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THE PROTEIN SYNTHESIS SPECTRUM DURING THE INDUCTION PHASE OF SOMATIC EMBRYOGENESIS IN CARROT (*DAUCUS CAROTA* L.) CULTURES AND THE ROLE OF NITROGEN FORMS FOR EMBRYO DEVELOPMENT

By

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic Acid
2-DE	Two Dimensional Electrophoresis
ABA	Abscisic Acid
ADH	Alcohol Dehydrogenase
ADP	Adenosine Diphosphate
AP	Alkaline Phosphatase
ASA	American Standard Association
ATP	Adenosine Triphosphate
B5	Gamborg Basal Medium
BPB	Bromphenol Blue
BSA	Bovine Serum Albumine
CAP	Chloramphenicol
CBB	Comassie Brilliant Blue
CH	Casein Hydrolysate
Ci	Curie 3.70 X 10^{10} dps
CW	Coconut Water
Da	Dalton = Mass of an Atom
DAO	Diamine Oxidase
DFMO	Dimethyl Sulphoxide
DHA	Dihydroalanine
DNA	Deoxyribonucleic Acid
dpm	Disintegration Per Minute
DTE	Dithiothreitol
DW	Dry Weight
EC	Enzyme Commission, Enzyme Nomenclature
EDTA	Ethylendiaminetetraacetic Acid
FAD	Flavin Adenine Dinucleotide
FADH	Reduced Flavin Adenine Dinucleotide
FW	Fresh Weight
G	Glucose
GA	Gibberellic Acid
GABA	Gamma Amino Butric Acid
GD	Glutamate Dihydrogenase
GOGAT	Glutamin OxoGlutarat AminoTransferase
GS	Glutamine Synthetase
http	Hyper Text Transfer Protocol
IAA	Indole-3-Acetic Acid
pI	Isoelectric point
KD	Kilo Dalton
KIN	Kinetin (6-Furfurylaminopurine)
Km	Michaelis Constant
M	Mol
MAS	Marker-Assisted Selection
MGBG	Methylglycooxyl-Bbs Guanylhydrazone
mRNA	messenger RNA
MS	Murashige and Skoog Basal Medium
MW	Molecular Weight
NAA	Naphthalene Acetic Acid
NAD	Nicotinamid Adenine Dinucleotid

NADH	Reduced Nicotinamid Adenine Dinucleotid
NADPH	Reduced Nicotinamid Adenine Dinucleotid Phosphate
NiR	Nitrite Reductase
NL	Neumann Basal Medium
nm	Nano Meter
NMR	Nuclear Magnetic Resonance
NR	Nitrate Reductase
PCIB	Phosphochlorophenoxy Isobutric Acid
PDC	Pyruvate Decarboxylase
PEMs	Pre-Embryonic Mass of Cells
PGRs	Plant Growth Regulators
ppm	Parts Per Million
PPO	2,5-Diphenyloxazole
PVP	Polyvinylpyrrolidone
r	Coefficient of Correlation
RF	Relative to front
RNA	Ribonucleic Acid
RPM	Rotation Per Minute
SAM	S-Adenosyl-L-methionine
SDS	Sodium Dodecyl Sulphate
t	Time (Days)
TDZ	Thidiazuron
TRIS	Tris(hydroxymethyl)-aminomethane
U/g	Unit Per Gram
Vm	Maximum Velocity
W/V	Weight Per Volume
www	World Wide Web
X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid
ZEA	Zeatin

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Abstract

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THE PROTEIN SYNTHESIS SPECTRUM DURING THE INDUCTION PHASE OF SOMATIC EMBRYOGENESIS IN CARROT (*DAUCUS CAROTA* L.) CULTURES AND THE ROLE OF NITROGEN FORMS FOR EMBRYO DEVELOPMENT

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The goal of the investigation was to broaden our knowledge about the pattern of protein synthesis during the induction phase of somatic embryogenesis in *Daucus* cultures using petioles as the source material implementing histological, biochemical, molecular and bio-informatics methods to achieve this aim for a better understanding of the somatic embryo-genesis in the model system carrot.

During the induction phase, histological examination of cross sections of petiole explants of *Daucus carota* in Gamborg medium with a concentration of 0.5 ppm 2,4-D liquid media shows three different zones, namely, a rhizogenic area with the appearance of cytoplasm

rich cells and first cell division located around the vascular bundles 2 days after culture initiation in an auxin containing medium (Schäfer et al., 1988). Root primordia appear 5 days after culture, and at 7 days of the culture adventitious roots appear. Second, a caulogenic area which arises from the parenchyma, with first cell divisions 5 days after culture in an auxin containing medium. Shoot primordia appear 12 days after culture, and finally embryogenic areas appear which are characterized by cytoplasm rich cells and first cell divisions in subepidermal regions 12 days after culture in auxin containing media. These cytoplasm rich embryogenic cells, when transferred to an auxin free medium, divide and give rise to globular structures which proceed to torpedo and finally mature embryo forms. This trend can be seen through staining the petiole transection with hematoxylin.

To study the pattern of the protein synthesis, the cultured petiole explants were labelled with ^{14}C-Leucine. Comparison of the historadiogram of t0 (t5h), t7 and t14 section indicates a preferential accumulation of ^{14}C-Leucine in the active morphogenic areas at different stages of culture.

Coomasse brilliant blue R-250 stained and ^{14}C-Leucine labelled 2-DE of protein spots in a 5 hours, 7 days and 14 days old carrot petiole culture showed 91, 250 and 256 spots respectivly (Grieb et al., 1977). 71 spots appeared at all periods, however, each period had its own specific proteins. Some spots were merely stained, some merely labelled, and some stained and labelled.

Global protein analysis is a procedure to identify proteins and their functional analysis using an identification data base, in this case Swiss Prot, through subtractive analysis and comparison on the basis of protein spots variation, additional spots, missing spots, using MW and pI of the protein spots for identification.

To examine the role of nitrogen, particularly during the realization phase of somatic embryogenesis in carrot petiole and suspension cultures and the role of pH, inorganic reduced and oxidized forms such as $(NH_4)_2SO_4$ and KNO_3 and an organic form of nitrogen in form of

casein hydrolysate were used. According to the results obtained, ammonium sulfate as the reduced form of nitrogen in higher concentrations reduces pH of the liquid medium to a range of 4.00 on the pH scale, which leads to the arrest of embryo development. By using potassium nitrate, globular structures developed into heart, torpedo, mature embryos, and finally plantlets were formed. Potassium nitrate increased the pH of the liquid medium to 7.2. Using casein hydrolysate at the same nitrogen concentration as the inorganic nitrogen source, embryo development was arrested at the late torpedo stage. This form of nitrogen, having a close relationship between its pH and pK, acts as a buffer, therefore, pH of the liquid solution medium was not strongly subjected to variation.

A very important character of the selected nitrogen form used is their effect on the pH of the culture medium. A mixture of three different nitrogen forms in the solution had a pH capacity between the organic and inorganic nitrogen containing solution. So the solution makes use of different characteristics of each nitrogen form. Diammonium sulfate reduces pH of the solution, potassium nitrate exerts an opposite effect by increasing pH, and finally casein hydrolysate acts as buffer to stabilize the pH of the system.

2 LITERATURE REVIEW

The science of plant cell culture, linking developmental, cellular, and molecular genetics with conventional plant breeding, enjoys a pivotal position in the general area of agricultural biotechnology, as seen in the commitments being made by major academic institutions, research foundations, and institutional corporations. Agricultural planners are depending on plant cell culture and molecular genetics to accelerate the pace of plant breeding and allow food production to meet the needs of a world population that will potentially increase, from 6 to 10 billion, in the next 20 years. Moreover, this new technology fits within the 5 to 7 year corporate research time-frame of industry, by allowing the production of value-added consumer and industrial products with an agricultural base.

2.1 Carrot

Carrot [*Daucus carota* L. subsp. sativus (Hoffm)] is a temperate climate plant grown for its edible storage taproots throughout much of the world. Carrot grows optimally at 15-20° C and is cultivated in the spring, summer, and autumn in temperate climates and in the winter in subtropical climates. For a good many years, it was a minor crop, the taproots serving primarily as an ingredient in soups and sauces. In recent times, its popularity as a vegetable in itself has increased markedly.

Carrot is a member of the Apiaceae, the parsley family, which includes such vegetables and spices as parsley (*Petroselinum sativum* L.), celery [*Apium graveolens* L. var. Dulce (Mill.) Pers.], celeriac [*A. graveolens* L. var. Rapaceum (Mill.) Gared. Beaup.], parsnip (*Pastinaca sativa* L.), caraway (*Carum carvi* L.), coriander (*Coriandrum sativum* L.), anise (*Pimpinella anisum* L.), fennel (*Foeniculum vulgare* Mill.), cumin (*Cumin cyminum* L.), and dill (*Anethum graveolens* L.). A number of wild species are extremely poisonous, including the poison hemlock (*Conium*

maculatum L.). The family is large and taxonomically complex. All cultivated carrots are forms of the wild carrot (*Daucus carota* L. subsp. carota).

Almost 80 species have been described for the genus Daucus, half of which are subspecies or forms of the species *D. carota.* Chromosoms numbers of around 25 Daucus species are known (Bell and Constance, 1960; Moore, 1971); the basic number ranges from n = 7 to n = 11. The species, *D. carota* L., to which all cultivated and wild carrots belong, is diploid, with nine pairs of chromosomes (n = 9, 2n = 2X = 18). The only other species with n = 9 are *D. capillifolius* gilli and *D. sytricus* Murb. from North Africa. Two polyploid species have been reported: *D. glochidiatus* Labill. (2n = 4x = 44) and *D. montanus* Humb. et Bonpl. (2n = 6x = 66). Polyploidy and structural changes in the chromosomes apparently have not played a major role in the differentiation of the species (Whitaker, 1949).

The domestic carrot is typically biennial, although annual forms are known. It produces a rosette of leaves and the fleshy taproot during the first growing season, and the flower stalk and seed in the second. The taproots need a cold period, or vernalization, in order to sprout and flower in the second season. The inflorescence is a terminal compound umbel: a primary umbel with a system of second-, third-, and fourth-order umbels, defined by the sequence in which they form on the main stem. The primary umbel is the largest, and each order is progressively smaller in size. The flowers are small, and bear white petals. There are five petals, five stamens, and a single, inferior ovary, bearing two locules, each containing a single ovule. The seed is a “mericarp”, one-half of a dry, indehiscent fruit. Carrots are natural outbreeders, showing severe inbreeding depression.

2.2 Callus Initiation

The first cultures of nontumorous plant cells were from carrot roots (Gautheret, 1939; Nobecourt, 1939. In the same year, White (1939a,b) reported the growth of genetic tumors from the interspecific hybrid *Nicotiana glauca x N. langsdorffii.* The cultures were initiated and maintained on a medium composed of a Knoop solution supplemented with Berthelot's mixture of accessory salts, glucose, gelatin, thiamin, cysteine hydrochloride, and

indoleacetic acid. On such a medium, carrot callus tissue could be maintained for many years without apparent diminution of growth rate and with the only sign of differentiation being occasional lignified cells.

The responsiveness of carrot tissue to growth induction has made it a model system for studies of factors promoting cell division (Steward, Mapes, and Ammirato, 1969). The facility with which the carrot cells can be grown in liquid media in suspension cultures has made it useful for studies of cell growth, including scale-up using bioreactors, secondary product synthesis and mutant selection.

2.3 Morphogenesis in Carrot Cultures

Nobecourt (1939), studying the growth of carrot taproot tissue, reported the appearance of roots from the callus mass. Callus tissue could be removed from stationary cultures and grown in a liquid medium as suspension. Roots were frequently seen in carrot tissue cultures; shoots also developed, but less frequently (Levine, 1947). Within a short period, both Steward (Steward et al., 1958), working with suspension cultures, and Reinert (1959), working with callus cultures on semisolid medium, described the development of somatic embryos and ultimately plants from carrot tissue. In Steward's work, suspensions were grown in a culture medium with 10 % CW in which multicellular masses arose. If the masses remained in the liquid medium, root meristems formed and roots emerged. If, before root growth occurred, the multicellular aggregates were transferred to medium solidified with agar, “meristematic nodules” very closely resembling developing carrot embryos arose and buds and leaves emerged. Reinert (1959) grew carrot callus on medium containing 7% CW and IAA (57 μM) and observed root formation if tissue was transferred to auxin-free medium or allowed to grow on the same medium for a long period. However, callus maintained for several months on a complex medium (Reinert and White, 1956) underwent a change in texture and morphology; the outer surface became covered with “horny nodules” that upon close examination appeared similar to “normal bipolar carrot embryos”.

2.4 Embryogenesis

One understands somatic embryognesis as a developmental process of somatic cells, which resembles morphologically zygotic embryogenesis (Neumann, 1995).
This process can be initiated using cells from different plant organs, for example, carrot explants of roots, leaf petiole, hypocotyle, leaf lamina and other plant parts. Even if somatic and zygotic embryogenesis morphologically resembles one another, some morphological and anatomical deviations exist. By using a suspension culture through application of a bioreactor, the whole process for industrial means can be automatised, which has economical advantages over meristem culture and adventive organogenesis. The other aspect of somatic embryogenesis is the greater cytogenetic stability of the plants in comparison to adventive organogenesis, although the number of plants produced through somatic embryogenesis could be less than using adventive organogenesis. The plant species plays a role too. In some plants, this process cannot produce embryos. These are called recalcitrant plants, the number of which is large.

The process of somatic embryogenesis is not only important for the production of plants and secondary products, but also for the transgenic plants and fussed cells. One of the most important uses of somatic embryogenesis is in studying biochemical processes in the cell. The aim is to establish a system applicable for nearly all plant species. The system needs differentiation of the totipotent cells, and it occurs on a medium supplemented with the plant hormone Auxin. Different researchers use different auxins to achieve this. The most common is the synthetic auxin 2,4-D, which originally is a herbicide. This auxin is very persistent and photo-non-sensitive, some others use the auxin IAA, which is photosensitive and degrades within 4-6 days (Neumann, 1995). There is an advantage to using IAA, because the cells can be kept in the same culture vessel and there is no need for subculturing the cells in a fresh hormone free vessel, through which the chance of contamination is reduced and it is industrially more favored. After removing the differentiated cells or plant material, the cells undergo subsequent embryonal stages, namely PEMS, globular, heart and torpedo stage and ultimately small plantlets with two cotyledonary leaves and roots will be visible.

2.4.1 Some Fundamental Aspects of Embryogenesis

Apart from the zygotic embryogenesis, some plants develop asexual embryos, which is called apomixis. In case of adventive embryo formation or also called nucellar embryo formation, the generation change and formation of the embryo sack does not occur, so that the diploid embryo (somatic) arises directly from the diploid cells, present in embryo, nucellus or the integuments. The sporophytic form of apomixis often leads to polyembryony, for example, as in case of citrus (Maheswari, 1979). By contrast, in gametophytic apomixis a generation change and the formation of embryo sack occure. The diploid embryo sacks as formed in the case of Apospory, which is also called somatic apospory, is formed from the vegetative cells of the nucellus, and distinguished from diplospory, which is also called vegetative apospory from the embryo sack mother cell as a consequence of an uncompleted meiosis. In both cases, the embryo contains 2n, which is parthenogenetic either from the egg cell or from the synergides or antipodes (diploid apogamy). Haploid parthenogenesis or haploid apogamy are the processes of the so called "non recurrent" apomixis (Maheswari, 1979). So it seems not only the embryo sack mother cells, but also some cells of embryo have some kind of embryogenic competence (Neumann, 1995). Finally, there is the possibility of embryo development from micro- and macrospores (androgenesis, gynogenesis).

Induction of embryonal development in these cells needs stimulus. This stimulus, in the case of zygotic embryogenesis, is pollination. In apomictic embryogenesis, this process can come into action through pollination or through other factors like temperature shock or changes in the photoperiod (Nogler, 1984). The chemical nature of such a stimulus is not yet known. The prerequisites for embryo development are the embryonic competence of the cells, a stimulatory factor for triggering embryogenesis and a proper chemical surrounding for the embryonically induced cells. This chemical environment is present as the endosperm of zygotic and apomictic embryos. Different non zygotic development traits in the embryo originate from the cells of nucellus or integuments. The first sign of embryonal development after receiving the stimulus is an intensive plasma growth of the originally vacuolized single cell.

These competent cells are more or less randomly scattered in the nucellus or in the integuments, so that afterwards embryogenic competent cells in a given time are restricted to only some special cells of the embryo and not to all the cells of this tissue. The megaspore mother cell itself develops from a vacuolized cell through accumulation of cytoplasm (Maheswari 1979).

2.4.2 Variation in Carrot Cultures

Genetic changes in cultured plant cells are well known and include a wide spectrum of alterations, from changes in chromosome number and karyotype (cf. Krikorian et al., 1983) to single gene changes (e.g., Evans et al., 1984). Changes that appear when sporophytic tissue is cultured have collectively been termed "somaclonal variation" (Larkin and Scowcroft, 1981) and can generate substantial genetic variability in plants regenerated from these cultures (Reisch, 1983).

