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Budding and Cleavage Division of Tobacco Mesophyll Protoplasts in Relation to Pseudo-wall and Wall Formation

Yves Meyer and Wolfgang O. Abel

Botanisches Institut der Universität, Hofmeisterweg 4, D-6900 Heidelberg, Federal Republic of Germany

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Summary. Two saline media, differing primarily in the presence or absence of NH_4^+ but also in the concentration of sucrose, were developed for culture of tobacco (*Nicotiana tabacum* L.) mesophyll protoplasts. In the R0.6 medium, which does not contain NH_4^+ and only 1 g/l sucrose, protoplasts divide 2–3 times by budding and form only a pseudo-wall, *i.e.* a non-rigid structure containing polysaccharides. Later the cells degenerate, and sustained division does not take place. In the W0.6 medium, which contains NH_4^+ and 30 g/l sucrose, the protoplasts form a rigid wall and divide by cleavage of the cells. After a few divisions, the walls of practically all of the newly formed cells degenerate into pseudo-walls, and the divisions cease. Only a few cells keep a wall, continue to divide, and form colonies. A very high frequency of colony formations from protoplasts is obtained by culturing protoplasts for a week in R0.6 or W0.6 and then diluting the culture with a sugar medium. A detailled study of the inorganic and organic components of the saline media showed a strong interaction between the nitrogen supply and the cytokinin requirement. The advantages of the saline media in obtaining cell colonies from protoplasts, the problems associated with budding-type division, the causes of the cessation of division when no complete wall is formed, and the conditions necessary for wall formation are discussed.

Introduction

Maintaining the osmotic pressure in the culture medium of tobacco mesophyll protoplasts with salts results in divisions of protoplast batches which do not divide and degenerate when they are cultured in a sugar medium (Meyer, 1974). We have systematically analysed the influence of the components of the saline media in order to optimize the proportion of surviving protoplasts and the number of divisions. Two different saline media were developed which induce two different types of development of the protoplasts. The composition of one of these media was already given in a preceding paper (Meyer and Abel, 1975): we use the same terminology in order to differentiate a wall from a pseudo-wall.

Material and Methods

Protoplasts of *Nicotiana tabacum* L. (cv. Maryland, originally obtained from Bundesanstalt für Tabakforschung, Forchheim, FRG), both diploid ($2n = 48$) and haploid ($n = 24$), were isolated by the following method. After sterilization of the leaves and removal of the lower epidermis (Meyer, 1974), leaf parts were plasmolyzed in a solution containing KCl (2.5%) and $MgSO_4 \cdot 7H_2O$ (1%). After 30 min, the solution was replaced by a solution containing 11% mannitol, 0.4% glycine as buffer, and 0.05% PATE, a crude preparation of pectin acid trans-eliminase obtained from *Bacillus polymyxa* (Buschmann *et al.*, 1973), and adjusted to pH 8.0 with KOH. After 1 h of maceration without shaking the solution was replaced with 2.5% KCl plus 1% $MgSO_4 \cdot 7H_2O$ containing 0.4% Cellulase Onozuka R10 (All Japan Biochemicals,

Table 1. R0.6 and W0.6 media

Major nutrients (mg/l):	R0.6	W0.6	Other nutrients common to both media	
KNO_3	5400	5000	$FeSO_4 \cdot 7H_2O$	3.72 mg/l
KCl	7200	4000	Na_2EDTA	2.78 mg/l
NaCl	2000	2000	Minor nutrients according to Murashige and Skoog (1962)	
$CaCl_2\,2H_2O$	10800	10800		
KH_2PO_4	250	250		
$MgSO_4 \cdot 7H_2O$	1500	1500	Vitamins:	
NH_4Cl	0	250	*Meso*inositol	1 g/l
			Thiamin-HCl	1 mg/l
Sucrose (g/l)	1	30	Folic acid	1 mg/l
			Hormones:	
			2,4-D	1 mg/l
			6-BA	1 mg/l
			Tween 80	0.02%

Nishinomiya) and 0.02% Pectinol fest (Röhm, Darmstadt, FRG), adjusted to pH 5.2. The maceration was completed after 90 min. After 3 washes in the KCl-$MgSO_4$ solution, the protoplasts were resuspended in this same solution in order to obtain a dense suspension. In experiments conducted in order to test the influence of Mg^{2+}, SO_4^{2-} and K^+ in the culture medium, an NaCl (2.0%) plus $CaCl_2 \cdot 2H_2O$ (0.6%) solution was used instead of the KCl-$MgSO_4$ solution. 0.2–0.3 ml of the protoplast suspension was mixed with 2.5 ml of the culture medium in order to obtain a final concentration of $5–10 \times 10^4$ protoplasts/ml. The modification of the medium caused by the introduction of the protoplast suspension was not considered. This was justified by the results which will be shown in Table 3.

