Chapter 8
Some Endogenous and Exogenous Factors in Cell Culture Systems

The performance of defined processes of differentiation forms the basis to use cell and tissue cultures for propagation, and the production of valuable compounds on a commercial scale. To ensure reliability in both these domains, a thorough understanding of the procedure is a prerequisite. The core of this is an understanding of cellular growth and differentiation, and based on this, to develop ways and means to exert influences on productivity. In commercial production, the systems should work reliably and reproducibly every day. As long as more knowledge on differentiation is not available, our only option are empirical assessments based on trial and error. Indeed, from the newly emerged fields of genomics, proteomics, and metabolics, to date only very limited contributions have been made to achieve a better understanding of growth and differentiation. Still, these new approaches are in their infancy.

The many parameters exerting an influence on growth, development, and the biochemical performance of cells can be tentatively grouped in terms of "endogenous and exogenous factors". Genetic influences, the developmental status of the "mother plant" used to obtain primary explants (or the state of the subculture used as origin), and also the developmental status or age of the plant organ from which primary explants are obtained are all endogenous factors. Nutrition, the hormonal supplement, and physical environmental factors like light, temperature, or humidity of the ambient air are grouped as exogenous factors. Certainly, the list, especially of endogenous factors, is not complete yet. An all-encompassing discussion of these factors also will not be attempted here within the limited space available in this volume, and in view of the tremendous wealth of literature available on the internet. Still, some examples, mainly from our own research program, will be given to indicate tendencies in the significance of such factors.

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8.1 Endogenous Factors

8.1.1 Genetic Influences

In callus growth performance, it is difficult to distinguish between genetic influences, and those stemming from the status of the organ serving as origin of the explants. Nevertheless, clear genetic influences can usually be observed by comparing the growth performance of explants from a given organ in a given developmental status in different varieties of a given species. One example of such strong influences is the ability to perform somatic embryogenesis in *Daucus* (Table 8.1), as has also recently been described for, e.g., *Medicago trunculata* following proteomic analysis of recalcitrant and readily embryogenic lines (Imin et al. 2005, see above).

In Table 8.1, some examples on the differentiation of cultured root explants from three carrot varieties cultivated under identical conditions are given. The explants of one variety produced only callus, those of the two others differentiated roots, and one variety could additionally be induced to somatic embryogenesis.

Differences can also be observed in pith explants of *Datura* plants from two different species, derived by androgenesis using anthers of a given flower of each. As a result of meiosis, these strains would differ in their genetics, and due to this, variations in growth and in the compactness of the developing callus material can indeed be observed (Table 8.2).

For a more detailed discussion of the topic, see Chapter 13.

8.1.2 Physiological Status of "Mother Tissue"

Often, clear relations of the physiological status of the original tissue, and the mode of reactions of explants taken thereof can be observed. This could be shown for callus growth of explants obtained from different parts of the stele of tobacco (Table 8.3). Best growth was obtained in explants from the upper third of the tobacco plant, which would represent the physiologically youngest part. A position effect can also be observed for differentiation (Fig. 8.1). With increasing distance to the apex, the ability of the explants to produce flower buds is reduced (Van Tran Than 1973).

Table 8.1 Growth (number of cells $\times 10^3$/explant), and development (rhizogenesis, somatic embryogenesis) in NL medium (see Table 3.3) of cultured root explants (cambium) of some carrot varieties (*rhiz.* adventitious roots, *s. e.* somatic embryogenesis)

Variety	No horm.	IAA + inositol				IAA + inositol + kinetin
	t0			Rhiz.	S. e.	
Frühbund	73.0	270.1	320.6	-	-	1,394.9
Zino	43.2	217.0	257.1	+	-	913.1
Rotin	41.6	124.8	109.2	+	+	1,157.1

Table 8.2 Fresh weight (mg/explant) of stem sections of some strains of haploid plantlets of *Datura innoxia* and *Datura meteloides* (six strains each) cultured in NL+IAA+inositol+kinetin (3 weeks of culture; Kibler 1978)

Strain	Basis	Middle	Upper third	Growth characteristics of callus
Datura innoxia				
i1	115	162	97	Compact callus
i2	137	130	136	Friable callus
i3	203	196	168	Compact callus
i4	115	77	–	Friable callus
i5	120	92	98	Friable callus
i6	195	–	–	Sec. callus formation
Datura meteloides				
m1	67	72	54	Compact callus
m2	65	38	38	Sec. callus formation
m3	66	57	45	Compact callus
m4	–	68	54	Sec. callus formation
m5	34	37	49	Friable callus
m6	80	92	92	Compact callus

Table 8.3 Fresh weight (mg/explant) of some sections of the shoot of haploid plants (8–10 leaves) of *Nicotiana tabacum* var. Xanthi (2 weeks of culture in NL medium)

Growth regulator applied	Basis	Middle	Upper third
0	11.3	9.3	37.3
Inositol+IAA	23.2	16.8	76.2
Inositol+IAA+kinetin	33.6	39.1	123.6

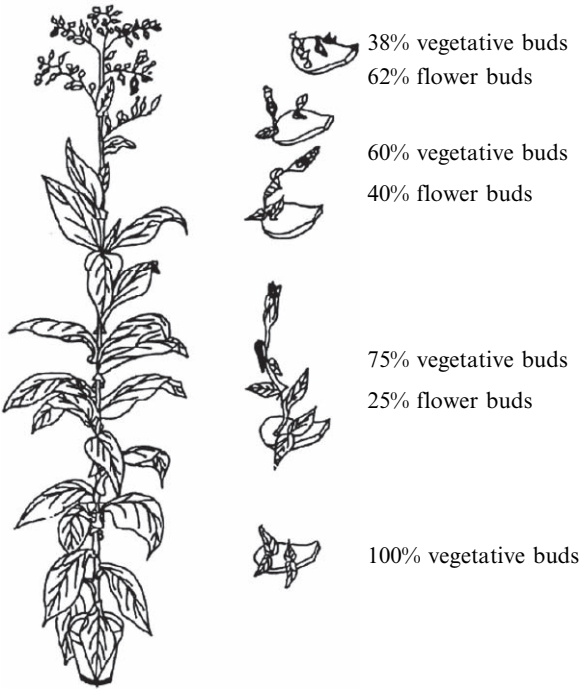


Fig. 8.1 Influences of the region of tissue used for explantation on the development of flower buds in culture (*Nicotiana tabacum*; Van Tran Than 1973)

Table 8.4 Influence of kinetin on the fresh weight, number of cells per explant, root formation, and somatic embryogenesis of cultured explants of various root tissues of *Daucus carota* (I, II denote tissue of two carrot roots; NL medium, 21 days of culture)

