency of protoplast-derived cells was usually in the range of 35–45%. Plating-efficiency determinations were done on duplicate cultures. The standard deviation of measurements in medium AG was 6% in a control experiment where ten identical cultures were scored.

Incorporation Experiments with Labelled Hormones. [1-^{14}C] Naphtaleneacetic acid ($2.3 \cdot 10^{12}$ Bq mol^{-1}) was a gift from Pr J. Guern. Protoplasts were incubated in an hormone-deprived T_0 medium supplemented with $1.13 \cdot 10^3$ Bq ml^{-1} of the tracer and cold NAA up to a final concentration of 3 mg l^{-1}. For the determination of incorporated radioactivities, protoplasts were collected by low-

pressure filtration through Whatman glass-fiber filters (GF/C) and the filters were immediately rinsed with 2 ml cold medium T_0. Care was taken to avoid dehydration of the filter during the procedure. NAA and derived metabolites were analyzed, according to the procedure of Leguay and Guern (1977), in the culture media or in ethanol extracts from the cells. Culture media were brought to pH 2.5 with phosphoric acid and then extracted twice with ether at room temperature. After evaporation, the extracted metabolites were dissolved in ethanol and separated on silica gel TLC plates by chromatography in a mixture of chloroform, ethyl acetate, and formic acid (50:40:10, v/v/v). NAA spots were located by autoradiography, carefully scraped, and counted after ethanol elution. The scintillation cocktail was composed of toluene (1 l), triton X-100 0.5 l), PPO (4 g) and dimethyl POPOP (0.1 g). All radioactivity measurements were corrected for quenching.

Results

Our first attempts to grow protoplasts directly at low cell densities in medium T_0 showed us that a sharp decrease in plating efficiency occurred when protoplasts were plated at concentrations below 10^4 protoplasts ml^{-1}. Protoplasts appeared to collapse before regeneration of the cell wall was completed. We thus tried to culture cells at low densities after cell-wall regeneration and initiation of the first division of the protoplasts had taken place, and we decided to use four-day-old, protoplast-derived cells.

Preliminary Attempts to Characterize a Defined Medium Supporting Growth at Low Cell Densities. When protoplast-derived cells were diluted at low densities in medium T_0 (less than $5 \cdot 10^3$ cells ml^{-1}), they became brownish and died. The inability of plant cells to grow at low concentrations may be caused by excessive diffusion of metabolites into the medium, resulting in their intracellular decrease to a level below that required for cell survival (Ham 1965). Our first attempts to increase cell survival consisted therefore of adding various substances to the culture medium (vitamins, aminoacids, bases, casein hydrolysate, and yeast extract). These attempts were unsuccessful. A significant increase of plating efficiency was obtained, however, when organic acids such as succinate, malate, or citrate were added to the medium, confirming the observations of Kao and Michayluck (1975). For instance, addition of 1 mM succinate in the medium resulted in a three- to ten-fold decrease in the minimal cell density which still allowed growth. Since organic acids were shown by Gamborg and Shyluk (1970) and by Behrend and Mateles (1976) to interfere with ammonium metabolism, we examined the influence of the nitrogen source on growth at low densities. Indeed, media containing only 10 mM ammonium chloride and 1 mM ammonium succinate as the sole nitrogen supply allowed more reproducible results than the mixed standard ammonium+nitrate source of medium T_0.

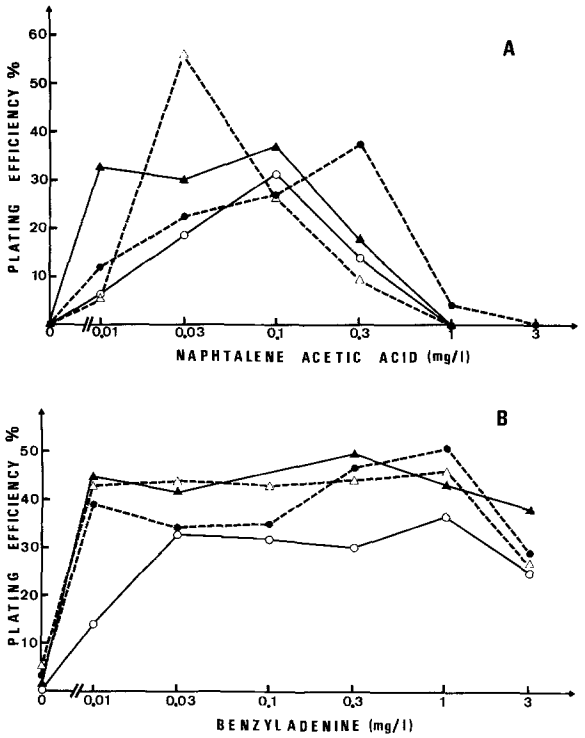

Fig. 2A and B. Effect of naphtaleneacetic acid and benzyladenine concentrations on the plating efficiency of cells grown at low densities. Protoplast-derived cells were washed in medium A deprived of nitrogen source and hormones. Cells were then plated at low cell density (10^2 cells ml^{-1}) in various hormone-free media containing either 0.3 mg l^{-1} benzyladenine and variable amounts of naphtaleneacetic acid (**A**) or 0.1 mg l^{-1} naphtaleneacetic acid and variable amounts of benzyladenine (**B**). After incubation for one month visible colonies were counted and plating efficiencies estimated. Hormone requirements were compared with various nitrogen sources by modification of medium A. ▲ Ammonium and nitrate (Medium A, containing 10 mM NH_4NO_3 and 10 mM NO_3K); △ Nitrate alone (Medium A minus 10 mM NO_3NH_4); ● Ammonium alone (Medium N^- containing 10 mM NH_4Cl and 1 mM potassium malate); ○ Glutamine alone (Medium N^- containing 1 mM glutamine)