The protoplasts were cultured in plastic Petri dishes. These were sealed with parafilm, maintained at a temperature of 25° and illuminated continuously at 800 lx (fluorescent tubes Osram 65 W/15 and Philips 65 W/33, mounted alternately). Subcultures were maintained under the same conditions.

The saline media were sterilized by filtration in a SM 16510 Sartorius filter with 0.45 μm pore-size filtermembrane.

The criteria used for the selection of the saline media were (1) the proportion of protoplasts which were alive after 3 days in culture, and (2) the proliferation rate = number of the cells present after 12 days in culture divided by the number of protoplasts alive after 3 days. The capability of the protoplasts to form colonies was also tested by the following method. A protoplast suspension after 7 days culture in a saline medium was mixed with 5 volumes of the Linsmaier and Skoog (1965) medium supplemented with 8% mannitol and containing 0.1 mg/l 6-BA, 1 mg/l 2,4-D and 8 g/l agar, maintained liquid at 35°.

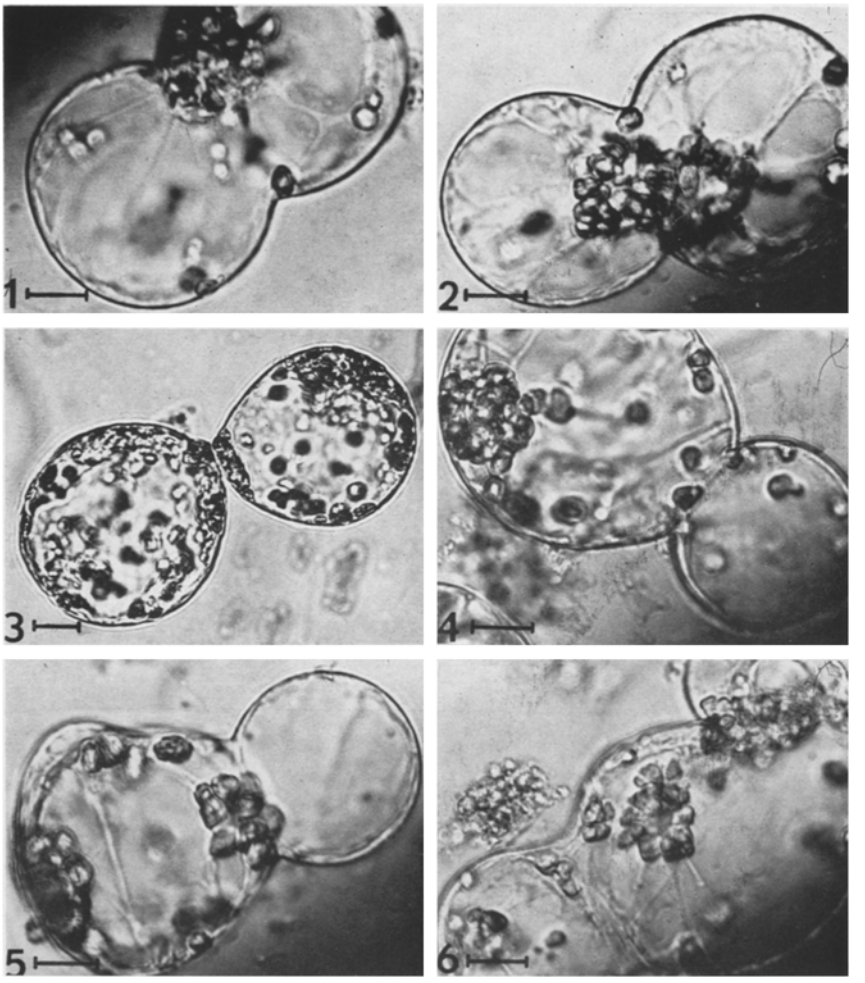
2,4-dichlorophenoxy-acetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-pyruric acid (IPyA), indole-3-propionic acid (IPrA), indole-3-butyric acid (IBA) were puchased from Serva (Heidelberg, FRG), naphthalene-1-acetic acid (NAA) from Sigma (St. Louis, Mo., USA), p-chlorophenoxyacetic acid (CPA), 4-chlorophenylurea (4-ChlPhU) from Fluka (Basel, Switzerland), 6-benzyladenine (6-BA) and kinetin from Roth (Karlsruhe, FRG). Benazolin (4-chloro-2-oxobenzothiazolin-3-yl acetic acid) was a generous gift from Boots Pure Drugs Co. (London, U.K.).