It was recognized quite early that carrot cells in culture can exhibit substantial variation in chromosome number, both polyploidy and aneuploidy, and chromosome morphology (Mitra et al., 1960). However, plants regenerated from those cultures were almost exclusively diploid, with the exception of a few tetraploids, and showed no cytological abnormalities, at least as observed with the techniques of the day (Mitra et al., 1960). Large numbers of plants were grown, all of which were phenotypically normal (Steward et al., 1964). At that time, it was thought that only cells with an unaltered chromosome complement could develop into somatic embryos and plants. Some years later, Mok et al. (1976) also reported recovering normal diploid plants from carrot root callus from a population of cells with varying chromosome numbers.
Research work has confirmed the tendency of carrot embryos and plants to be selectively regenerated from cells with the normal (diploid) chromosome number, but aneuploids as well as polyploids (tetraploids) have also been seen (Toncelli et al., 1985).

It is now known that chromosome counts are not entirely reliable indicators that genetic change has not occurred. Changes in chromosome karyotype rather than complement have been seen in somatic embryos and plants, as in *Hemerocallis* (Krikorian et

al., 1981). Tomato plants that appeared both phenotypically and chromosomally normal produced progeny with a range of mutations (Evans and Sharp. 1983).

In studies of somatic embryogenesis in long-term carrot cultures, embryos and plants could be grown, but they were often sterile (Sussex and Frei, 1968). Also, morphological variants have been observed in carrot plants regenerated from culture, with erect stems and leaf dissection and modifications in leaf thickness and leaf color, but changes were epigenetic rather than genetic (Ibrahim, 1969). More recently, through the use of specific selection procedures, genetic variants have been observed in carrot, both in cultured cells and, in a number of cases, in the plants regenerated from them (Sung and Dudits, 1981, Widholm, 1984b). In many cases, these lines have been isolated from existing variations in cell cultures, i.e., without the use of mutagens. The selection procedures and the mutants isolated are discussed in the next section. A second change that can occur in carrot cultures is the loss of the ability to regenerate somatic embryos. This occurs gradually during progressive sub culturing (Syono, 1965). Also, changes in karyotypic structures occur with increasing time in culture, i.e., as tissue is progressively subcultured (Bayliss, 1977, 1980). In one study, the loss of potential was traced to a change in chromosome complement when aneuploids gradually replace diploid cells (Smith and Street, 1974). However, the loss in a particular culture may not be permanent. In habituated *Citrus sinensis* cultures, embryogenesis was restored by eliminating sucrose from the medium or by aging of the tissue (Kochba and Button, 1974). By moving carrot cells to a medium with elevated KIN concentration, a nonembryogenic line became embryogenic (Chandra, 1981). Whether this was due to a promotion of cells that had insufficient endogenous cytokinins, a selective enrichment of a small number of embryogenic cells in the suspension, or de novo induction of embryogenic cells was not determined. The addition of activated charcoal has permitted somatic embryogenesis in carrot cultures that failed to regenerate when auxin was eliminated (Fridborg and Eriksson, 1975;Drew, 1979).

The question is the control of genetic and epigenetic variations, i.e., the retention of chromosomal and genetic fidelity in cases when cloning is required and alterations in the genome, when variants are needed. There are indications that careful attention to procedures, such as the frequent establishment of fresh cultures, the use of proper media,

and the subculture schedule, can maintain clonal fidelity in both cultures and regenerated plants (Evans and Gamborg, 1982, Krikorian, 1982). Embryogenic cultures can be maintained for long periods (e.g., Sheridan, 1975). In addition, changes in the genome have arisen spontaneously in carrot cultures and have also been induced by mutagens. These can be selected for IN VITRO culture to provide useful variant carrot cells and plants.

2.4.3 Selection of Variants In Carrot Cultures

Carrot cells are easily grown as fine suspensions in culture and can readily be planted out following somatic embryogenesis. In addition, cell lines have been maintained over a long period with a relatively stable karyotype. Because of these features, carrot cell cultures have proven very amenable to the selection of variant cell lines (Flick, 1983). The isolation of variant cells has been achieved through the use of a number of selection procedures that have produced a wide range of variants. Many of these are stable through subculture. There are a few studies where plants regenerated from the lines show the trait, as do cells removed from the plants and grown in culture. In some cases, the trait is expressed by the cells in culture but not in the regenerated plants. In others, the trait does not seem to be the consequence of a genetic change.

The wide range of variant cell lines isolated include those resistant to inhibitors such as amino acid analogs (e.g., 5-methyltryptophan; Widholm, 1974), purine and pyrimidine analogs (e.g., 5-fluorouracil;Sung and Jacques, 1980), and antibiotics (e.g., cycloheximide, Sung et al., 1981). Color variant mutants have been isolated, including those with substantial levels of anthocyanin (Dougall et al., 1980) or carotinoids (Mok et al., 1976), or those lacking chlorophyll (Miller et al., 1980). So developmental mutants have been isolated (Breton and Sung, 1982; Terzi et al., 1982).

Because of the relative ease of selection, most carrot variants have been selected as resistant to a particular growth-inhibitory compound, the largest group of these being those resistant to amino acid analogs, such as ethionine (Widholm, 1976), 5-methyltryptophan (Widholm, 1972, 1974), and hydroxyproline (Widholm, 1976). These

offer potential practical applications, since resistance to the analog may be gained by the overproduction and/or accumulation of high levels of an amino acid, such as increased free methionine after ethionine selection, increased free proline after hydroxyproline selection (Widholm, 1976), and increased free tryptophan after 5-methyltryptophan selection (Widholm, 1972). A mutant selected against the latter demonstrated resistance due to decreased uptake than to overproduction (Widholm, 1974). This trait was expressed in the regenerated plants and then in the cultured cells removed from the plants (Widholm, 1974). In another study, a carrot line not resistant to inhibition by lysine plus threonine generated plants that were also resistant to these two amino acids (Ammirato, 1985). Both callus and plants of the resistant lines contained at least 8 times more free threonine and 2.5 times more free isoleucine than the wild type (Cattoir-Reynaerts et al., 1983).

In a number of cases, cell lines resistant to 5-methyltryptophan and which accumulate high levels of free tryptophan are also auxin autotrophic (Widholm, 1977a). This appears to be due to high levels of indoleacetic acid, of which tryptophan is a precursor (Sung. 1979). However, cell lines selected for auxin autotrophy (ability to grow without exogenous auxin in the medium) were not resistant to 5-methyltryptophan (Widholm, 1977a).

Cell lines resistant to other inhibitors have also been selected, and some of these have been useful in protoplast fusion studies, e.g. 8-azaguanine resistant cultures that are also sensitive to HAT (hypoxanthine, aminopterin, thymidine, and glycine; LoSchiavo et al., 1983) and cycloheximide resistance (Sung et al., 1981). In addition, albino cells and plants that were isolated (e.g., Miller et al., 1980) have proven useful in protoplast fusion product selection procedures (Dudits et al., 1977). The appearance of highly colored cells in carrot cultures did not lead to regenerated plants with elevated levels of carotinoids (Mok et al., 1976) or anthocyanins (Dougall et al., 1980). The latter study of specific anthocyanin producing clones showed that the synthesis of increased anthocyanin was not caused by a mutant gene. However, anthocyanin production in cultures of other species can be selected and is consistent (Yamamoto et al., 1982), in carrot suspension cultures, pigment levels do respond to cultural variables (Ozeki and Komamine, 1985; Neuman, 1995). Stable color variants may one day be produced in carrot. In addition, variant carrot cells hold much promise in secondary product synthesis (Hess, 1992).

Also of interest are variant lines that may lead to varieties with increased tolerance to environmental extremes, such as heat, cold, drought or salinity. Although there has been some success in selecting for variant cell lines, such as with cold tolerance (Templeton-Somers et al., 1981), the characters have not passed through to the regenerated plants as yet. However, a variant cell line tolerant to aluminum, which is harmful to plants when present in excess in the soil, did regenerate carrot plants with a similar tolerance (Ojima and Ohira, 1982). Furthermore. the tolerance was exhibited in seedlings grown from seeds of these regenerated plants.

Developmental mutants have been selected by using a filtration-enrichment procedure (Breton and Sung. 1982;Terzi et al., 1982;Giuliano et al., 1983). In this technique, cells that are unchanged develop into embryos in the maturation medium and these can be removed by passing the suspensions through filters. Cells that cannot grow or cannot differentiate pass through the filter; the filtrate is therefore enriched in cells blocked in growth and development. Using these techniques, mutant cells have been selected that can either arrest development at a particular stage, or disrupt organized development completely. Among the more interesting are temperature sensitive mutants. Somatic embryogenesis and maturation can proceed in wild type populations at a range of temperatures from 18 to 32° C. Temperature-sensitive mutants will form embryos at low temperature (18° C) but fail to do so at high temperature (32° C). These are, then, conditional developmental mutants. Investigations are in progress looking for a temperature-sensitive protein accompanying somatic embryogenesis. Sung and Okimoto (1981) found two specific proteins that appeared in carrot suspension cultures after embryo development had begun. The proteins disappeared if the embryos were transferred to media that caused callus formation. The proteins were absent in carrot lines incapable of embryogenesis. The pattern of proteins synthesized by carrot embryonic cells after heat shock has also been analyzed (Pitto et al., 1983). These studies hope to provide markers for somatic embryogenesis per se and for the various stages of embryonic development. Mutagens have been used in many variant selection studies. However, only in a few cases, including one study involving resistance to 5-methyltryptophan (Widholm, 1977b) and another concerned with resistance to cycloheximide (Sung, 1981), were the frequencies of variants substantially higher in mutagen-treated cultures. Variants can be isolated from nonmutagenized cultures, tapping the variation that appears spontaneously (Evans et al., 1984).

2.5 Somatic Embryogenesis

It was evident from the first observations, that the development of the somatic embryos in carrot cultures strongly resembles the zygotic embryogenesis. Somatic embryos often show the same developmental sequence, progressing through globular, heart, torpedo and cotyledonary stages. Like zygotic embryos, they develop from single cells (McWilliam et al., 1976). Large numbers of somatic embryos can be produced in a small volume of liquid, and from them large numbers of plants can be grown (Ammirato. 1984).

There are, however, a number of problems. Suspension cultures are a mixture of embryogenic and non-embryogenic cells and clusters, and these are of varying sizes and numbers. During successive subculture regimes, the cultures may become composed solely of non-embryogenic cells, thereby losing the ability to produce somatic embryos and plants. Because there is a proembryo decrease when embryo maturation begins, the resulting populations of somatic embryos develop asynchronously. In addition the normal pathway of development may be diverted (Ammirato, 1985) resulting in a range of structurally aberrant forms. These are epigenetic changes, for normal carrot plants can be grown from them. Since Steward and Reinert independently discovered somatic embryogenesis in carrot cultures (Steward et al., 1958; Reinert, 1959), it has been a model system for investigating many aspects of plant cell cultures, in particular morphogenesis, and especially somatic embryogenesis cell mutant selection, and somatic hybridization via protoplast isolation-fusion techniques.

2.5.1 Different Forms of Somatic Embryogenesis

There are two different forms of somatic embryogenesis. Direct embryogenesis, arising from a single cell e.g. of the hypocotyl or the petioles, without the necessity of callus formation, and indirect embryogenesis, in which a precallus formation is needed. In direct embryogenesis, it is obviously the parenchymatic cells that transform into

embryogenic cells. In case of indirect embryogenesis, by contrast, a callus will be formed and it is from this callus culture that embryos arise. Somaclonal variation of the embryos arising through direct embryogenesis is lower than that of indirect embryogenesis, but frequency of direct embryogenesis in different plant species is lower than that of indirect embryogenesis. For example, till 1990, only 8 plant species showed the characters of direct embryogenesis in which 5 of 8 species used embryo cells as the source material for the formation of the embryos. In carrot plants, direct embryogenesis is observed in hypocotyl and in petiole cells, also in *Triticum rubens* embryogenesis is observed in hypocotyle and petioles cells (Lui et al., 1988). In *Dactylis glomerata* (Conge et al., 1983) direct embryogenesis occurred in cells of the leaf base. In some plants, both forms are observed and carrot belongs to this category. Direct embryogenesis can be seen in an obvious form in the petiole cells of carrot (Neumann and Grieb, 1992). The material used is a one- cm-long petiole explant of 6-8 week old carrot plants, sterilized and cultured in a defined nutrition solution.

2.5.2 Mechanisms of Somatic Embryogenesis in Cell Cultures: Physiology, Biochemistry and Molecular Biology

Somatic embryogenesis is an ideal system for investigation of the whole process of differentiation of plants, as well as the mechanisms of expression of totipotency in plant cells. The attempt by Haberlandt to establish plant tissue culture systems provided support for a better understanding of the totipotency of plant cells. Thus, the mechanism of somatic embryogenesis is one of the most fundamental problems in plant physiology. Recently, somatic embryogenesis has attracted attention in plant biotechnology, because it provides useful systems to produce transgenic plants, as well as material for the production of artificial seeds.

2.5.3 High Frequency and Synchronous Somatic Embryogenesis Systems

The first and epoch-making reports on somatic embryogenesis were published in 1958 by Steward et al. (Steward, Mapes, and Mears, 1958) and Reinert, 1959. In the following twenty years, however, little progress was made in understanding the mechanisms of somatic embryogenesis, because somatic embryogenesis occurred in vitro only at low frequency and asynchronously, in the system used at that time. In such systems, biochemical and molecular events specific for embryogenesis should be diluted by the activities of cells not engaged in embryogenesis. Furthermore, only average values for biochemical parameters related to various stages of embryogenesis could be determined when asynchronous embryogenesis systems were used. Thus, high frequency and synchronous embryogenesis systems were required for investigation of mechanism of somatic embryogenesis at the molecular level. Embryogenic cell clusters were selected by sieving with nylon screen and density gradient centrifugation in Ficoll Solution, and then transferred to media lacking auxin and containing zeatin at 10^{-7} M. In this system synchronous embryogenesis occurred from cell clusters at about 90% frequency (Komamine et al., 1992). This system is useful for investigating the process of embryogenesis from embryogenic cell clusters, which are designated as cell cluster stage. However, since these clusters can differentiate to embryos in an auxin-free medium without any trigger, embryogenesis can be considered to have already been determined in embryogenic cell clusters, State 1. Thus, the process of formation of State 1 cell clusters from single cells is also important for analyzing the process of embryogenesis, and a system is required in which high frequency embryogenesis occures from single cells. The competent single cells, which were small, round and cytoplasm rich and were designated state zero cells, were collected by sieving with a nylon screen, density gradient centrifugation and manual picking. When State zero cells were pretreated with auxin (2,4-D at $5X10^{-8}$ M) for 6 days and then transferred to the auxin-free medium, embryos were formed at high frequency (85-90%). Auxin pretreatment was essential and zeatin (10^{-6} M), mannitol (10^{-3} M) and a high concentration of oxygen (40%) were promotive (Komamine et al., 1992). This system provides a useful method to investigate the whole process of somatic embryogenesis from single cells to plants. Furthermore, when State

zero cells were cultured directly in auxin-free media, cells were elongated and they could not differentiate to embryos even if they were transferred to a medium containing auxin. Therefore the process in which State zero cells were cultured in auxin-free media can be regarded as the process of controlling of totipotency, while the process in which State zero cells were cultured in media containing auxin and differentiated to embryos at high frequency, can be regarded as the process of expression of totipotency. These two processes are useful to investigate what events occur during expression or losing of totipotency.

2.5.4 Phases in Somatic Embryogenesis

Detailed morphological observations revealed that four stages, namely zero, 1, 2 and 3, were recognized in the early process of embryogenesis (Fujimura and Komamine, 1980).

In stage zero, competent single cells (state zero) form embryogenic cell clusters (state 1) in the presence of auxin. During this stage, the cell clusters formed from single cells gain the ability to develop into embryos when auxin is removed from the medium, giving rise to stage 1 cell clusters. The subsequent stage is induced by the transfer of state 1 cell clusters to an auxin-free medium. During stage 1, cell clusters proliferate slowly and are apparently undifferentiated. After stage 1, rapid cell division occurs in certain parts of cell clusters, leading to the formation of globular embryos. This stage is designated as state 2. In the following stage, state 3, plantlets develop from globular embryos via heart-shaped and torpedo-shaped stages.

2.5.5 Synchrony of Development

Embryogenic carrot suspensions contain a range of proembryonic structures and nonembryogenic free cells and clusters. For studies of both basic mechanisms and the use of carrot somatic embryos for Crop improvement, it is important to separate the various

proembryonic structures and reduce the populations of non-embryogenic cells. Two basic procedure have emerged and these can be used separately or sequentially to manipulate large populations of carrot suspension cultures.

One procedure is based on separation or fractionation by size. This can be done by sieving. e.g., by means of a graded series of stainless steel mesh sieves (Halperin, 1966; Ammirato, 1974; Kamada and Harada, 1979a), or nylon mesh (Fujimura and Komamine, 1975), or by passing the cells through glass beads (Warren and Fowler, 1977).