Tissue	mg F. wt./explant		Cells × 10^3/explant	
	Exp. I	Exp. II	Exp. I	Exp. II
Secondary phloem (4–6)[a]				
T0	2.0	2.0	8.9	9.1
NL	13.0	12.0	37.8	20.5
NL+I+IAA	26.4 R	62.0 R	80.0	156.6
NL+I+IAA+K	106.0	236.0	488.4	792.0
Cortex				
T0	2.0	2.0	9.4	8.8
NL	10.0	7.0	16.7	23.1
NL+I+IAA	24.0 R	35.0 R	66.2	113.1
NL+I+IAA+K	111	213.0	440.0	1,183.2
Xylem (10–12)[a]				
T0	2.0	2.0	6.3	7.3
NL	10.0	37.0	36.2	76.7
NL+I+IAA	11.0	38.0	54.5	168.0
NL+I+IAA+K	142.0	201.0	399.6	2,450.0
Cambium (2–3)[a]				
T0	2.0	2.0	11.1	9.8
NL	16.0	16.0	30.0	30.9
NL+I+IAA	23.0 R	22.0 R	59.5	42.6
NL+I+IAA+K	95.0	214.0	279.0	1,484.0

[a] Duration of preculture for somatic embryogenesis, in weeks; R, adventitious roots; I, 50.0 ppm m-inositol; IAA, 2.0 ppm; K, 0.1 ppm kinetin

Table 8.5 Influence of iron, manganese, and molybdenum on the fresh weight, number of cells per explant, and average cell weight of carrot callus cultures (BM medium, see Table coconut milk, 3 ppm Fe, 3.6 ppm Mn, 0.25 ppm Mo; 3 weeks of culture; Neumann and Steward 1968)

	0	Fe	Mn	Fe+Mn	Mo	Fe+Mo	Mo+Mn	Fe+Mn+Mo
mg Fresh weight	8	94	18	150	20	152	24	175
Number of cells × 10^3 per explant	18.6	650.0	78.6	742.0	68.6	486.0	68.3	951.2
µg per cell	0.43	0.14	0.23	0.20	0.29	0.31	0.35	0.18

Variation was also observed in explants of various tissues from the same organ of carrot plants (Tables 8.4, 8.5). Using identical conditions of culture, somatic embryogenesis was observed 2 weeks after prior rhizogenesis in cambium explants, in explants of the secondary phloem after 4–6 weeks, and in explants of the xylem area after 10–12 weeks. Also explants of different organs of the same plant vary in growth, as shown in Table 8.6 for young poppy plants. These variations are certainly related to the number of meristematic cells and of parenchyma cells of an explant inducible to cell division, at least as far as callus growth is concerned.

Table 8.6 Influence of kinetin on the fresh weight, and number of cells per explant of cultured explants of various tissues of *Papaver somniferum* L. (var. Scheibes Ölmohn) in NL3 medium (see Table 3.3, 73 days of culture)

Tissue	Original tissue		No kinetin		+0.1 ppm Kinetin	
	F. wt.[a]	No. cells	F. wt.	No. cells	F. wt.	No. cells
Root	0.3	2.9	1.0	22.2	9.0	275.2
Hypocotyl	1.0	2.1	7.0	132.2	24.0	368.9
Cotyledons	1.0	6.6	1.0	6.6	11.0	122.1
Leaves	0.4	3.1	1.0	16.0	17.0	168.9

[a] F. wt., mg fresh weight per explant; no. cells, number of cells $\times 10^3$ per explant

Particularly the differentiation of the explants, but also "plain" growth of cultured explants are strongly influenced by the phytohormone supplement to the nutrient medium (see callus cultures in Chap. 3, and Chap. 11). Therefore, some relation of the endogenous hormonal status to the reaction of explants in culture would be expected. Evidence of this is rather scarce, and some examples will be discussed later.

The physiological status of cell suspensions is also important for the growth performance of subcultures derived thereof. In Fig. 8.2, the influence of duration of pre-culture (before setting up subcultures) on the growth of haploid and diploid callus cultures is presented. Particularly for the haploid cultures, two clear maxima can be observed.

8.1.3 Growth Conditions of the "Mother Plant"

The reaction of explants often correlates with the growth conditions of the mother plant used to obtain explants for culture. In our laboratory, rhizogenesis in an IAA-containing, cytokinin-free nutrient medium (as described in Chap. 3) could be induced only if the mother plants grew for several weeks under short day conditions (Fig. 8.3). This unexpected result was repeated in 3 successive years. In temperate climatic zones, the sowing of carrots is usually done at the end of February or in March, and therefore during early development under natural conditions, the carrots obtained for investigation pass through several weeks of short day conditions. This agrees with the formation of adventitious roots in the NL medium. Unfortunately, systematic investigations of influences of growth conditions of the "mother plant" on the reaction of explants in culture are hardly available.

This example shows how important the physiological status of cells of explants for reaction in culture can be. Neglecting this may cause problems in repeating experiments. Seemingly, the various tissues used for explantation, with their individual molecular and biochemical architecture, vary in their competence to receive and respond to the stimuli associated with explantation and in vitro culture. To which extent such variation determines the response in culture has been discussed in Chapter 7, dealing with somatic embryogenesis in cultured petiole explants.

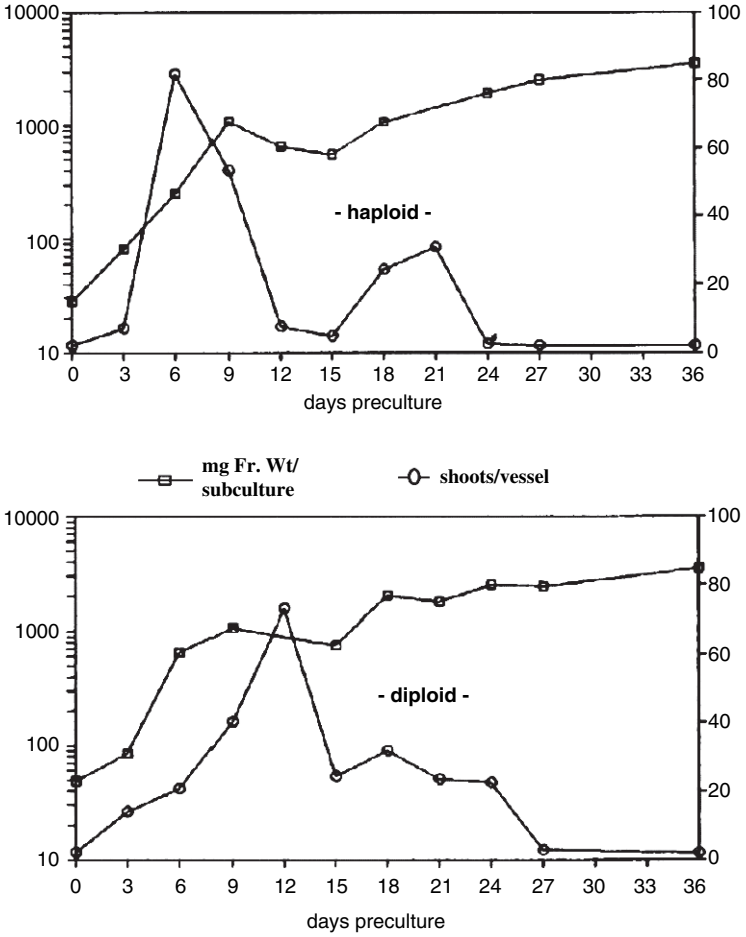


Fig. 8.2 Fresh weight and shoot differentiation of haploid and dihaploid callus cultures of *Datura innoxia* as a function of time of transfer from an MS medium with 2.4D, to an MS medium with 10 ppm kinetin (induction medium, 54days of culture, 22°C, continuous illumination; Forche et al. 1981)