Results

1. Development of the Protoplasts in Culture

Two different saline media, inducing two distinct types of protoplast development, were developed in this study.

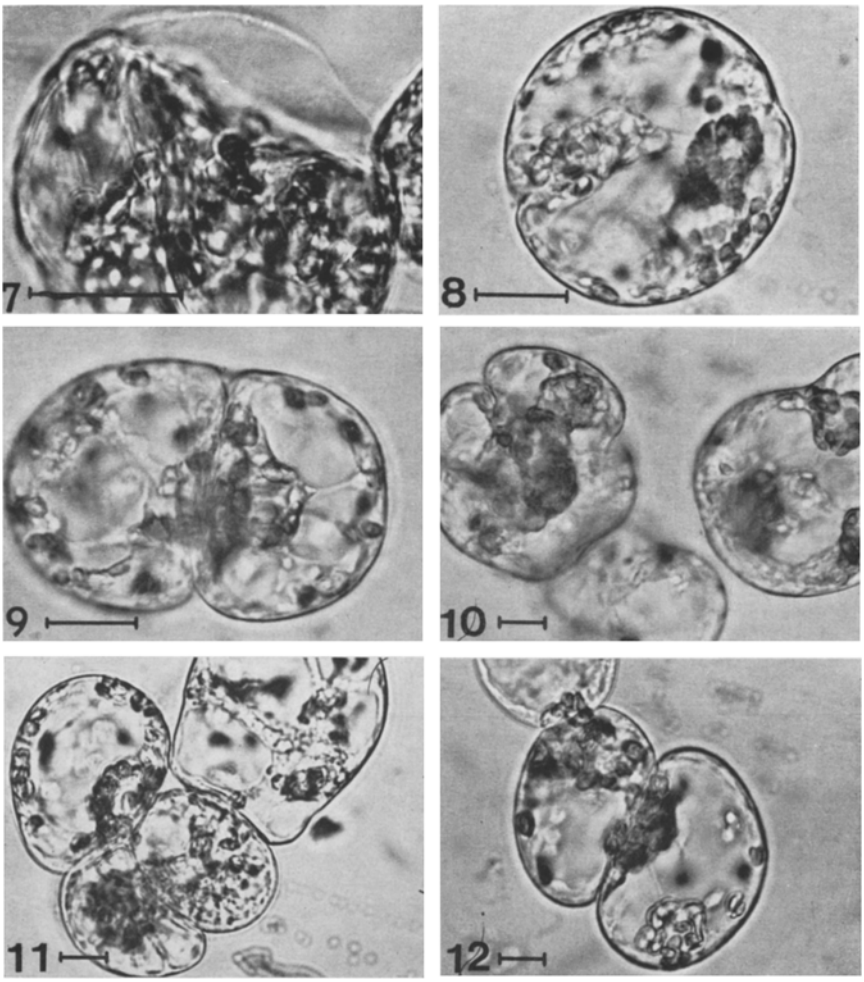
In R0.6 (Table 1), the protoplasts increase in size, dedifferentiate during 4 days, and many chloroplasts cluster around the nucleus. Budding divisions



Figs. 1—6. Development of tobacco protoplasts in R 0.6. Bar in all figures = 20 μm

Fig. 1. Bud formation.—Fig. 2. Karyokinesis.—Fig. 3. Cell separation at the end of the budding division.—Fig. 4. Formation of a bud non localised at the level of the nucleus.— Fig. 5. Karyokinesis preceeding the bud formation.—Fig. 6. Cell forming 3 buds

appear after 5 or 6 days. Generally, a bud appears at the level of the nucleus (Fig. 1) and caryokinesis occurs at the constriction (Fig. 2); afterwards the cells separate (Fig. 3). However, in many cells caryokinesis takes place before the budding (Fig. 5), and in some cases the bud is not localized at the level of the nucleus (Fig. 4). A few cells form several buds simultaneously (Fig. 6). Generally, the division results in a mother cell with numerous plastids at the periphery of its cytoplasm and one or more daughter cells with few or no plastids at their periphery. However, the daughter cells inherit a part of the plastids localized around the nucleus. The dividing cells do not possess rigid walls which can be separated from the cytoplasm by plasmolysis, but only pseudo-walls which can