A second technique is to separate cells by differences in specific gravity, often after sieving (Fujimura and Komamine, 1979b). This can be done, first, by density fractionation, in which the cells suspended in 10 % Ficoll solution with 2% sucrose are layered on a Ficoll discontinuous density gradient (12-18% in water, 8 ml total volume) containing 2% sucrose and centrifuged at 50 g for l min, and then at 150 g for 4 min. This is followed by velocity sedimentation, in which each Ficoll gradient is suspended in a culture medium and centrifuged at 50 g for 30 sec. to remove nonembryogenic cells. The resulting populations contain many small proembryos, from 3 to 10 cells each. Upon transfer to the maturation medium, more than 90% of the structures form embryos synchronously. An alternative technique has been devised by Giuiliano et al. 1983. The suspension culture is passed through a nylon sieve with 120-µm pores and then through a second sieve with 50-µm pore size. The cell masses that are retained on the second sieve are resuspended in a maturation medium. After 6-8 days, during which 10% of the somatic embryos have reached the torpedo stage, the population is filtered through a l70 µm nylon mesh sieve into a petri-dish. The suspension that passes through the sieve consists of at least 95% single embryos. Nonembryogenic cells are removed by first allowing the suspension to sediment for 15-30 sec. and then aseptically separating most of the medium. Next, the suspension is stirred (thus concentrating the embryos in the center) and the surrounding medium is separated. This can be repeated to enrich the population of globular proembryos, upon addition of fresh medium and growth under appropriate conditions. The population consists of 60% heart-shaped embryos in 2 days, and 70% torpedo-shaped embryos in 6 days. Each population can be enriched by filtration with sieves of appropriate size. The technique yields large quantities of somatic embryos of specific sizes. It does not appear to inhibit further embryo maturation and plant development.

2.5.6 Expression of Polarities in Early Stages of Somatic Embryogenesis

Rapid cell division occurs in certain parts of cell clusters in stage 2, leading to the formation of globular embryos. Cell division is very rapid in this stage, the doubling time being 6.3 hr, while it is 51 hr and 36 hr in stage 1 and 3, respectively (Fujimura and Komamine, 1980). The polarity of DNA synthesis in cell clusters was confirmed during Phase 1-2 by autoradiography using H-thymidine. However, the polarity was lost when cell clusters were cultured under the non-embryogenic condition, i.e., in the presence of auxin. The polarized rapid cell division or DNA synthesis is, therefore, considered specific to embryogenesis. It is important to investigate the mechanisms of expression of polarity of active DNA synthesis and rapid cell division to understand embryogenesis.

2.6 Factors Affecting Carrot Somatic Embryogenesis

Since the earliest successes were achieved in media supplemented with coconut milk or coconut water, attention was focused on the role of complex naturally occurring liquid endosperms that normally bathe zygotic embryos in nourishing young somatic embryos (Steward and Shantz, 1959; Steward et al., 1969). Subsequent investigations showed that both the induction of embryogenic growth and the promotion of maturation in carrot cultures could be achieved with totally defined media lacking CW (e.g. Kato and Takeuchi, 1963). However, it was during this early period of research that the basic requirements for somatic embryogenesis in carrot were demonstrated:

(1) An auxin or auxin-like substance was critical for embryo initiation and the lowering of the auxin concentration, or its complete absence fostered maturation (Halperin and Wetherell, 1964; Halperin, 1966; Steward et al., 1967).
(2) Reduced nitrogen was important for both initiation (Halperin and Wetherell, 1964b; Halperin, 1966) and maturation of somatic embryogenesis (Ammirato and Steward, 1971).

2.6.1 Physiological Factors Affecting Somatic Embryogenesis

Auxin is the most important factor in the regulation of induction and the development of embryogenesis, and it has different effects in different phases of embryogenesis. The presence of 2,4-D or other auxins is required for the formation of embryogenic cell clusters (state 1) from single cells (state zero). This indicates that auxin is an essential factor in the induction of embryogenesis (state zero). In other words, auxin is necessary for "competent" cells (state zero) to express their endogenus totipotency. However, auxin is inhibitory on embryogenesis in Phase 1 and in following phases. Auxin is most inhibitory in Phase 1. Since the original single cells cannot differentiate directly to form embryos in auxin-free media, there are at least two stages in somatic embryogenesis; stages requiring auxin and those inhibited by auxin.

Anti auxins, 2,4,6-trichlorophenoxyacetic acid and Phosphochlorophenoxyiso-butyric acid (PCIB) inhibited embryogensis after Phase 1 (Fujimura and Komamine, 1979b). These findings suggest that auxin is required for the induction of embryogenesis (the process from competent cells to embryogenic cell clusters), but is inhibitory for the development of embryogenesis (the progress from embryogenic cell clusters to plantlets).

A cytokinin, zeatin, shows a promotive effect on embryogenesis in every phase. It is most promotive in Phase 2, in which active cell division occurs. Zeatin may be involved in the promotion of cell division. Other phytohormones, e.g. gibberellin and abscisic acid, inhibited embryogenesis of cell clusters in carrot culture (Fujimura and Komamine, 1975).

Besides phytohormones, cell-to-cell interaction is another important factor in somatic embryogenesis. A rather high cell density (10^{+5} cells/ml) is required for the formation of embryogenic cell clusters from single cells (Nomura and Komanine, 1986b), whereas a lower cell density (2×10^4 cells/ml) favors the development of embryos from embryogenic cells (Fujimura and Komanine, 1979).

2.7 Histological Observation in Cultured Petiole Explants

Histological observation of the petiole explants shows not only different parenchymatic cells in different locus of the petiole transsection in the tissue with competence for different morphogenetic processes, but also the morphogenetic process occurs at a specific time interval. If the callus medium contains IAA as in the case of the NL media, or 2,4-D in the case of B5 media, as the stimulus, 2-4 days after the transfer of the petiole into the media, the transformation of paranchymatic cells surrounding the vascular bundle into cytoplasm rich, rhizogenic competent cells can be seen. The further development is not the same. In the culture supplemented with IAA as the auxin source, root primordia and adventive roots differentiate 4-6 days after the culture. If however, 2,4-D is the only auxin source, only rhizogenic centers will be formed. This is a sign of a specific growth, but root primordia do differentiate when the stimulus is the auxin IAA. After the removal of the 2,4-D, it is also possible that adventive root will be formed. This indicates that the competence for rhizogenesis is always there, and the further development and the realization stop in the culture supplemented with auxin 2,4-D. In NL medium, the formation of the caulogenic centers in the large parenchematic cells of the petiole can be observed after a few days. During further culture with both auxin forms, after 12-14 days sub-epidermal cell layers transform directly without predivision into cytoplasmic rich embryogenic competent cells. Interestingly enough, these cells are located near the vicinity of the glandular channels (Neumann, 1995). Hence the process of embryogenesis differes if different auxines are used, as use of IAA leads mostly to direct embryo development. A single cytoplasmic rich cell divides into 2 and then 4 parts, and then this leads to pre-globular, globular, heart, torpedo and finally plantlet forms. Using 2,4-D as the auxin supplement with a concentration of 0.5 ppm leads to indirect embryo formation. Many cell aggregates form embryogenic meristems, also called PEMs (Pro-Embryogenic Masses). The masses of cells disattach themselves from the ground tissue after splitting of the petioles and rupturing of the epidermis, and suspend in the liquid solution. After transfer to an auxin free medium, these structures go through the globular, heart, torpedo stages and form plantlets. In both cases, if the auxin used is IAA or 2,4-D, malformed embryos can be seen. The reason for this phenomenon is not clear. After the stimulus has functioned, induction of embryogenesis follows. At this stage the

cells are embryogenic competent and they divide. This phase is called the reproduction phase. Most probably, the realization of the embryogenesis program is blocked through the addition of auxin, for example, when the cells of a cell suspension culture are transferred into auxin containing media.

Parallel to the morphological and histological studies, there are some studies, which have been conducted regarding the developmental pattern in the petiole explants of carrot dealing with the hormone system and the pattern of the protein synthesis (Grieb, 1992). Characterizing the hormone system dealing with IAA, ABA and six different cytokinines during the induction phase shows changes in the hormone concentration during different developmental stages (Grieb et al., 1997). In the first days of culture in a petiole system, IAA and ABA dominate the process, but later the concentration of cytokinines increases. Studies on protein synthesis during the culture of petiole explants give valuable hints, such as the specific pattern of the protein synthesis during the induction phase. The protein synthesis pattern linked with the use of radioactive isotope and two dimensional electrophoresis gives relevant information about the formation and the characteristics of proteins during different embryonal stages.

2.8 Nitrogen Metabolism

There are two ways to cover the nitrogen requirements of the cells in a liquid culture. First, the application of ammonium or nitrate or both as the source of inorganic nitrogen, and second, the use of amino nitrogen present in different amino acids. The amino acids can be added separately or as a mixture of different amino acids e.g. addition of casein hydrolysate. Amides in form of urea or glutamine can be used as a nitrogen source in the cell culture too. Ammonium or nitrate each can be used as the only source of nitrogen in the cell culture. Experiments with tobacco cell culture showed that cells proliferate better in a medium containing nitrate as the only nitrogen source through which there is a marked increase in cell dry weight. Cell proliferation is higher, and there is an increase of the pH value in the medium by comparison with the ammonium treatment. (Elsner, unpublished, Institute for Plant Nutrition, Department of Tissue Culture JLU,

Giessen). This is an indication of lower cell division activity of the ammonium treatment. Cell development of both treatments is nearly the same. The assimilation of ammonium is against exchange for H^+-ions in the culture medium. This aspect makes it interesting to investigate how important the pH value is, and what role the pH plays in the process of ammonium assimilation. In the meantime, one must not forget the competitive process of ammonium against other cations present in the nutrition solution. In some liquid media, both forms of inorganic nitrogen are used e.g. in the MS medium. In this medium, it seems there is a timely preferential selectivity for the amino form of nitrogen. Later on, cells use nitrate for their metabolic activities (Neumann, 1995). Ammonium is a reduced form of nitrogen, hence it can be readily used for the synthesis of amino acids. Nitrate as an oxidized form of nitrogen must first be reduced. This process needs some energy source from metabolism of the cell. The reaction process is managed by two enzymatic processes. The enzyme nitrate reductase (NR), which is localized in the cytoplasm, reduces nitrate to nitrite. Further reduction to ammonium occurs through an in plastids localized enzyme, nitrite reductase (NIR). After this glutamic acid can be produced through glutamine synthetase and glutamate synthetase (GS-GOGAT). In this way inorganic nitrogen changes to an organic form in which glutamic acid is formed. In rose suspension culture, nitrate is used as the only source of nitrogen, two days later, the culture concentration of the ammonium in the cells were 0.4 µ mol/g fresh weight and the nitrate 1.2 µ mol/g fresh weight. Four days after the culture began, nitrate concentration increased to 2.3 µmol/g fresh weight, and five days after the culture amide concentration reached its maximum level of 5.9 µ mol/g fresh weight. Increase in the amide concentration shows that the amount of the reduced nitrogen is more than the actual need for nitrogen, so that the amino acid synthesis mechanism cannot process the produced surplus of the reduced nitrogen. Determination of the pattern of the enzyme activity measured in relation to the application of the nitrate form of nitrogen shows that the enzyme glutamine synthetase processes more nitrogen than is the nitrogen requirement of the cell ("Pauls Scarlet Rose" after Fletscher, 1982). According to this data, the restricting enzyme is probably glutamine synthetase or GOGAT. To find out how far the accumulation of ammonium in the vacuole and the cytoplasm influences the feed-back mechanism of nitrate- and nitrite reductase, the culture is supplemented with nitrate and the glutamine synthetase blocking agent methionin sulfoximin. After 30 minutes of application, the concentration of

ammonium in the cell becomes ten-fold and after 4 hours it is increased to seventy-fold of the normal concentration. Despite this concentration of ammonium the activity of nitrate reductase was reduced. It is not quite sure to postulate that the preference of the cells for ammonium nitrogen and not nitrate is due to high levels of ammonium concentration in the cell and subsequently blocking of the enzyme nitrate reductase in which nitrate uptake is minimized or stopped. There must be some other reason and a specific mechanism for this phenomenon (Neumann, 1995). The transfer of an amino group of a glutamine to oxoglutarat needs energy. In this process, two molecules of glutamic acid will be produced. This energy can be provided either through the electrons from ferredoxin localized in chloroplast (Hill-Reaction), or it can be gained from $NAD(P)H_2$.

Tissue culture experiments with carrot and soya bean suspension culture show that only the pyridin nucleotide dependent glutamine synthetase is of importance. Tobacco cultures showed some deviation from this. An experiment is conducted using etiolated and non-etiolated cells. Activity of the $NAD(P)H_2$ after 21 days of culture remained the same in both culture forms. Ferredoxin enzyme-dependent activity however increased many fold. Ferredoxin-dependent enzyme activity depends more or less on chlorophyll formation and the formation of chloroplasts, and so correlates to the intensity of the photosynthesis. In both etiolated and non-etiolated treatments, ferredoxin-dependent enzyme activity influenced the $NAD(P)H_2$-dependent enzyme activity. Enzyme immunological assays show that these two processes deal with two quite different enzyme proteins. Activity of the pyridin nucleotide $NAD(P)H_2$-dependent glutamine synthetase in non-etiolated cells was 10 % of the activity of ferredoxin-dependent enzyme. It was nearly as active as the NADH and $NAD(P)H_2$-dependent enzyme presuming that both can serve as an energy donor for the reduction equivalent. In etiolated cultures the relationship was 3:1 with the fact that the ferredoxin dependent enzyme was dominant (Suzuki and Nato, 1982).

In cell culture, the determined activity of glutamine dehydrogenase in the first instance needs NADH as a reduction equivalent to be able to use the amino group. NADPH as co-factor showed an activity of 20 % in comparison to NADH. Experiments with tobacco callus culture and intact plant show that the enzyme localized in the mitochondria of the cell which is responsible for the conversion of nitrogen into organic form plays a minor role quantitatively, but is nevertheless related to the excess of

ammonium concentration in the cell. In many cases, blocking of the glutamine synthetase with the use of methionine sulfoximin shows an increase in the enzyme glutamate dehydrogenase.

Application of ammonium as the reduced form of nitrogen can lead to growth of a more friable callus, which from a technical standpoint is of importance. Application of nitrate in addition to ammonium leads to an increase of the cell suspension vitality, as in the case of the MS medium. By keeping a constant concentration of the nitrogen, one can obtain the same cell growth using an organic form of nitrogen or even using a mixture of organic and inorganic nitrogen forms. The source of the organic nitrogen used in the media is either urea or a mixture of different amino acids, added separately or in combination as casein hydrolysate. There are many reports in the literature concerning the beneficial effects of using amino acids e.g. glutamic acid and its amid glutamine. On the basis of the experiments conducted by the Steward group at Cornell in the early sixties, it has been postulated that right after the beginning of the experiment the protein content of the carrot root explants increases. These series of experiments were carried out, using coconut milk as a supplement to the liquid media. Later it was shown that the quantity and the maximum levels of protein synthesis could be influenced by the application of cytokinin, in this case in the form of kinetin (Neumann, 1995).

2.8.1 Reduced Nitrogen

The initial observations of somatic embryos by Steward and Reinert were with cultures containing complex media, including CW and casein hydrolysate, both of which serve as sources of reduced nitrogen. The specific requirement for ammonium in carrot somatic embryogenesis was reported by Halperin and Wetherell (1964b). Most culture media used for somatic embryogenesis contain ammonium nitrate (Ammirato, 1984). The source of reduced nitrogen may vary, and complex agendas (e.g., CW), mixtures of amino acids (Kato and Takeuchi, 1966) and single amino acids have all been successfully employed (Wetherell and Dougall, 1976). Alanine and glutamine were superior when nitrate was also present (Kamada and Harada, 1979b). In media lacking nitrate, glutamine was superior to

other amino acids added singly, and ammonium could serve as the sole nitrogen source if the proper pH was maintained (Dougall and Verma, 1978). Studies of changes in endogenous amino acid levels (Kamada and Harada, 1984) have shown that glutamic acid and glutamine accumulated in carrot cells during embryo maturation, but alanine did not. Alanine, which was far superior to ammonium in promoting somatic embryogenesis (Kamada and Harada, 1979b), occupies a central position in amino acid metabolism and was most likely transformed into other amino acids during embryo development. Restricting access to reduced nitrogen may be one way to control synchrony (Kamada and Harada, 1979b).

Proline has been shown to a have a beneficial effect on the course of somatic embryogenesis in alfalfa (Stuart and Strickland 1984b). The effect of proline is dependent on ammonium levels (Stuart and Strickland, 1984b). At certain concentrations, it serves to improve the "quality" of mature embryos, i.e., to produce a more normal structure and facilitate higher levels of germination or the conversion of embryos to plantlets. Proline and serine added to culture media during the growth of carrot suspensions stimulated growth, but also markedly altered the normal development of the embryos (Nuti Ronchi et al., 1984). Polyamines have also been shown to play a role in carrot somatic embryogenesis. Embryonic cells, in comparison to non-embryonic cells, show increased levels of polyamines, in particular putrescine and spermidine (Montague et al., 1978). The level of arginine concentration of arginine decarboxylase is important since arginine serves as a precursor in polyamine synthesis (Montague et al., 1979). A cell line resistant to the inhibitor 5-fluorouracil, which regenerated poorly, also had significantly less arginine during proliferative growth (Sung and Jacques, 1980). Cell suspensions treated with putrecine in the presence of 2,4-D and arginine produced globular embryos that failed to develop further when transferred to media lacking 2,4-D and putrescine, but did develop if subsequently transferred to a medium lacking arginine (Bradley et al., 1984). Feier et al. (1984) were able to inhibit embryogenesis by using difluoromethyl arginine, an inhibitor of polyamine synthesis. A mutant cell line that grows at the same rate as the wild type line in embryogenic medium does not show the characteristic increase in spermidine and spermine levels found in embryogenic cultures (Feinberg et al., 1984). In addition, exogenous auxin (2,4-D), which prevents embryo maturation but not proliferation of the suspension, suppresses the activities of two polyamine biosynthetic enzymes, arginine

decarboxylase and S-adenosylmethionine decarboxylase. Increases in polyamines appear to be specific to embryo maturation rather than cell growth.