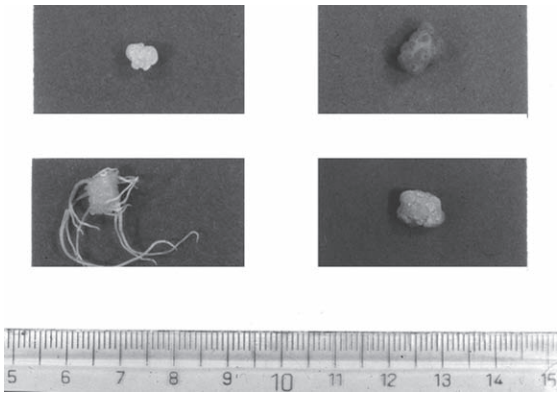


Fig. 8.3 Influence of the photoperiod during early development of carrot plants (until 6 weeks after seedling emergence) on the development of cultured explants of mature plants: NL2 with IAA and inositol; NL3 with IAA, inositol, and kinetin *top row* long day conditions, *bottom row* short day conditions

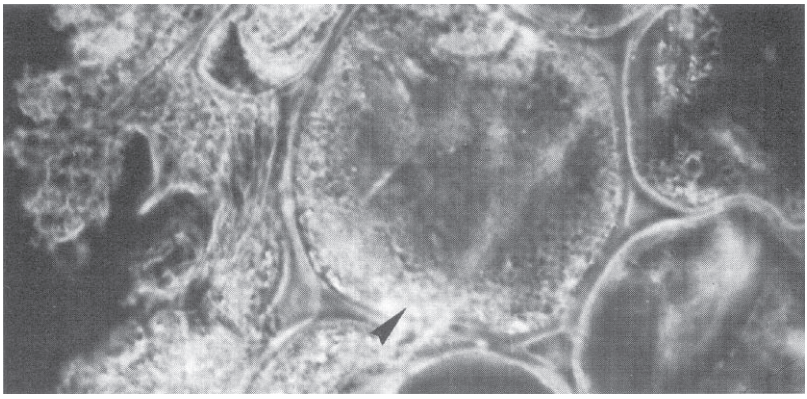


Fig. 8.4 Some histological images of cultured petiole explants (*Daucus carota*). Cytoplasm-rich subepidermal cell

Here, only a short recapitulation shall be given. Under suitable conditions (NL2, B5), cultured petiole explants are able to differentiate adventitious roots and shoots, as well as somatic embryos. As summarized in Section 7.3, for these different tissues serve as origin. An additional important factor is time. Indeed, 2–3days after initiation of the experiment, adjacent to the conductive cells, and between the conductive elements and the glandular channel, after vigorous production of cytoplasm cell divisions are initiated in some cells, which develop first into root primordials, and eventually into adventitious roots. After 5–6 days of culture, cell division is initiated in the large parenchymatous cells after a prior growth of cytoplasm, and later the differentiation of adventitious shoots can often be seen. After about 2–3 weeks, the differentiation of somatic embryos from originally vacuolated subepidermal cells can be observed. Here again, the initial histological indication is a vigorous growth of cytoplasm (Fig. 8.4, Table 8.7).

A careful peeling of the epidermis connected to two or three subepidermal cell layers cultured under the same cultural conditions results also in the initiation of somatic embryogenesis. This indicates the capacity of these subepidermal cells to differentiate somatic embryos independently of other parts of the petiole, which would be related to the differential status of these cells at explantation. Important is the increase in cytoplasm in all three cases, as the first cytologically observable sign. The different morphogenic processes would subsequently be related to differences in the composition of the newly produced cytoplasm. It would be of interest to investigate the significance of the glandular channel for these processes.

8.2 Exogenous Factors

In this section, phytohormones and growth regulators, the mineral nutrition of cell cultures, and influences of light and temperature will be discussed. Most literature currently available deals with the significance of phytohormones and growth

Table 8.7 Flow sheet of somatic embryogenesis in cultured petiole explants of *Daucus carota* (Li and Neumann 1985)

Days after explantation to the next dev. stage	Developmental stage	Hormonal supplement at transition
t0	Somatic cells	High auxin conc., 2 ppm IAA
3–5	Meristematic cells near conductive elements	High auxin conc., 2 ppm IAA
ca. 10	Adventitious roots	Less auxin, 0.1 ppm 2.4D
ca. 15	Embryogenic cells (subepidermal region), densely filled with cytoplasm[a]	Low auxin conc., 0.01 ppm 2.4D
18–20	Four-cell stage of embryogenic cells, pre-globular stage	Low auxin conc., 0.01 ppm 2.4D
ca. 24	Globular stage	Low auxin conc., 0.01 ppm 2.4D
ca. 28	Heart-shaped stage	Low cytokinin conc. (0.02 ppm zeatin), low auxin conc., 0.01 ppm 2.4D
30–40	Torpedo-shaped stage[a]	No growth regulators
50–60	Mature embryo	No growth regulators
80–90	Young plant	

[a] Transfer to a new medium with the concentration of growth regulators indicated

regulators, and also here only some examples will be given to indicate tendencies—for more information, the internet is recommended. Again, some empirical ideas of more general significance will be considered, exemplified by research results mostly from our own laboratory. The same approach is taken for the significance of nutrition and physical factors.

8.2.1 Growth Regulators

Let us start with some remarks on terminology. In the literature, some confusion exists on the use of the terms phytohormones and growth regulators. In this book, mainly phytohormones are defined as natural occurring regulators of growth and development native to plants; the term growth regulators includes phytohormones, and synthetic substances with influences similar to those of phytohormones.

Nutritional factors are generally rather unspecific with respect to growth and differentiation, and predominately recognizable in quantitative terms. However, growth regulators exert rather specific influences usually at low concentrations in the medium. Some exceptions to this have been discussed in Chapter 7, and will be discussed in Chapter 11.

As a general principle, cell division and cellular differentiation are counteracting processes. Independently of the classification of a compound as, e.g., an auxin or a cytokinin, if its application promotes high cell division activity, then usually differentiation will be inhibited at the same concentration. The application of IAA,

a native auxin, to cultured carrot root explants induces the differentiation of adventitious roots within about 2 weeks. Under these conditions, its activity to promote cell division is relatively low. After a simultaneous application of kinetin, a synthetic cytokinin, high cell division is induced, and root formation is either prevented or sometimes delayed for about 3 weeks. The same delay can be observed for an equimolar application of 2.4D, a synthetic auxin that strongly promotes cell division at suitable concentrations.

Another important factor is the concentration of the growth regulators applied. As an example, if kinetin is applied at 0.1 ppm to the nutrient medium of *Datura* explants, a strong stimulation of cell division activity can be observed; an application of 10 ppm inhibits cell division, and the differentiation of shoots is induced; brushing a solution of 30 ppm onto isolated leaves slows down senescence. Very important are interactions of the various growth regulators. As demonstrated in Fig. 8.5, a separate application of IAA, kinetin, or m-inositol induces only small growth responses, and even a combination of any two of these increases growth only slightly. A growth rate of callus cultures comparable to that recorded with a supplement of coconut milk is achieved only by a combination of all three components. There is evidence suggesting an enhanced multiple interaction of these growth regulators, rather than simply a summation of individual effects (see Chap. 11).