Figs. 7—12. Development of tobacco protoplasts in W0.6. Bar in all figures = 20 μm

Fig. 7. Wall formation after 4 days (plasmolysis test).—Fig. 8. Beginning of a cleavage division.—Fig. 9. Later phase of a cleavage division.—Fig. 10. Direct formation of polycellular masses.—Fig. 11. Small colonies after 10 days in culture.—Fig. 12. Cleavage division followed by a budding division after 12 days in culture

be stained with calcofluor. We have shown before that this pseudo-wall cannot be interpreted as a thin but otherwise typical wall (Meyer and Abel, 1975). After 2–3 divisions the division activity stops and the cells become drastically vacuolized. Many cells become polynucleate, indicating that caryokinesis continues although cytokinesis has stopped.

In W0.6 the development of the protoplasts is the same as in R0.6 during the first 3 days, but after 4 days a wall can be seen after plasmolysis (Fig. 7). From day 5 on, the course of development in W0.6 is markedly different from that in R0.6, consisting of a period of regular (cleavage) divisions followed by a period

of budding. Cleavage division begins after 5 days in culture (Fig. 8); this division is apparently similar to that observed in sugar medium (Nagata and Takebe, 1970) and consists in the partition of the dividing cells into two approximately equal parts (Fig. 9). The divided cells do not separate, forming small colonies (Fig. 11). In this period, there are also a few atypical divisions which lead directly to the formation of multicellular masses (Fig. 10). After 8–10 days in culture, the majority of the newly formed cells do not possess a wall but only a pseudo-wall. These cells are formed by budding divisions (Fig. 12). The division of these cells stops after a few cycles as in R0.6. At this time, a very small proportion of the newly formed cells possess walls, continue to divide by cleavage, and form colonies and later callus. The proportion of cells which show this development was variable, differing from one protoplast batch to another, but was always less than 1 % of the initial number of protoplasts.

2. Influence of the Composition of the Medium on the Development of the Protoplasts

Influence of Osmotic Pressure. The saline medium proposed in a previous publication (Meyer, 1974) contains a salt concentration equivalent to 0.966 M sugar plus 0.146 M of sucrose, *i.e.* an osmotically active particle concentration equivalent to 1.112 M sugar. By means of a dilution series, it was shown that the minimal osmotic pressure which did not induce bursting of the protoplasts was dependent on the physiological state of the tobacco plants and on the isolation procedure. For example, using our isolation method, as described above, with plants cultured in our greenhouse, the minimal pressure varied from 0.45 to 0.60 M sugar (osmotic equivalent) with Maryland diploids and from 0.50 to 0.60 M sugar (osmotic equivalent) with Maryland haploids. The use of a higher osmotic pressure than 0.6 M results in retardation of dedifferentiation and reduction of the proliferation rate. The lower osmotic pressure (0.6 M) allows one to obtain colonies simply by diluting a protoplast culture in a saline medium with 5 volumes sugar medium. This is not possible when the culture is started at a high osmotic pressure because the concentration of cells become too low for self-conditioning. A lesser dilution of the culture with the sugar medium does not permit wall formation. In this case it is only possible to obtain colonies after eliminating part of the saline medium by centrifugation before diluting the culture with the sugar medium (Meyer, 1974).

Influence of Sugar. Protoplasts dedifferentiate and divide without sugar being present in the medium. However, a low concentration of sucrose, 1–10 g/l, augments the proliferation rate. A series of mixtures of R0.6 with sugar medium II (Meyer, 1974) showed that dedifferentiation was optimal when the osmotic pressure was maintained only with salts. Therefore, the majority of experiments were performed with 10 g/l sucrose in the media. After media R0.6 and W0.6 had been developed the influence of sucrose was restudied. An increase to 50 g/l sucrose in R0.6 did not modify the budding division, but an occasional genuine cell wall was synthesized during the degeneration phase. A decrease to 1 g/l did not reduce the proliferation rate, but reduced the fluorescence of the pseudo-wall after calcofluor staining. An increase to 30 g/l sucrose in W0.6 increased the

proliferation rate and retarded the transition from the cleavage division to the budding division. Increasing sucrose concentration further resulted in a reduction of the proliferation rate.

