SOME STUDIES ON SOMATIC EMBRYOGENESIS, A TOOL IN PLANT BIOTECHNOLOGY*

K.-H. Neumann, Institut für Pflanzenernährung, Justus-Liebig-Universität, 35390 Gießen, Germany

Introduction

Biotechnology is an area of production and research in which biological systems and biological principles are employed to solve technological problems. This includes beer brewing using yeast as well as the production of pharmaceuticals by immobilized enzymes. Also some systems in biotechnology were developed using cultured cells of higher plants to produce economically valuable chemical compounds, mostly of pharmaceutical interests, in fermenter systems up to some ten thousand liters. Still, in spite of great efforts during the last decades only rather few applications of such systems in commercial companies became known. Here economic considerations require that the product for the market should not only have the same price as the conventionally produced one from raw material obtained from agricultural products. In order to initiate a change in the production process the fermenter product has to be at least at halve the production costs as the conventionally produced compound.

Another area of biotechnology is micropropagation of plants. The aim of this technique is a fast production of a great number of genetically identical plants from a highly valuable mother plant or e. g. monosexual male and female plants. These plants can be either directly sold on the market for planting, used for breeding purposes, for genetechnology or the technique is used as a method for basic science studies.

To this end three broad techniques are available in many variations for micro propagation:

- **meristem cultures**, in which apical or axilary meristems are induced to produce shoots, which later are rooted in a suitable medium, usually containing an auxin
- **organogenesis** in callus material in which the differentiation of shoots and roots is induced and the two parts grow together to form plants, or possibly on which shoots are grafted following rhizogenesis
- **somatic embryogenesis**, during which a program of differentiation is induced in somatic cell material resembling closely the development of an embryo out of the zygote, i.e. the fertilized egg cell, though some differences exist (Ammirato, 1985).

The first two methods require the manual isolation of the original explant and of the plants produced and consequently are labor intensive with high production costs. However, for somatic embryogenesis the possibility of automatization exists. Further, by this method also artificial seeds for some purposes desirable are envisaged, although here again the costs involved will limit its application. Still, for some species methods are already available to produce embryos or to raise plantlets in fermenter culture. Here as an example within two weeks out of a 3g innocculum of induced cells ca 100 000 plantlets can be produced (Imani, 1999).

Another application of plant cell cultures in combination with gene technological methods is the production of medical agents like antigens to be used for vaccination. In our laboratory we introduced e.g. the gene for a coat protein of Hepatitis B into the carrot genome. Through somatic embryogenesis plants were raised containing the gene and expressing this protein in the tap root of mature plants ready for harvest. Clinical tests feeding these roots are now taken up.

** Based on a lecture at the 87th Indian Science congress Jan. 2000 in Pune, India.*

Somatic embryogenesis – General considerations

This article, however, will concentrate on somatic embryogenesis as described originally by Reinert (1958) and reported by the Steward group at Cornell University in the same year (Steward et al. 1958), in which the production of somatic embryos out of cells derived from root explants of the carrot were described and which were eventually raised to intact plants. From these plants explants were obtained to be used again as a source of a second generation of plants (Fig. 1).

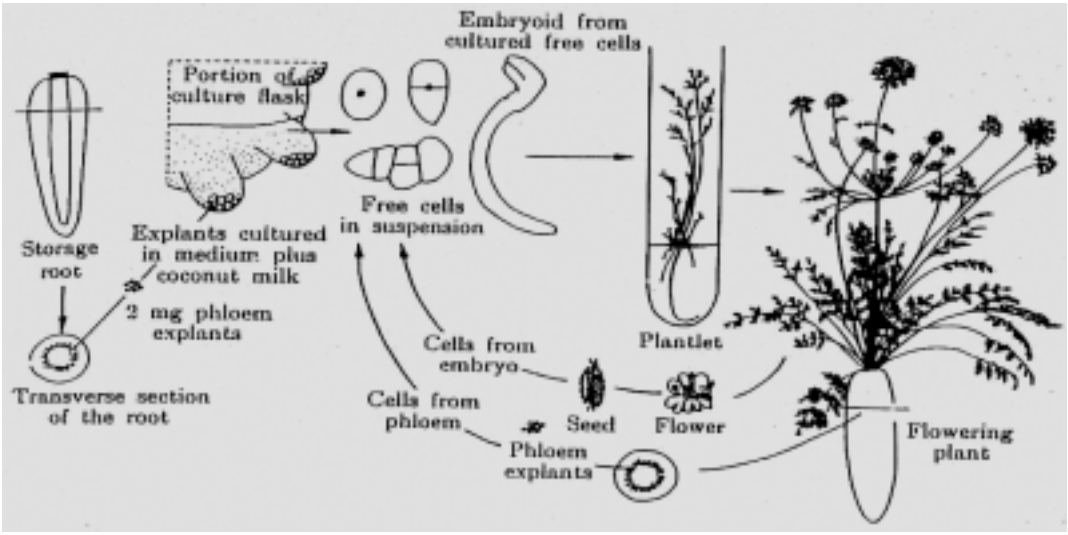


Fig.1 The growth of carrot plants continuously maintained through cultured cells. (Drawing by M. O. Mapes.) (See Science, 143, p. 20-27, 1964).

A peculiarity of eucaryontes is the propagation by zygotic embryogenesis following the fusion of the male and female gametes upon fertilization. However, for higher plants detours to zygotic embryogenesis became known for some species, generally summarized as apomixis (Fig. 2).

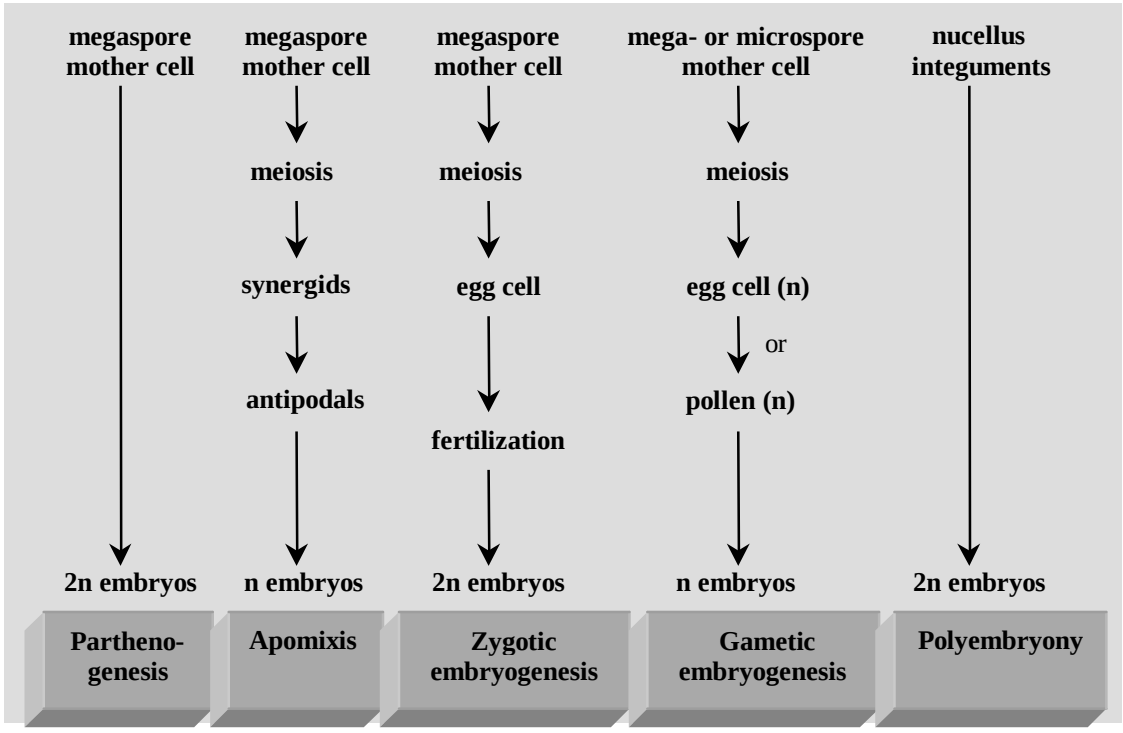


Fig. 2 Some pathways to produce haploid or diploid embryos in plant material.

These detours, however, are confined to the generative apex. Still, this natural competence of cells to somatic embryogenesis seems to exist more generally, though usually camouflaged in the intact plant. Here, somatic embryogenesis as practiced in many tissue culture laboratories, however, has to be induced by special conditions as can be provided in vitro by a suitable environment, consisting of a nutrient medium containing a stimulus, usually an auxin, sometimes also a cytokinin or both, and some requirements for appropriate temperature and illumination should be fulfilled. In some protocols also an ABA supplement is beneficial. In most plant species, however, this competence is lost during ontogenesis and somatic embryos can not be produced from explants of other parts of the plant. Thus, these species are heuristically defined as recalcitrant. Still, using cells of embryonic origin often proofed to be successful. This e.g. is the case for some economically important cereals. Here mature seeds are germinated in a medium containing high concentrations of 2.4-D (e.g. 10 -15 ppm 2.4-D) followed by excessive callus formation from which a great number of embryos can be derived after a transfer to an auxin free nutrient medium (Imani 1999, Fig. 3, unpublished results of our laboratory).

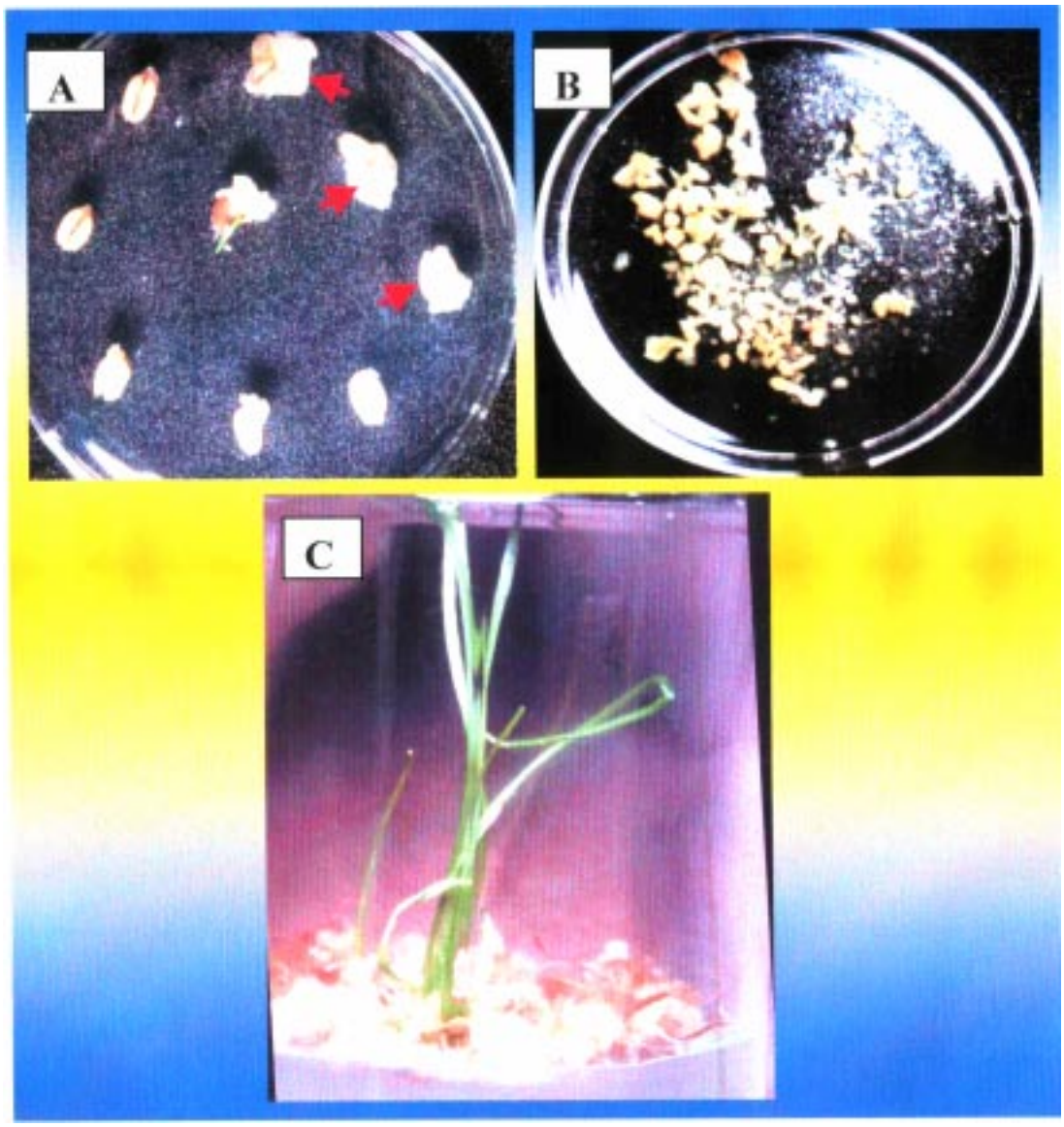


Fig. 3. A) Callus formation on germinating seeds of wheat; B) Development of somatic embryos in a wheat cell suspension; C) Young wheat plant derived by somatic embryogenesis (Imani, 1999)

Generally speaking, some hierarchical order within the plant seems to exist for many species, with highest success using embryonic cell material, followed by that of the hypocotyl, the petiole, the young leaves and finally the root (Fig. 4).