An important factor in such relations is certainly an endogenous hormonal system that evolves during culture of the explants, which will be dealt with in Chapter 11. Also genetic influences have to be considered, and it is open to which extent there exist relations between these and the endogenous hormonal system of cultured tissue.

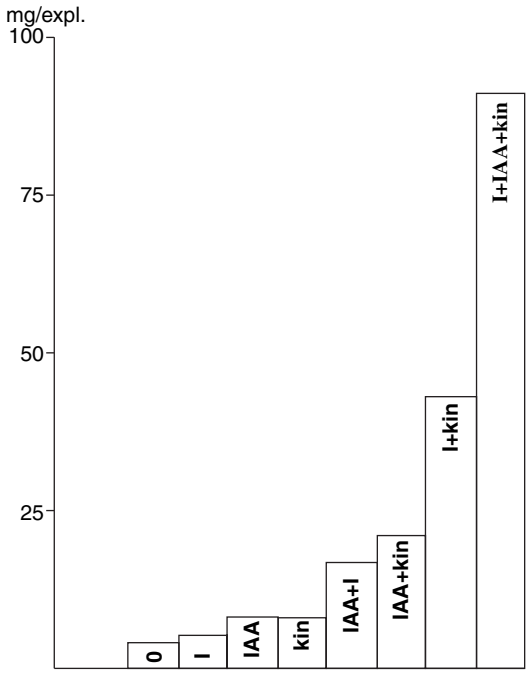


Fig. 8.5 Multiple interactions of several growth regulators influencing the fresh weight of cultured explants of the secondary phloem of the carrot root (NL, see Table 3.3, 22°C, continuous light at ca. 4,000 lux; Bender and Neumann 1978a)

Such relations of cell division and differentiation, as described for the growth and development of cultured cells, can be also observed for biochemical differentiation, and consequently for the production of components of secondary metabolism that could be of commercial interest (Chap. 10). Compared with highly active, proliferating cell populations, the development of the secondary metabolism usually requires a certain age of cells, i.e., a longer interphase in the cell cycle.

8.2.2 Nutritional Factors

As can be seen from the composition of nutrient media (Sect. 3.4), cell cultures require all the mineral nutrients as intact plants for optimal growth and development. Also in terms of growth performance, dose/response relations tend to be similar to those known for intact plants since a long time (cf. Figs. 8.6, 8.7, 8.8). If a tangent is projected on the ascending curve, an angel of ascent can be observed that is characteristic for each nutrient in cell cultures as well as for intact plants, certainly due to the specific function of the nutritive element investigated. Here, differences can be observed for callus growth, and for the number of cells per explant. Evidently, cell division activity and cellular growth are influenced differently by the nutrient.

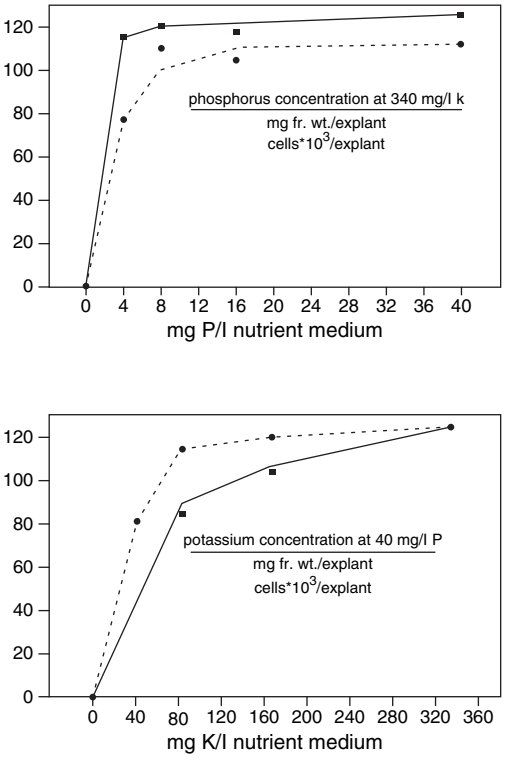


Fig. 8.6 Influence of potassium and of phosphorus in the nutrient medium on the fresh weight and number of cells per explant of cultured explants of the secondary phloem of the carrot root (NL, see Table 3.3, supplied with 50 ppm m-inositol, 2 ppm IAA, 0.1 ppm kinetin)

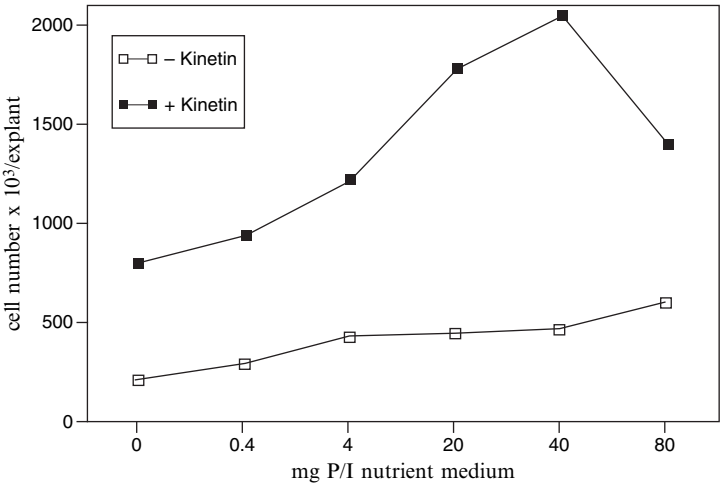


Fig. 8.7 Influence of various phosphorus concentrations and kinetin (0.1 ppm) on the cell number of explants of cultured carrot root explants after 3 weeks of culture (cell number at $t0 = 15 \times 10^3$/explant). The nutrient efficiency rate was derived by interpolation of the increment of cells per explant/ng of nutrient between the two lowest nutrient concentrations, and amounts to 131×10^3 for minus kinetin, and 343×10^3 for plus kinetin treatments (Stiebeling and Neumann 1987)

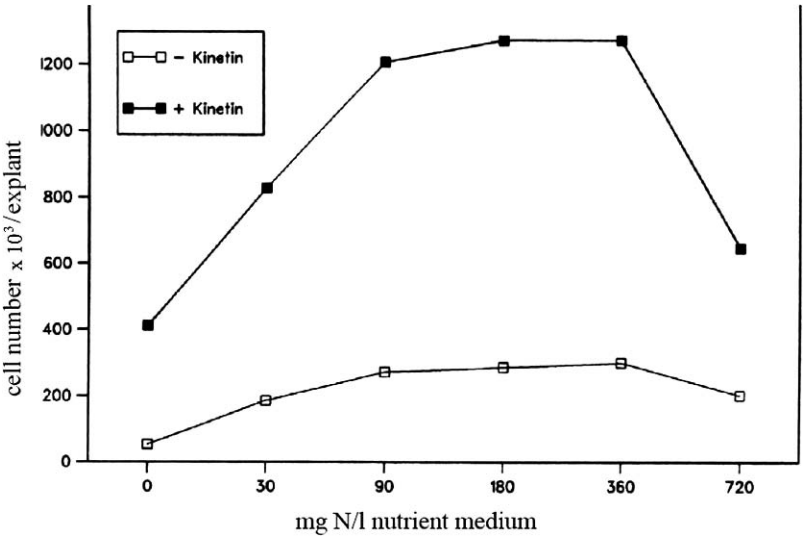


Fig. 8.8 Influence of various nitrogen concentrations and kinetin (0.1 ppm) on the cell number of explants of cultured carrot root explants after 3 weeks of culture (cell number at $t0 = 15 \times 10^3$/explant)

For phosphorus, the angle of ascent for callus growth is greater than for the number of cells per explant, the reverse being the case for potassium.