2.9 Protein Synthesis

According to the experiments conducted by Neumann in the year 1975, kinetin negatively influences the amount of the protein and RNA in the cell suspension culture. In this experiment, however, the content of the protein and RNA density of the cell suspension culture increased per cell through the application of kinetin in a 28-days-old carrot cell suspension culture. Kinetin, according to Neumann, has not only an influence on the protein and the RNA content of the culture, but also affects the activity of some enzymes. According to his experiments, through the application of 0.1 ppm kinetin in NL medium, activity of the enzyme aldolase increases until the seventh day of the culture, but decreased rapidly afterwards as the culture proceeds further. By contrast enzyme activity of Rubisco increased very slightly till the 21st day of the culture but accelerates rapidly afterwards. Fumerase shows the same pattern as aldolase, that is, it reaches its peak at the seventh day of the culture, but beyond that day the activity of the enzyme drops drastically. The fate of PEPcase is quite different from the others. It shows a moderate increase till the 21st day of the culture and decreases in the same moderate manner. In this experiment, Neumann showed in both the cultures with and without kinetin supplementation to the nutrient solution, that there is an increase in the protein content of the cells in the log phase, and a decrease in protein content per cell in the linear phase of dividing, active cells. Kinetin, according to him, decreases the protein and RNA content of the dividing cells. This decrease is correlated, however, to the decrease in cell size in this growth stage. The fresh weight of the cells after the seventh day of culture decreased, but the number of the cells increased. With the increase in the cell size at the end of the log phase and after the transition to the stationary phase of the cell division, however, the protein content increases. This pattern is identical for both of the cultures, whether kinetin is added to the culture or not. This increase in the protein content is based on fresh weight, but if the dry weight is measured, it can be shown that the kinetin supplemented treatment, although showing less protein content on fresh weight measurement, shows a higher protein content

on the basis of dry weight per cell measurements. This phenomenon can be explained through the fact that the more intensively dividing, small cells of the kinetin treatment possess more cytoplasm per weight unit. Activity of the protein synthesis is not only controlled through plant hormones, other factors influence it too. Iron, for example, plays an important role in the protein synthesis. Iron deficient, kinetin supplemented carrot cell cultures show lower cell division activity than the kinetin-free treatment supplemented with sufficient iron. However, average cell fresh weight and cell size become five-fold. If the iron in the kinetin free treatment is deficient, this increase is less significant. Less proliferated growth in the kinetin free treatment in the first instance can be the result of lower cell division activity, in which the cell growth is not affected. In the iron deficient treatment, as a result of a lower cell division activity, however, cell growth is affected. In the iron deficient treatment, however, concentration of the soluble amino acids is higher. It can be postulated that iron deficiency specifically prohibits protein synthesis and subsequently cell growth. Less proliferation of cell growth in the kinetin free treatment, can in the first instance, be the result of lower cell division stimulation with a rather qualitative impact on protein synthesis. A comparison of the protein turnover of these two different treatments shows a lower metabolic stability in the proteins of the kinetin free treatment, as compared to the control. In the iron deficient treatment, protein metabolic stability is nearly identical with the standard, the quantitative level, however, varies. It can be concluded that iron plays a significant role in the primary synthesis of proteins. On the basis of this experiment, in which the protein synthesis is measured according to fresh weight of the cells, one can postulate that the decrease in the cell division activity of the iron deficient treatment, in comparison to the control, is the consequence of blocking protein synthesis and in the kinetin-free treatment, mainly the consequence of higher protein turnover of a preferential synthesis of metabolic unstable, soluble, cytoplasmic protein fractions. In the kinetin-free treatment, the formation of adventive roots is observed plus a lower cell division activity. This was not the case in the iron-deficient treatments, so that it can be stated that the reason for the lower cell division activity and metabolic processes can also be embedded in the morphogenic potential of the cultured cells. Not only kinetin but also 2,4-D suppresses the formation of the adventive roots in the culture (Neumann, 1995).

2.10 Other Inorganic Nutrient Sources

In addition to nitrogen, other elements are also required by cells in culture. These include inorganic salts that are provided in relatively large amounts (macro nutrients) such as potassium, magnesium, calcium and sulfur (usually as sulfate), phosphorus (as phosphate) and iron. Both sodium and chloride are usually present. In addition, there are traces of other elements (micro nutrients), and these include copper, zinc, manganese, iron, boron and molybdenum. Most basal media supply all the essential macro- and micro-nutrients. With reference to carrot cell cultures, specific requirements for potassium ion (Reinert et al., 1967; Tazawa and Reinert, 1969) and phosphate (Tazawa and Reinert, 1969) have been shown.

2.11 Carbohydrates

Many mono- and disaccharides can support the initiation and development of carrot somatic embryos (Verma and Dougall, 1977), although sucrose appears to be most effective and is the most widely used (Ammirato, 1983). Glucose has also been a superior carbohydrate source (Homes, 1967). Elevated sucrose levels and increased osmolarity can prevent early germination, but these treatments tend to increase the frequency of secondary or accessory embryo formation (Ammirato and Steward, 1971). Increased osmotic concentration of the medium lead to plasmolysis of explanted cells and enhanced somatic embryogenesis (Wetherell, 1984). By increasing the inositol concentration while lowering sucrose concentrations, both germination and extraneous proliferation were prevented (Steward, et al., 1975). Such control of somatic embryo development can also be affected by ABA.

2.12 Growth regulators

2.12.1 Auxin

Although somatic embryos can arise from explanted cells without an exogenous auxin source, particularly if the cells are embryonic, auxin appears essential for initiating carrot somatic embryogenesis (Ammirato, 1983). Many sources of auxin have promoted embryogenic cell proliferation in carrot, including IAA (Sussex and Frei, 1968), NAA (Ammirato and Steward, 1971) and 2,4-D (Halperin, 1966). However, the stronger auxins such as 2,4-D seem particularly effective (Fujimura and Komamine, 1980; Ammirato, 1985). Auxin appears essential for the initiation of embryonic growth, but inhibits embryonic maturation.

The usual procedure is to move the cells to a medium lacking auxin, containing the same auxin at a lower concentration, or containing a different auxin, usually at a lower concentration (Ammirato, 1983). However, in wild carrot suspensions, it is possible to get large numbers of globular embryos in a medium with 2,4-D by diluting the suspension with fresh medium, thereby lowering the density to 20 000 cells per ml (Sung and Okimoto, 1981). However, the embryos do not proceed through the remaining stages of maturation. Somatic embryogenesis in carrot has been inhibited by 2,4,6-trichlorophenoxyacetic acid and p-chlorophenoisobutyric acid (Fujimura and Komamine, 1979b). However, the authors pointed out that responses to anti-auxins were at the early stages of embryogensis, when young embryos are also sensitive to auxin in the medium. The embryo maturation occurs when embryos are removed to an auxin-free medium.

2.12.2 Cytokinins

Carrot somatic embryos will mature fully in a medium free of exogenous growth regulators provided the density of cells is correct (Ammirato, 1985). However, the addition of exogenous growth regulators can benefit development, particularly within certain parameters. Cytokinins have been shown to be necessary for carrot somatic embryo development (Fujimura and Komamine, 1980) and particularly for cotyledon development (Ammirato and Steward, 1971). If present in the medium but not required for growth, they can stimulate aberrant maturation, such as in caraway cultures (Ammirato, 1977). They also counter the growth inhibitory effects of ABA (Ammirato, 1977). The specific type of cytokinin may be important, e.g., in one study, only ZEA but not BA or KIN benefited carrot somatic embryogenesis (Fujimura and Komamine, 1975).

At low cell densities, proembryos typically will not mature (Halperin, 1967). ZEA promoted maturation in low-density caraway cultures, being most effective in combination with ABA (Ammirato, 1983a). ZEA in combination with NAA fostered carrot somatic embryo formation directly from protoplast-derived cells, without an intervening callus phase (Dudits et al., 1976a).
Although not required for cell growth, KIN was shown to maintain an embryo forming potential in long-term cultures (Wochok and Wetherell, 1971;Reinert, 1970). In another study contrasting the responses of embryogenic and non-embryogenic lines (Chandra, 1981), one passage of the non-embryogenic cells on a medium with high levels of KIN (9.3 µM KIN + 0.45 µM 2,4-1) resulted in somatic embryogenesis when the cells were transferred to a 2,4-D-free medium. If the line was maintained on the standard medium for embryogenic cells (0.93 µM KIN + 0.22 µM 2,4-D), only roots formed when transferred to a 2.4-D-free medium. The embryogenic line readily formed somatic embryos with this latter procedure.

2.12.3 Gibberellins

These growth-regulating compounds are rarely used in carrot somatic embryogenesis. GA1 can inhibit embryogenesis in carrot suspension cultures (Fujimura and Komamine, 1975;Tisserat and Murashige, 1977). However, in caraway somatic embryos, GA3 can promote embryo maturation in combination with ABA, and interacts with ZEA and ABA in modulating the course of development (Ammirato, 1977).

2.12.4 Abscisic Acid

This naturally occurring growth-inhibitor selectively inhibits certain morphogenetic events during carrot somatic embryogenesis. First observed in caraway cultures (Ammirato, 1974), ABA will also affect carrot somatic embryo maturation (Ammirato. 1983a). It inhibits abnormal embryo development, including cotyledon malformation, prevents new centers of embryo initiation, and represses precocious germination. It does not inhibit the progression of the small, globular proembryo through the regular sequence of development. The effect of ABA, then, is to foster normal embryo maturation.

2.12.5 Ethylene

This naturally occurring growth regulator has been shown to inhibit the initiation of carrot somatic embryogenesis (Tisserat and Murashige, 1977). Some studies have suggested that ethylene is not found in embryogenic wild carrot suspension cultures, and that the addition of low levels (l-10 μM) of l-aminocyclopropane carboxylic acid, a

compound that triggers ethylene synthesis, enhances embryo maturation (Verma et al., 1985).

2.13 Perspectives

The final goal of studies on somatic embryogenesis is an understanding of its mechanisms at the molecular level. The establishment of a high-frequency and synchronous embryogenesis system promoted the elucidation of the mechanisms of somatic embryogenesis.

However, it is still a hard way. More and more genes involved in embryogenesis have been and will be revealed. Regulation of expression of these genes will also be elucidated by molecular biological techniques. It can be investigated whether or not a gene isolated as a specific one for embryogenesis plays a critical role in embryogenesis by the introduction of anti-sense RNA or at specific stages using microinjection or other techniques and by observation of the following fate of treated cells or cell clusters. Mutants with various developmental stages of embryogenesis provide useful tools for the genetic analysis of influence on embryogenesis (Lo-Schiavo et al., 1988). But it will still be difficult to know the biochemical function of a gene if it is a novel one. Furthermore, several or rather many genes can be involved in the induction and development of embryogenesis, which may be regulated by the balance of expression of the genes. This will make the analysis of the mechanisms of embryogenesis at molecular level more complicated.
Thus, we have many problems to solve the mechanisms of somatic embryogenesis However, the most fundamental and characteristic function of plant cells, totipotency, should be elucidated using high frequency and synchronous somatic embryogenesis in cell cultures.

3 MATERIAL AND METHODS

3.1 Plant Material

When not stated otherwise experiments are conducted with *Daucus carota sativus* L. German variety Rotin. Seeds are sown in soil in a tray of 32 cm x 25 cm x 5 cm at 22°C and a relative humidity of 55 % under constant light conditions using ca. 4000 Lux (Osram, Lumilux White).

3.2 Tissue Culture

3.2.1 Culture Condition

The temperature during the culture was maintained at 28°C under constant illumination using ca. 4000 Lux (Osram, Lumilux White). The vessels used for the liquid cultures were either normal Erlenmeyer flasks or T-tubes with a capacity of 15 ml basal nutrient medium. The opening of the tubes were closed using three-fold aluminum foil and hung on the Auxophyton, modified by Neumann in year 1965 with a revolution of 1 RPM, to enable more supplementation of the basal medium with oxygen.

3.2.2 Modified B5 (Gamborg et al., 1968) Basal Medium for Plant Cell and Tissue Culture

The basal medium used is a modification of B5 (Gamborg et al., 1968). The pH of the medium is adjusted to 5.7-5.8 and autoclaved at 134 °C with a pressure of 2.1 bar for 40 minutes.

Composition of Modified B5 (Gamborg et al., 1968) Basal Medium

B5 Major salts (10 X), g/1 Aqua dest

NaH_2PO_4 X 2 H_2O	1.50
KNO_3	30.00
$(NH_4)_2$ SO_4	1.34
$MgSO_4$ X 7 H_2O	5.00
$CaCL_2$ X 2 H_2O	1.50

B5 Minor salts (10 X), mg/1 Aqua dest

$MnSO_4$ X H_2O	100.00
H_3BO_3	30.00
$ZnSO_4$ X 7 H_2O	20.00
Na_2MoO_4 X 2 H_2O	2.50
$CuSO_4$ X 5 H_2O	0.25
KI	0.25

Iron-EDTA solution (10 X), mg/100 ml Aqua dest

Fe - EDTA	463.00

Mg Salt	**g/1 Aqua dest**
$MgSO_4$ X 7 H_2O	36.00

B5 Vitamins (10 X), mg/100 ml Aqua dest

Nicotinic acid	50.00
Thiamin	10.00
Pyridoxine	10.00

Stock Solution of Growth Regulators (10 X), mg/100 ml Aqua dest

myo - Inosit	5000.00
2, 4-D	100.00

Preparation of Basic modified B5 Medium, l

	B5+	B5-
Sucrose (g)	20.00	20.00
Casein hydrolysat (mg)	250.00	250.00
Major and Minor Salts (ml)	100.00	100.00
Iron-EDTA Stock (ml)	10.00	10.00
Mg Stock (ml)	7.00	7.00
Vitamin Stock (ml)	1.00	1.00
Inosit Stock (ml)	10.00	10.00
2, 4-D Stock (ml)	5.00	-------

Adjust the pH to 5.7 - 5.8

3.2.3 B5 (Gamborg et al., 1968) Basal Medium for Plant Cell and Tissue Culture

Basal medium of B5 (Gamborg et al., 1968).

The pH of the medium is adjusted to 5.7 -5.8 and autoclaved under 134 °C with a pressure of 2.1 bar for 40 minutes.

Composition of B5 (Gamborg et al., 1968) Basal Medium

B5 Major salts (10 X), g/1 Aqua dest

NaH_2PO_4 X 2 H_2O	1.50
KNO_3	25.00
$(NH_4)_2SO_4$	1.34
$MgSO_4$ X 7 H_2O	2.50
$CaCL_2$ X 2 H_2O	1.50

B5 Minor salts (10 X), mg/1 Aqua dest

$MnSO_4$ X H_2O	100.00
H_3BO_3	30.00
$ZnSO_4$ X 7 H_2O	20.00
Na_2MoO_2 X 2 H_2O	2.50
$CuSO_4$ X 5 H_2O	0.25
KI	7.50
$CoCl_2$ X 6 H_2O	0.25

Iron-EDTA solution (10 X), mg/100 ml Aqua dest

Na - EDTA	373.00
$FeSo_4$ X 7 H_2O	278.00

B5 Vitamins (10 X), mg / 100 ml Aqua dest

Nicotinic acid	100.00
Thiamin HCl	100.00
Pyridoxine HCl	100.00
myo - Inosit	1000.00

Stock Solution of Growth Regulators (10 X), mg/100 ml Aqua dest

2, 4-D	100.00

Preparation of Basic B5 Medium, l

	B5+	B5-
Sucrose (g)	30.00	30.00
Major and Minor Salts (ml)	100.00	100.00
Iron-EDTA Stock (ml)	5.00	5.00
Vitamin Stock (ml)	10.00	10.00
2, 4-D Stock (ml)	5.00	-------

Adjust the pH to 5.7 - 5.8

3.2.4 Petiole Culture

5-6 cm long petioles of a 2.5 - 3.5-weeks-old carrot plant are washed in distilled water, then, for the purpose of surface sterilization, immersed first in 70 % ethanol for one minute, then the petioles are immersed in a solution of Na - Hypochlorid (1 : 2 water with 5 % active chloride). One drop Tween 80 is added to lower the surface tension. The 1 cm terminal ends of the petioles are cut and the rest is washed five times with sterile distilled water. The petioles are cut into pieces 1 cm long, and 4-6 pieces are placed in a T-tube containing 15 ml of the B5 basal medium and 0.5 ppm of the synthetic auxin 2.4-D for two weeks. For the realization of somatic embryogenesis, the petioles are washed with a hormone-free basal medium for 30 to 60 minutes and then transferred to a hormone-free basal medium, where they produce globular, heart, torpedo stage and finally small plantlets.

For the histological examination, the petioles are cultivated with different time intervals in an auxin containing basal medium. These intervals are t0 (zero) which means the petioles excised freshly from the carrot plant are cultured for only five hours in an auxin-containing basal medium. t7, t14 means the petioles were cultured for 7 days or 14 days in an auxin containing basal medium.

3.2.5 Establishment of Cell Suspension Cultures

3.2.5.1 Callus Culture

As above mentioned, the desired plant part is surface sterilized and placed in sterile condition on a solidified basal medium. After a few weeks, the callus can be observed macroscopically. The formed callus is cut into small pieces and transferred to a liquid medium containing auxin.