Embryo > Hypocotyl > Petiole > Leaf lamina > Root

Fig. 4 Competence to somatic embryogenesis in carrot (*Daucus carota* L.)

To this loss of embryogenic competence during ontogenesis, however, some exceptions exist and one of these is *Daucus carota*, the common carrot. Here it is possible to culture intact six to eight weeks old plants aseptically and partly submerged in an appropriate medium containing an auxin, and within about 4 weeks somatic embryos from all parts of the shoot appear (Fig. 5). If IAA is used as the auxin adventitious roots emerge about two weeks earlier (Schäfer et al. 1988). In this system the competence to somatic embryogenesis is apparently preserved beyond the embryo stage and it can be activated rather easily by a suitable environment.

Fig. 5 Development of somatic embryos on cultured intact carrot plantlets (ca. 6 weeks old)

Such favorable conditions to initiate somatic embryogenesis apparently can be also invoked in special genetic circumstances in intact plants on an inorganic Agar medium. This was observed on about 6 to 8 weeks old plantlets derived by somatic embryogenesis from fusion products of protoplasts obtained from transgenic plants of the wild carrot and transgenic plants of a domestic variety of *Daucus carota*. Into the wild carrot strain hygromycine resistance was introduced and 5-methyl-tryptophan resistance into the strain of the domestic variety (Rotin). On the petioles or also on roots of these cybrids a small callus developed on which later somatic embryos appeared. These could be used to obtain plantlets on which this process was observed again. – this was repeated for three “generations“ (Fig. 6, Chinachit 1991; De Klerk et al. 1997).This kind of development was not observed on sexual crosses of the “parents“ of the cybrids or of protoplast fusion products of the genetically unaltered parent genomes. In what ever way the introduction of the foreign DNA should have changed the developmental control system of the cybrids, possibly related to the

hormonal system, is unclear and no further explanation of these observations can be given at present.

Fig. 6 Development of embryogenic callus structures on petioles of fusion products of two transgenic carrot lines (Wild carrot = Hygromycin resistent, domestic carrot = 5-Methyl-tryptophane resistant on an inorganic Agar medium)

Results of histological examinations of embryogenic systems require a distinction between a **direct** and an **indirect** induction (Fig. 7). In **direct** somatic embryogenesis the embryos develop out of distinct cells without an interfering callus phase, whereas in the **indirect** process a callus phase characterized by high cell division activity is required during which apparently some process of dedifferentiation occurs. In the latter often genetic obstruction in the " offspring " are reported, usually called somaclonal variation. In some species like the carrot both of these developmental processes can be observed. This review will be mainly concerned with **direct** embryogenesis and a few results describing this process from our own research program will be reported.

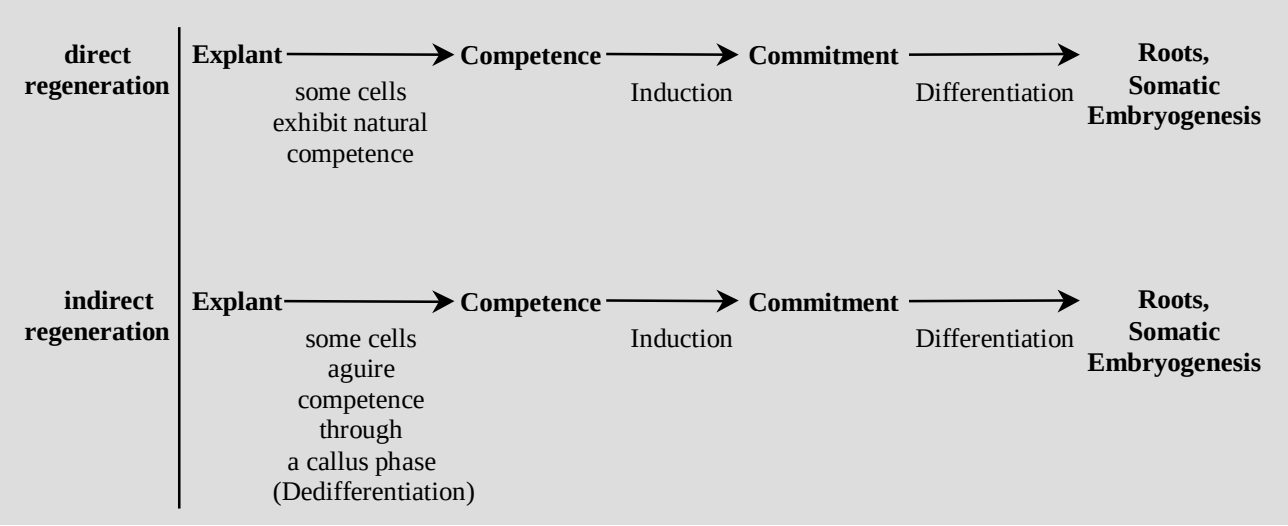


Fig. 7 Phases of direct and indirect regeneration in cultured plant tissue

For further studies to understand this developmental process it is not suitable to use cultured entire plants in vitro but it is more practical to use explants from the various organs. In our laboratory we mainly use explants of petioles of ca. 6 weeks old plants. Here two culture systems are in use (Fig. 8). If the rather stable 2.4-D is used as the auxin the cultured explants have to be transferred to an auxin free medium two weeks after the beginning of culture and about two weeks later the various stages of embryo development can be observed. Though rhizogenic centers can be observed histologically comparable to the IAA treatment as described below, rarely also some adventventitious roots develop. In the other system using IAA as an auxin after about 10 days adventitious roots appear and again at about 4 weeks after the isolation of the explants embryo development occurs. Since the IAA of the nutrient medium is destroyed after 2 or 3 days in the light (Bender and Neumann 1978) a transfer to an auxinfree medium is not required. In the former system the number of embryos developed is usually higher then in the second and for many biochemical investigations often the inclusion of root tissue is not desirable. Therefore in many studies the first system is preferred, for others the second (Neumann, 1995). A culture of 48 h in the 2.4-D-medium, however, is sufficient to initiate embryo development at t12 to t14 after initiation of the culture (Grieb, 1991/92).

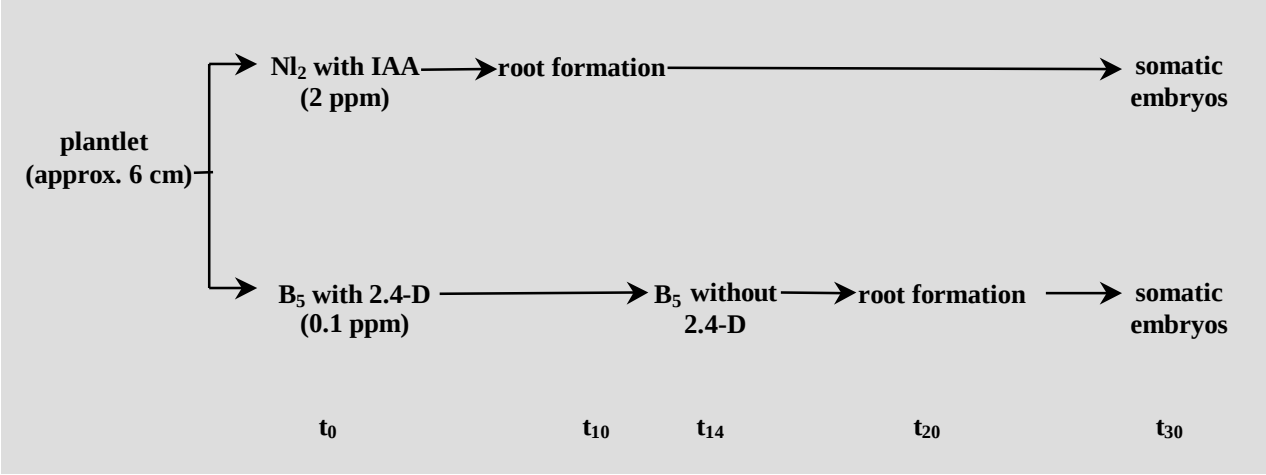


Fig. 8 Developmental processes in cultured carrot petiole explants

The various developmental processes are confined to special areas in the petiole and only the subepidermal cells (some times in near proximity to a glandular canal) are truly totipotent and competent to produce somatic embryos in a direct way without a prerequisite for a callus phase (Li and Neumann 1985, Neumann and Grieb, 1992, Neumann, 1995; Fig. 9).