Further progress in experimental reproducibility was made by increasing calcium chloride and magnesium sulfate concentrations to 3 mM each (see mineral composition of medium A, Table 1). Tween 80, used in the medium T_0 to prevent protoplast bursting in plastic Petri dishes, also appeared in some experiments to be toxic at low cell concentrations and was thereafter omitted. However, all these modifications did not allow reproducible clonal growth conditions below 10^3 cells ml^{-1}. Thus, we considered the possibility that our culture media might be toxic for cells plated at low concentrations rather than deficient in any component, and we therefore decided to re-assess the optimal requirements of the various compounds included in medium T_0.

Growth-substance Requirements for Growth at Low Cell Densities. These requirements were studied in medium A (Table 1). NAA concentration strongly affected the plating efficiency of cells in this medium, as shown in Fig. 2A. The standard NAA concentration of medium T_0 (3 mg l^{-1}) was strongly inhibitory. The plating efficiency of cells was highly increased if the NAA concentration was lowered below 0.3 mg l^{-1}, the BA concentration being kept at 0.3 mg l^{-1}. When NAA concentration was lowered to 0.1 mg l^{-1}, growth was possible at cell densities as low as 10^2 cells ml^{-1} with a plating efficiency in the range of 30%. However, auxin requirements were not abolished, since in the absence of NAA growth stopped after a few divisions.

The composition of the nitrogen source clearly affected the optimal auxin requirements (Fig. 2A). When cells were grown on nitrate as the sole nitrogen source, highest plating efficiencies were observed for NAA concentrations in the range of 0.03 mg l^{-1}. On the other hand, when cells were grown on a mixture of ammonium and malate, a significant increase in plating efficiencies at high NAA concentrations was observed. This must be related to previous observations on the improvement of plating efficiencies at low cell densities in medium T_0 (in the presence of 3 mg l^{-1} NAA), when organic acids were added to the medium. Growth on a mixture of ammonium and nitrate or 1 mM glutamine alone led to intermediate optimal NAA requirements.

Plating efficiency at low cell densities was not strongly dependent on BA concentration. When NAA concentration was kept to 0.1 mg l^{-1} and BA concentration was varied from 0.01 to 3 mg l^{-1}, only limited toxicity was observed at the highest concentrations, whatever nitrogen source was used (Fig. 2B). The absence of cytokinin in the culture medium drastically decreased plating efficiency, but small colonies were occasionally observed in cytokinin-deprived cultures.

Effect of Cell Density on Plating Efficiency of Protoplast-derived Cells. Plating efficiency, although it increased when NAA concentration was reduced, might be dependent on cell density, a specific ratio between NAA concentration and the number of cells per milliliter of medium being required for growth. To test this hypothesis protoplast-derived cells were prepared as described in Materials and Methods, and their plating efficiencies were measured at various dilutions in medium A containing 0.1 mg l^{-1} NAA. Plating efficiencies did not depend on cell density, even as low as 1–4 cells per ml (Fig. 3). At increasing cell densities, apart from the shorter period of growth probably resulting from auxin deprivation, the plating efficiency of cells was significantly decreased only above

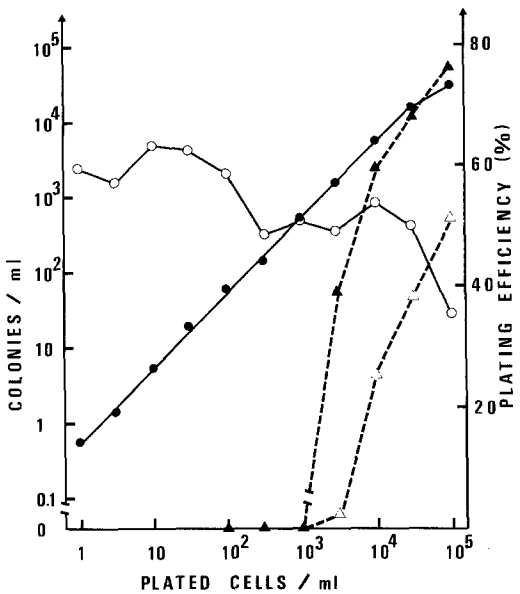

Fig. 3. Plating efficiency at variable cell densities. Protoplasts derived cells were plated either in medium A (● ○) or in the same medium supplemented with 3 mg l^{-1} NAA (▲ △). The abscissa indicates the number of cells plated per ml of culture medium and the left ordinate (● ▲) gives the number of resulting colonies. The right ordinate gives the plating efficiency as a function of initial cell density (○ △) in the same experiment

$5 \cdot 10^4$ cells ml^{-1}. Identical results were obtained when cells were plated in medium A containing 1 mM glutamine (medium AG). In this medium, the growth rate of colonies was increased so that the number of colonies could be scored three weeks after plating instead of five weeks after plating in medium A. A 20–25% reduction of plating efficiency was observed, however, at all cell densities in this medium as compared to medium A.