Clear influences on the equilibrium of cell division and cellular growth can also be observed for micronutrients. Especially iron promotes cell division, whereas Mn and Mo (at the concentrations applied) seem to preferentially promote cellular growth (Table 8.5). Some data on the consequences of nutrient deficiencies for metabolism will be discussed in Chapter 9, dealing with primary metabolism.

To characterize the function of an individual nutrient element in terms of yield production of cereals, a so-called c-value was introduced over 50years ago by Mitscherlich (1954):

$$dy/dx = k\,(A - y)$$

or, after integration,

$$\ln\,(A - y) = c - kx$$

where y is the fresh weight, dry weight, or cell number per explant, x the variable of the experimental system (e.g., the concentration of the nutrient), A the maximum achievable growth, and k is a constant.

Here, c is represented by an integration constant of invariable components of a system, except for k. After transformation into Brigg logarithms, we have

$$\log\,(A - y) = \log A - cx$$

and c is proportional to k, which is based on the transformation to Brigg logarithms ($c = kx\ 0.434$). Often, the Mitscherlich formula is written in a non-logarithmic form:

$$y = A\,(1 - 10^{-cx})$$

The value c describes the angle of ascent of the tangent in experiments on mineral nutrition. Such calculations can also be applied to cell and tissue cultures. Comparing c values of mineral nutrients for intact plants in pot experiments with those calculated for cell cultures, the latter are considerably higher. This would be due to the meristematic character of cell and tissue cultures; in intact plants, meristematic areas are "diluted" by tissue with low or no proliferation, and consequently low or no requirements for mineral nutrients (Stiebeling and Neumann 1987).

Beside influences of individual nutrients on growth, also interactions of these have to be considered for macro- as well as for micronutrients. In Table 8.5, examples are given for interactions of Fe with Mn and Mo. A supplement of the latter two, either alone or in combination, in addition to Fe clearly increases growth more than when summing their individual effects.

Nitrogen nutrition is satisfied by providing nitrate or ammonia, mostly as salts (Sect. 3.4). Starting with White's nutrient medium, glycine, as an organic nitrogen source, contains reduced nitrogen. Nowadays, organic nitrogen in reduced form is

Table 8.8 Influence of casein hydrolysate (CH, 200 ppm) on the fresh weight, and number of cells per explant of cultured carrot root tissue (secondary phloem) in NL medium (see Table 3.3), supplemented with 50 ppm m-inositol, 2 ppm IAA, and 0.1 ppm kinetin (21 days of culture)

	Fresh weight (mg/explant)	No. of cells (cells × 10^3/explant)	Aver. cell weight (µg/cell)
Without CH	99.00	866.13	0.13
With CH	206.00	1,683.00	0.10

Table 8.9 Influence of nitrogen form (360 mg N/l) on the growth, total nitrogen content, pH of the nutrient medium, and concentration of nicotine for tobacco cell cultures (var. Xanthi 8/11, NL medium, see Table 3.3), supplemented with 50 ppm m-inositol, 2 ppm IAA, and 0.1 ppm kinetin (28 days of culture; Elsner, unpublished results of our institute)

	g Dry wt./250 ml NL	Number of cells × 10^3/ml	Cell wt. (µg)	pH[a]	N uptake (mg/100g)[b]	Nicotine (µg/g dry wt.) in cell material	Nicotine (µg/g dry wt.) in nutrient medium
Nitrate ($NaNO_3$)	2.628	348	0.030	5.8	461	25.5	8.75[c]
Ammonia (NH_4Cl)	1.235	184	0.027	4.4	486	11.7	6.90[c]

[a] pH 5.6 at t0
[b] Kjeldahl-N
[c] Difference highly significant

supplied to most nutrient media as casein hydrolysate. Carrot root explants grow quite well on only nitrate in the NL medium (Table 8.8), but callus weight is nearly twice as high following an application of casein hydrolysate. All amino acids of this mixture can be utilized by cell cultures, but often a selective preference in uptake can be observed—in carrot cultures, this is for leucine. Thus, this mixture of several amino acids, obtained by hydrolysis of the naturally occurring protein casein, can be replaced by one (usually glutamic acid) or a few amino acids.

Compared to media containing only ammonia as nitrogen source, growth of tobacco cell suspensions is higher in nutrient media containing only nitrate as nitrogen source (Table 8.9). For both, average cell weight is essentially identical, and therefore differences would be due to a reduced cell division activity in the ammonia treatment, in which also the concentration of nicotine is at a considerably lower level. Ammonia is taken up by plant cells as a cation in exchange for protons, which accounts for the lowering of the pH of the medium. Nitrogen uptake was at the same level for both nitrogen forms, suggesting that the differences in growth performance are due to differences in metabolism of the two, and possibly to the differences in pH (see later).

Only reduced nitrogen can be utilized by heterotrophic plant cells. The high energy requirement to reduce nitrate is fulfilled by photosynthesis in intact plants, and for cell cultures usually by some carbohydrate in the medium, commonly sucrose. Beside influences on growth, the nitrogen form exerts influences on morphogenesis. Already in the mid-1960s, Halperin and Wetherell (1965) reported a requirement for

ammonia in addition to nitrate, i.e. reduced nitrogen, in the nutrient medium to induce somatic embryogenesis. This was later confirmed using other species (e.g., Gleddie et al. 1982 for *Solanum melanogena*). In this system, the nitrate to ammonia ratio of 2 is optimal up to a concentration of 60 mM of total nitrogen in the medium. With the exception of the NL medium, all other media used to induce somatic embryogenesis contain ammonia in addition to nitrate. In the NL medium, nitrate is the only source of inorganic nitrogen. Reduced nitrogen, however, is supplied as amino acids in casein hydrolysate. As will be reported elsewhere in detail, here a strict requirement of ammonia to induce somatic embryogenesis does not exist.

Employing the more recent methods of proteomics, some investigations using intact plants may shed more light on the differences in the function of the two nitrogen sources. A nitrate supply to nitrogen-starved tomato plants results in an upregulation of 115 genes, including nitrate transporters, nitrate and nitrite reductase, and also some of those involved in general metabolism, like transaldolase and transketolases, malate dehydrogenase, asparagine synthase, and histidine decarboxylase (Y.H. Wang et al. 2001). Similar results have been reported for *Arabidopsis* (R. Wang et al. 2000). Here, beside an upregulation, also repressions of some genes were observed, like for AMT1;1, encoding an ammonium transporter. Evidently, as of its first entry into metabolism, a molecule as small as nitrate is able to initiate a whole family of genes with possibly remote functions.