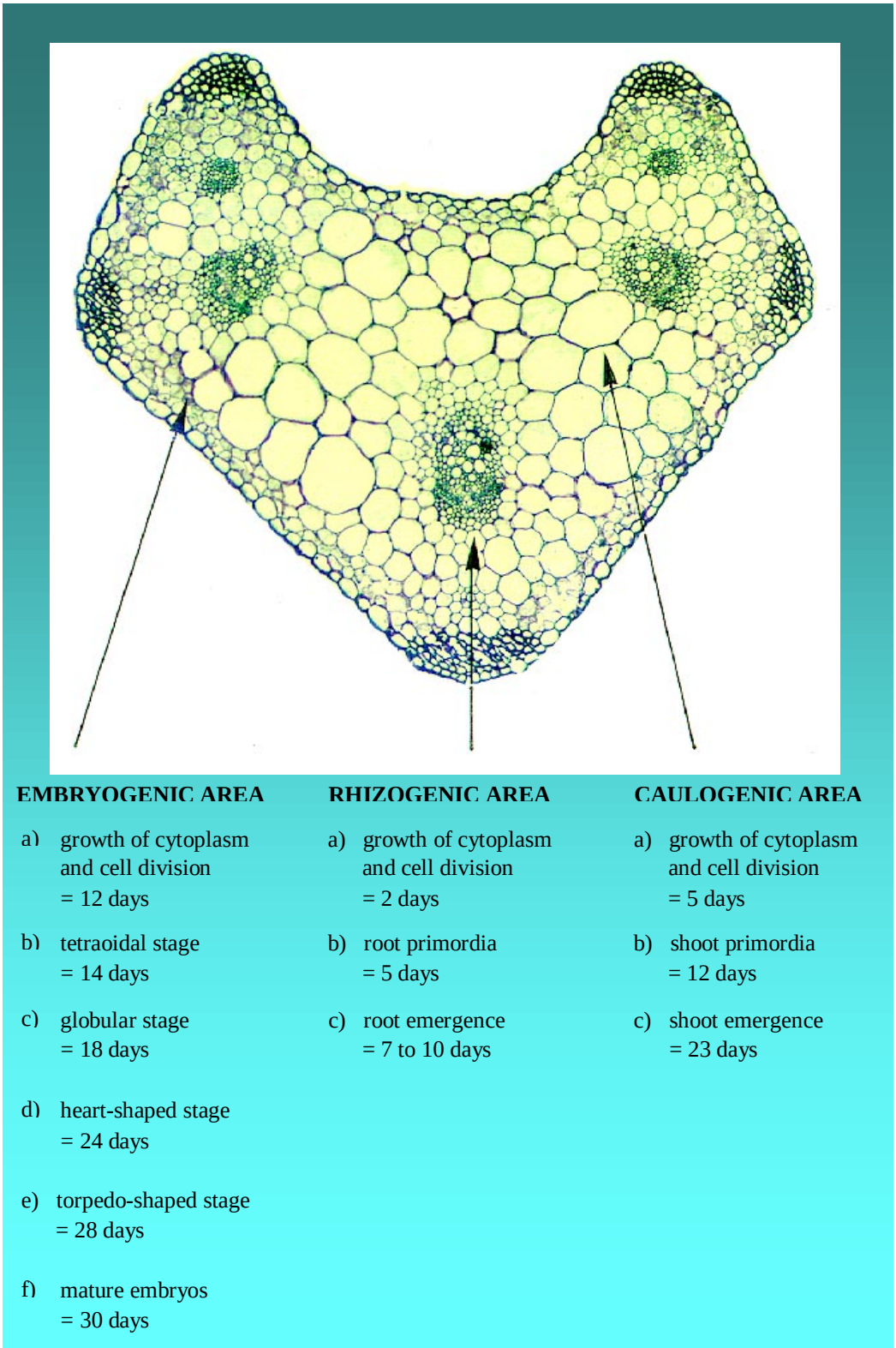


Fig. 9 Regenerational responses of various parts of cultured petiole explants of *Daucus carota* (Schäfer et al., 1985).

Interestingly, the cells forming the glandular canal contain high concentrations of auxins as shown by using transgenic plants containing the auxin sensitive MAS-promoter coupled to the GUS-gene (s. Fig. 16 for more details, see below), whatever the significance (Grieb et al. 1997). Rhizogenic

centers develop near vascular bundles prior to those embryogenic centers. In both cases some originally vacuolated cells start to produce new cytoplasm before the initiation of cell division. This contrasts with the initiation of cell division in cultured explants of the carrot tab root. Here the first responses are associated with the formation of phragmosomes localized in strings of cytoplasm traversing the vacuoles (Neumann, 1995). In these strings of cytoplasm also nuclear division takes place. The cytological events described in Fig. 9 are summarized from an extensive histological investigation and not all stages could be observed in all section obtained. Looking at longitudinal cuttings of cultured petiole regenerative centers are irregularly distributed along the axis.

Apparently the regenerative cells in these petiole explants contain at least a second competence to that brought about in the original petiole hidden during its development on the intact plant i.e. rhizogenesis in the cells near the vascular bundles or embryogenesis in those subepidermal cells. A rough summary for the 2.4-D-system is given in Fig. 10.

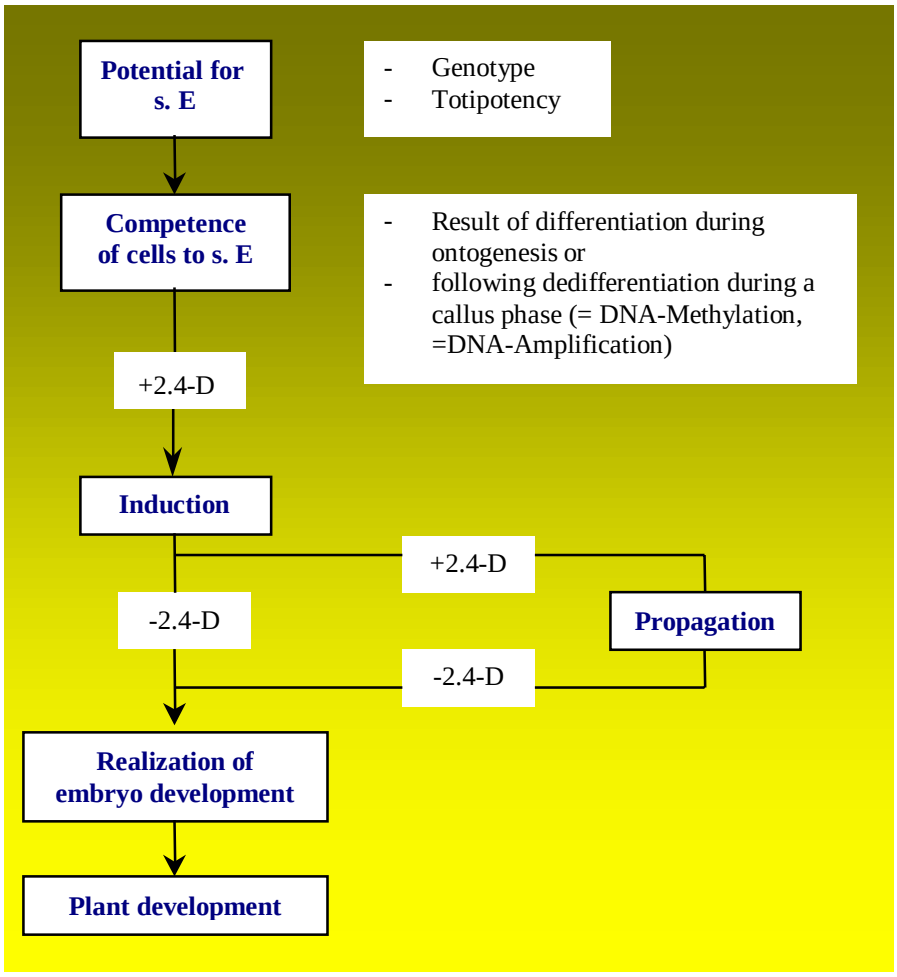


Fig. 10 Some steps in somatic embryogenesis

Although many laboratories use somatic embryogenesis nowadays mostly as an essential part of raising transgenic plants, neither its cytological nor its physiological or biochemical background is really understood. Although its practical application are obvious, still it is also an object of basic studies to understand differentiation as one of the great features of biological systems. What do we know now and on what lines should research concentrate?.

Below, first some essential requirements for somatic embryogenesis are given and these in general apply also to zygotic embryogenesis, and if one comes right down to it, actually to all differentiation pathways.

Requirements to induce somatic embryogenesis:
- competent cells
- suitable environment
- stimulus

Based on these requirements, the following questions were formulated for further investigations:

- What constitutes embryogenic competence at the cytological and the molecular level?
- How are embryogenic competent cells produced during ontogenesis of plants?
- Molecular organization of the program of somatic embryogenesis and its realization
- Of what consists the stimulus to induce the program of embryogenesis in competent cells?

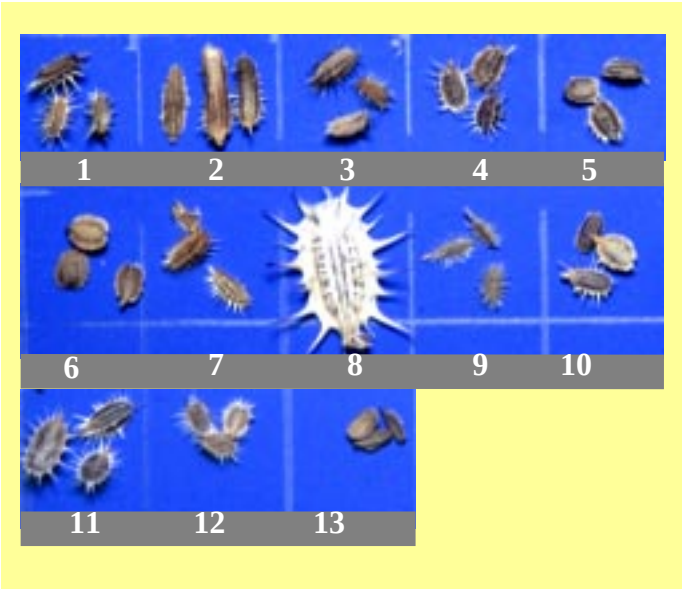
Of these four question complexes only the third and the fourth will be considered in this article.

Genetic aspects – DNA organization

Genetic factors play a central role to induce somatic embryos, i.e. to provide the competence of the species for the process. Here, great variation can be found even within a genera such as *Daucus*. Eight of twelve *Daucus* species cultured in identical conditions produced somatic embryos (*D. halophilus, D. capillifolius, D. commutatus, D. azoricus, D. gadacei, D. maritimus, D. maximus, D. carota*), whereas four species (*D. montevidensis, D. pussillus, D. muricatus, D. glochidiatus*) were not competent to do so (Fig.11).

Fig. 11 Seeds from some *Daucus* species

Species	Origin
1 = *D. halophilus.* (e)	Mediterean
2 = *D. capillifolius* (e)	North africa
3 = *D. montevidensis* L. (n)	Mediterean/ South america
4 = *D. commutatus* (e)	Mediterean
5 = *D. azoricus* ssp. (e)	Azores, Iran
6 = *D. gadacei* ssp. (e)	France
7 = *D. pusillus* Michx.(n)	North and South america
8 = *D. muricatus* L. (n)	Mediterean
9 = *D. glochidiatus* (n)	Australia
10 = *D. maritimus* (e)	Mediterean
11 = *D. maximus* ssp. (e)	Mediterean
12 = *D. carota* (wilde Möhre) (e)	Germany
13 = *D. carota* vari. (Rotin) (e)	Germany



(e) = embryogenic; (n) = non embryogenic

Since two of the recalcitrant species are native to the Mediterranean as well as three of the competent, broadly speaking the geographic location of origin seems to be of secondary importance (Le Tran Thi, unpublished results of our laboratory, in preparation).

We used several molecular approaches to characterize the DNA of competent and recalcitrant species and one is the RAPD-Technique which gives Random Amplified Polymorphic DNA sequences employing PCR, i.e. the Polymerase chain reaction. Here selected primers with special sequences of about 10 nucleotides are applied as starters for replication and the number of nucleotides between the primer sequences yields DNA stretches of various length which can be separated on a gel by electrophoresis. The location of the primer sequences on the DNA is genetically fixed and characteristic of the species.

The RAPDs of these *Daucus* species were compared, using some 30 primers and for one of these two areas were identical in the embryogenic species, missing in the recalcitrant, i.e. the areas with RAPDs at about 1100 bp and at about 650 of primer 3. We do not know yet what the function of these " marker DNA " sequences for an embryogenic potential could be and whether they have anything to do with somatic embryogenesis at all. The two conspicuous bands were isolated and sequenced. Without going into all details of this study which will be published soon in detail, both bands were quite similar in the embryogenic species with an identity between 70 and 95 % in the nucleotide sequence. Neither indicated an open reading frame and it is safe to conclude that no sequences of genes occur in these stretches of DNA. A search in data banks did not help for further characterization, these DNA-stretches were apparently not described previously. Further investigations are required to see whether these bands can be regarded as markers for the ability to produce somatic embryos, at least for the genera *Daucus*.

Lets go from species to varieties. Also here differences exist in respect to competence to produce somatic embryos as reported some years ago (Tab. 1).