In the same experiment, the plating efficiency of protoplast-derived cells was also estimated in a modified medium A containing 3 mg l^{-1} NAA. When cell densities were lower than $3 \cdot 10^3$ cells ml^{-1}, no colonies were formed, but when cell densities were kept above 10^4 cells ml^{-1}, plating efficiencies in this medium were similar to the controls in medium A (Fig. 3). When protoplast-derived cells were washed twice in medium AG before dilution and then plated at low density in the same medium, their plating efficiency was not significantly affected by the washing procedure.

Effect of the Conditions of Preincubation on the Plating Efficiencies of Protoplasts and of Subcultured Protoplast-derived Cells. The duration of the preincubation of protoplasts in medium T_0 at a high cell density ($7 \cdot 10^4$ protoplasts ml^{-1}) strongly affected the subsequent plating efficiency of cells diluted in medium

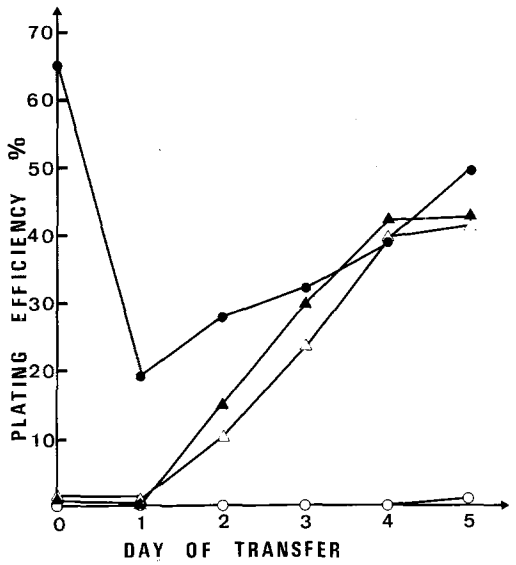

Fig. 4. Relations between the length of the period of preincubation of protoplasts in medium T_0 and their subsequeht plating efficiencies at various cell densities in T_0 and AG media. Protoplasts were incubated under standard conditions ($7 \cdot 10^4$ protoplasts ml^{-1}) in medium T_0 and thereafter washed and plated either in AG medium (▲, 10^2 cells ml^{-1} or △, 10^3 cells ml^{-1}) or in fresh medium T_0 (○, 10^3 cells ml^{-1} or ●, $2 \cdot 10^4$ cells ml^{-1}) for various times after the beginning of the preincubation period. After three weeks of incubation plating efficiencies were estimated

Table 2. Relations between auxin and cytokinin concentrations during preincubation of protoplasts in T_0 medium and their subsequent plating efficiency in AG medium at low cell density

Auxin and cytokinin concentrations in the preculture medium (mg l^{-1})		Plating efficiencies (%)	
NAA	BA	in the preculture medium	after transfer to AG medium
3	1	67	29
3	0.3	58	28
3	0.1	63	29
1	1	46	22
1	0.3	54	25
1	0.1	51	26
0.3	1	35	6
0.3	0.3	33	12
0.3	0.1	30	4
0	1	0	0
1	0	0	0

Protoplasts were isolated from haploid tobacco leaves as described under Materials and Methods except that auxin and cytokinin were omitted during overnight maceration treatment.

Protoplasts were then washed and incubated in medium T_0 containing naphtaleneacetic acid (NAA) and benzyladenine (BA), as specified, at a final cell concentration of $7 \cdot 10^4$ protoplasts ml^{-1}. Part of the cultures were incubated for ten days and kept for direct determination of plating efficiencies by counting under the microscope in the preculture medium. Four days after the beginning of preincubation the remaining cultures were washed and diluted in medium AG (0.1 mg l^{-1} NAA and 1 mg l^{-1} BA) to a final concentration of 10^2 cells ml^{-1} and further incubated for three weeks up to the appearance of visible colonies. Plating efficiencies in medium AG were then measured

AG (Fig. 4). When protoplasts were directly plated in medium AG after isolation, plating efficiency was very low although not nil (1%). After two days of incubation in medium T_0, the plating efficiency of protoplast-derived cells increased sharply. This increase was correlated with the appearance of mitoses in the protoplast cultures. When the period of preincubation was further lengthened plating efficiency reached a plateau. An optimal preincubation period of four days was therefore used in further experiments. If protoplast-derived cells were transferred to fresh medium T_0 instead of medium AG after preincubation, their plating efficiency at 10^3 cell ml^{-1} was nil (Fig. 4). Even at a higher cell density (10^4 cell ml^{-1}), transfer to medium T_0 induced a sharp reduction of plating efficiency, suggesting an increased sensitivity of preincubated cells to the high concentration of NAA included in medium T_0.