The function of mineral nutrients depends on the supplement of growth regulators to the medium. In Fig. 8.8, results of an experiment on the influence of kinetin on the growth of carrot root explants at various nitrogen concentrations are summarized. It is obvious that a kinetin supplement induces a higher efficiency of nitrogen for callus growth. Similar results can be obtained for phosphate with, however, some variation, possibly specific for this nutrient (Fig. 8.7). Nitrogen and phosphorus in casein hydrolysate were not considered in the two nutrient media given in the tables. These and similar results indicate an influence of growth regulators on the nutrient efficiency rate. It remains to be seen to which extent this influence, here of phytohormones, exists also for intact plants—some preliminary results dealing with this aspect are positive.

The nutrient efficiency rate was derived by interpolation of the increment of cells per explant/ng of nutrient between the two lowest nutrient concentrations. This amounts to 2.2×10^3 for minus kinetin, and 13.6×10^3 for plus kinetin treatments (Stiebeling and Neumann 1987).

The influence of growth regulators on nutrient efficiency can not be explained easily. Each amino acid requires its characteristic number of nitrogen atoms, and each nucleotide needs at least one phosphorus. Although no explanation is available for nitrogen, some first lines of evidence exist for phosphorus (see also Chap. 9). Although the explants of the experiments in Figs. 8.7 and 8.8 can be considered as mixotrophic, photosynthesis contributes considerably to fulfill the demands in energy and carbon. The export of assimilates from chloroplasts, however, is mediated mainly by a phosphate translocator that requires inorganic phosphate in the cytoplasm for operation. At phosphorus deficiency, the transport of assimilates through chloroplast membranes is less; assimilates will accumulate in the chloroplasts, and be initially stored as starch. The starch storage capacity of the plastome is limited, and if this is

exhausted, then the assimilates reduce the activity of the Calvin cycle enzymes to fix carbon dioxide by feedback. Neither NADPH nor ATP can be transported through the chloroplast membranes directly to the cytoplasm. To the Calvin cycle, a second route of assimilate export exists that is independent of inorganic phosphate—a dicarboxylate shuttle. A main function of assimilates in the cytoplasm is to provide substrates to produce reduction equivalents, mostly NADPH and NADH. This function can be at least partly substituted by the dicarboxylate shuttle. Some first results indicate a dependence of this shuttle on kinetin (Neumann and Bender 1987). Should these be confirmed, the influence of kinetin on phosphate efficiency could find some explanation. At low phosphate concentrations in the medium, kinetin could promote the operation of this shuttle, as supplement for a low activity of the phosphate translocator, thereby increasing the assimilate export of chloroplasts.

It has to be checked to which extent such conditions could influence also secondary metabolism. As could be expected, generally many influences of the nutritional status of the culture can be observed on the concentration and the composition of the protein, concentrations of free amino acids, as well as of carbohydrates and other components of primary and secondary metabolism. For influences of nutrients on secondary metabolism, the anthocyanin concentration in cultured carrot root explants as influenced by Mo shall serve as an example (Neumann 1962). Cultured explants of some carrot roots are able to synthesize and accumulate anthocyanins. An increase results from higher iron concentrations in the medium. By contrast, a dramatic decrease is associated with high molybdenum levels (Fig. 8.9). The synthesis of

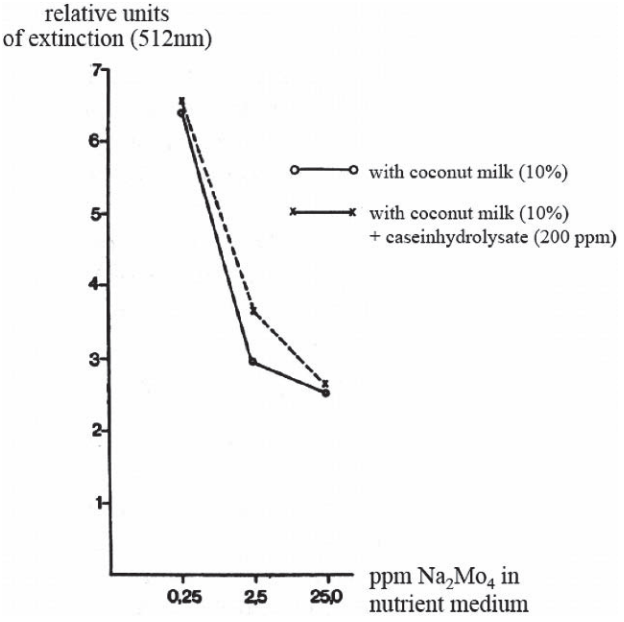


Fig. 8.9 Influence of various concentrations of molybdenum on the concentrations of anthocyanin in cultured carrot root explants (BM, see Table 3.3, with 10% coconut milk; after Neumann 1962)

anthocyanin is closely related to an interaction of carbohydrate and nitrogen metabolism, and often its accumulation can be observed in situations favoring an accumulation of carbohydrates. Iron increases the uptake of sugars from the nutrient medium, and molybdenum, as a cofactor to nitrate reductase, should increase the synthesis of amino acids and other nitrogen-containing compounds (Neumann 1962; Neumann and Steward 1968). Consequently, due to requirements of carbohydrates for amino acid synthesis, the concentration of carbohydrates in cultures would be reduced by molybdenum. Also at phosphorus deficiency, usually anthocyanin will accumulate, which is used for diagnosis of phosphorus deficiency in intact plants. One explanation could be the requirements of phosphate for optimal operation of the phosphate translocator. A disturbance of this endogenous transport system would promote an accumulation of carbohydrates in the cells, and anthocyanin would accumulate. As expected, an accumulation of anthocyanin can also be induced by elevated sucrose levels in the nutrient medium.

The courses of uptake of the two twin nutrient pairs potassium/phosphorus and calcium/magnesium are quite similar, and also the influence of kinetin is comparable (Fig. 8.10). The nutrients of the former pair are used up to a greater extent than those of the latter. This can be observed also for intact plants. The highest rate of uptake, at least for K, P, and Mg, takes place during the log phase from the 10th to

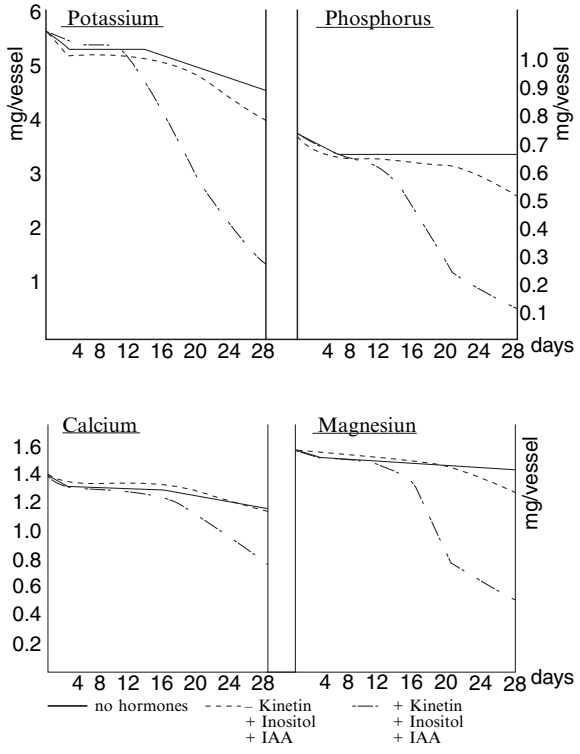


Fig. 8.10 Influence of kinetin (0.1 ppm), inositol (50 ppm), and IAA (2 ppm) on the concentrations of potassium, phosphorus, calcium, and magnesium in the nutrient medium of cultured carrot root explants during 28 days of culture

the 20th day of culture (see also Chap. 3). During the stationary phase, uptake is slowed down again. The uptake follows growth intensity, and at least for P and Mg, a "luxury" consumption can be excluded. Although the concentrations of all four nutrients are lower if compared to those at t0, a deficiency of these should not be responsible for the transition of the cultures from the log to the stationary phase.