Tab. 1 Somatic embryogenesis in cultured petiole explants of some carrot varieties (B5-System, 32 days of cuture)

Wild carrot	Vosgeses	Lobbericher	Rote Riesen	Rotin
+	+	⬬	+	++

⬬ = no s. E.; + = < 50 s. E./15 ml; ++ = > s. E./15ml

Whereas petioles from wild carrots, a French variety (Vosgeses) and the old German variety Rote Riesen are moderately competent under the conditions employed the variety Lobbericher is not embryogenic, the more recent German variety Rotin, however, is highly competent. The DNA of these varieties was compared by density gradient centrifugation (Tab. 2, Dührssen and Neumann 1980).

The GC content of DNA sequences obtained by mechanical sheering of total DNA varies and the density increases with GC content. GC rich sequences appear as heavy satellites of the main band DNA. The highest number of GC rich satellites (Cs_2SO_4/Ag^+ density gradient centrifugation) was found for wild carrots and in the domestic varieties always one or the other of the satellites are missing. This indicates that during domestication some DNA sequences are lost or altered in concentration (Dührssen et al. 1984).

Tab. 2 DNA components in some carrot varieties after Ag^+/Cs_2SO_4 density gradient centrifugation of sheared DNA.

	Density ($g.cm^{-3}$)						
	I Main	**I 1.422**	**II 1.448**	**IV 1.498**	**V 1.502**	**V 1.520**	**VI 1.539**
Wild carrots	**1.485**	+	+	+	+	+	+
Lobbericher	**1.482**	◂	◂	◂	+	+	+
Rote Riesen	**1.478**	+	◂	◂	+	+	+
Italian	**1.484**	◂	+	◂	◂	+	+

+ = present; ◂ =

The wild carrot is embryogenic and also the variety Rote Riesen, whereas Lobbericher and an Italian variety are not. The common denominator of wild carrots and Rote Riesen is the satellite with a density of 1.422 g/cm^3, missing in the nonembryogenic. Here again we do not know yet whether the DNA of this satellite has anything to do with somatic embryogenesis, or if it could be only regarded as a ”marker“ for the potential.

These GC rich satellites are either due to highly or middle repeated sequences generally not coding for proteins and are nowadays often defined as so called „junk DNA“. This holds probably true also for the RAPDs discussed before and the function of this “junk DNA” is unknown nowadays. But one has to keep in mind that this junk DNA can represent more than 90 % of the DNA of an organism. The genes proper could be similar or even identical in these varieties and even species with different embryogenic potential, what distinguishes them could be the organization of the genetic system, i.e. the ways and sequences in which they are activated to produce proteins and enzymes. Here possibly junk DNA plays a crucial role.

Some remarks on the physiological situation of cultured cells in vitro

Before turning to the endogenous hormonal system as related to the induction of somatic embryogenesis in cultured petioles, lets first consider the physiological situation cultured plant cells are in generally. They are isolated from their natural environment which provides them with water, nutrients and growth regulators generally supplied by the surrounding cells or even remote tissue in the intact plant as a result of the many interactions of the various cell and tissue types produced during the development of the intact plant in its environment. This, however, is in vitro replaced by the nutrient medium. This aspect is of some disadvantage if results and conclusions derived from tissue culture experiments are used to explain and understand problems of the physiology of intact plants. The advantage of cultured cells in vitro, however, can be seen in the opportunity to investigate the significance of one or the other ingredient in plants or other physiological aspects like the pH or osmotic phenomena for the performance of plant cells.
Secondly, by obtaining primary explants a wound is set which provokes physiological reactions by the explanted tissue and actually it was quite a natural consequence of tissue culture experiments for Haberlandt, the father of plant tissue culture at the end of the second last century, later to get interested in wound healing. Since many years it is known that wounding promotes the synthesis of ethylene and that in turn the synthesis of ethylene can be induced by auxins (e.g. Bender and Neumann, 1978 b; Fig. 12). Already after a few days of culture the amount of ethylene produced is closely related to the number of cells produced after a culture period of three weeks. Still, the basis of the phenomenon is not understood yet. Also rarely data are available on influences of decaying cells on the environment of viable cells in the same culture vessel. In the intact plants the number

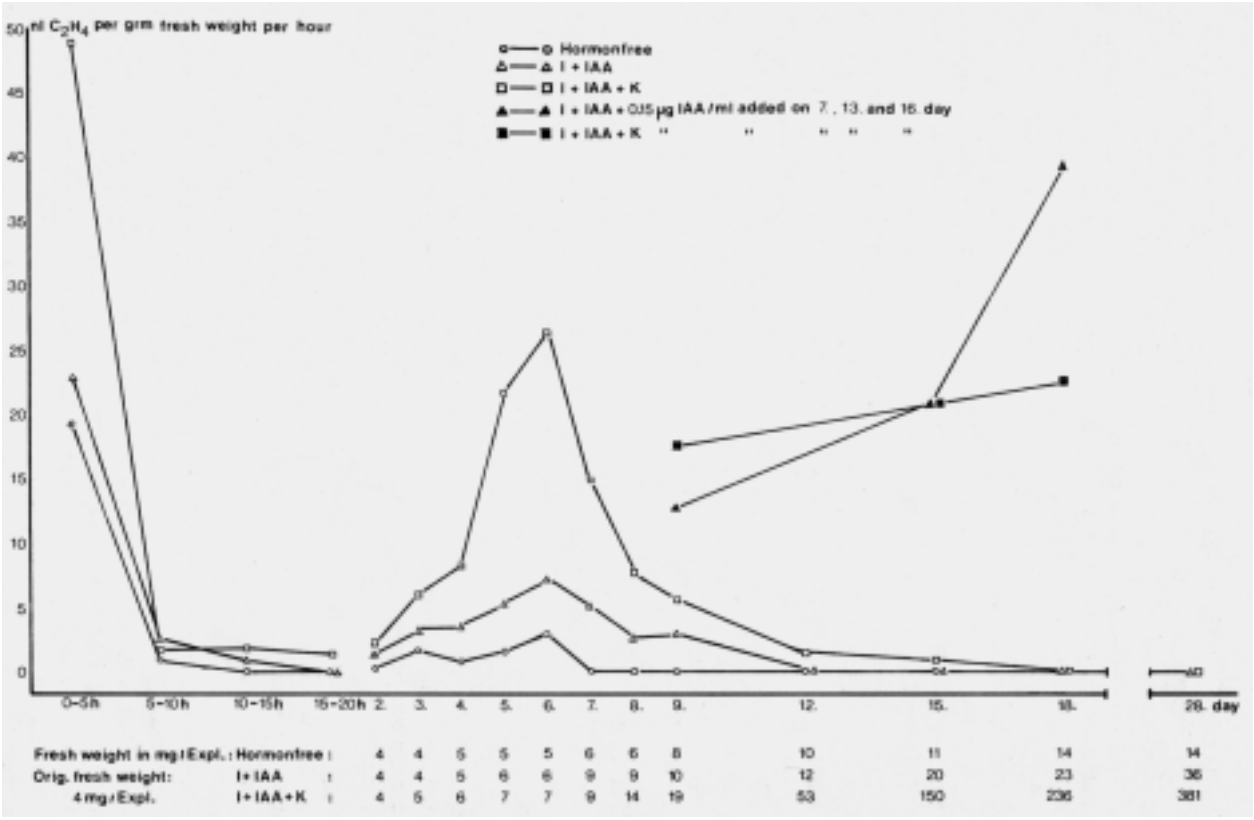


Fig. 11 Ethylene production and growth of cultured carrot root explants (sec. phloem) during a 28-day cultured period with various hormonal treatments. In two experiments 0.15 µg IAA/ml nutrient solution were added on the 7th, 13th and 16th day, respectively and ethylene production was determined 2 days later.

of dieing cells in lets say a leaf, should be much less in percentage then in cell suspensions and their influence should be more restricted than in a closed system as represented by cultured cells in vitro in its original sense of the word, namely in „glass“. Still, reactions observed in vitro, like somatic embryogenesis can be also observed on intact plants in vitro in special circumstances without wounding (Fig. 5, 6; s.above, Schäfer et al. 1988, Chinachit 1991, De Klerk et al. 1997). This indicates that wounding is not an essential prerequisite for growth of primary explants as often discussed.

The fate of cultured cells is determined basically by three physiological phenomena common also to all cells in general. These are cell growth, cell division and cell differentiation. They are interconnected and it is difficult to separate one from the other in experiments and in the interpretation of results derived from tissue culture experiment, let alone those obtained using intact plants as experimental systems. Still, an attempt shall be made by referring to some data obtained by using one of the most ancient and prominent experimental systems in the field of cell cultures, cultures of carrot explants and cell suspensions derived there off. This system was originally introduced by the Steward group at Cornell University in Ithaca, N.Y. in the late forties of the last century.

The phytohormone system

Competent cells have to be induced to somatic embryogenesis by a **trigger**, which in the carrot system is an auxin in a suitable nutrient medium such as a modified B5 medium or the NL-Medium developed in our laboratory (Neumann 1966, 1995). If these induced cells continue to grow in an

auxin free medium either brought about by photooxidative destruction of IAA or by a transfer into an auxinfree medium as in the case of 2.4-D embryo development will proceed. If, however, these induced cells are continuously subcultured in the 2.4-D medium this commitment will be preserved for many years and the realization of the embryogenic program will be prevented till these cells are transferred into an auxin free medium (Fig. 10).

Lets first turn to the induction process. As shown for many cultures the hormonal system plays a key role in the induction of somatic embryogenesis. It is known from earlier investigations that cultured cells develop an endogenous hormonal system different from that of the original explants (Bender and Neumann, 1978 a, Stiebeling and Neumann, 1987) and therefore as a first approach the concentration of some phytohormones was determined at various stages during the induction of somatic embryogenesis in cultured petiole explants (Fig. 13; Grieb et al. 1997). Of those phytohormones determined ABA dominates in the original petiole explants followed by a much lower concentration of IAA and of cytokinins. Whereas the ABA concentration more or less continuously decreases during culture, the IAA concentration reaches a maximum 6 days after the start of the experiment, concurrently with the start of the development of adventitious roots. After this the concentration of this native auxin continuously decreases till at the 14th day again a small peak appears. At the tenth or eleven day, though at a lower level, also a maximum of the cytokinin

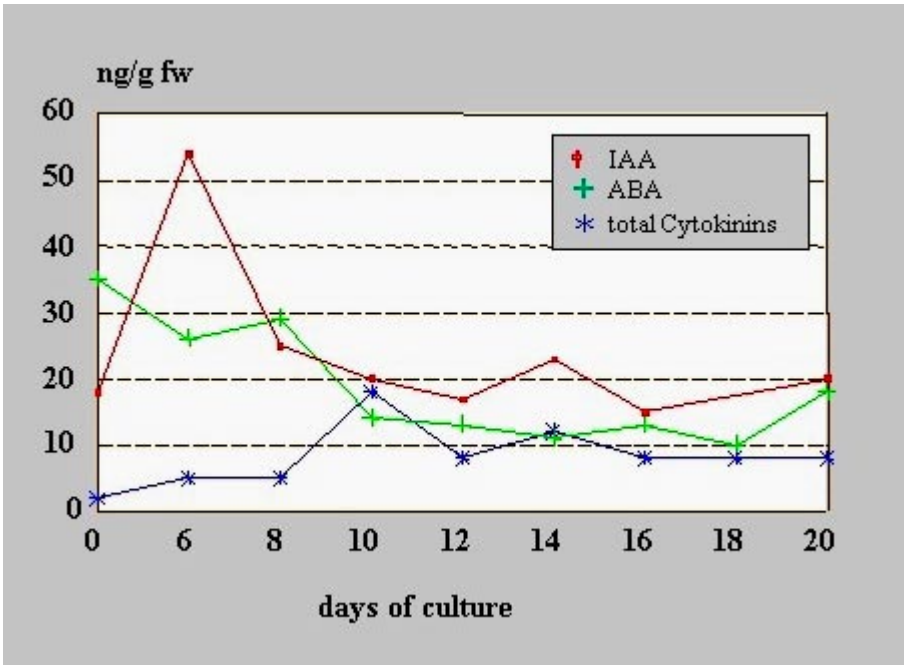


Fig. 13 The concentration of some endogenous phytohormones in cultured carrot petioles (NL-System) at several stages during the induction and realization of somatic embryogenesis

fraction occurs, dominated by 2iP and its riboside coinciding with the onset of cytoplasmic growth in small originally vacuolated subepidermal cells to become embryogenically induced as described above. In summary, the cultured petiole explant produces its own hormonal system with continuous changes of the ratio of concentration of these phytohormones investigated to each other. The significance of such changes to plant development were recognized many years ago by Skoog and Miller (1957) and could possibly play a decisive role also in cultured petiole explants to become embryogenic. This is to some extend confirmed by comparing these data obtained from a highly embryogenic variety with those of a recalcitrant variety with a completely different course of the concentration of IAA and of cytokinins as published earlier (Li and Neumann, 1985). Also in cultured cells the concentration of phytohormones follows a circadian rhythm which has to be

considered in interpretation of results on the phytohormone system in vitro. This was at least demonstrated for callus cultures of root origin (Fig. 14).