The auxin concentration during preincubation, but not the cytokinin concentration, affected the plating efficiency of cells after transfer at low cell density to medium AG (Table 2). Plating efficiency of protoplasts was also clearly affected by NAA concentration, as already reported by Schilde-Rentschler (1973), but not by BA concentration in the range of 0.1–1 mg l^{-1}. In conclusion during preincubation a high initial auxin concentration (3 mg l^{-1}) was required to induce high plating efficiencies both in preculture medium T_0 and in medium AG after dilution to low cell densities.

Effect of Inorganic Components of the Medium on the Growth at Low Cell Densities. Cell culture at low density, under conditions minimizing conditioning of the culture medium by the cells, was used to investigate more carefully their nutritional requirements and to elaborate a minimal essential medium.

Nitrogen requirements were studied in a modified medium A deprived of ammonium and nitrate (Fig. 5A). Cells were able to grow in the complete absence of ammonium and optimal nitrate feeding was obtained with 1–5 mM potassium nitrate. Conversely, cell growth was possible in the absence of nitrate, provided the ammonium chloride concentration was kept between 1 and 20 mM. In agreement with previous reports, e.g., Gamborg and Shyluk (1970), ammonium-fed cells could grow at the usual

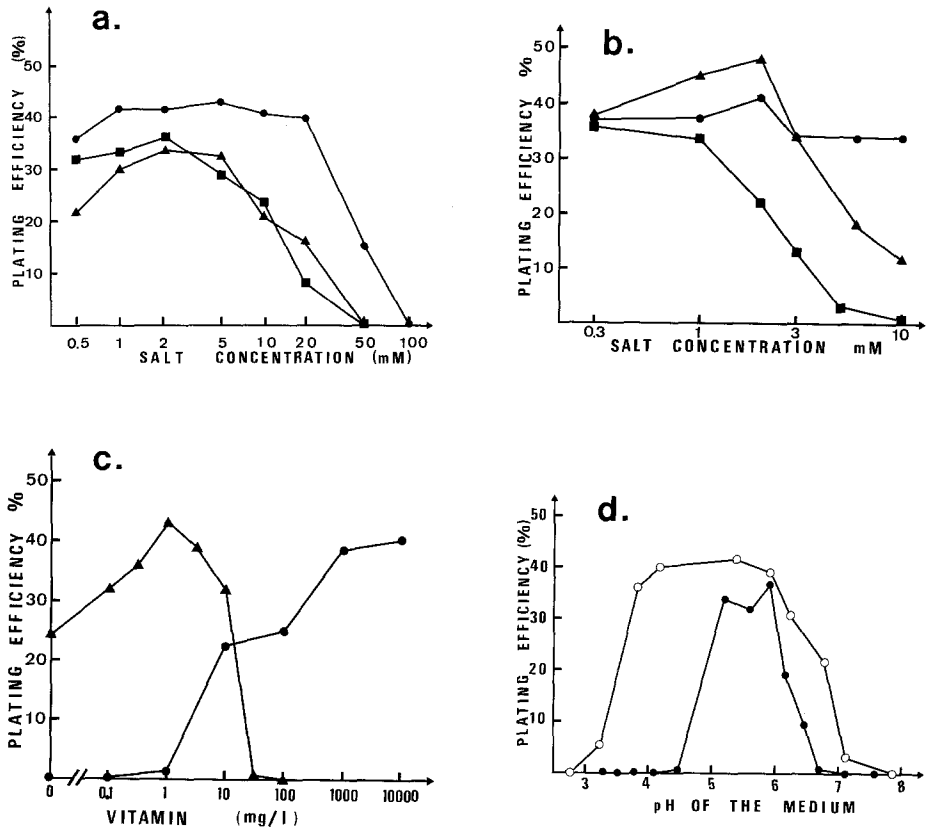

Fig. 5a–d. Optimal requirements for low cell density growth. **a** Nitrogen source. Protoplast-derived cells were washed twice in medium N^- (medium A deprived of nitrogen source) and then plated at 10^2 cells ml^{-1} in the same medium. Variable concentrations of potassium nitrate (▲) or ammonium chloride (●) or ammonium nitrate together with equimolar amounts of potassium nitrate (■) were then aseptically added to the Petri dishes. When cells were incubated in the presence of ammonium as the sole nitrogen source media were supplemented with 1 mM potassium malate, pH 5.5. After incubation for one month plating efficiencies were measured. **b** Other macronutrients. Protoplast-derived cells were washed and plated in medium AG deprived of $CaCl_2$ (▲), $MgSO_4$ (●) or K_2HPO_4 (■) at a cell density of 10^2 cells ml^{-1}. The required salt was then added at increasing concentrations and cultures were incubated for three weeks before estimation of plating efficiencies. **c** Vitamins. Protoplast-derived cells were washed and plated at low cell density (10^2 cells ml^{-1}) in medium AG deprived of vitamins and containing increasing amounts of inositol (●) or in medium AG containing 100 mg l^{-1} of inositol and increasing amounts of pyridoxine (▲). Filter-sterilized vitamins were used. After a three week incubation plating efficiencies were estimated. Pyridoxine not only increased plating efficiencies but also the average size of resulting colonies. **d** pH optimum. Medium A was prepared and autoclaved under standard pH conditions. Protoplast-derived cells were than plated in this medium at low cell density (10^2 cells ml^{-1}), ●) or high cell density (10^4 cells ml^{-1}, ○) and the cultures were then brought to the required pH by aseptic addition of 0.1 M hydrochloric acid or sodium hydroxide. After incubation for one month plating efficiencies were estimated