In the experiments described above, nutrient uptake was estimated by determination of the concentration of the nutrients in the nutrient medium at various stages of culture. If, however, the nutrient concentration is calculated on the basis of cell number of the cultures, as in Fig. 8.11 for potassium and in Fig. 8.12 for phosphorus, then a high accumulation occurs during the lag phase of callus growth up to the 6th or 7th day of culture. A kinetin supplement increases the concentration of both nutrients on the 6th day by about 25%. At this day, cell number is approximately the same as in the kinetin-free treatment. Therefore, the nutrient uptake rate would not be related to the growth-promoting capacity of kinetin, but rather to changes in metabolism initiated by kinetin during the lag phase.

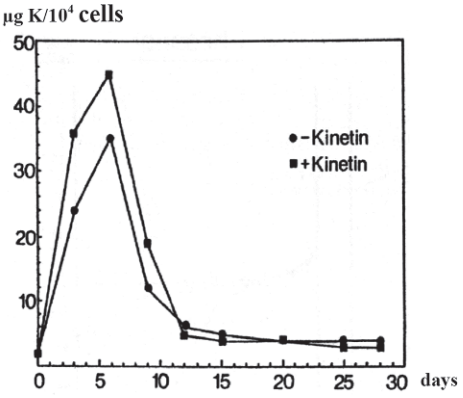


Fig. 8.11 Influence of kinetin (0.1 ppm) on the potassium concentration of carrot callus cultures during a 28-day culture period (Krömmelbein, unpublished results of our institute)

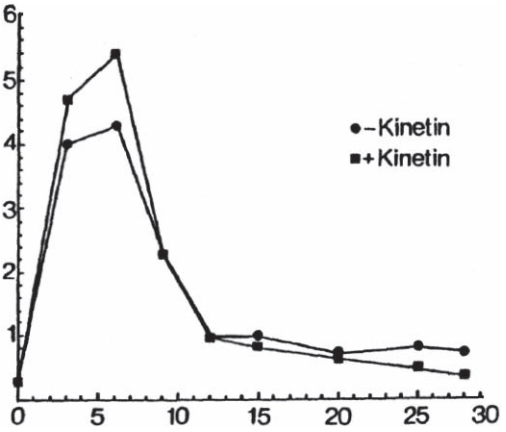


Fig. 8.12 Influence of kinetin (0.1 ppm) on the concentration of phosphorus (µg P/10^4 cells, Y axis) in carrot callus cultures during a 28-day culture period (X axis; Krömmelbein, unpublished results of our institute)

This maximum of K and P is followed by a steep decrease in concentration, which certainly would at least be partly due to a "dilution" induced by the strong increase in cell number per explant during the log phase of callus growth. From the 12th day onward, the concentration of these two mineral nutrients remains more or less constant, and no influence of kinetin can be observed. In other experiments, sometimes the concentration is somewhat elevated in the kinetin treatment.

Although the experiments on mineral nutrients discussed above clearly show their significance for cell cultures, only a limited number of investigations are known dealing with this aspect. Except for the early investigation performed at the time to establish the mineral composition of nutrient media, systematic studies of the significance of mineral nutrition in cell cultures are not available. Also, it is only rarely that investigations on the influences of mineral nutrients on metabolism, and the composition of cultured cells can be found in the literature. This should be also of commercial interest. The importance of such investigations shall be demonstrated by, e.g., the results of Fujita and Tabata (1987). A nitrogen supplement as ammonia reduces the production of shikonin by *Lithospermum* cultures; this is in agreement with results obtained for nicotine production by cultures of tobacco (see above). Another example is an increase of products of the secondary metabolism by a general reduction of nitrogen in the nutrient medium. A reduction of nitrogen results also in an increase in the production of capsaicin in pepper cultures, and the formation of serpentine and ajmalicin is increased in *Catharanthus* cultures by lowering the phosphate level. An increase of rosmarinic acid could be the result of a reduced growth rate by cell division in the cultures, and the accumulation of older cells. In Chapter 10, more details will be discussed. Influences of mineral nutrients on morphogenesis have already been discussed in Chapter 7.

8.2.2.1 Improvement of Nutrient Uptake by Transgenic Carrot Cultures

Phosphorous (P) is an essential nutrient for plant growth, development, and production, as part of key molecules such as nucleic acids, phospholipids, ATP, and other biologically active compounds. The total amount of P in the soil may be high, but often it is unavailable for plant uptake.

To adapt to phosphate (Pi) deficiency, plant roots release citrate or malate, or both, which mobilizes Pi from sparingly soluble Pi sources (Penaloaza et al. 2005). Phosphoenolpyruvate carboxylase (PEPCase) is an important enzyme that regulates the generation of some organic acids, such as oxalacetic acid and malic acid, by carboxylation of phosphoenolpyruvate (PEP, see below). The transcriptional activation of PEPCase genes is also regulated by P deficiency (Toyota et al. 2003).

As described later, at phosphorous deficiency the activity of the phosphate translocator is reduced, and the energy export from the chloroplasts is substituted by a dicarboxylate shuttle. The initial step of this shuttle is a carboxylation

of PEP by PEPCase; oxalacetic acid is produced, which is reduced to malate after uptake by the chloroplast (see also Chap. 9). Malate and also citrate accumulate in the cells, and in the nutrient medium. To improve the utilization of phosphorus by increasing the production of malate and citrate in the nutrient medium, we have generated transgenic carrot cultures containing an additional PEPCase gene (ppcA, Accession Z48966) from *Flaveria pringlei* (C3 plant; Swenson et al. 1997; Westhoff et al. 1997), under control of the MAS promoter with the methods described later (Sect. 13.2). In nutrient media, usually water-soluble Na-bis-phosphate is supplied as phosphorus source. To check the efficiency of this foreign gene, bis-phosphate was substituted by Thomas phosphate in which phosphate is hardly water-soluble. As shown in Fig. 8.13, only transformed cells carrying the second PEPCase gene are able to grow in the nutrient medium with Thomas phosphate as only P source (Natur et al., unpublished data of our laboratory). No data are available yet for transgenic carrot plants growing on P-deficient soil.