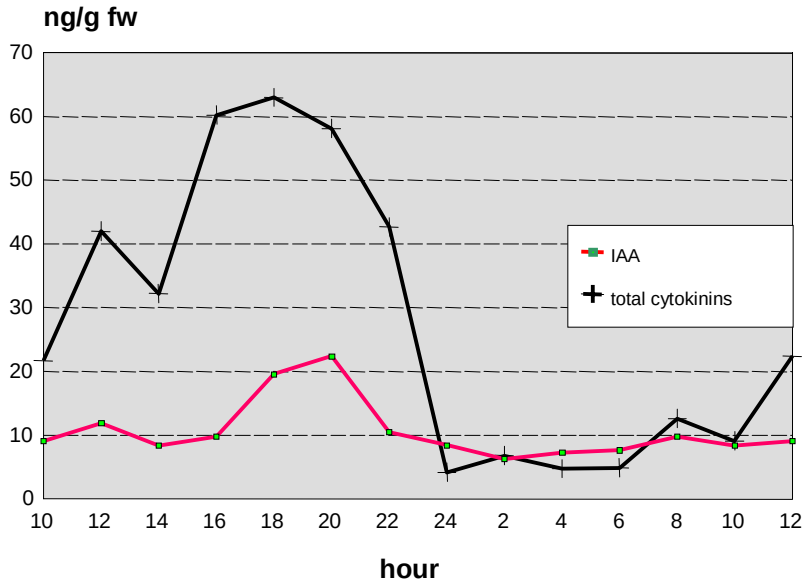


Fig. 14 The concentration of IAA and of cytokinins in cultured carrot root tissue (constant environment: continuous illumination of ca. 5000 lx, 22°C) during a 26 h experimental period. The samples were taken at 2 h intervals.

Using petiole explants from transgenic plants containing the auxin responsive MAS promoter linked to the GUS reporter gene (Fig. 15, 16) the distribution of auxin within the cultured petiole could be followed during the induction phase of somatic embryogenesis.

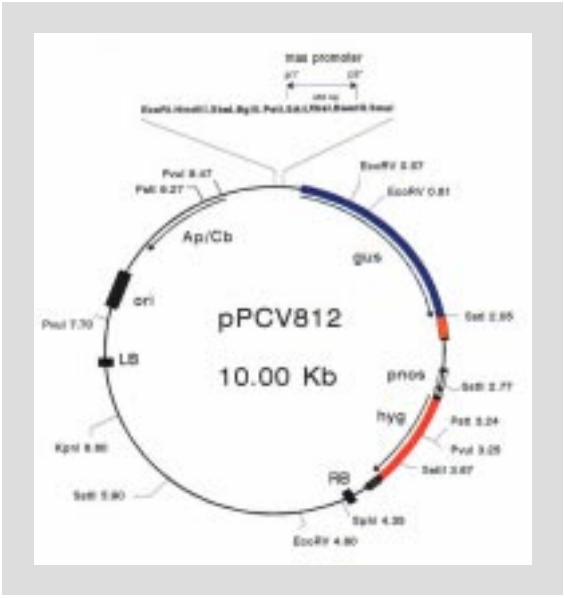


Fig. 15 Plasmid pPCV812 with the MAS promoter and the GUS reporter gene, hyg=Hygromycin resistance, Ap/Cb=Ampicillin/Carbenicillin resistance (courtesy of Dr. Z. Koncz, Max-Planck- Institut Cologne, Germany, for providing the plasmid)

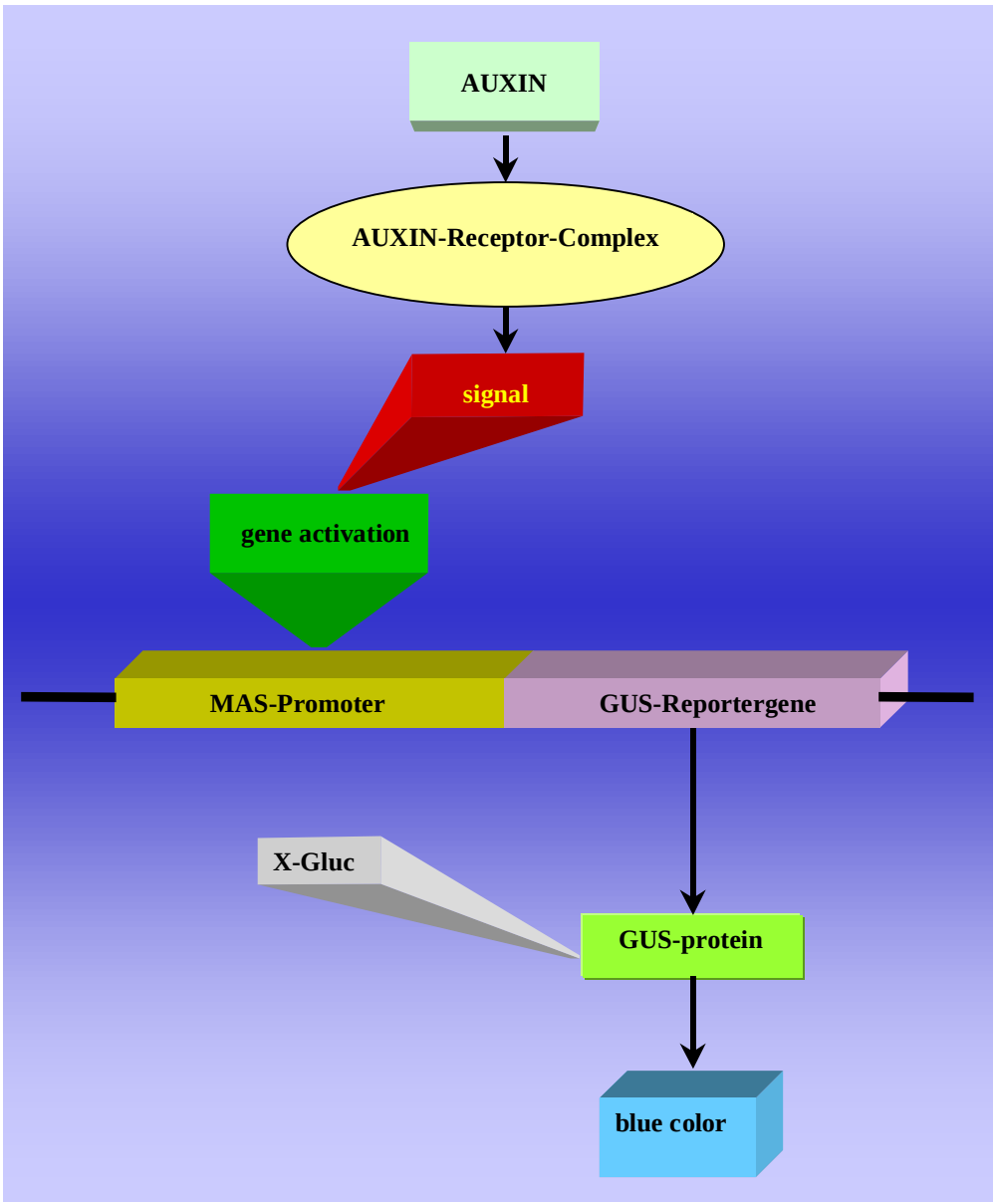


Fig. 16 Reaction system of the MAS-promoter coupled to the GUS-reporter gene

Whereas in the original petiole explant at explantation the auxin is more or less evenly distributed throughout the petiole, after 5 to 6 days in culture, concurrently with the formation of root primordia near vascular bundles, IAA is now accumulated in this area of the petiole (Fig. 17). After 9 days of culture immediately before root development can be observed, in this area the auxin concentration is drastically reduced and IAA accumulates now in the emerging embryogenic areas. Apparently in the cultured petioles not only changes in the total concentrations of IAA occurs, but also distingued changes in its distribution related to histogenic events can be observed (Grieb et al. 1997, Imani 1999).

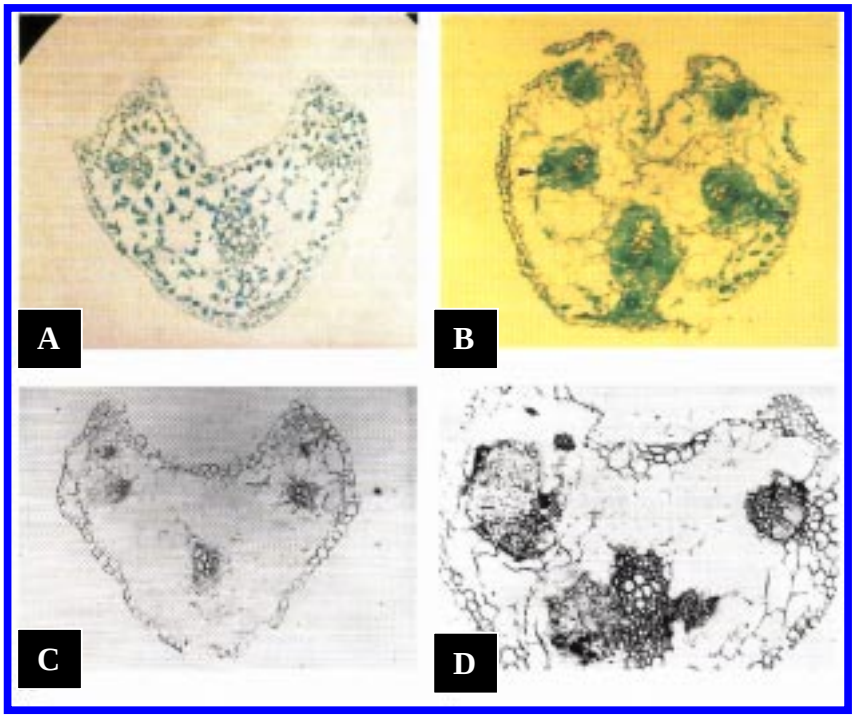


Fig. 17 Section of petiole explants of transgenic carrots. The explants were obtained from plantlets containing the MAS-promoter (auxin sensitive) coupled to the GUS reporter gene. GUS activity was detected after application of X-Gluc to indicate the occurrence of auxin. A = at explantation, B = after 5 days of culture. Note the high response of cells forming the glandular canals (arrows), C = historadioautogram at d0 after labelling for 3h with ^{14}C-leucine, D = the same labelling duration as in C after 7 days of culture.