Use of Tween 80: During the first hour in culture, a large proportion of the protoplasts (up to 20%) burst when they come into contact with the plastic of the Petri dish. This does not happen when glass Petri dishes are used; however, these are impractical. Chupeau *et al.* (1974) succeeded in preventing protoplast bursting by using Tween 80 at a level of 0.04% in the culture medium. This concentration was found to be slightly toxic for our protoplasts; we therefore used 0.02%, obtaining the same protection.

Influence of the Nitrogen Supply (Table 2). Very few protoplasts divide in the absence of nitrogen and those that do, divide by budding. NO_3^-, glutamine, and a low concentration of urea result in a more frequent division of the protoplasts but these do not form complete walls and division is by budding. In contrast, NH_4^+, high concentrations of urea, and the simultaneous addition of NO_3^- plus glutamine result in wall formation and cleavage division.

Influence of the Major Elements (Table 3). The basal medium 0.6 without NH_4^+ contains KNO_3 (6000 mg/l), KCl (7456 mg/l), $CaCl_2 \cdot 2H_2O$ (10800 mg/l), KH_2PO_4 (250 mg/l), $MgSO_4 \cdot 7H_2O$ (1500 mg/l) and 10 g/l sucrose. The other elements are indicated in Table 1 (Meyer, 1974). In order to maintain a constant osmotic pressure, a cation the effect of which was to be studied was used in place of K^+, or was replaced by K^+, an anion to be studies replaced Cl^- or was replaced by Cl^-. These experiments were performed 2 times with different batches of diploid protoplasts, and once with haploid protoplasts. The basal medium 0.6 with NH_4^+ is R 0.6 in which 250 mg NH_4Cl have been substituted for 400 mg KNO_3 and 9 g/l sucrose for 1.250 g/l KCl. These tests were performed once with diploid protoplasts.

The results were similar in the presence or in the absence of NH_4^+ for both the diploids and the haploids. The toxic level, the level of deficiency, and the development of the protoplasts in the absence of these elements are shown in Table 3. The most important finding was the need for a high concentration of Ca^{2+}. In some protoplast batches, a deficiency of Ca^{2+} could be compensated by a high concentration of Mg^{2+}. However, this high Mg^{2+} concentration was toxic for other protoplast batches, causing a decrease in the proportion of protoplasts still alive after 3 days. Those that did survive developed normally. The requirement for a high concentration of divalent cations probably explains why other authors were not able to obtain protoplast divisions in saline media (Erikson *et al.*, 1974). Na^+ was generally unnecessary but in some tests, it stimulated the division activity slightly. In addition, it can largely replace K^+ without modifying the development. Phosphate and sulfate are not necessary, probably because of the quantity already contained in the protoplasts.

Influence of the Minor Elements and of Iron. Neither absence of the minor elements, nor a 10 times higher level modified the development of the protoplasts. We used the minor elements according to Murashige and Skoog (1962). Absence of iron reduced significantly the proliferation rate. The Fe level of the Murashige and Skoog (1962) medium was toxic for some protoplast batches. We therefore used a level 10 times lower.

Table 2. Influence of the nitrogen supply on the development of tobacco protoplasts

	meq.N								
	0	1.4	2	7	10	30	60	200	
KNO_3	+B			++B		3 g/l	+++B	20 g/l	++BT
L-glutamine	+B	100 mg/l	++B	500 mg/l	++BT				
Urea	+B			++B		300 mg/l	+++C	1800 mg/l	++CT
NH_4Cl	+B	++C	100 mg/l	+++C	500 mg/l		++CT		
KNO_3 (5.4 g/l) + glut. (variable)	+++B	+++BC	+++C		++BC				

Symbols: + = a few divisions, +++ = numerous divisions; B = budding division, C = cleavage division; T = toxic level.