By transferring an embryogenic carrot cell suspension into a hormone-free B5 medium, the development of somatic embryos is decreased by P deficiency. Figure 8.14 shows that the transgenic cells as well as the control are growing normally in B5 with water-soluble hydrogen-P, whereas only the transgenic cells can grow in B5 with water-insoluble Thomas phosphate.

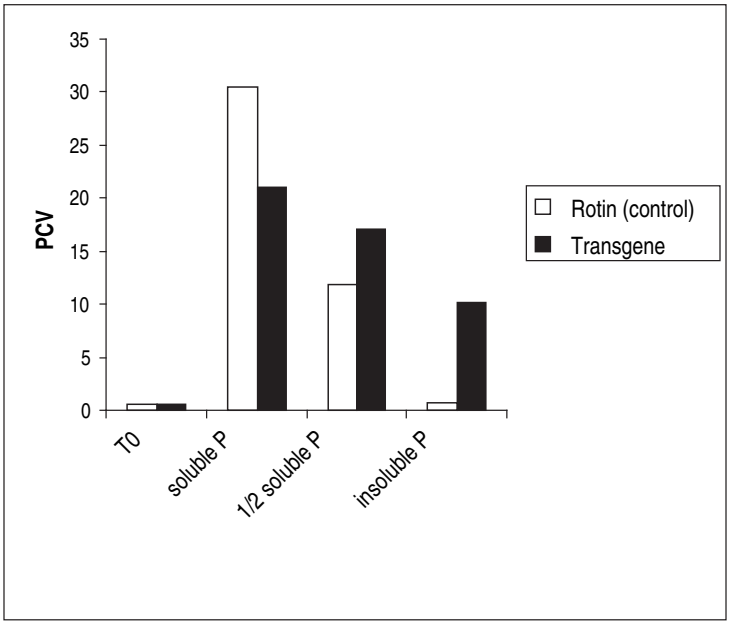
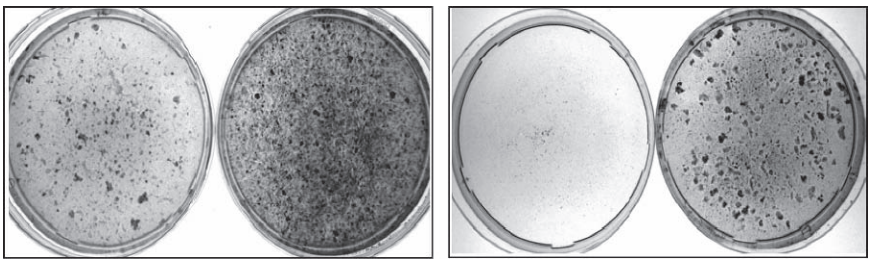


Fig. 8.13 Growth of carrot cell suspension during 5 weeks of culture in B5 medium with different P sources ($NaH_2PO_4.H_2O$ as soluble P, and Thomas phosphate as insoluble P). Cell density is shown as pcv (packed cell volume, ml cells/100ml suspension)



A

Culture in B5 containing soluble Phosphate (Na-bis-phosphate)

B

Culture in B5 containing insoluble Phosphate (Thomas phosphate)

Fig. 8.14 Development of somatic embryos during 28 days of culture in the hormone-free B5 medium with Na-bis-phosphate or Thomas phosphate: *A left* Rotin, *right* transgenic strain; *B left* Rotin, right transgenic strain

8.3 Physical Factors

Here, some examples on influences of temperature and illumination on cultured cells shall be discussed. Although marked influences of these factors can be expected for the performance of cell cultures, data of systematic studies on this aspect are rather rare.

Like all biological systems, also for cell culture there exists a profound influence of temperature on growth and development. Up to 30–35°C, an increase in growth performance of cell cultures of a number of species has been described. If possible, the optimal temperature for each cell culture system should be determined, and this could be expected to range between 20 and 30°C. Usually, the temperature is kept constant during an experiment. As an example, growth of tobacco shoot cultures at three temperature levels is given in Table 8.10. Also morphogenetic processes can be controlled by temperature, as shown for caulogenesis of cultured lily bulb explants (van Aartrijk and Blom-Barnhoorn 1983), which is strongly increased by elevating the temperature from 15 to 25°C (Fig. 8.15). Even relatively small genetic differences, as between varieties of the same species, will be significant in determining the optimal temperature. The anthers of the tobacco variety "Wisconsin" produce abundant haploid plantlets at 22°C, a temperature at which androgenesis could not be induced using anthers of "Xanthi". Here, temperatures of 27–28°C are required for androgenesis. Also, the positive influence of a short storage at low temperatures to induce androgenesis described in Chapter 6 should be recalled. Eventually, the optimal temperature for rhizogenesis and caulogenesis could be different. As an example, influences of temperature on callus growth are given in Table 8.10.

For light, several factors have to be considered. Beside light intensity, which can vary between darkness and continuous illumination by 8,000–10,000lux, also light quality, and the variation in the daily duration of illumination are of significance.

Table 8.10 Influence of temperature on callus growth (mg fresh weight/explant) of *Nicotiana tabacum* (var. Xanthi 8/11 = n, 8/12 = 2xn) on MS medium, and 0.8% agar, 0.2 ppm 2.4D, and 0.1 ppm kinetin, 21 days of culture (Zeppernick, unpublished results of our institute)

	6°C	22°C	28°C
8/11	10	170	270
8/12	10	270	360

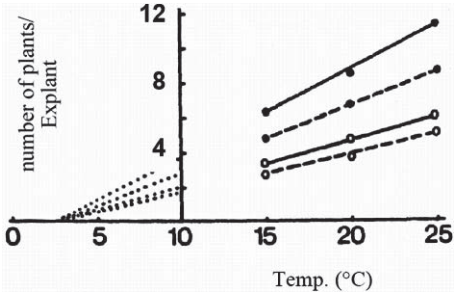


Fig. 8.15 Interactions of temperature, NAA, and wounding in the formation of adventitious shoots of explants of lily buds (van Aartrijk and Blom-Barnhoorn 1983): *closed circles* application of 0.5 μM NAA; *open circles* no NAA; *dotted lines* no wounding; *continuous lines* wounded explants

Table 8.11 Influence of light on the cell division activity (cell number × 10^3/explant) of cultured carrot root explants, cultured with m-inositol (50 ppm), IAA (2 ppm), and kinetin (0.1 ppm) in NL medium (see Table 3.3, 3 weeks of culture; Neumann and Raafat 1973)

	Dark	Light
Original tissue	8.0	18.0
No growth regulators	12.5	35.2
m-Inositol+IAA	25.4	146.1
m-Inositol+IAA+kinetin	444.0	752.4

Green cultures with photosynthetic activity will improve growth at higher light intensities. Also the response to growth regulators will be modified by illumination. In the experiment in Table 8.11, however, it is difficult to distinguish between light influences on photosynthetic activities, and direct influences on the function of the growth regulators. Sometimes an increase in growth can also be observed in the dark, and rhizogenesis in some systems can be promoted in darkness, or at low light intensities. The significance of illumination for protoplast cultures was discussed before (Chap. 5). With respect to light quality, Fluora-lamps would be preferred to the usual fluorescent lamps, because of a light spectrum close to that of sunlight. In our laboratory, however, only Osram lamps are used (15W/21, Lumilux White), with success.