Although not required for the *Daucus carota* system to be supplied to the medium to induce somatic emryogenesis, cultures of other plant species often require an ABA supplement for the process. In *Daucus carota* cultures an ABA supplement actually seems to be slightly inhibitory. If ABA plays a role in the process then it should do so during the first week of culture when its concentration in the petiole explants is high. Of all the *Daucus* species investigated (s. above) *Daucus carota* exhibits the highest embryogenic potential. To investigate the role of ABA in somatic embryogenesis first its concentration in the original petiole explants of the various *Daucus* species embryogenic and nonembryogenic was determined. The highest concentration of free ABA was found in the highly embryogenic domestic carrot variety Rotin. The other embryogenic species with a much lower potential for the process had considerably lower concentrations of this phytohormone. The number of embryos produced was greatly increased and the time required for the initiation of embryogenesis was clearly reduced by application of ABA to the medium used for culture (Le, unpublished results of our laboratory). In still unknown ways ABA seems to be just as involved in embryogenesis as auxins. Possibly the high potential of *Daucus carota* for somatic embryogenesis could be related to the high concentration of free ABA in the original explants of this species at explantation. Studies on the distribution of ABA in analogy to those on IAA using the SalT promoter linked again to the GUS reporter gene or the GFP-gene are taken up. Such transgenic plants will be also excellent tools in general to study the concentration of hormones during the development of intact plants. Some samples are given in Fig. 18.

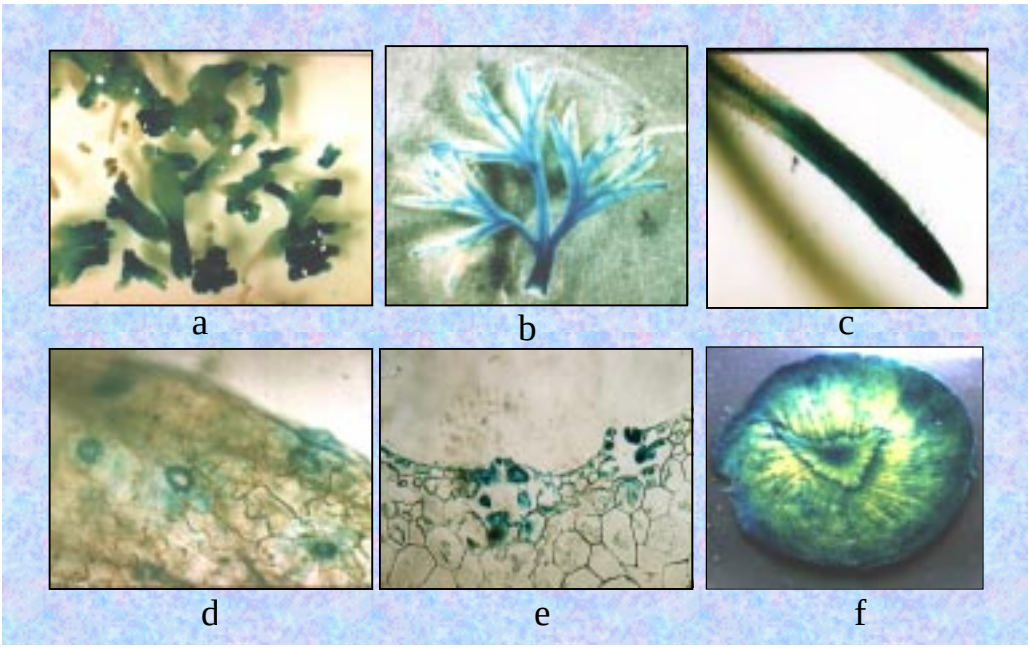


Fig. 18 IAA distribution in various parts of transgenic carrot plants after introduction of the system MAS-promoter and GUS reporter gene (Imani, unpublished results of our institute s. Imani et al. 1999).
a) somatic embryos; **b)** leaf lamina, after chlorophyll extraction; **c)** root tip; **d)** leaf with open stomata; **e)** mechanical wound on petiole; **f)** cross section of storage root

The protein system

At some of these stages of the petiole system used for hormonal investigations also the protein synthesis pattern were determined (Grieb, 1991/2, Neumann and Grieb, 1992; Grieb et al. 1997). For localization of protein synthesis activities within the cultured petioles ^{14}C leucine was applied and its distribution was followed by histoautoradiography and electrophoresis after protein extraction. Most of the labelling was concentrated in those parts of the petiole which indicated also an accumulation of auxin, i.e. the protein synthesis at one stage or the other should occur in cells engaged in differentiation (Fig. 17). For these elaborate studies the cultured petiole explants in the induction medium, i.e. the B5 medium supplemented with 0,5 ppm 2.4-D, were labelled for 3 hours each with ^{14}C leucine, starting at 0 h,5 h then the next was at day 7 and the last labelling was at day 14 of culture. The soluble protein was extracted as described earlier (Gartenbach-Scharrer and Neumann 1990), the extracts were separated by 2D-gelelectrophoresis followed by either staining with coomassy brilliant blue to visualize proteins on the gels or were subjected to fluorography to detect the distribution of ^{14}C to the various proteins. In all ca. 280 proteins were detected on the gels in these investigations by either one detection method or by both. According to the staining and labelling pattern and the occurrence at the various labelling periods the proteins were arranged in 9 groups and set into relation to cytological events during the induction of somatic embryogenesis (Fig. 19). A continues change in the composition of the protein moiety occurs with the initiation or termination of the synthesis of proteins of one or the other group in a sequential and hierarchical pattern during the induction of somatic embryogenesis. Some proteins, however, were detected throughout the whole experimental period and should represent so called household proteins. Of special interest are the 17 proteins which could be only detected at day 14 and which should be somehow related to the occurrence of the cytoplasm-rich subepidermal cells destined to be the origin of somatic embryos and/ or to the initiation of embryo development (Grieb 1991/92).

As a first approach to understand the physiological significance of these proteins for somatic embryogenesis based on the isoelectric point and the molecular weight a search for analogs published in data bases like Swiss-Prot or Trembl was undertaken. Of those 17 proteins only synthesized at day 14 after start of the cultures for only 3 analogs could be found. Interestingly all

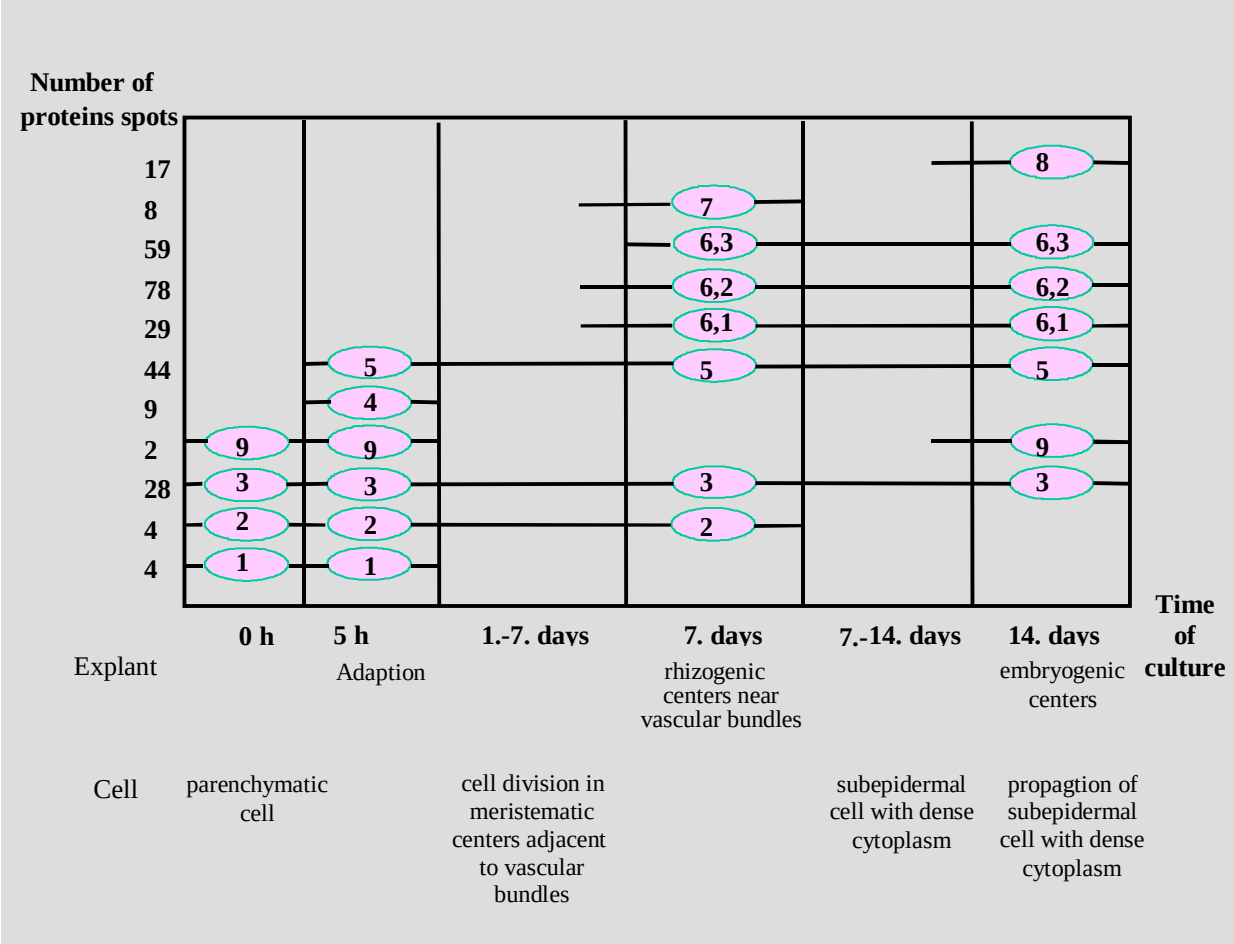


Fig. 18 The occurrence of various protein groups in cultured carrot petiols at some periods of the cultural cycle (B5-Medium with 0,5 ppm 2.4-D, Neumann & Grieb 1992, Neumann 1995)

three are related to carbohydrate metabolism, namely alpha-amylase, phosphofructokinase and alcoholdehydrogenase (Mashayekhi-Nezamabadi, unpublished results of our laboratory). Another protein described by the Komamine group as characteristic for an embryogenic status was already synthesized at the seventh day of culture prior to the cytoplasmic growth of subepidermal cells destined to become embryogenic, usually observed from the 10. or 12. day of culture onward. This indicates that the initiation of the embryogenic program starts quite some time before its histological evidence.

For characterization of the function of alpha-amylase synthesis starch content and starch distribution by histochemistry on the 12. and the 14. day was compared. Whereas up to the 12. day high starch accumulation in parenchyma cells of the petiole were observed, the starch concentration on the 14.day was reduced, presumably the result of the action of the newly synthesized alpha-amylase molecules (unpublished results of our laboratory). This was in tendency confirmed by enzymatic determination starch. The glucose resulting from starch break down should be phosphorylated by Hexokinase in preparation for further metabolic processing.

As described for other plant species also cultured cells of carrot can perform photosynthesis (Neumann, 1962, 1995; Neumann and Raafat, 1973; Bender et al.1981).For further studies on the function of free glucose a photoautorophic cell culture strain also embryogenic was employed. The cells of this strain are able to grow slowly at ambient CO_2 in the light without differentiating somatic embryos. This, however, is the case after a supply of sucrose, of glucose and less pronounced of fructose or mannose at low concentrations (Pleschka 1995, Grieb et al. 1994, Tab. 3).