3.2.6 Maintenance of Cell Suspension Culture (0.5 ppm 2,4-D)

Under sterile conditions the suspension culture has been sieved through a 90 μ sieve and the suspension let to stand for 30 minute. Afterwards, the supernatant solution is decanted, keeping only the cells and a portion of the basal solution. The cells are transferred into a graduated cylinder. Add as much of the previously prepared sterile basal media, so that the cells constitute 10 % of the whole volume. The petiole explants were examined macroscopically and microscopically for the presentation of the embryonal structures.

3.3 Somatic Embryogenesis

Somatic embryogenesis has four phases
- Induction of somatic embryogenesis
- Preservation and multiplication of the embryogenic potential
- Realization of somatic embryogenesis
- Regeneration of plants

3.3.1 Somatic Embryogenesis in the Petiole Explants

For the induction of somatic embryogenesis, the petioles are first cultivated for 14 days in an auxin containing B5 basal medium and for the realization of somatic embryogenesis the cultivated petioles are washed thoroughly with the B5 basal medium without auxin for 30 to 60 minutes and later subcultured in T-tubes containing B5 basal medium without the auxin 2,4-D.

The section of the petioles are examined at different time intervals, e.g. 7 and 14 days. In some cases, time intervals of 21 and 28 days have been considered too. During the microscopic examination of the petioles, different histological methods and staining agents, e.g. Hematoxyline, Eosin, neutral red and Safranin fast green, were used to show changes in tissues and cells caused by different treatments, e.g. increase of the cytoplasm content of the cells, etc.

3.3.2 Somatic Embryogenesis in Cell Suspension Culture

As mentioned above the desired plant part is surface sterilized and placed under sterile conditions on a solidified basal medium. After a few weeks, callus can be observed macroscopically. The callus is cut into small pieces and transferred to an auxin containing liquid medium to obtain cell suspension culture.

Under sterile conditions, suspension culture is sieved through a 90 µ sieve, 10 ml of the culture is transferred into a sterile, conical, calibrated centrifuge tube and spun at 300 g for

10 minutes. The supernatant solution is poured off. Then as much of the sterile solution is added to the conical, calibrated centrifugal tube so that the total volume of the cells and the solution reaches 10 ml. This is repeated 3 times. The required amount of packed cell volume of the cell suspension for the realization phase without auxin is 0.5 % PCV. For the evaluation of the realization phase, tissue culture is macroscopically and microscopically examined.

3.3.3 Investigations Related to the Role and Effect of Different Nitrogen Forms During the Realization Phase of Somatic Embryogenesis in Carrot Petiole and Suspension Culture

- Casein hydrolysate as the sole nitrogen form in the nutrient solution during the realization phase of the somatic embryogenesis

- Ammonium as the sole nitrogen form in the nutrient solution during the realization phase of somatic embryogenesis

- Nitrate as the sole nitrogen form in the nutrient solution during the realization phase of the somatic embryogenesis

- Determination of pH and pK values

- Determination of chlorophyll and Anthocyanin in plant material

3.3.3.1 Modified B5 (Gamborg et al., 1968) Basal Medium for Plant Cell and Tissue Culture Containing Different Nitrogen Forms

The basal medium used is a modification of B5 (Gamborg et al., 1968). There were three different nitrogen sources which are used in preparing the stock solution, namely, casein hydrolysate as an organic nitrogen source, $(NH_4)_2SO_4$ as the reduced form of nitrogen, and KNO_3 as the oxidized form of nitrogen. The pH of the medium is adjusted to 5.7-5.8 and autoclaved under 134 °C with a pressure of 2.1 bar for 40 minutes.

3.3.3.2 Composition and Concentration of Different Nitrogen Sources in Modified B5 (Gamborg et al., 1968) Basal Medium

3.3.3.2.1 Modified B5 (Gamborg et al., 1968) Basal Medium

KNO_3	2.5000 g/l
$(NH_4)_2SO_4$	0.1340 g/l
Casein hydrolysate	0.2500 g/l

3.3.3.2.2 Ammonium Based Modified B5 (Gamborg et al., 1968) Basal Medium

KNO_3	0.0000 g/l
$(NH_4)_2SO_4$	3.4293 g/l
Casein hydrolysate	0.0000 g/l

The missing K and S are compensated through the addition of $K_2(SO_4)$

3.3.3.2.3 Casein Hydrolysate Based Modified B5 (Gamborg et al., 1968) **Basal Medium**

KNO_3	0.0000 g/l
$(NH_4)_2SO_4$	0.0000 g/l
Casein hydrolysate	3.6610 g/l

The missing K and S are compensated through the addition of $K_2(SO_4)$

3.3.3.2.4 Nitrate Based Modified B5 (Gamborg et al., 1968) **Basal Medium**

KNO_3	3.3743 g/l
$(NH_4)_2SO_4$	0.0000 g/l
Casein hydrolysate	0.0000 g/l

The missing K and S are compensated through the addition of $K_2(SO_4)$

3.3.3.3 Composition and Concentration of Different Nitrogen Sources in B5 (Gamborg et al., 1968) **Basal Medium**

3.3.3.3.1 B5 (Gamborg et al., 1968) **Basal Medium**

KNO_3	2.5000 g/l
$(NH_4)_2SO_4$	0.1340 g/l
Casein hydrolysate	0.0000 g/l

3.3.3.3.2 Ammonium Based B5 (Gamborg et al., 1968) Basal Medium

KNO_3	0.0000 g/l
$(NH_4)_2SO_4$	3.4293 g/l
Casein hydrolysate	0.0000 g/l

The missing K and S are compensated through the addition of $K_2(SO_4)$

3.3.3.3.3 Casein Hydrolysate Based B5 (Gamborg et al., 1968) Basal Medium

KNO_3	0.0000 g/l
$(NH_4)_2SO_4$	0.0000 g/l
Casein hydrolysate	3.6610 g/l

The missing K and S are compensated through the addition of $K_2(SO_4)$

3.3.3.3.4 Nitrate Based B5 (Gamborg et al., 1968) Basal Medium

KNO_3	3.3743 g/l
$(NH_4)_2SO_4$	0.0000 g/l
Casein hydrolysate	0.0000 g/l

The missing K and S are compensated through the addition of $K_2(SO_4)$

3.3.3.4 Vitality Test (Staining with Neutral Red)

This method can be used to show the vitality of the cells in the petiole and in cell suspensions. Neutral red stains the vacuole of the cells. If the vacuoles are stained red, it means that the cells are living, otherwise if the cells are dead they do not absorb the neutral red and remain transparent.

The solution of neutral red has been made with a concentration of 0.002-0.01 % (W/V) by dissolving 2 mg of neutral red in 100-1000 ml Aqua dest. It is very important that the pH of the medium containing neutral red is between 6.8 -8.0. The pH can also be adjusted by using NaOH or HCl when distilled water or liquid solution is not used. The plant material is incubated for 30 - 60 minutes in the neutral red solution. The incubation time can be prolonged to even 3 hours or shortened to 15 minutes. The plant material is washed later with some 100 ml liquid solution for the microscopic examination.

3.3.3.5 Determination of Dry and Fresh Weight of Plant Material

Before measuring the fresh weight of the plant material, it is placed on a piece of filter paper. Then the fresh weight is determined by using a digital balance. For the determination of the dry weight, the plant material was air-dried.

3.3.3.6 Counting Cells in Callus, Petiole and Suspension Culture

A maceration solution of chromic acid and HCl in 1:1 ratio (10 % chromic acid : 0.1 N HCl) has been made. 10 ml of maceration solution is added to 1 g fresh weight of callus,

cells of the suspension or petiole. The plant material mixed with the maceration solution is kept for 24-48 hours at room temperature (Neumann, 1995).
After the maceration solution has dissolved the pectin between the cells, and when the cells are completely separated from each other, then one ml of the mixture is taken and at least 0.1 ml of the mixture is dropped on each field sight on the hemacytometer and counted 6-10 times. An average is taken from the counts and substituted in the following formula:

$$N = X (M V + F G) : VK n$$

Where:

N	Number of cells
X	Mean of number of cells
MV	Volume of the maceration solution being used in µ l
FG	Fresh weight in mg
VK	Volume of the chamber in µ l (0.1 ml or 100 µ l)
n	Number of the examined glasses or tubes etc.

3.3.3.7 Determination of the Osmotic Potential

3.3.3.8 Preparation of the Plant Material (Extraction of Cell Sap)

Using a surgical syringe, the cell sap has been extracted from single cells. 50 µl of the cell sap is used to measure the osmotic pressure with the help of Digital Mikro-Osmometer Type 11 from Roebling Messtechnik Co.

3.3.3.9 Determination of pH Value of Different Basal Media and the Culture Solution

Before the measurement of the pH, the culture is filtered through a blue band filter, and then the pH of the culture solution is measured with the help of a digital pH meter.

3.3.3.10 Determination of pK Value of Different Basal Media

10 ml of the basal medium is added to a 100 ml glass beaker. 60 ml distilled water is added to it, plus 5 ml of HCl 2 mol/l. Then the pH is measured constantly while adding 100 µl of NaOH 2 mol/l. The pH change is measured till the solution reaches the pH of 12.

3.3.3.11 Determination of Chlorophyll and Anthocyanin in Plant Material

1 % solution of HCl in 96 % ethanol in a graduated Erlenmeyer flask has been prepared. After determining the fresh weight of the plant material, 10 ml of a solution consisting of 1 % HCl/ethanol (W/W) is added to 1 g of the plant/cell material kept over night at 4° C in absolute darkness. The liquid phase is separated from the solid phase by means of blue band filter. The extinction of Anthocyanin and chlorophyll is measured using a digital spectral photometer. The peak of the absorption spectrum is 647,664 and 750 nm for chlorophyll, 500 and 512 nm for Anthocyanin.

3.3.4 Nitrogen Form and its Relation to the Enzyme Nitrate Reductase

3.3.4.1 Extraction of Total Soluble Protein (After Bender et al., 1978)

Extraction Buffer (After Bender et al., 1978)

Tris HCl pH 7.5	0.15 M
Na-EDTA	0.01 M
KCl	0.01 M
$MgCl_2$ X 6 H_2O	0.001 M
DTE (fresh)	0.01 M
Saccharose	0.05 M
Na-Ascorbat	0.04 M

1 ml of the extraction buffer is added to 0.100 g of insoluble PVP in a mortar, covered with parafilm and kept over night in the refrigerator (4°C).
Using a Buchner funnel, the plant material is washed with double distilled water. 1 g of plant material is crushed with a pestle along with 3 ml of extraction-buffer and sea sand, then centrifuged for 30 minutes at 12000-14000 rpm. The supernatant solution is collected and the residue discarded. The amount of the supernatant solution is measured for further calculations. The extracted soluble protein is precipitated with ammonium sulfate and immediately used for the detection of the enzyme activity, whereas for the measurement of the protein content the sample was stored in a refrigerator at -80°C.

3.3.4.1.1 Purification of Protein Using Ammonium Sulfate

682 mg ammonium sulfate are weighed in a microfuge tube and 1 ml of the protein extract is added. After gentle shaking dipped in liquid nitrogen, the supernatant solution is discarded by inclining the microfuge tube. 2 ml of the extraction solution are added to the sample and recentrifuged under 4°C at 13000 rpm for 15 minutes. The supernatant solution is discarded. 1 ml of the extraction buffer is added to the microfuge tube and shaken gently till the protein is completely dissolved in the solution.

3.3.4.2 Detection of Constitutive and Inductive Nitrate Reductase (NR)

Nitrate is reduced by reduced nicotinamid-adenine dinucleotid phosphate (NADPH) to nitrite in the presence of the enzyme nitrate reductase (NR).

Nitrate + NADPH + H^+ --- NR ---> nitrite + $NADP^+$ + H_2O

The amount of NADPH oxidized during the reaction is stoichimetric to the amount of nitrate. The decrease in NADPH is measured by means of its light absorbance at 340 nm. For the determination of both, the inductive and the constitutive NR activity, in a cuvette, 0.5 mg NADPH, 1.850 ml double dist water, 100 µl KNO_3 in a concentration of 10 ppm is added to 1 ml of the imidazole buffer (pH 7.8) and stirred well. Then after 3 minutes the absorption extinction is measured (DE1) at 340 nm. 200 µl extraction solution is added and stirred well. The absorption extinction is measured every minute against the blank for 120 minutes (DE2). The temperature during the experiment is kept at 25 °C.
The activity of NR exhibits a daily rhythm, peaking at the light phase (Lopes et al., 1997, Hunter J. J. and Ruffner, H. P. 1997). The activity of NR, at posttranscriptional level is regulated by light and photosynthesis and is one of the mechanisms responsible for the short-term coupling between photosynthesis and leaf nitrate reduction in the light (Lejay et al., 1997). Additionally the levels of NR activity are affected by nitrate nutrition and plastid

integrity (Cabello et al., 1998). The activity of both forms of NR follows circadian rule, based on either an endogenous (internal) rhythm, behaving as if it is driven by a biological clock, this behavior is said to be *free-running* continuing in a regular cycle even in the absence of N or an exogenous (external) timer (Singh, 1995). To distinguish between the activity of constitutive and inductive (present only in KNO_3 treatment) NR enzyme, considering nitrogen source of the culture medium and the diurnal rhythms of enzyme NR, additionally the extraction of the sample protein from cell culture has been conducted for the determination of constitutive NR enzyme between 12-14 PM and for inductive NR enzyme 22-24 PM.

3.3.4.3 Determination of Intensity of the Enzyme Nitrate Reductase

V	Enzyme activity (U/ml)
DE	Extinction difference (DE1 - DE2)
Ve	Volume of the enzyme used in µl
Vt	Total volume (3.05 ml)
e	6.3 $mmol^{-1}$ X cm^{-1} = 6300 µ mol^{-1} X cm^{-1}
t	Extinction time difference in minutes
d	Light path in cm

Enzyme activity (U/ml) = V = DE .Vt . 1000 : e . d . Ve . t

$$V = [DE * (3.05) * 1000] / [(6300) * (1) * (0.1) * (1)]$$

3.3.4.4 Determination of Optimum pH of the Enzyme Nitrate Reductase

The optimum pH of the enzyme NR is determined by changing the pH of substrate solution right before measuring the absorption extinction in the DE1 phase. The pH values used to find out the optimum pH of the enzyme NR were 5.5, 6.0, 6.5, 7.0 and 7.5.

3.3.4.5 Determination of K_m Value of the Enzyme Nitrate Reductase

To determine the K_m value of the enzyme NR, which is a constant that reflects the affinity of an enzyme for its substrate or that is, the strength by which the enzyme binds to its substrate, different concentrations of KNO_3, namely, 1, 10, 20, 40, 50, 80, 160, 320 and 1000 ppm is added to the reaction, then the enzyme activity is measured.

3.3.4.6 Determination of Protein Content (After Bradford, 1978)

Tris Buffer (pH 8,5)

Tris	50 mM
EDTA	1 mM
DTT (Dithiothreitiol)	5 mM

Bradford Reagent

Comassie Blue G-250	0,01 %
Ethanol	4,7 %
H_3PO_4	8,5 %

Protein Standard

Protein Calibration Standard (BSA) is mixed with Tris Buffer to a total volume of 100 µl. 5 ml of the Bradford solution is added to it and after 5 minutes the absorption spectrum of the protein is measured at the wavelength of 595 nm. The amount of the protein is calculated in µg per gram plant material.

3.3.4.7 Calculation of Protein Content of Cells

To 100 µl of the extracted soluble protein of the plant cells 5 ml of the Bradford reagent is added. After 5 minutes the absorption spectrum of the protein is measured at the wavelength of 595 nm. The amount of the protein is calculated in µg per gram FW plant material.

3.3.5 Histological Examination

The carrot petioles are harvested at room temperature, fixed by immersing in a mixture of ethanol : glacial acetic acid, so the inorganic matter is extracted, then imbedded in a paraffin block. The paraffin block is cut with a wedge shape microtom blade (NO. C sliding microtom OmE, Fa. Reichert). The thickness of the cut sections were 10-15 µm. Depending on the size of the sections, 15 to 30 sections are placed on a 40°C pre-warmed metal plate and kept overnight on a metal plate with a temperature of 30°C. Because the formation of the meristematic cells is characterized by an increase in protein synthesis intensity in the cytoplasm, the transections are dyed with hematoxylin (modified after Heidenhain and Gerlach, 1977)

A Leitz light microscope is used to examine the specimen and Agfapan ASA 25 for the documentation.

3.3.5.1 Procedure for Preparation of Petiole Transection and Staining for Histological Examination

The petioles are washed with 90 ml basal medium and about 50 ml distilled water, and placed in small glass or plastic bottles for fixation.