Table 3. Influence of the major mineral nutrients on the development of tobacco protoplasts (mM)

	R0.6				W0.6			
	Toxic level	Deficiency level	R0.6 level	Development without	Toxic level	Deficiency level	W0.6 level	Development without
K^+[a]	No	20	151	A few divisions	No	20	104	A few divisions
Na^+	No	No	35	As control	No	No	35	As control
Ca^{2+}	120	40	74	Dead after 3 days	120	40	74	Dead after 3 days
Mg^{2+}	20–100[b]	2	6	A few divisions	20–100[b]	2	6	A few divisions
NO_3^-	80	30	54	A few divisions	80	0	50	As control
PO_4^{2-}	8[c]	0	2	As control	8[c]	0	2	As control
SO_4^{2-}	20[c]	0	6	As control	20[c]	0	6	As control
NH_4^+	—	—	—	—	10	2	5	Budding divisions

[a] Replaced by Na^+.
[b] Depending on the protoplast batch.
[c] Precipitation in the medium; no modification of protoplast development.

Table 4. Development of tobacco protoplasts in presence of different hormones. Numbers in the table = mg/l. The cytokinins were tested in the presence of 1 mg/l 2,4-D, the auxins in presence of 1 mg/l 6-BA

Hormone	R0.6				W0.6			
	Toxic level	Deficiency level	Optimal level	Development at optimal level	Toxic level	Deficiency level	Optimal level	Development at optimal level
Cytokinins								
6-BA	20	0.1–0.2	0.5–2	+++	20	0.02	0.05–2	+++
Kinetin	20	0.1–0.2	0.5–2	+++	20	0.02	0.05–2	+++
4-ChlPhU	150	20–30	50–90	+++	100	5–10	25	+++
Auxins								
2,4-D	20	0.2	0.5–2	+++	20	0.2	0.5–2	+++
CPA	30	1	5	+ +	30	1	5	+ +
NAA	20	0.2	0.5–2	+++	20	0.2	0.5–2	+++
Benazolin	30	1	5	+ +	30	1	5	+ +
IAA	15	—	—	0	5	0.2	0.5–1	+++

Symbols: +++ = Development as in the control; + + = cells more vacuolised, numerous divisions; 0 = as without auxin.

Influence of Vitamins and Hormones (Table 4). Absence of vitamins did not alter the development of protoplasts in R0.6. In contrast, *meso*-inositol, folic acid, and thiamin increased the proliferation rate in W0.6.

Both an auxin and a cytokinin were necessary for dedifferentiation and division. In the absence of auxin, the protoplasts increased in size, the clustering of the chloroplasts around the nucleus did not take place, and the protoplasts died after 10–12 days. We tested in both R0.6 and W0.6 a large number of substances hav-

ing auxin-like properties. All the synthetic auxins tested were effective, and the optimal concentration level was the same in either medium. IAA was not effective in R0.6, but was effective in W0.6. In addition, IBA and IPyA tested at a level of 1 mg/l were ineffective in both media; IPrA at 1 mg/l was ineffective in R0.6 and only slightly effective in W0.6. This slight effectiveness could be related to traces of IAA in the IPrA sample.

In the absence of cytokinin, the development of the protoplasts was the same as in the absence of auxin, but their life time was longer; the protoplasts died after 15–20 days. We tested two cytokinin derived from adenine (6-BA and kinetin) and one derived from urea (4-ChlPhU). Both cytokinin types were able to induce divisions.

Quantitatively the cytokinin requirement was lower in W0.6 than in R0.6. The relation between the quantity of cytokinin necessary and the nitrogen supply was determined by using NO_3^-, NH_4^+, glutamine or urea as the only source of nitrogen, or one of the latter three in association with NO_3^-. When nitrogen was supplied solely as NO_3^-, 0.5 to 2 mg/l 6-BA were necessary for optimal development of the protoplasts. In the presence of NH_4^+ (250 mg/l) or glutamine (250 mg/l), with or without nitrate, only 0.05 to 0.2 mg/l 6-BA were required. In the presence of urea (1 g/l) the majority of protoplasts divided without any cytokinin in the medium.

Influence of the Culture Conditions on the Protoplasts (Temperature, Light, pH, Cell Concentration). The protoplasts were cultured routinely at a temperature of 25°. When they were maintained between 27° and 29° divisions took place one day earlier.

In total darkness, divisions did take place but the proliferation rate was less than in light of 800 lx. When used continuously, higher light intensities were injurious for many protoplast batches, but higher proliferation rate could be obtained by maintaining the protoplasts at 800 lx for 2 days followed by exposure to 2000 lx. This is in agreement with previous experiments by Enzmann-Becker (1973) using a sugar medium.