Tab. 3 Influences of various carbohydrates on the development of somatic embryos in a photo-autotrophic cell suspension (*Daucus carota* L., var. Vosgeses, hormone free medium, ambient CO_2)

A)	**0.06 M**		**B)**	**0.003 M**	
	Sucrose	+		Sucrose	+
	Glucose	+		3-OM-Glucose	-
	Fructose	+			
	Galactose	+			
	Ribose	-			
	Xylose	-			
	Arabinose	-			
	Pyruvat	-			

To distinguish nutritive from regulatory effects of these sugars on somatic embryogenesis, cell suspensions of this autotrophic strain were cultured at an elevated CO_2-concentration of 2,3 %. Here growth is comparable to that after a supplement of 2% sucrose, still also here somatic embryogenesis could not be observed (Pleschka, 1995, Grieb et al. 1994, Tab. 4).

Tab. 4 Influences of sucrose and an elevated CO_2 concentration on fresh weight, cell number/g Fr. Wt and somatic embryogenesis of a photoautotrophic carrot cell suspension culture (*D. carota*, var. Vosgeses), 42 days of culture.

	ambient CO_2			**2.34 % CO_2**		
sucrose concentration, in % in the medium	mg Fr. Wt increment per 100 mg inoculum	cell number per g Fr. Wt • 10^6	s.E.	mg Fr. Wt increment per 100 mg inoculum	cell number per g Fr. Wt • 10^6	s. E.
0	288.6	2.90 ± 0.5	-	753.9	10.69 ± 0.39	-
0.1	436.8	3.48 ± 0.44	+	644.3	7.49 ± 2,.28	+
0.5	1282.1	5.62 ± 0.38	+	1151.8	9.68 ± 1.71	+
1.0	2109.4	6.39 ± 1.4	+	1174.6	8.14 ± 1.61	+
2.0	2341.1	7.28 ± 1.8	+	1875.4	10.85 ± 2.67	+

This, however, is the case after an additional supply of 0.1% of sucrose to the nutrient medium. These results indicate that the requirement of sugar should not be its only function as a nutrient but also as a regulator in the development of embryos (Pleschka 1994, Grieb et al.1994). Glucose can be substituted by Glucose-6-Phosphate but not by Glucose-1-Phosphate in petiole cultures which is

the result of starch break down by starch-phosphorylase and bypasses Hexokinase for further break down. Apparently for embryo development Hexoskinase activity with free hexose or glucose-6-phosphate as substrates as described for animal systems seems to be required for embryo development. To substantiate these ideas the occurrence and the metabolic activity of hexokinase has to be investigated further. Especially important shall be investigations on the histological and cytological distribution of the enzyme. Such investigations are presently in progress.

Cell cycle studies

To understand the regulation of embryo development in more detail the relation of some aspects of the cell cycle to differentiation shall be considered. However, first some related data on rhizogenesis in cultured explants of the secondary phloem of the carrot tap root will be discussed as a basis. As already shown earlier a kinetin supplement to a nutrient medium containing IAA greatly increases growth of cultured cells, mainly by cell division (Linser and Neumann, 1968) and concurrently suppresses regeneration, e.g. adventitious root formation. Whereas those explants growing in a hormone free nutrient medium show, besides mainly growth by cell expansion, also a few cell divisions (data not given), those supplemented with IAA and m-Inositol without kinetin show about a tripling of cell number per explant during a three weeks cultural period indicating a clear, though rather small log phase of cell number (Fig. 20).

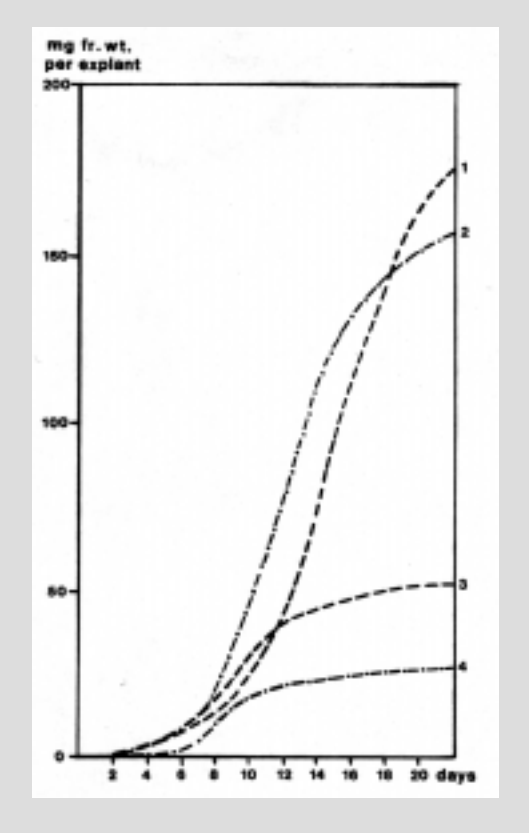
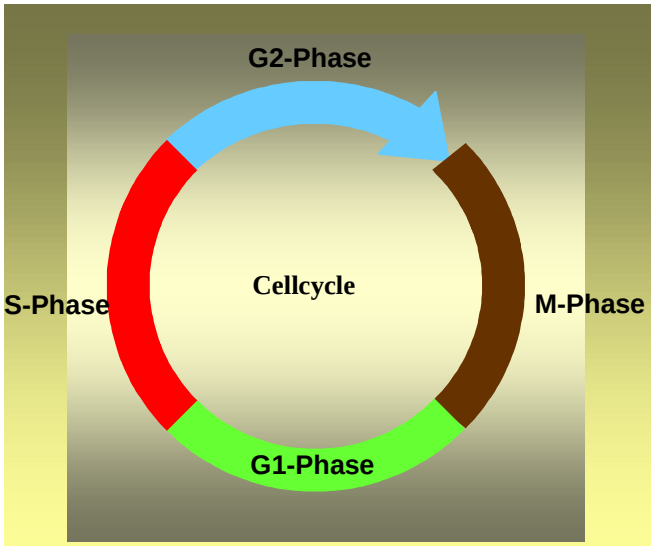


Fig. 20 The influence of kinetin on fresh weight and number of cells during a culture period of 21 days. 1: mg/fr. wt. per explant, + kinetin; 2: cells per explant x 10^3 + kinetin; 3: mg/fr.wt. per explant, - kinetin; 4: cells per explant x 10^3, -kinetin; kinetin = 0,1 ppm (Neumann 1968).

The samples supplemented additionally also with Kinetin are characterized by an extensive log phase of high cell division activity and a decrease of cell size. Most remarkably only the treatment containing IAA and m-Inositol in the medium is able to differentiation of cells leading to the formation of adventitious roots. Both other treatments remain as morphologically undifferentiated cell material. Still, also the production of a callus could be defined as a kind of differentiation and after about 4 - 5 weeks of culture in a medium originally also supplemented with kinetin during a stationary phase of cell division also here some roots appear. A similar course of the cell number per explant can be also observed if kinetin and IAA are replaced by the rather stable auxin 2.4-D, which strongly promotes growth by cell division. Here also the formation of adventitious roots is prevented. Apparently some correlation between the possibility of root formation, i.e. differentiation and cell division activity seems to exist.

The number of cells produced per cultured explant during a given unit of time is either due to the number of cells engaged in active cell division or the length of time between successive divisions, i.e. the duration of the cell cycle, or both. This could be demonstrated for a kinetin supplement resulting in the reduction of the duration of the cell cycle for *Datura* as well as for carrot cell cultures by about 60 – 90 minutes. This was due mainly to a reduction of the G1 phase of the cell cycle (s. Fig. 21, Blaschke et al. 1978, Froese and Neumann 1997).



Cellcycle-phase	Duration (h) without kinetin		with kinetin	
	Datura	*Daucus*	*Datura*	*Daucus*
Cellcycle (total)	10,4	9,5	9,2	8,5
G1-Phase	6,7-7,2	7,0	5,0	5,5
S-Phase	2,0	1,5	2,0	2,0
G2/M-Phase	2,2	1,0	2,2	1,0

Fig. 21 The influence of kinetin on the duration of the cell cycle and its various phases

Although many information are still lacking, the following hypothesis was formulated many years ago to explain the correlation between cell division activity and differentiation (Neumann 1968, s. also Neumann 1995). After the completion of the M-phase during the G1-phase genetic information are utilized in a hierarchical sequence till this is interrupted at the onset of the S-phase and it depends on the duration of the G1-phase which part of the genetic information potential can be realized during the cell cycle. In our system apparently those information related to differentiation are localized later in this sequential activation of the genetic information. The initiation of the S-phase and consequently the potential to differentiation is here determined by kinetin. In plus-kinetin

treatments the length of the G1-phase is just not sufficient to activate those information required to bring about root or embryo development. Theses ideas are illustrated by the following scheme.

Kinetin, cell cycle phases and differentiation: (********* genetic information in G_1)

§--------M--------§*-*-*-*-*-*-*-*-*-*§--------S--------§--------G_2--------§--------M----
G_1

Without kinetin: Cell cluster, PEMs, rhizogenesis, somatic embryogenesis

§--------M--------§*-*-*-*-*-§--------S--------§--------G_2--------§--------M----
G_1

With kinetin: cell cluster, PEMs

The results reported for cell suspension cultures below confirm this old hypothesis to some extent. In the regulation of the hierarchical activation of genetic information during the G1-Phase certainly the cycline system which comes to be known in more details recently seems to play a central role, which , however, will not be dealt with here.

A prolongation of the cell cycle duration alone is not sufficient to promote differentiation. Just as an omission of kinetin, as an example at iron deficiency the number of cells produced by the cultured explants is also reduced, however, cell growth is also retarded and no root formation could be observed (Neumann 1972).

As mentioned before also in the hormone free treatment no differentiation was observed. Here only about two rounds of cell division occurred in some areas of the explant and than growth takes place only as cell expansion. Cell division activity is also strongly reduced in the treatment supplemented with IAA and m-Inositol as compared to the plus kinetin treatment, however, here a short log phase of cell division activity with a reduced duration of the cell cycle occurs. During this log phase apparently some dedifferentiation takes place in dividing cells, later to become rhizogenic. This dedifferentiation may result in some reprogramming of the genetic machinery as well as the cells in toto, possibly both. In cells a continuous protein turn over takes place and if the hypothesis of the relation between the duration of the cell cycle and realization of genetic information has some meaning then some proteins with coding positions later in the G_1-Phase, however present at the beginning of this short log phase with high cell division activity, could be removed from the cells by break down without being replaced by newly synthesized molecules due to the reduction of the G1-Phase. If the protein moiety of a cell represents its differentiational status then this should be responsible for changes in differentiation at the molecular level of cultured cells engaged in active cell division.

Reprogramming of the genetic machinery could be due to a change in DNA methylation (LoSchiavo et al. 1989.). Also in our system during a log phase of cell division an increase in methylation can be observed and only at the stationary phase of cell division also the amplification of some DNA sequences was found, usually associated with de novo differentiation. Here DNA methylation is reduced again. As was shown later, this increase in methylation during the log phase of cell division is not directly related to a kinetin application but due to the reduction of cell cycle duration (Dührssen and Neumann 1980, De Klerk et al. 1997, Arnholdt-Schmitt et al. 1998).Variation in the amplification of DNA sequences requires also mechanisms of DNA break down. Years ago the occurrence of such “metabolic” DNA was reported in particular for differentiating cells employing the pulse/chase technique (Schäfer et al. 1978).