Fixing Processes for Histological Studies

- Glacial acetic acid 100 % + Ethanol 96 % (1:3) 2- 48 hours
- Ethanol 70 % for at least 4 hours
- Dehydration solution H_2O + Ethanol 96 % + Tert. Butanol (15:50:35) 2 hours
- Ethanol 96 % + Tert. Butanol (45:55) 2 hours
- Isopropanol 100 % + Tert. Butanol (25:75) 2 hours
- Tert. Butanol overnight or 12 hours
- Tert. Butanol + liquid Paraffin (1:1) at 60°C, until there is no smell, 12-24 hours.
- Orienting the object in paraffin block 24 hours
- Fixing of paraffin blocks on wooden blocks 24 hours
- Trimming of the paraffin (0.5 cm * 0.5 cm)
- Washing the slide glasses with diluted chrome sulfuric acid or with 40 % ethanol
- Washing the slide glass with tap or distilled water

- Cleaning the slide glasses with a good quality clean cloth to avoid cloth fiber sticking on the slide glass surface
- Dipping the slide glasses in gelatin solution (gelatin 5.0 g + chromalaun 0.5 g + 1000 ml water)
- Drying the slide glasses on a hot plate or in the air or under suction chamber
- Adjusting the cutting thickness on the Reicherd-Microtom to 10 micron at an angel of 7 degrees.
- Placing 3 bands each consisting of 12-15 cuts on the slide glass.
- The microtom knife is cleaned from time to time with xylol
- Placing the slide glass on a hot plate at 40° C to spread shrunken cells 4 hours
- Placing the slide glass on a hot plate at 30° C for evaporation of the water 12 hours
- Placing the slide glasses in a holder, then in a cuvette
- Bathing the slide glasses in xylol for 5 min

Ethanol 96 % for 5 min, ethanol 70 % for 5 min
Ethanol 50 % for 5 min, distilled water for 5 min
Fe-Alaun-Beize (3 %) for 12 hours
Flowing water for 10 minutes
Haematoxylol for 24 hours
Flowing water for 10 min
Ethanol 50 % for 5 min
Ethanol 70 % 5 min
Ethanol 96 % 5 min
Xylol 5 min.

Placing the slide glasses on a piece of blotting paper or toilet paper to dry, then with the help of a glass rod adding 3 drops of Malinol on the slide glass and cover it or use liquid cover slip. After about a week, the slide glasses are in the position to be kept for an unlimited period of time.

3.3.6 Observing Protein Spectrum and Pattern of Protein Synthesis in Carrot Petiole Transection (^{14}C-Leucine Labelled) During Induction Phase

3.3.6.1 Culture Condition for Labelling Petioles with ^{14}C-LEUCINE

Petioles were cultured in tubes containing modified Gamborg basal medium with 0.5 ppm 2, 4-D. After different incubation times (5 hours,7 and 14 days) under sterile conditions, the petioles are harvested and labelled with ^{14}C-Leucine.

3.3.6.2 Labelling of Petiole with ^{14}C-LEUCINE

Under sterile conditions the petioles are washed with 90 ml Gamborg basal medium without casein hydrolysate (casein hydrolysate contains 52.9 μg L- Leucine / mg), then for the purpose of adaptation incubated further with 15 ml in a 50 ml Erlenmeyer flask in Gamborg basal medium without casein hydrolysate on a rotary shaker for 2 hours.

5 μ Ci L - [U ^{14}C] Leucine (» 100 μ lit) with a specific activity of 282 μ Ci / μ Mol is added to the incubation medium and shaken for 3 hours on a rotary shaker. Finally, the plant material is sieved and washed first with 50 ml of distilled water, then with 120 ml of ^{12}C L-Leucine solution (0.15 mol). The collected wash fraction is used for the detection of the absorption of the radioactive material from the plant material.

For the autoradigraphic examination of the petioles, they are incubated and fixed in ethanol : glacial acetic acid at 21°C, then embedded in paraffin blocks

3.3.6.3 Procedure for Preparation of Labelled Petiole Transection for Histological Examination

- Taking the petioles out of the tube and washing the petioles three times with 90 ml $B5^{+}$ (without casein hydrolysate) in laminar airflow.
- Transferring the content of Erlenmeyer flask over a sieve to filter the liquid media out.
- Sub culturing the petioles in 15 ml $B5^{+}$ (without casein hydrolysate) by putting the plant material in a 50 ml Erlenmeyer flask and shaking it for a period of 2 hours

- Measuring the fresh weight of the petioles
- Adding ^{14}C-Leucine to media. There must be 2 different concentrations of ^{14}C-Leucine for a better contrast. These concentrations are 1 and 5 micro Curie 20 and 100 µl respectively (0.1 micro Curie per 1 ml of the liquid media).
- Shaking the Erlenmeyer flask for 3 hours
- Transferring the content of the Erlenmeyer flask over a sieve to filter the liquid media out and washing the petioles while on the sieve with about 50 ml distilled water, 120 ml 0.15 molar ^{12}C-Leucine and 40 ml distilled water. Then transferring the petioles to a scintillation tube.

3.3.6.4 Fixing Processes for Histological Studies

Same as 3.3.5.1

3.3.6.5 Storage of NTB2 Emulsion (Fa.Technomara, Kodak NTB 2, Mean Grain Diameter 0,26 µm)

If frozen or kept at room temperature, this results in a deterioration of the emulsion and fog formation or loss of the ability to coat the slides. After coating, the slides can be stored in a refrigerator at 4-13°C.

3.3.6.6 Emulsion Handling and Slide Preparation

The liquid emulsion is actually in solid form and must be liquefied under safelight conditions (use a Kodak safelight filter NO. 2 with a 25 watt bulb at 4 feet distance from the emulsion).

- Removing the emulsion bottle under the safelight conditions from the box and placing it in a water bath of 43°-45°C. Liquidification takes place in about 45-60 min.
- Gentle movement of the bottle speeds the process, but too much agitation leads to the formation of microscopic bubbles. It is recommended or approved to add nothing to the emulsion, otherwise there is the possibility of fog formation on the slides.

The emulsion layer thickness and uniformity depend on:
a - Temperature of emulsion and the slides.
b – Speed rate of withdrawal of the slides from emulsion to form a uniform layer.
c - Whether the slide is allowed to drain in a vertical position or immediately placed horizontally
d - Whether the emulsion has been diluted.

The researcher must adjust the parameter to suite his specific requirement for thickness and the uniformity of the emulsion.
- Under safelight conditions using a Kodak safelight filter NO. 2 with a 25 Watt bulb at 4 feet distance from the emulsion, dip the slide glasses very gently in the emulsion. The slides are kept 30-60 minutes in a horizontal position to dry.

3.3.6.7 Exposure Consideration

- Keeping the prepared slides in a light-tight box in a refrigerator with a drying agent at a temperature of 5-10° C during the exposure period. Low temperature and high humidity decrease latent image fading and effect chemical reaction between the specimen and the emulsion. The exposure time must be determined empirically.
- After the exposure period is over, allow the box reach room temp for 2-3 hours, then unseal it. This is to prevent moisture condensation on the surface of the cold slides. Exposure period depends on the intensity of the radioactivity used, and if the emulsion is diluted or not. In this way, petioles labelled with 1 µCi of the L - [U ^{14}C] Leucine need an exposure period of 7-9 days, whereas with water diluted emulsion (1:1) they require 14 days.

3.3.6.8 Exposure Period (Days) in Relation to Intensity of Radioactivity and Dilution

Intensity	NTB2 (pure) (Days)	NTB2 + Aqua dest. (1:1) (Days)
1 µCi	2 - 3	7 - 9
2,5 µCi	1 - 1.5	2 - 3
5 µCi	0.5 - 1	1 - 1.5

3.3.6.9 Processing Instructions

For Kodak autoradiography solutions, use a temperature of 15° C.
Under safelight conditions (use a Kodak safelight filter NO. 2 with a 25 watt bulb at 4 feet distance from the emulsion)
- Develop the slides with Kodak Dektol developer (1:1) for 2 min.
- Dip the slides in stop bath filled with distilled water (do not use acid) for 10 sec.
- Fix the slides with Kodak fixer for 5 min.
- Wash the slides with distilled water for 5 min.
- Let the slides dry in dust free atmosphere for 2-4 hours.

3.3.6.10 Determination of Absorption Rate of ^{14}C-Leucine

1 ml of the collected fraction obtained through washing the labelled plant material is added to 10 ml of scintillation cocktail (Emulsiva 199TK, Fa. Packard) and the amount of isotope present in the nutrient solution is measured with the help of a liquid scintillation counter (Packard TRI - Carb 300 C / 460 C).

Petioles absorbed only some amount of the applied isotope (L-[U ^{14}C] Leucine). Absorbed amount of isotope by the plant is calculated considering the amount of the isotope present in the collected fraction obtained through washing.

Days in Culture	**Absorption of L-[U ^{14}C] Leucine in %**
t0	7.00 %
t7 (B5, + 2,4-D)	36.20 %
t14 (B5, + 2,4-D)	42.63 %
t19 (B5, + 2,4-D)	53.30 %
t19 (B5, - 2,4-D)	42.50 %
t21 (B5, + 2,4-D)	69.43 %
t21 (B5, - 2,4-D)	49.50 %

3.3.7 Investigations Related to Protein Spectrum and Pattern of Protein Synthesis in Cultured Petiole of Carrot Using 2-Dimensional Gel Electrophoresis (2-DE, Grieb, 1992)

3.3.7.1 Labelling of Petiole with ^{14}C-Leucine

Under sterile conditions after 7 and 14 days the petioles are washed with 90 ml Gamborg basal medium without casein hydrolysate (casein hydrolysate contains 52.9 µg L- Leucine /mg), then for the purpose of adaptation incubated further in a 50 ml Erlenmeyer flask with 15 ml Gamborg basal medium without casein hydrolysate on a rotary shaker for 2

hours. In the same manner the control "t0" is adapted for the further examination to all treatments. 25 μ Ci L-[U 14C] Leucine (» 100 μl) with a specific activity of 282 μ Ci / μ Mol is added to the incubation medium and shaken for 2 hours on a rotary shaker. Finally the plant material is sieved and washed, first with 100 ml of distilled water, then with 240 ml of ^{12}C-Leucine solution (0.15 mol), and finally with 90 ml distilled water. The collected wash fraction is used for the indirect detection of difference of absorption rate of the radioactive isotope from the plant material. On the basis of the fresh weight of the petioles, a defined amount of acetone hexane mixture is applied. Detection of the absorbed isotope is performed using wash fraction of acetone hexane mixture.

3.3.7.2 Determination of Absorption Rate of ^{14}C-Leucine

1 ml of the collected fraction obtained through washing of the labelled plant material is added to 10 ml of scintillation cocktail (Emulsiva 199TK, Fa. Packard) and the amount of isotope present in the solution is measured with the help of a liquid scintillation counter (Packard TRI-Carb 300 C / 460 C).

3.3.7.3 Sample Preparation for 2-DE

3.3.7.4 Protein Extraction using Acetone Powder

15 ml of Acetone-Hexane mixture with the following composition: 10 mM Tris/HCl (pH 8.5), 77 mM Glycin, 0.2 mM EDTA, 15 mM DTT, 9 M urea, 2 % Triton X-100 (w/v), 2 % Ampholyte at a temperature of -18°C is added to 1 g of plant material and homogenized for 1 min using a commercial homogenizer with a rotation velocity of 30,000 RPM. The homogenate is placed on blue ribbon filter paper, pressed using a glass rod, then washed frequently with ice-cold Acetone-Hexane mixture. For the complete evaporation of the organic solvent, this mixture is kept over night in an incubation chamber at a temperature

of 30°C. This mixture can be kept for further processing in a desicator. Using this method, not only are the soluble proteins extracted, but also some of the membrane bound proteins (SCOPES 1982).

3.3.7.5 Calculation of Protein Content (after Bradford, 1976)

To 100 µl of the extracted soluble protein of the cells 5 ml of the Bradford reagent is added. After 5 minutes the absorption spectrum of the protein is measured at the wavelength of 595 nm. The amount of the protein is calculated in µg per gram FW plant material. BSA is used as protein calibration standard.

3.3.7.6 2-Dimensional Gel Electrophoresis (2-DE) by Grieb

2-DE performed according to O´Farell (1975) and modified for the tissue culture by GARTENBACH-SCHARRER (1988).

3.3.7.7 First Dimension (Isoelectric Focusing (pI))

3.3.7.7.1 IEF (pI) Run

Round gel is used as described by GARTENBACH-SCHARRER (1988) with a height of 120 mm and a diameter of 4 mm. Havana electrophoresis with 2 % concentration of the ampholyte and the ampholyte pH range of 3-10 (1.2 %) and 4-6 (0.8 %).

100-250 μg protein is applied to the gel, which corresponds to a specific absorption of ^{14}C-Leucine ca. 25 x 10^4 dpm / gel rod. IEF (pI) in itself is performed at a temperature of 18°C for 17 hours and a constant current of 300 Volt, finally, for obtaining a clear band contrast, for 1 hour with a constant current of 500 Volt. Measurement of the gradient is done by the elution method using 20 mM KCl, 3 parallel for each 3 gels. For the second dimension, the gel is equilibrated with SDS buffer 3 times each time for 30 minutes.

3.3.7.8 Second Dimension (SDS-PAGE) and Staining with CBB R-250

3.3.7.8.1 SDS-PAGE Run

After LAEMMLI (1970), a flat gel 18 cm long and 1.5 mm thick is used. The method is described by GARTENBACH-SCHARRER (1988).

SDS-electrophoresis is performed at a temperature of 15°C for 7 hours and a constant current of 400 mA. LMW-Calibration Kit (MG 14-94 kd) is used as molecular marker.
The gel is fixed for 2 hours on a rotary shaker and stained with Coomassie Brilliant Blue-R 250 for 45 minutes, finally, it is distained with the help of rapid distainer for 1 hour, photographed and kept in 7% acetic acid before the fluorography.

3.3.7.9 Fluorography

The gel is gently shaken 3 times, each time for 30 minute, in a solution of dimethylsulfoxid (DMSO), later for 3 hours in PPO/DMSO (BONNER and LASKEY 1974). By washing the gel 1 hour PPO will be removed from the gel and the gel can be dried in a gel dryer for

7 hours at 60°C. The fluorography film (KODAK X-OMAT AR) is brought into direct contact with the gel and exposed for 7 days at -80°C. The exposed film is developed according to the producer's instructions (KODAK).

Stained and labelled protein spots can then be examined qualitatively. CBB stained spots show the protein pattern and the fluorograph shows the pattern of the protein synthesis. From both procedures, protein spots obtained are overlapped on one another, specifying the spot either stained with CBB or labelled with radioisotope, and the spots are stained and labelled and a number allocated to each of them.

3.3.7.9.1 Procedure for Calculation of MW of Protein Spots on 2-DE

The MW of the protein spots is determined using marker proteins as the orientation point. Polynomial regression of the 3rd grade ($Y = a + bx + cx^2 + dx^3$) is the applied statistical method for this purpose with a coefficient of correlation $r = 0.999\ 996$.
Calculation of MW of the protein spots and comparison of the spots at different stages of induction are conducted by a self-written computer application program on Borland Turbo Basic interpreter.

3.3.7.9.2 Marker Proteins

Marker Protein	MW in Dalton
Rabbit Muscle	94 000
BSA	67 000
Ovalbumine	43 000
Bovine Erythrocyte	30 000
Bovine Alpha-Lactalbumin	20 100

3.3.7.9.2.1 Multiple Standard Error of Estimate

For marker proteins, the multiple standard error of estimate is calculated using the following formula:

Standard Error of Estimate = $Y^2 / (n - 1)$

Where:

Y^2 = Sum of square of residues
n = Number of independent variables

Marker Protein	MW Deviation in Dalton (residue)
Rabbit Muscle	± 57
BSA	± 139
Ovalbumin	± 121
Bovine Erythrocyte	± 54
Bovine Alpha-Lactalbumin	± 21

3.3.8 Global Protein Analysis Information Resource Search Database ExPASy Server

Subtractive analysis was performed on the basis of comparative variation of protein spots at t0, t7 and t14 using TagIdent SWISSP-ROT Identification Database, on the EXPASY server http://www.expasy.ch/ (app. M, released Dec. 1995, 360 000 proteins)

considering pI, MW, standard error of estimate and residues of the marker proteins for each protein spot as follow.

Range of MW of each protein spot = (standard error of estimate + residue of the nearest marker protein) / 2

The homologue proteins in the database of the server are selected according to their MW, pI values and their function.

4 RESULTS

4.1 Somatic Embryogenesis in Carrot Petiole Explants

As a preliminary, a system was established giving the opportunity to observe the physical induction site of somatic embryogenesis to follow the protein formation in different plant cells using a common, well-established procedure.

To achieve this objective, the petiole explants of the carrot has been used, for in one instance many experiments have been conducted since the first reports of somatic embryogenesis with this plant. As a model plant, it gives the possibility of using the experience of the past and allows a greater degree of comparison. The reason for selecting petiole as the explants source was to see the physiological changes in different cell types for a better understanding of the induction process, coupled with the examination to find out the possible changes in the synthesis of the proteins (app. A, Fig 1; app. D).

As regards the role of nitrogen as a keystone for the protein structure and synthesis, during the realization stage mostly cell suspension culture has been used. Our efforts were concentrated on the cell fractions observed by histological examination, which during the whole process of somatic embryogenesis, from induction till formation of the plantlets, were presumed to be embryogenic competent cells.