rate with good plating efficiency only if organic acids were present in the medium. Growth-promoting effects of various organic acids and amino acids were compared in the presence of 10 mM ammonium chloride: the organic acids directly related to the Krebs cycle were the most effective at low concentrations (Table 3). Among the amino acids tested at 1 mM, aspartate, glutamate, and alanine were uneffective, whereas glutamine promoted growth but the plating efficiency thus obtained was lower than with glutamine (1 mM) used as the sole nitrogen source (results not shown).

The concentration of *other macrosalts* of the medium A were varied separately (Fig. 5B). Optimal calcium chloride requirements were in the range of 1–2 mM. Magnesium sulfate optimal requirements were difficult to estimate: concentrations could be varied between 0.3 and 10 mM without notably affecting the plating efficiency. However, high concentrations (10 mM) of these two salts reduced growth rates of the colonies. Phosphate (KH_2PO_4) was toxic when its concentration exceeded 1 mM. Although potassium requirements were not precisely examined, it is noteworthy that 1 mM potassium brought as phosphate salt in ammonium medium allowed high plating efficiency.

Table 3. Action of organic acids on the plating efficiency of cells grown on a medium containing 10 mM ammonium chloride as the sole nitrogen source

Organic acid concentration	Plating efficiency (%)			
	0 mM	0.1 mM	0.3 mM	1 mM
Acetic acid	5[a]	3[a]	0	0
Citric acid	–	15	5	0.1
Fumaric acid	–	13	17	27
Isocitric acid	–	18	26	13
α-ketoglutaric acid	–	28	38	39
Lactic acid	–	2[a]	0	0
Malic acid	–	26	38	29
Malonic acid	–	12	19	36
Oxalic acid	–	0	0	0
Pyruvic acid	–	13	17	22
Succinic acid	–	30	43	36

Protoplast-derived cells were washed and plated in medium N^- supplemented with 10 mM ammonium chloride (see Materials and Methods) at a final concentration of 10^2 cells ml^{-1}. Organic acids were brought to pH 5.5 with potassium hydroxyde before filter sterilization and introduction into the medium at the specified concentration. After a three week incubation visible colonies were counted and plating efficiencies calculated

[a] Very small colonies were observed in the control and in the presence of low amounts of acetic or lactic acid

Table 4. Effect of the composition of carbohydrate source on plating efficiency

Carbohydrate source	Concentration ($g\,l^{-1}$)	Plating efficiency
Sucrose	20	56
–	2	51
–	0.2	42
–	0	0
Arabinose	10	0
Fructose	10	0.3[a]
Galactose	10	0
Glucose	10	35
Inositol	10	2[a]
Lactose	20	36[a]
Mannose	10	39
Ribose	10	0.1[a]
Xylose	10	0

Protoplast-derived cells were washed in sucrose-free medium AG containing 80 $g\,l^{-1}$ mannitol and then plated at 10^2 or 10^3 cells ml^{-1} in the same medium supplemented with various sugars. After incubation for three weeks plating efficiencies were estimated.

[a] Very small colonies were observed when cells were incubated in the presence of fructose, inositol, ribose or lactose

Micronutrient requirements were also studied: since we did not use specially purified macrosalts and sugars, the aim of this work was rather to test an eventual toxicity of micronutrients at the standard concentration used in our medium, such as that found for potassium iodide by Eriksson (1965). Preliminary observations showed that Murashige and Skoog's micronutrient solution (1962) was less effective than the modified solution of Heller (1953) used in T_0, and that a three-fold increase in the concentration of this last formula further increased plating efficiency. This latest solution completed with molybdenum and cobalt salts from Murashige and Skoog's solution was therefore used in standard experiments with medium AG (Table 1).

A systematic study of the requirements for individual micronutrients was performed later. No clear requirements were observed for zinc, cobalt, molybdenum, iodine, aluminium, and nickel. These last three micronutrients were generally considered non-essential (Price et al. 1972) and were thereafter omitted. Boron, iron, and possibly copper increased plating efficiency and were supplied in the minimal medium at the final concentrations of 100, 100, and 0.1 μM, respectively. Manganese and zinc appeared toxic above 3 μM and were supplied at 1 μM each in the medium. Molybdenum and cobalt were supplied at the lowest tested concentration 0.1 μM (see micronutrient composition of medium M, Table 1).