The pH of the media was adjusted to 5.6–5.8 before filtration. After 1 week of protoplast culture it had decreased to 5.0 in R0.6, and to 4.8 in W0.6. Using a pH of 4.5–6.0 from the start did not modify the development of the protoplasts nor the pH value after 1 week in culture. Supplementing either medium with 500 mg/l citric acid prevented the pH change during culture without modifying the development of the protoplasts.

Fig. 13 shows the influence of cell concentration on the development of diploid protoplasts. For comparison, we have included also the results of protoplasts cultured in a sugar medium. The minimal concentration which permitted division is the same in the sugar medium as in saline media. However, the maximal concentration which did not inhibit divisions was lower in the sugar medium than in the saline media.

3. Obtaining Colonies from Protoplasts

One advantage of R0.6 and W0.6 in comparison with our previous saline medium (Meyer, 1974) is the possibility of obtaining colonies simply by mixing a 1-week-old culture with 5 volumes of a sugar medium. It is no longer necessary

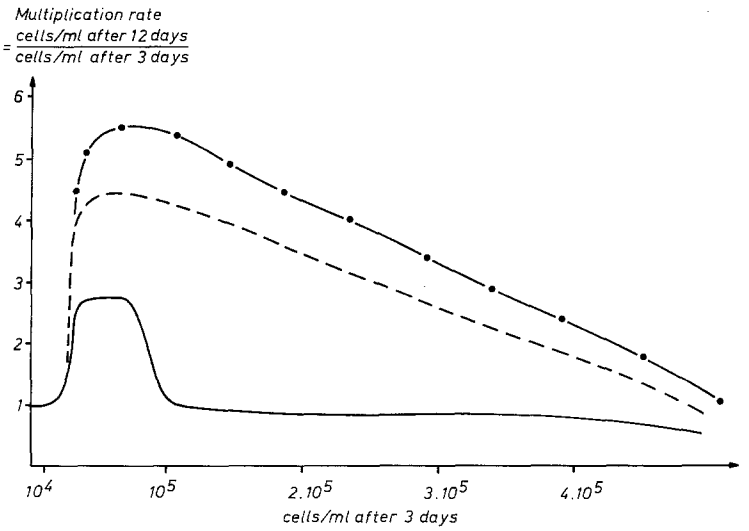


Fig. 13. Influence of the cell concentration on the proliferation rate in different media. —— Sugar medium according to Nagata and Takebe (1971) but with a ten times lower Fe-EDTA concentration; ------ R0.6; •——• W0.6

to remove the saline medium partially by centrifugation. We routinely used as the "dilution sugar solution" the Linsmaier and Skoog (1965) medium containing 1 mg/l 2.4-D, 0.1 mg/l 6-BA, 8% mannitol and 0.8% agar, autoclaved and maintained liquid at 35°. Under these conditions protoplasts form a wall and begin to divide after 2 days; they form macroscopic colonies after 2–3 weeks. The plating efficiency is about 90–100% of the cells present in the culture at the time when the sugar medium is added. NAA can replace 2.4-D in the subculture medium, but we did not obtain colonies using IAA. However, IAA is effective in W0.6, and it supports the growth of tobacco callus.

Discussion

Advantages of the Saline Media for Protoplast Culture

The advantage of the isolation method used since 1973 in our laboratory, and the modification proposed in this publication permit us to use practically all tobacco leaves independent of their age, position on the stem, and lighting conditions. The difficulties in the isolation and culture of tobacco protoplasts using the classical method (Nagata and Takebe, 1971) were discussed by Watts *et al.* (1974). Power and Coking (1970), Motoyoshi *et al.* (1973), Chupeau *et al.* (1974) and Kassanis and White (1974) have developed methods of isolating protoplasts which are variations of the original method of Takebe *et al* (1968) but the success still very much depends on the conditions under which the plants from which the protoplasts are prepared have been cultured.