4.1.1 Investigations Related to Role and Effect of Different Nitrogen Forms During the Induction and Realization Phase of Somatic Embryogenesis in Carrot Petiole Culture

One cm long petioles were cultured in a modified B5 auxin-containing medium to observe the role of different nitrogen treatments during induction and realization of somatic embryogenesis in carrot. Eight different media were prepared containing one or a mixture of the following nitrogen forms added to the basal medium, namely:

Casein Hydrolysate	**= 32.5 ppm N**
$(NH_4)_2SO_4$	**= 28.4 ppm N**
KNO_3	**= 415.6 ppm N**

I	**Casein Hydrolysate + $(NH_4)_2SO_4$ + KNO_3**
II	**Devoid of any Nitrogen Source**
III	**$(NH_4)_2SO_4$ + KNO_3**
IV	**KNO_3**
V	**$(NH_4)_2SO_4$**
VI	**Casein Hydrolysate**
VII	**KNO_3 + Casein Hydrolysate**
VIII	**$(NH_4)_2SO_4$ + Casein Hydrolysate**

After transfer to an auxin-free medium, macroscopic examination was conducted 6, 16, 22, 29, 37, 47, 52 and 84 days after the culture, examining splitting of petioles, number of cells in the suspension and formation of embryonic bodies e.g., globular, heart, torpedo and formation of plantlets, pH of culture media (app. H, table 1).

In these series of experiments it was shown that the performance of somatic embryogenesis varies using different nitrogen forms as follows:

I- Casein Hydrolysate + $(NH_4)_2SO_4$ + KNO_3

Petiole culture with all three different nitrogen sources showed moderate growth. Six days after the culture, globular stage emerged, pH of the medium remained 5.6. Twenty days after the sub-culture in auxin-free media plantlets were visible. The number of globular stages produced using three types of nitrogen was the highest at the end of the culture compared to the other treatments (app. H, table 1).

II- Devoid of any Nitrogen Source

Without any source of nitrogen petioles literally starved. There was no sign of growth and development. Petioles were beige at the beginning and their color became very pale 16 days after the culture, on the 22nd day of culture it became beige-brown and were practically dead. The pH of the medium falls to 3.96 twenty-two days after the culture, and due to the mechanical friction a few cells could be seen in the culture solution without any sign of growth (app. H, table 1).

III- $(NH_4)_2SO_4$ + KNO_3

This treatment was similar to treatment number one (I), with a slight sign of advancement in the realization stage, so that 13 days after sub-culture torpedoes could be seen macroscopically, which lead to the formation of plantlets (app. H, table 1).

IV- K NO_3

When nitrate as the oxidized form of nitrogen is used as the sole nitrogen form, pH of the media increased from the initial value of 5.7 to as high as 6.26. Petioles split six days after sub-culture in an auxin-free medium. At first the globular stage appeared, followed by the heart stage on the 13th day of culture and ultimately 21 days after the culture small plantlets were formed. Sixty-eight days after the sub-culture, an average of 72 plants were formed per tube using merely nitrate as the source of nitrogen. It was observed that nitrate increases the pH of the culture. A similar trend was observed using different nitrogen concentrations in cell suspension culture (app. H, table 1).

V- $(NH_4)_2SO_4$

Using only $(NH_4)_2SO_4$, the reduced form of nitrogen caused a drastic decrease in culture pH, so that 6 days after the sub-culture the pH of the milieu reached a low value of 3.94. Petioles showed a very pale color. The pH of the solution reached a minimum of 3.72 sixty six days after sub-culture. This treatment was nearly similar to the treatment where no nitrogen was supplied to the solution. The reason behind the restriction in growth and development of the petiole culture can be the drastic reduction of pH affecting the function of metabolically important enzymes (app. H, table 1).

VI- Casein Hydrolysate

Casein hydrolysate acts as a buffer system stabilizing the pH of the medium. Petioles split profoundly and went through all embryonal stages, but development ceased at the late torpedo stage. Petioles showed pale green color, which later turned to yellow. On the basis of these observations, casein hydrolysate stimulates cell division at the cost of differentiation (app. H, table 1; app. K, fig. 4).

VII- KNO_3 and Casein Hydrolysate :

Using a mixture of oxidized and organic form of nitrogen leads to an increase of splitting with the highest number of plants per tube. The pH of the solution reached 6.27. The amount of 32.5 ppm casein hydrolysate added to the media could hardly buffer the system and neutralized the pH increase induced by the application of 415.6 ppm KNO_3 (app. H, table 1).

VIII- $(NH_4)_2SO_4$ and Casein Hydrolysate :

In this system, the concentration of casein hydrolysate was not enough to buffer the system. The presence of ammonium reduced the pH to 4.31 six days after sub-culture. This value remained nearly the same till 66 days after sub-culture. In this treatment, no embryonal bodies were observed during the induction period, and there was no splitting of the petioles (app. H, table 1).

To summarize the role of different nitrogen forms in B5 for petiole culture during the realization phase, it can be stated that ammonia reduces the pH of the solution and nitrate increases the pH. Caseine hydrolysate has a buffer effect on the solution in higher concentrations. It can, however, cause stabilization of the pH even at lower concentrations. Casein hydrolysate can induces dormancy, which stops development of the embryonal bodies at the late torpedo stage. With an oxidized form of nitrogen, such as KNO_3 petioles went through all embryonic stages during the realization phase, which were more advanced in time and ultimately produced plants. In these experiments nitrogen was supplied at the various nitrogen concentrations as in B5. To elucidate the influence of these various nitrogen forms on embryo development during the realization phase the complete B5 nitrogen concentration for each form was applied separately to cell suspensions previously induced to somatic embryogenesis in a complete B5 medium.

4.1.2 Investigations Related to Role and Effect of Different Nitrogen Forms During the Realization Phase of Somatic Embryogenesis in Carrot Cell Suspension Culture

For studying the role of different nitrogen forms, three auxin-free culture solutions were prepared which were merely supplied either with casein hydrolysate or $(NH_4)_2SO_4$ or KNO_3 as the sole source of nitrogen at identical nitrogen concentrations (app. H, table 2, 3).

Observations concerning the realization of somatic embryogenesis using different nitrogen forms showed:

Using 476 ppm casein hydrolysate led to intensive cell division, but differentiation of the dividing cells slowed down. The cell culture went through different embryonic stages, namely, globular heart and torpedo. The realization stops, however, at late torpedo stage (app. L, fig. 1,2,3). The concentration of casein hydrolysate did not play a role in its specific mode of realization of carrot somatic embryogenesis. In the casein hydrolysate treatment, globular stage appeared 6-8 days, heart 12-14 days and torpedo 16-18 days after the sub-culture in auxin-free medium.

By using 476 ppm $(NH_4)_2SO_4$ somatic embryogenesis was not realized. This phenomenon was probably as a result of a pH change induced by the application of ammonium. Reducing the concentration of ammonium in this system, however led to realization of somatic embryogenesis so that treatments using concentrations of 0.1, 0.2, 0.4, 1.0 and 2.0 mM ammonium sulfate prove this hypothesis. Cell cultures supplemented with such low amounts of ammonium nitrogen went through different embryonal stages. The pH of the solution remained between 4.9 and 5.0, but did not fall below 4.0 as when greater concentrations of ammonium were used. In all the treatments using low concentrations of ammonium nitrogen, somatic embryogenesis terminated at the torpedo stage (app. H, table 6).

By using KNO_3, as an oxidised nitrogen form, during the realization phase as the sole source of nitrogen, contrary to the hypothesis stating that "somatic embryogenesis necessarily needs a reduced form of nitrogen" (Halperin et al., 1965; Kamada et al., 1979, 1984b;Wetherell et al., 1976), globular stage was formed already 5-6 days after the culture,

10-12 days thereafter heart structures appeared. Torpedoes emerged 13-16 days thereafter leading to the formation of plantlets 15-18 days from beginning of the sub-culture in auxin-free medium. To verify this phenomenon, different parallel experiments were conducted using modified and original Gamborg B5 media differing in their nitrogen form and using different cell material. The independent experiments were conducted in our institute, in which the same results were obtained (app. H, table 1, 10a-10d).

Studying the role of nitrogen is connected to the chemical changes, which that very specific nitrogen form exposes to the culture. A very important feature of the selected nitrogen form used is its effect on the pH of the culture medium. To find out this characteristic, it is advisable to determine the buffering capacity of each nitrogen form in the culture solution (app. H, table 7). Starting with the ammonium sulfate as the reduced form of nitrogen, it is well known that the potential of the redox processes, in which H^+ ions participate in the reaction are pH dependent. When the pH value increases the redox-potential decrease.

NH_4^+ <==> $NH3^-$ + H^+/e^- (oxidation form)

NO_3^- ---> NO_3^- + $[8H^+/8e^-]$ --> NH_3 + OH^-

Thus, due to the release of protons in diammonium sulfate treatment the pH of the culture medium is reduced, and because of the release of OH^- in KNO_3 treatment the pH of the medium increases. In the case of casein hydrolysate, it could be seen that the casein hydrolysate acts as a buffer.

As a definition, the buffering capacity of a solution is dependent on the relation between the pH and pK of the system. A solution is considered as having a good buffering capacity if this difference is within a range of 1 on the pH scale. It has been observed that the buffering capacity of the diammonium sulfate and potassium nitrate lay within a range of 2. Casein hydrolysate, but showed a narrow difference between its pH and pK values. It is indeed a suitable buffer, and as will be discussed later, it stabilizes the pH of the culture significantly. A mixture of three different nitrogen forms in the solution had a pH capacity between the organic and non-organic nitrogen containing solution. So the solution makes use of different features of each nitrogen forms (app. H, table 1, 3, 7, 8, 9). Diammonium sulfate reduces the pH of the solution, potassium nitrate exerts an opposite effect by increasing the pH, and finally casein hydrolysate acts as a buffer to stabilize the pH of the system.

Experiments with petiole culture ($B5^-$) under different pH conditions showed that in the induction phase the pH of the solution had a maximum fluctuation of 1 pH range 12 days after the culture. In general the pH tended to reach a value around 6 (app. H, table, 3, 8). After subculture in an auxin-free medium, the pH value decreased for the next two weeks. This can be a result of the release of H^+ (protons) in exchange for readily available and absorbable cations like Ca and K. and N H_4^+ After 14 days, however, the pH of the culture solution rose, so that 30 days after sub-culture in an auxin-free medium the pH of the nutrient solution tended again to reach 6 on the pH scale. The pH difference between the lowest adjusted pH of 4.5 and the highest adjusted pH of 7.2 at this time was about 0.5 on the pH scale. This difference became less as the culture proceeded, so that 43 days after the sub-culture it was 0.45 on the pH scale. Considering the realization of somatic embryogenesis on the basis of the occurrence time and the number of embryonal bodies, cultures with higher adjusted pH values showed a better performance (app. H, table 8, 9). In one treatment, petioles were incubated in stock solutions with an initial pH of 4.5, 5.0, 5.8, 6.5 and 7.2. Petioles with a pH of 4.5 failed to produce any embryo. Petioles cultured in stock solution with a pH of 5.0 showed a restricted embryogenesis. Other treatments with higer pH went through all stages of realization of somatic embryogenesis and produced small plants (app. H, table 8; app. J).
To distinguish pH influence on induction of somatic embryogenesis during the realization, the same experiment was conducted using induced cell suspension in modified B5 as the source material with pH variations of 4.5, 5.0, 5.8, 6.5 and 7.2 (app. H, table 8). The measured pH of the culture solution after 28 days ranged betwen 6.8 and 7. The increase in the pH of the solution was 1 pH scale more than that of the petiole culture, however, showing the same trend. Cell suspension adjusts its pH after transfer in an auxin-free solution. Regarding the realization of somatic embryogenesis, different treatments behaved in the same way as in the petiole culture. Treatment with low solution pH failed to give rise to advanced stages of embryogenesis. Somatic embryogenesis in treatments with a pH of between 5.8 and 6.5 led to a better development. This is evident because the optimum pH of most of the enzymes is in this pH range.
To determine the role of pH and its relation to the kind of nitrogen, three stock solutions with initial pH ranges of 4.2, 5.8 and 7.2 were prepared. With a pH of 4.2 there were no sign of realization of somatic embryogenesis regardless of the type of nitrogen. With a pH

of 5.8 and 7.2 casein hydrolysate and KNO_3 treatment showed normal embryo development. The development was better under the pH of 5.8, indicating that an optimum pH for somatic embryogenesis is around 6. As for $(NH_4)_2 SO_4$, however, it showed slight improvement under pH of 7.2 suggesting a relation between pH and the occurrence and degree of realization during carrot somatic embryogenesis. So that, first, a pH readjustment can be forced on the system when the pH reducing nature of diammonium sulfate encounters the adjusted pH of 7.2. Secondly, under higher pH ranges plant cells can more readily use the nitrogen of diammonium sulfate for its vital metabolic activities e.g. growth and development (app. H, table 8, 9).

A parallel experiment conducted with different pH ranges of 5.8, 6.8, 7.2 and 8.0 showed a similar trend, indicating an optimum pH of 5.8 - 6.8 as a general rule (app. H, table 10a-d). The optimum pH range of control B5 medium lies between 5.8 and 6.8. Casein hydrolysate treatment follows the same trend. In $(NH_4)_2SO_4$ treatment, only under a high pH of 8, the cells divided more rapidly as compared to culture with lower pH. The vitality of cells was determined by using neutral red. The cells were vital even under a pH of 4.2. Nitrate treatment showed a retardation trend as the pH reached the scale of 8.0.

Concerning the pH of the suspension solution, as a general rule, diammonium sulfate lowers the pH of the solution to 3.5, nitrate by contrast increases it up to around 7.0, and casein hydrolysate keeps the pH of the solution nearly constant, around 6.0. Treatments with low concentration of $(NH_4)_2SO_4$ led to embryo formation. This can be interpreted as follows: decreasing the concentration of the supplied amount of $(NH_4)_2SO_4$, pH is less effective.

Different nitrogen nutrition does not only have an effect on the realization of somatic embryogenesis and pH, but also on the chlorophyll content, Anthocyanin production, fresh weight, soluble protein content and osmotic pressure of the cell sap (app. H, table 2, 4, 10a-10d). Taking the fresh weight of the plants into consideration, the treatment using casein hydrolysate showed the maximum fresh weight even if the source material was petiole or cell suspension, and cell division and growth were more when compared to the other treatments.

Diammonium treatment decreases the pH to a critical level at which physiological and biochemical processes stagnate. Fresh weight of potassium nitrate was less than that of casein hydrolysate treatment, but on the other hand, realization of somatic embryogenesis

in this treatment was better. The control treatment with all three different types of nitrogen ranked between casein hydrolysate and potassium nitrate treatment. This is regardless of the number of embryonal bodies e.g. number of young plants. The casein hydrolysate treatment produced the highest number of embryonal bodies, the formation of plantlets, however, failed in this treatment. This phenomenon is of importance in itself. The existing hypothesis proclaiming that all torpedoes will be transferred to plantlets comes under question.

The total chlorophyll content of KNO_3 treatment was the highest, followed by the control and casein hydrolysate treatment (app. H, table 2, 4). The $(NH_4)_2SO_4$ treatment produced a very low amount of chlorophyll because as a result of low pH, enzyme activities were reduced and the growth of the cells was affected extremely negative. An interesting point, however, is the ratio of chlorophyll a to chlorophyll b in photosystem II, in which molecules of chlorophyll a are arranged with a different geometry, so that shorter wavelength, higher energy photons are absorbed than in the ancestral photosystem I. Chlorophyll a is the main photosynthetic pigment and is the only pigment that can act directly to convert light energy to chemical energy. However, chlorophyll b, acting as an accessory or second light-absorbing pigment, complements and adds to the light absorption of the primary pigment, chlorophyll a. Chlorophyll b has an absorption spectrum shifted towards the green wavelength. Therefore, chlorophyll b can absorb photons, which chlorophyll a cannot (Mengel, 1991;Raven and Johnson, 1999). In the control treatment, it was a 2:1 ratio, which became considerably different in casein hydrolysate and KNO_3 treatment in which this ratio changes in favour of chlorophyll b when the concentration of KNO_3 decreases (app. H, table 2, 4).

This effect was not merely a matter of the form of nitrogen nutrition, but also the concentration of nitrogen in the treatment solution (app. H, table 4; app. K, fig. 4). The ratio of chlorophyll a to chlorophyll b reaches the normal ratio of 2:1 as in the control treatment, with an increase in the KNO_3 concentration in the treatment solution.

This level already reached a ratio of 1:1 when 276 ppm KNO_3 was supplied to the solution. A reverse trend was observed in casein hydrolysate treatment in which the ratio of chlorophyll a:b became 1:1.9 (app. H, table 4). In the KNO_3 treatment the ratio of fresh weight to dry weight was greater than that of the casein hydrolysate treatment; this ratio, however, was the greatest in the control treatment. Anthocyanin production differs too,

according to the form and intensity of the nitrogen nutrition. Anthocyanin production in KNO_3 treatment, for example, showed a 20-fold increase as compared to the casein hydrolysate treatment, the production of anthcyanin increased with the increase of nitrogen supplied to the solution.

The difference in the osmotic pressure of the cell sap showed that KNO_3 treatment had the highest osmotic pressure, followed by casein hydrolysate treatment and control (app. H, table 2). Measuring osmotic pressure of the liquid solution showed that solution media of KNO_3 treatment had the lowest osmotic pressure, followed by casein hydrolysate and standard treatment. Since osmotic pressure results from the concentration of molecules in the solution, it can be stated that the nitrogen form alters the osmotic pressure of the plant cell and of the media.
The soluble protein content of different nitrogen treatments showed that the highest rate of protein was produced when casein hydrolysate was supplied to the system, followed by modified B5 having all three types of nitrogen (app. H, table 2; app. I). The lowest values were determined in the KNO_3 treatment. These results did not show the same trend regarding dry weight proportions. It can be presumed that the protein content in the casein hydrolysate treatment is high, but the non-soluble protein substances are less as compared to KNO_3 treatment, which has less protein content but a higher dry weight percentage.
During the realization phase, the activity of the inductive form of the enzyme NR was highest when KNO_3 was the only source of nitrogen, followed by the control and casein hydrolysate treatment. Measurement of the constitutive enzyme NR showed an extremely small difference between different treatments (app. I).