The influence of pH on growth at low cell density was studied in medium A (Fig. 5D). When the pH was adjusted before autoclaving the media, salt precipitation associated with a shift toward pH 6 occurred. The media were therefore autoclaved after adjusting the pH to 5.5 and were then aseptically brought to the required pH by addition of hydrochloric acid or sodium hydroxyde. Under these conditions a relative instability of alkaline media in the incubation conditions was still observed, their pH decreasing towards pH 6 at an average rate of 0.5 units per day. The optimal initial pH value appeared to be in the range 5–5.5. Growth of cells plated at higher concentration (10^4 cells ml^{-1}) in the same media was observed in a larger range of pH values (from pH 3.5 to 7), suggesting that cells can rapidly modify the pH of the culture medium, in agreement with previous observations (Rossini 1973).

Organic Requirements for Growth at Low Cell Densities. A *carbohydrate source* was required for growth in medium AG (Table 4). Sucrose, glucose, and mannose supported cell growth with good plating efficiency. Contrarily, arabinose, fructose, galactose, ribose, and xylose were not efficient carbohydrate sources. The minimal concentration of sucrose allowing growth was 0.2 $g\,l^{-1}$ but this low concentration resulted in an earlier arrest of colony growth, probably by carbohydrate deprivation.

The *vitamin supply* included in medium A is composed of myo-inositol, thiamin, pyridoxin, pantothenic acid, nicotinic acid and biotin, according to Morel and Wetmore (1951). Among those vitamins, only inositol was strictly required for growth at low cell densities. In its absence colony growth stopped after a few divisions. The standard concentration of this vitamin in medium A (100 mg ml^{-1}) was found to be suboptimal and a gradual increase in plating efficiency was observed up to concentrations as high as 1 g l^{-1} (Fig. 5C). However, at high cell densities (10^4 cells ml^{-1}) inositol was not absolutely required, although colonies grew slowly and appeared partially necrotic in its absence. Biotin, thiamin, pyridoxin, pantothenic acid, and nicotinic acid were not required for growth (results not shown). When supplied in the medium, together or separately, these vitamins did not affect growth, except for pyridoxin which induced a significant increase in plating efficiency. The effect of pyridoxin concentrations on plating efficiency in the presence of 100 mg l^{-1} inositol is presented in Figure 5C. Maximal plating efficiency was observed at a concentration of 1 mg l^{-1} pyridoxin, and this was associated with an increase in the growth rate of colonies. Thus, only inositol and pyridoxine were supplied in the minimal medium M (Table 1).

Among various organic substances (aminoacids, bases, vitamins, and organic acids) and mixtures (yeast extract, casein hydrolysate, and coconut milk) which were also tested, only coconut milk increased plating efficiencies in medium A. 1 mM glutamine, 1 mM malic acid, or 30 mg l^{-1} casein hydrolysate increased growth rates in medium A but had a limited effect on plating efficiency. Various aminoacids supplied at 0.1–0.3 mM were strongly toxic.

We have modified the composition of medium A according to the results of the above studies (see Table 1, medium M). In medium M the plating efficiency of protoplast-derived cells was reproducibly higher than in medium A (50% instead of 38%), and the growth rate of colonies was increased.

Effect of Light and Agar on Low Density Growth. Protoplast-derived cells were usually grown under light. Light was not absolutely required for low cell density growth. In medium A the absence of light during growth resulted in a 2.5-fold decrease of plating efficiency. When glutamine was added in the medium (medium AG) plating efficiency was more strongly reduced in the absence of light (1% instead of 38% in the control grown under light). Similar results were observed in medium M.

Various attempts to grow protoplast-derived cells on medium solidified with 0.8% agar were performed: cells were cultured in 0.5 ml of liquid medium spread on the top of 5 ml agar media in Petri dishes. Difco bacto-agar and Noble agar did not allow reproducible growth with good plating efficiencies below $5 \cdot 10^4$ cells/plate. When purified agar such as Indubiose A37 from IBF or Agarose C from Pharmacia was used, good plating efficiencies were obtained at a cell density of $5 \cdot 10^3$ cells/plate, but low plating efficiencies were observed at lower densities. We suggest that toxic substances or modification of the medium by adsorbtion of components on the agar might be involved in this inhibition of growth on agar media.

Table 5. Comparison of the plating efficiencies of protoplast-derived cells in AG medium and in freshly prepared or conditioned T_0 medium

Tested medium	Additive	Plating efficiency (%)	
		10^3 cells ml^{-1}	$2 \cdot 10^4$ cells ml^{-1}
T_0	–	0	17
T_0, conditioned	–	32	38
T_0, conditioned	1 mg l^{-1} NAA	0	19
AG	–	34	49
AG	1 mg l^{-1} NAA	0	39
T_0 and T_0 conditioned (1:1)	–	0	32
AG and T_0 conditioned (1:1)	–	29	54
AG and T_0 (1:1)	–	0	51

The plating efficiencies of protoplast-derived cells were estimated at low and high cell densities (10^3 or $2 \cdot 10^4$ cells ml^{-1}) in mixtures of various media. Medium T_0 was conditioned by incubation with freshly prepared protoplasts at high density ($7 \cdot 10^4$ protoplasts ml^{-1}) during four days. Cells were then removed by centrifugation and debris were further discarded by filtration on millipore filters (0.45 μm). The resulting medium was used for growth experiments