As mentioned above, the higher cell division activity in the embryogenic carrot cell suspension due to the Kinetin supplement to the nutrient solution results in a reduction of the duration of the cell cycle by about one hour (Fig. 20, Froese and Neumann 1997), again basically due to a reduction of the duration of the G1-phase as was shown for *Datura* cultures already many years ago (Blaschke et al. 1978).Whereas in the carrot system in the kinetin free medium supplemented with IAA and m-inositol (2 mg/l) rhizogenesis and the development of embryos up to plantlets was observed, in the medium supplemented with kinetin (0,1 mg/l) in addition to IAA and m-inositol no roots were produced and embryo development did not proceed beyond the stage of PEMs (pre embryogenic masses). It was assumed that also here a correlation between the duration of the G1-phase and the regeneration capacity exists. The determination of protein synthesis pattern during the prolonged G1-phase by labelling of proteins using synchronized cultures (FDU/Thymidine System (s. Fig. 22) with ^{14}C leucine as described above for the petiole system indicated the synthesis of 132 additional proteins in the minus Kinetin cultures, prevented to be synthesized in the Kinetin treatment due to the immediate

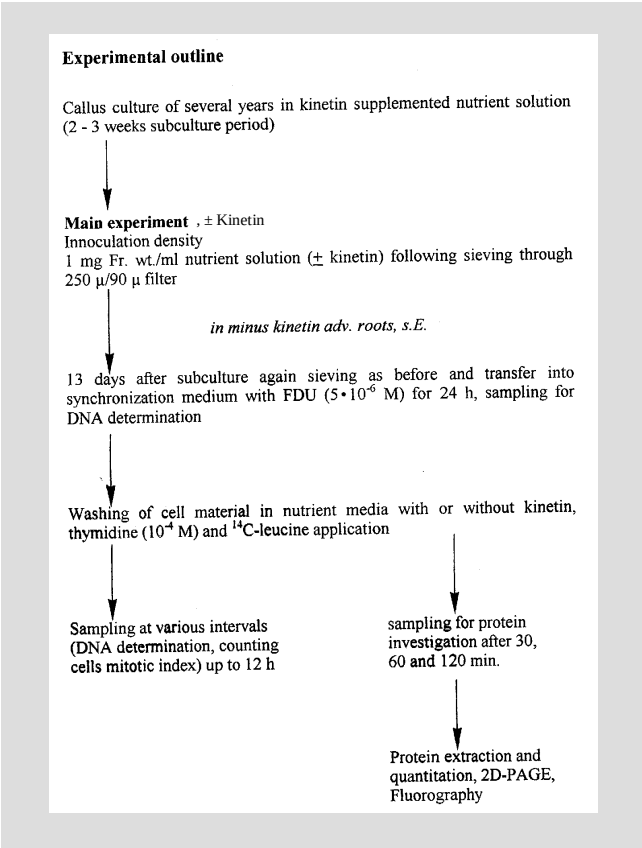


Fig. 22 Protocol for cell cycle synchronisation and labelling with ^{14}C –leucine of an embryogenic *Daucus* cell suspension

transition from the G1-phase to the S-Phase of this treatment. These cells in the Kinetin supplemented medium pass immediately upon the addition of thymidine into the S-phase with strongly reduced protein synthesis activity whereas those of the kinetinfree treatment remain in G1 for up to another 60 to 90 or even 120 minutes (varies from one experiment to the other). During this prolonged G1-phase the synthesis of these additional proteins occurred in a hierarchical sequence with initiation and termination of the synthesis of individual specimen (Tab. 5).

Tab. 5 Summary of ^{14}C-labelled protein spots (^{14}C from ^{14}C-labelled leucine) in the soluble protein fraction at various stages of the cell cycle of synchronized carrot suspension cultures. The numbers between the sampling times indicate either that a given labelled protein spot was missing at the next sampling (-), it was an additional one (+) or it occured already at the former sampling (id.).

duration of labelling	**30 min**		**60 min**		**120 min**
			Kinetin free treatment		
phase of the cell cycle	G1		G1		G1
		(-/+/id.)		(-/+/id.)	
No. of labelled protein spots	14	(3/117/11)	128	(54/75/74)	149
			plus 0.1 ppm Kinetin		
phase of the cell cycle	(G1)S		S		G2
No. of labelled protein spots	0	(0/43/0)	43	(18/26/25)	51

It remains to be seen if and to which extend one or the other of these additional proteins are required for embryo development of these induced cultures. At present a similar search in data banks as described for the petiole system above is taken up.

Acknowledgement:

Thanks are due to Dr. J. Imani for the technical finish of this article, to Dr. B. Pauler for its preparation for publication on the internet and to J. Korb-Müller for many hours spent on the computer (all of our group in Gießen).

Literature:

Ammirato, P.V. (1985): Patterns of development in culture – In: Tissue culture in Forestry and Agriculture. Henke, R. R., Hughes, K. W., Constantin, M. I. and Hollaender, A. (eds.) New York: PLENUM, 9 – 29

Arnholdt-Schmitt, B., Schäfer, C., Neumann, K.-H. (1998): Untersuchungen zum Einfluß physiologisch bedingter Genomvariationen auf RAPD-Analysen bei *Daucus carota*. Vortr. Pflanzenzüchtg. 43, 107 - 113

Bender, L., Neumann K.-H. (1978, a): Investigations on Indole-3-Acetic Acid metabolism of carrot tissue cultures. Z. Pflanzenphysiol. 88, 209 - 217

Bender, L., Neumann K.-H. (1978, b): Ethylene production and growth of carrot root tissue cultures (*Daucus carota* L.) as influenced by ß-indole-3-acetic acid and kinetin. Z. Pflanzenphysiol. 88, 227 - 234

Bender, L., Kumar, A., Neumann, K.-H. (1981): Photoautotrophe pflanzliche Gewebekulturen in Laborfermentern. In: Fermentation. (Lafferty, R.M. ed.) Springer-Verlag, Wien, 193-203

Bender, L., Kumar, A., Neumann, K.-H. (1985): On the photosynthetic system and assimilate metabolism of *Daucus* and *Arachis* cell cultures. Primary and Secondary Metabolism of Plant Cell Cultures (Neumann, K.-H., Barz, W., Reinhard, E., eds.). Springer-Verlag, Berlin, Heidelberg, 24-42

Blaschke, J.R., Forche, E., Neumann, K.-H. (1978): Investigations on the cell cycle of haploid and diploid tissue cultures of Datura innoxia Mill. and its synchronization. Planta 144, 7-12

Chinachit, W. (1991): Die somatische Hybridisierung von Wildkarotten und Kulturkarotten (*Daucus carota* L.) und die Charakterisierung der Hybriden. Diss., Justus-Liebig-Universität Gießen

De Klerk, G.-J., Arnholdt-Schmitt, B., Lieberei, R., Neumann, K.-H. (1997): Regeneration of roots, shoots and embryos: physiological, biochemical and molecular aspects. Biologia Plantarum 39 (1), 53-66

Dührssen and Neumann (1980): Charakterisation of satellite-DNA of *Daucus carota* L. Z. Pflanzenphysiol. 100, 5, 447-454

Dührssen, E., Lanzendörfer, M., Neumann, K.-H (1984): Comparative investigations on DNA organization of some varieties of *Daucus carota* L. Z.Pflanzenphysiol 113, 223 – 229

Froese, C., Neumann K.-H. (1997): The influence of kinetin on the protein synthesis pattern at some stages of the cell cycle of regenerative synchronized cell suspension cultures of *Daucus carota* L. Angew. Bot. 71, 111 - 115

Gartenbach-Scharrer, U., Habib, S., Neumann , K.-H. (1990): Sequential synthesis of some proteins in cultured carrot explant (*Daucus carota* L.) cells during callus induction. Plant Cell, Tissue and Organ Culture 22, 27-35

Grieb, B. (1991/2): Untersuchungen zur Induktion der Kompetenz zur somatischen Embryogenese in Karottenpetiolenexplantaten (*Daucus carota* L.) - Histologie und Proteinsynthesemuster. Maraun Verlag, Frankfurt/M. (Dissertation, Justus-Liebig-Universität Gießen)

Grieb, B., Groß, U., Pleschka, E., Arnholdt-Schmitt, B., Neumann, K.-H. (1994): Embryogenesis of photoautotrophic cell cultures of *Daucus carota* L. Plant Cell Tissue and Organ Culture 38, 115-122

Grieb, B., Schäfer, F., Imani, J., Nezamabadi Mashayekhi, K., Arnholdt-Schmitt, B., Neumann, K.-H. (1997): Changes in soluble proteins and phytohormone concentration of cultures carrot petiole explants during induction of somatic embryogenesis (*Daucus carota* L.). Angew. Bot. 71, 94 - 103

Imani, J., Nezamabadi Mashayekhi, K., Neumann, K.-H. (1999): Gentechnische Verfahren als Werkzeuge in der pflanzlichen Hormonforschung. http://www.tzm.uni- giessen.de/unigi/ifz/ beitrag.cfm?key=30

Imani, J., (1999): In situ- Nachweis der Auxinverteilung in kultivierten Petiolenexplantaten von transgenen Pflanzen während der Induktion der somatischen Embryogenese bei *Daucus carota* L. Diss. Justus Liebig Universität, Gießen, Germany

Li,T., Neumann,K.-H. (1985) Embryogenesis and endogenous hormone content of cell cultures of some carrot varieties (*Daucus carota* L.). Ber.Dt.Bot.Ges.,98,227-235

Linser, H., Neumann, K.-H. (1968): Untersuchungen über Beziehungen zwischen Zellteilung und Morphogenese bei Gewebekulturen von *Daucus carota* L. I. Rhizogenese und Ausbildung ganzer Pflanzen. Physiol. Plant 21, 487-499

LoSchiavo, F., Pitto, L., Giulilano, G., Torti, G., Nuti Ronchi, V., Marazziti, D., Vergara, R., Orselli, S., Terzi, M. (1989): DNA methylation of embryogenic carrot cell cultures and its variation as caused by mutation, differentiation, hormones and hypomethylating drugs. Theor.Appl.Genet. 77, 325-331,

Skoog, F., Miller, C. O (1957): Chemical regulation of growth and organ formation in plant tissues cultured in vivo. Symp. Soc. Exp. Biol., 11, 118-131

Neumann, K.-H. (1968): Über Beziehungen zwischen hormonalgesteuerter Zellteilungsgeschwindigkeit und Differenzierung, ein Beitrag zur Physiologie der pflanzlichen Ertragsbildung. Habilitation, Justus-Liebig-Universität Gießen

Neumann, K.-H. (1972): Untersuchungen über den Einfluß des Kinetins und des Eisens auf den Nukleinsäure- und Proteinstoffwechsel von Karottengewebekulturen. Z. Pflanzenern. Bodenkunde 131, 211 - 220

Neumann, K.-H. (1995): Pflanzliche Zell- und Gewebekulturen. Ulmer Verlag, Stuttgart,304 pages

Neumann, K.-H., Grieb, B. (1992): Somatische Embryogenese bei höheren Pflanzen: Grundlagen und praktische Anwendung, Wiss. Zeitschrift der Humboldt-Universität zu Berlin, R. Mathematik/Naturwiss. 41, 63-80

Pleschka, E. (1995): Untersuchungen zum Einfluß von Saccharose und einigen weiteren Kohlenhydraten auf die somatische Embryogenese von mixotrophen und photoautotrophen Gewebekulturen von *Daucus carota* L. Diss., Justus-Liebig-Universität Gießen

Reinert, J. (1958): Üeber die Kontrolle der Embryogenese und die Induktion von Adventivembryonen an Gewebekulturen aus Karotten. Planta, 53, 318-333

Schäfer, A., Blaschke J. R., Neumann K.-H. (1978): On DNA metabolism of carrot tissue cultures. Planta 139, 97 - 101

Schäfer, F., Grieb, B., Neumann, K.-H. (1988): Morphogenetic and histological events during somatic embryogenesis in intact carrot plantlets (*Daucus carota* L.) in various nutrient media. Botanica Acta 101, 362-365

Steward, F. C., Mapes, M. O., Kent, A. E., Holsten, R. D. (1964): Growth and development of cultured plant cells. Science 143, 20-27

Stiebeling, B., Neumann, K.-H. (1987): Identification and concentration of endogenous cytokinins in carrots (*Daucus carota* L.) as influenced by development and a circadian rhythm. J. Plant Physiol. 127, 111-121