The frequency of division in sugar medium is also variable from one protoplast batch to another (Raveh *et al.*, 1974), even when our isolation method is used. In

R0.6 and W0.6 the percent of protoplasts which survive after 3 days shows some variation (50–95%), but later, after mixing them with the dilution sugar medium, practically all of the living protoplasts form colonies. In addition, the concentration of protoplasts necessary for a large number of divisions to take place may vary only between 2×10^4 and 5×10^4 protoplasts/ml in the sugar medium of Nagata and Takebe (1971) but between 2×10^4 and 20×10^4 protoplasts/ml in R0.6 and W0.6. This is particularly advantageous when large quantities of protoplasts are required for physiological studies. In addition, the use of saline media can be advantageous for the development of protoplasts having been treated for fusion (Keller and Melchers, 1973; Kao and Michayluk, 1974) or to transfer organelles (Potrykus and Hoffmann, 1973; Bonnett and Eriksson, 1974) because these treatments generally reduce the capacity to divide. However, we have presently no information on the stability of the chromosomes in these media. Particularly in R0.6, karyokinesis is relatively independent from cytokinesis and this might result in changes in the chromosome set.

Factors of Cell Division in Tobacco Protoplasts

The characteristics of the pseudo-wall were described in a previous publication (Meyer and Abel, 1975). Problems related to division without a complete wall and to the cessation of the division after a few cycles were discussed. The budding of protoplasts cultured in a sugar medium is a well-known phenomenon and has been generally interpreted as an extrusion of cytoplasmic material through a hole in the wall when wall regeneration is not complete (Horine and Ruesink, 1972). The budding division in R0.6 does not appear to be a phenomenon comparable to budding in sugar media, because of the lability of the pseudo-wall. In addition, budding division necessitates events which are more complicated than cytoplasm extrusion since one nucleus migrates from the mother to the daughter cell.

We have shown by culturing protoplasts in diluted saline media that the cessation of division in R0.6 is not because of an adverse effect of the saline medium on the cytoplasm, but is related to the absence of a complete wall. This is confirmed by the development of protoplasts in W0.6: only cells which are able to continue synthesizing a wall can sustain division. All of the cells in which the wall evolves into a pseudo-wall cease to divide.

The fact that the majority of the protoplasts cultured in W0.6 at first were capable of synthesizing a wall but later lost this potential, can be only partially explained by the disappearance of NH_4^+ from the medium: Adding NH_4^+ regularly during the culture increased the percent of cells forming colonies in W0.6 only slightly. It appears that a substance or substances necessary for wall formation is or are not synthesized in the majority of cells cultured in W0.6. This substance is (or these substances are) present in the freshly isolated protoplasts and permit wall formation during a few division cycles. Our interpretation of the development of the protoplasts in R0.6 and W0.6 can, then, be summarized as follows: In either medium, the cells are heterotrophic for a factor inducing wall formation but contain small amounts of this factor present at the time of protoplast isolation; this factor is only effective when NH_4^+ is present in the saline medium. In addition, the absence of a complete wall makes the cells heterotrophic for a division factor which is related to the presence of the wall. Both factors are

present in freshly isolated protoplasts and permit wall formation during a few cycles in W0.6 and a few budding divisions in R0.6. It should be possible to supplement R0.6 and W0.6 in order to obtain an the one hand sustained budding divisions, and on the other hand sustained wall synthesis.

The influence of nitrogen supply on wall formation can be interpreted as the result of the accumulation of NH_4^+ in the medium. In fact, urea can be metabolized to NH_4^+ and simultaneous addition of NO_3^- and glutamine can result in the accumulation of NH_4^+ produced by the reduction of NO_3^-, the utilization of this NH_4^+ being blocked because glutamine furnishes reduced nitrogen. The fact that glutamine alone cannot induce wall formation shows that the presence of not all sources of nitrogen in the medium lead to wall formation. The situation is comparable to results of Halperin and Wetherell (1965) showing that NH_4^+ was necessary for embryogenesis of carrot cells cultured *in vitro*, and that glutamine was ineffective in producing embryos.

Relations between Nitrogen Supply and Cytokinin Requirement in Tobacco Protoplasts

The influence of the nitrogen supply on the cytokinin requirement has already been observed by Poirier-Hamont *et al.* (1974) in snapdragon protoplasts and is comparable to the results of Sargent and King (1974) with soybean cells. Soybean cells are dependent on cytokinin when cultured in a medium containing only NO_3^- as nitrogen, but independent of cytokinin when NH_4^+ is present in the medium. Our results are similar in that NH_4^+ and glutamine reduce the cytokinin requirement and urea permits divisions of protoplasts without any added cytokinins. The fact that derivatives of urea act as cytokinins only at high concentrations is probably related to the influence of reduced nitrogen on the cytokinin requirement. This is independent of the effect of NH_4^+ on the wall formation, because glutamine alone lowers the cytokinin requirement but does not induce wall formation.

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