Conditioning of Medium T_0 during Incubation of Protoplasts at High Densities. The high auxin concentration in medium T_0 was found to be responsible for its toxicity toward cells cultivated at low density. However, medium T_0 conditioned by a four-day incubation period with high amounts of protoplasts supports the growth of protoplast-derived cells at low densities (Table 5). The process involved in this alteration of the medium was further studied. Three main hypotheses were considered to explain detoxification: (i) NAA is removed from the medium by protoplasts during preincubation; (ii) protoplasts induce the inactivation or degradation of NAA; (iii) NAA concentration is not directly affected by protoplasts, but substances are accumulated in the medium which al-

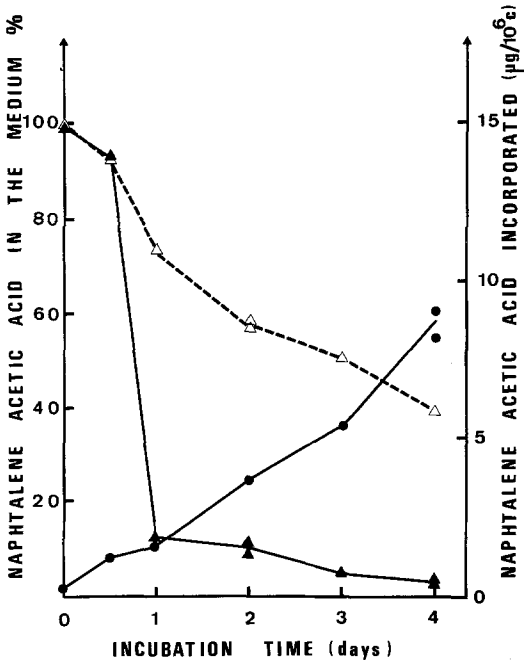

Fig. 6. Evolution of the amount of free and incorporated [^{14}C]naphtaleneacetic acid during the incubation of protoplasts at high densities in medium T_0. Protoplasts were incubated under standard conditions in medium T_0 containing [^{14}C]NAA ($1.13 \cdot 10^6$ Bq l^{-1}; 3 mg l^{-1}). Various times after the beginning of the incubations the radioactivity remaining in culture media (△) and incorporated into cells (●) was measured. The amounts of free NAA remaining in the media were also estimated after extraction and chromatography as described under Materials and Methods (▲)

low cells to be protected from the toxic action of NAA when they are grown in the conditioned medium at low concentrations. Whereas protoplast-derived cells grew at low density in a mixture of medium AG and conditioned medium T_0, a mixture of fresh and conditioned medium T_0 did not support low cell density growth (Table 5). The addition of 1 mg l^{-1} NAA to the conditioned medium also resulted in the loss of ability of this medium to support growth at low cell concentrations (Table 5). These observations allowed us to rule out the third hypothesis.

To test the validity of the first hypothesis we studied the metabolism of [^{14}C]NAA in medium T_0, in the presence of high concentrations of protoplasts conditioning the medium (Fig. 6). During the course of incubation the radioactivity of the medium progressively decreased. However, this decrease was not large enough to account for the detoxification of the medium. A four-day incubation period resulted in a 60% decrease in the amount of radioactivity in the medium equivalent to 1.2 mg l^{-1} NAA, a concentration still toxic for low density growth. In order to study the validity of the second hypothesis, NAA and its derivatives were separated by thin-layer chromatography and visualized by autoradiography. After incubation with protoplasts at the optimal cell density, the majority of radioactivity was detected in five spots: all of them were extracted by ether after acidification. The three spots showing low Rf values (0.44, 0.27, 0.11) probably corresponded to aminoacid conjugates and usually represented more than 80% of the extracted radioactivity. Free NAA was also detected and represented a very small proportion of total initial radioactivity (3–5%). The amount of free NAA in the medium decreased rapidly during the first day of incubation (Fig. 6). Simultaneously, a significant amount of radioactivity was accumulated by the cells but free NAA was almost undetectable in the cell extracts.

These observations were in agreement with our second hypothesis, suggesting that the detoxification of the medium requires the conversion of NAA to inactive or non-toxic derivatives of this synthetic auxin. The inability of protoplasts to produce colonies at low densities in medium T_0 is, therefore, probably a result of their inability to decrease the concentration of free NAA in the medium.

Discussion

Whereas the initiation of the division of tobacco mesophyll protoplasts requires a relatively high concentration of auxin in the culture medium, this auxin concentration appears toxic for protoplasts cultured at low cell population densities. By simply lowering the concentration of NAA after a few days of preculture, it becomes possible to reduce to very low levels (1–4 cells ml^{-1}) the population density of cells derived from the protoplasts, and the plating efficiency of cells cultured in these conditions is independant of the cell density. The high exogenous auxin concentration initially needed for the first division of mesophyll protoplasts is not further required to support sustained divisions of the protoplast-derived cells. This requirement could be related either to the dedifferentiation process in which mesophyll protoplasts may have to engage before entering new cycles of cell division or to the unusual needs of stressed, wall-less plant cells, or both.

The susceptibility to high levels of exogenous auxin of plant cells cultured at low cell densities could be a common phenomenon. Thus, as preliminary results indicate in the case of *Nicotiana sylvestris,* the procedure developed to support the growth of tobacco protoplast-derived cells at low cell population densities might be extended to cells derived from mesophyll protoplasts from various species or to cells from other types of tissues. In their study on the

growth requirements of tobacco callus cells plated at low densities (100–300 cells ml^{-1}), Logemann and Bergmann (1974) used a NAA concentration comparable to that of our medium A (0.2 mg l^{-1}). Conversely, however, the media developed by Kao and Michayluk (1975) to support the growth at very low cell densities (10–100 cells ml^{-1}) of cells and protoplasts from established cell suspensions of *Vicia hajastana* contained high amounts of auxins (0.2 mg l^{-1} 2,4 D and 1 mg l^{-1} NAA). But they also observed that an addition to their medium of organic acids, some of which are known to promote ammonium utilization (Gamborg and Shyluk 1970; Behrend and Mateles 1976) significantly improved plating efficiencies at low cell densities. This agrees with our observation of increased tolerance to auxin of protoplast-derived cells when cultured on ammonium malate as the sole nitrogen source. Similarly, Sargent and King (1974) observed in their experiments on nitrate-grown soybean cell suspensions that reduced nitrogen favored growth at low initial cell densities. It is, however, obvious that the use of low auxin concentrations will not be the only condition allowing growth at low cell densities of other types of cell suspensions.

Media initially containing a relatively high NAA concentration (3 mg l^{-1}) but conditioned by the growth of protoplasts at high cell densities also support the growth at very low densities of protoplast-derived cells. The rapid conversion of most of the free NAA initially present in the medium into NAA conjugates could account for this detoxification process. Besides, while auxin conjugates have already been detected in plant tissues (e.g., Andrea and Good 1955; Goren and Bukovac 1973), this is to our knowledge the first report of an accumulation of NAA conjugates in the culture medium itself. This rapid accumulation might result from the lysis of protoplasts in the culture medium. However, since (i) radioactive material is accumulated at a low and constant rate into protoplasts (4% of the input radioactivity accumulated per day) and (ii) a low proportion of protoplasts is unable to divide (less than 15% of protoplasts in optimal growth conditions), the amount of radioactive material released in the medium by protoplast lysis is not high enough to account for the accumulation of NAA conjugates in the medium (more than 40% of the input radioactivity after one day of incubation). The initial absence of cell walls around the protoplasts at the beginning of incubation may be responsible for that accumulation, since in a similar study using intact leaves of *Vigna sinensis*, no auxin conjugates could be detected in the culture medium after incubation with labeled auxins (Goren and Bukovac 1973).

The minimal nutritional requirements of tobacco protoplast-derived cells for growth at low cell population densities were defined. It is notable that they are relatively simple and that no undefined conditioning factors unintentionally carried over during the transfer can be involved, since washing of the protoplast-derived cells did not alter their plating efficiency in this defined medium. Thus, the hypothetical excessive diffusion of metabolic intermediates into the culture medium frequently assumed to be responsible for the inability of plant cells to grow at very low densities does not seem to play an appreciable role in the case of protoplast-derived tobacco cells. Only the study of vitamin requirements led to unexpected observations. Inositol appears to be the sole vitamin stringently required. Whereas addition of thiamin to the medium was considered by Linsmaier and Skoog (1965) to be essential to the sustained growth of tobacco callus, Dravnieks et al. (1969) observed that high cytokinin/auxin ratios in the medium promoted thiamin biosynthesis in the same material. The balance of growth substances used in our medium could thus account for the non-requirement of thiamin by protoplast-derived cells at low cell densities, and eventually this observation might facilitate the search for auxotrophic mutants requiring thiamin.

Plating efficiencies (30–40%) obtained with protoplast-derived cells grown at low cell densities could be considered as sub-optimal. However, taking into account (i) the reproducibility of these results and moreover (ii) their independence of the cell population density, the efficiency of the media established in this study will allow a quantitative estimation of the effectiveness of certain steps of the mutant selection from in vitro cultured somatic plant cells. Besides its simplicity, the procedure developed presents three practical advantages: (i) it permits without impairing proliferation rates the repeated washings often required in mutagenesis-selection experiments; (ii) it also permits a rapid eyescoring of actual plating efficiencies of cells cultured in various conditions; and (iii) the hormone balance of the medium promotes a rapid but induction (unpublished results), thus, allowing the actual use of this step in plantlet regeneration from selected mutant or otherwise genetically modified colonies.

Finally, one could regret that our procedure involves a mixture of single cells and of 2–4 cell units rather than an homogenous suspension of protoplasts. Studies are currently in progress to overcome this difficulty in the cases where it might hamper the recovery of mutant colonies.

I am grateful to J.P. Bourgin and Y. Chupeau for their support and encouragements throughout this work. I wish to thank Pr J. Guern and his colleagues Christiane Tyburn and J.J. Leguay

for their helpful suggestions concerning biochemical studies. I also wish to thank J. Goujaud for his careful raising of the plants.

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Received 25 June 1979; accepted 11 January 1980