4.2 Histological Examination

Histological examination of the petiole transection shows transformation of vacuolised cells into cytoplasm rich and division active meristemic cells (app. A). The use of Hematoxylin as the dyeing agent gave the opportunity to localize the cells and the pattern of protein synthesis during the induction phase (app. A, fig. 2, 4, 6). In the same manner, but with more precision, the application of a radio isotope e.g. in this context the

use of radio active leucine gave valuable indication not only about the location of the cells which later formed rhizogenic and embryogenic centers stimulated through auxin application, but also about the quantitative accumulation of leucine containing proteins in different cells and structures within specific culture periods (app. A, fig. 7, 8, 10, 11).

4.2.1 Histological Observations During the Induction Phase of Somatic Embryogenesis (culture in B5 with 2,4-D)

The petiole section of a 6-to-8 weeks old carrot plant is a heart-shaped structure and shows 5 collenchyma supporting structures adjacent to the periphery of the epidermis (app. A, fig. 1). Under the cuticle is a single-lined epidermis cell layer, under which is a small, vacuolized 2-to-3-lined sub-epidermal layer of vacuolized cells. The proportion of the nucleus to the whole cell is greater than that of ground and epidermal cells (Schäfer et al., 1985). The ground tissue by itself is composed of highly vacuolized cells varying in size. There are three or more collateral vascular bundles. The largest vascular bundle lies in the center, having xylem in the center and phloem tissues on the periphery. Glandular channels as a typical structure for plants belonging to Apiaceae are located between the conducting channels of vascular bundle and epidermis. There are three glandular channels in this phase of growth. Glandular channels are schizogen, secreting glands.

$B5^{+}$ cultured carrot petiole explants show the following histological changes:
As little as 4 days after incubation of the petioles in an auxin-containing medium, one or more meristematic centers are formed on the periphery of the vascular bundle (app. A, fig. 2). Individual cells of these meristems distinguish themselves from adjacent cells of the ground tissue in form, size and stainability with Hematoxylin. Regarding the form, these cells are round in shape and have a better-defined geometrical form. They are smaller in size and possess more cytoplasm with an obvious larger nucleus.
This process remains a mere microscopic event even after 6 days of petiole culture, but after 8 days of culture, splitting of the petioles can be seen macroscopically. The formation

of rhizogenic centers can be better observed in culture condition with less concentration of auxin. Further growth of the rhizogenic meristems finally leads to the rupture of the epidermis and some times adventitious roots appear (app. K) if the petioles are kept for more than 18 days in an auxin-containing medium.

Under the epidermis, but with a lapse of 5 days, almost the same developmental pattern can be observed. As early as 10 days after incubation of the petioles in an auxin-containing culture, some originally vacuolised sub-epidermal cells accumulate more cytoplasm and start to divide and form meristematic centers called embryogenic centers, resembling rhizogenic centers, but with smaller, more round and more compact cells (app. K, fig. B, C). These structures remain under the epidermis and with in the course of time rupture the epidermis. Some sub-epidermal cytoplasm rich cells show a specific developmental mode in which the cytoplasm of a single cell divides up first into 2, then into 4 cells and more, and in this way forms the pre-embryos (app. A, fig. 5).

The idea behind using a radioactive isotope was to show the differentiation and the changes relating to possible changes in the pattern of protein synthesis of cultivated carrot explants during the induction phase of somatic embryogenesis. So that for a specific change e.g. different culture times, some specific protein spectrum or some specific pattern for the protein synthesis related to that particular culture time and the developmental stage could be detected. The chosen time intervals for petiole culture were after 5 hours, denoted as 0, after 7, 14, 19 and 21 days, denoted as t0, t7, t14, t19 and t21 respectively (app. A fig. 1-6).

Histological observations strongly suggest that the protein content of the plant cell in an auxin-containing medium increases 7 days after culture (t7) as compared to t0 (app. A, fig. 1, 2). In the next 7 days in t14 this trend continues (app. A, fig. 6).

On the basis of the histological observations and the determination of the absorption the rate of ^{14}C-leucine, one can postulate that the accumulation of ^{14}C-leucine and its incorporation in the protein synthesis during the induction phase increases. In these series of observations, it was evident too, that the embryogenic centers in all the observation periods, namely t7, t14, t19 and t21, were labelled (app A, fig. 7, 8, 9, 10, 11). The rhizogenic centers, by contrast showed a selective absorption pattern, so that it can be postulated that the protein synthesis in embryogenic centers is more intensive than in the rhizogenic centers (app. A, fig, 8, 9, 10, 11).

A short comparison between rhizogenic and embryogenic meristems follows:

Development of Rhizogenic Centers:

Time of appearance = around 7 days
Location = near vascular bundles
Cell form = rather long
Cell size = small
Stainability with Hematoxylin = all structures are dyed
Absorption of radioactive leucine = some meristems are labelled and others not

Development of Sub-Epidermal Cytoplasm Rich Cells and Embryogenic Centers:

Time of appearance = 10-12 days
Location = sub-epidermal cell layers between epidermis and vascular bundles
Cell form = rather round
Cell size = small
Stainability with Hematoxylin = all structures are dyed
Absorption of radioactive leucine = all meristems are labelled

4.2.2 Histological Observations During the Realization Phase of Somatic Embryogenesis (culture in B5 with out 2,4-D)

After the petioles were 14 days in an auxin-containing medium, they were sub-cultured in a medium without an exogenous auxin. Using a very high concentration of auxin for the induction, however, is usually inhibitory to development of the somatic embryos in advanced stages. In the hormone-free medium, development of globular staged somatic embryos followed; heart, torpedo stage and finally plantlets were formed.
Thus induction of carrot somatic embryogenesis requires a single hormone signal to induce a bipolar structure (app. J, fig, 3) capable of forming a complete plant upon transfer to a hormone-free medium.

4.3 Protein Spectrum and Pattern of Protein Synthesis in Cultured Petiole of Carrot Using 2-Dimensional Gel Electrophoresis (2-DE, Adapted and extended from Grieb, 1992)

During the in-vitro culture of the petiole explants some changes in the tissue morphology and physiology were observed, so that more meristemic cells were formed followed by an increase in dry weight. The protein content of a 7-days-old (t7) petiole culture in an auxin-containing medium became three times more than the petioles that were only 5 hours in the culture (t0). Increase in the protein content of petioles that were 14 days in the induction medium was marginal, compared with the protein content of petioles that were 7 days in the culture. Comparable to this process was the ^{14}C-leucine accumulation, with the result that the accumulation of ^{14}C-leucine increased from t0 to t7, but decreased at t14 (app. B, table 1). The simultaneous increase in protein content and the ^{14}C-leucine accumulation, from t0 to t7, is an indication of an intensive synthesis of proteins. A decrease in ^{14}C-leucine accumulation from t7 to t14 can be due to the fact that either in this specific time proteins with less leucine are produced, or leucine is lost from the system through respiration in the form of CO2 (Dadhkahi 1978). It can be presumed that through using acetone powder, all the soluble proteins were extracted, but it is not clear if all, or some of the membrane-bound proteins were eventually extracted too. Keeping this in mind it can be presumed that the relation of soluble protein to the entire amount of synthesized protein from t7 to t14 has been changed, so that 14 days after the culture more ^{14}C-leucine was accumulated or incorporated in the membrane bound proteins (app. B, table 1). Experiments conducted by Neumann and Pauler (1969) and Pauler et al. (1977) with Daucus culture under the influence of kinetin suggest the tendency of the protein synthesis towards synthesis of non-soluble proteins in meristematic cells. The effect on the protein content of petiole culture and ^{14}C-leucine accumulation is similar to the histological observation (app. B, table 1, 2).

There are changes in the meristemic cells in the vicinity of the vascular bundles in t7, followed by an increase in the number of cells, accompanied with a more intensive protein synthesis (app. A, fig. 8). In a later stage of the culture the number of these meristems

increased, followed by the emergence of the first cytoplasm-rich embryogenic cells and centers (app. A, fig. 10).

A possible reason why this differentiation pattern cannot be localized clearly, considering protein content or ^{14}C-Leucine accumulation, can be either that the number of embryogenic cells is much smaller compared to the total number of cells, so that the protein synthesis of these cells is covered up by overlapping of cells, or the proteins required for the induction of somatic embryogenesis possibly exist already in a 7-days-old culture. The protein synthesized till this very stage possibly builds the prerequisite for the induction of somatic embryoghenesis. In a 2-DE, the emergence and absence of proteins should show which proteins are merely synthesized and which disappear, or which proteins are common comparing t0, t7 and t14. Furthermore, the changes occur till the end of induction of somatic embryogenesis in the petiole system (app. E).

4.3.1 Pattern of Protein Synthesis (Adapted and extended from Grieb, 1992)

A total number of 282 protein spots were identified using 2-DE and flurography. From the appeared spots, 211 were stained with Coomassie Brilliant Blue R-250 (CBB) and 241 spots were labelled (app. B, table 2). These protein spots can be categorized in three groups (app. C; app. E):

I- Protein spots, which were merely stained with CBB indicating proteins, were not newly synthesized at the time of investigation. These represent that part of the protein spectrum, which is present in that very particular differentiation stage.
II- Protein spots which were only detected by means of flurography. These proteins were newly synthesized at the time of investigation, so that they may correspond to a particular

differentiation stage. (They were not present before, or the CBB was not sensitive enough to show the existence of these spots.)

III- Protein spots which were both stained and labelled. These are proteins representative of a particular differentiation stage and their continuous synthesis could be vital for the maintenance of that very particular differentiation stage.

At the t0 stage, i.e. after 5 hours of culture only 91 proteins could be detected. This number becomes 2.7 fold by t7, when the petioles are 7 days in the induction medium. The number of spots of this stage amounts to 250. From this stage till t14, when petioles are 14 days in culture, there is a slight increase in the number of protein spots, namely to 256 spots.

New synthesis of proteins or proteins, which are representative for a particular stage resembles the total protein spectrum. It increases from t0 with 67 (13+54) proteins to 99 (6+93) proteins in t7. From t7 to t14 the synthesis of proteins, which appear during a specific period of differentiation and relate to a specific differentiation event decreases to 85 (17+68) protein spots (app. C). Protein spots stained only with CBB showed a continuous increasing trend. They increased from 12 proteins in t0 to 35 in t7, and later to 54 in t14. Considering the quantitative aspect of the protein spectrum, it provides the evidence confirming an increase of ^{14}C-leucine incorporation rate detected by scintillation counter measurement during the induction period from t0 to t7, and the decrease in ^{14}C-leucine incorporation from t7 to t14 (app. B, table 1).

Parallel to the histoautoradiographic studies (4.2), the soluble proteins of 2-DE of comparable treatments (Grieb, 1992) were identified. The identification of 52 homologue proteins and a summary of the possible function of the proteins in this investigation will be discussed.

Protein content, the number of protein spots and ^{14}C-leucine accumulation increased from t0 towards t7. ^{14}C-leucine accumulation and the number of labelled protein spots decreased from t7 to t14 (app. B, table 1, 2). The only increase later was an increase in total protein content and a marginal increase in total protein spots.

Observations showed the protein spots amounted to 91 spots 5 hours after the culture (t0), 13 % of which were merely stained with CBB. This means a part of these spots constitute proteins originally belonging to the petiole and its system, and the application of auxin did not play any role in their synthesis. These proteins appeared in t7 and t14 too. After 5 hours of culture, only 54 new proteins were synthesized (59 %), of which 9 spots were specific for this very stage. They did not emerge in t7 or t14 and so can be called t0 specific proteins. 44 proteins belonging to this induction stage were synthesized in other stages too. 71 protein spots in this stage emerged in t7 and t14, so they can be called household or house-keeping proteins, or proteins which play a role in the basic metabolism of the petiole or plant (app. B, table 2; app. C). To describe t0 protein spots: This stage had 13 protein spots belonging to this specific stage of induction, of which 9 protein spots were newly synthesized.

The protein spots that appeared 7 days after the culture amounted to a total of 250. Of this number, 122 (49 %) were specific for this differentiation period; they were both stained and labelled. 79 spots were present in this stage but absent in t0. 43 spots were identical with the spots in t0. 93 protein spots (37 %) were merely labelled, of which 65 spots were specific to this differentiation period/stage. 35 spots (14 %) were merely stained with CBB, meaning they were not newly synthesized at the time of observation.

256 protein spots were detected at 14 day of culture. There was not a highly significant difference between the protein spectrum on the whole and the rate of protein synthesis between the 7th and 14th day of culture, but there are differences in other respects. The number of stained spots at t7 increased from 14 % to 21 % in t14 (from 35 to 54 spots). The number of spots stained and labelled increased from 50 % to 53 % (135 spots). The number of spots merely labelled decreased from 37 % in t7 to 27 % in t14.
Of 54 spots, 6 spots were common with t0 and 43 with t7. The largest groups of protein spots (135) were spots, which were both stained and labelled; 4 spots appeared for the first time, 82 spots were present in t7 and 49 were present in t0 too. 68 protein spots in this stage were merely labelled, and of this batch 14 and 56 spots were merely labelled in t0 and t7 (app. C). It can be presumed that these proteins have either very low rates of synthesis

or that they emerge with a specific time sequence. Seventeen spots are t14 specific spots, of which 9 spots were stained, 4 labelled and 4 stained and labelled.
For a greater understanding of the possible function of the protein spots, considering stainabilty and ability to assimilate 14C-leucine in the structure, a classification system can be implemented. This classification is based on the nature and the time of appearance of the protein spots.

There are 9 groups and sub-groups (see 5.2.1; Grieb,et al 1997; app. G)*:*

A characteristic protein pattern is associated with the various stages of the cultural cycle investigated, (Grieb, 1992). After 5 hours of cultivation, proteins of the groups 1 - 5 and of group 9 could be detected, at t7 the proteins of groups 2, 3 and 5 – 7, and at tl4 proteins of groups 3, 5, 6, 8, and 9 occur simultaneously. Obviously, these changes in the protein complement should be part of an evolving program of molecular differentiation, as the basis of the histological events described earlier. Apparently, changes in differentiation of the petiole explants during the cultural cycle of 14 days involve a stepwise activation and termination of genetic subprograms. First, a subprogram for the petiole is terminated (group 1), which seems to be representative for the original tissue. At the same time, a subprogram obviously associated with explantsation and the inoculation procedure is activated (group 4) and terminated before t7. Another subprogram, however, which is active already in the petiole also continues during culture (group 3). Proteins of this subprogram should have some "house-keeping" function for the carrot petiole per se. Also the proteins of group 2 are already synthesized in the petioles, but at t7 of culture their concentration is reduced (only labelled), and at t14 they are no longer detectable. Additionally, a subprogram represented by 44 proteins is initiated at t0 (group 5), which continues throughout the cultural cycle. Apparently, these proteins are characteristic of cultured petiole explants. Associated with the formation of the rhizogenic centers near the vascular bundles (t7), proteins of some other subprograms are synthesized (groups 6 and 7). However, a large group of these proteins (166) is also present at t14 (group 6). Nevertheless, the 8 proteins of group 7 could only be detected at t7 and should be specific for this physiological state. This indicates that within the differentiation program in the cultured petiole, subprograms are switched on and off at defined stages in the cultural cycle. It remains to be seen which function each individual subprogram serves for the continuous progress of the differentiation program of the petiole as a whole. Although the extracts used for 2 D-electrophoresis contain proteins from all cells of the petiole explants, as historadioautograms indicate e.g at t7 most of the label is concentrated in and near adjacent vascular bundles, and to a lesser degree in the

epidermis area. Most of the proteins labelled should have been synthesized in these morphogenic parts of the cultured explants.

Only rather small changes in the protein pattern can be observed at t14, as compared to t7, which coincides with the appearance of the cytoplasm-rich sub-epidermal cells with embryogenic initiation, and of embryogenic centers. At t14, proteins of group 8 are absent, at t7 are only stainable, and should have been synthesized in the period between 7 and 14 days of the cultural cycle (spot No. 13, 74, 76, 97, 106, 142, 144, 145, 146; app. E)*. Others, however, are traceable by the stain and the label, and therefore the synthesis of these should have been initiated between t7 and t14 and should continue at t14 (*4 proteins: spot No. 85, 87, 88, 130; app. E)*. Eventually, 4 proteins of this group are only labelled at this stage* (spot NO. 117, 118, 119, 192; app. E)*. It remains to be seen whether and/or to what extent these proteins at t14 are specifically related to somatic embryogenesis.*

4.4 Global Protein Analysis Information Resource Search Database ExPASy Server

Subtractive analysis performed on the basis of comparative variation of protein spots of t0, t7 and t14 using **TagIdent SWISS-PROT** Identification Database, on the EXPASY server **http://www.expasy.ch/tools/#proteome_TagIdent** (app. M; released Dec. 1995, 360 000 proteins). The homologue proteins in the database of the server are selected according to their MW and pI values, and their function considering multiple standard error of estimate within and in between the marker proteins for each protein spot (app. E, F).

The soluble proteins of the treatments of 2-DE (Grieb, 1992) were identified. The identities of 52 homologue proteins are listed bellow and the possible function of the proteins in this investigation will be discussed.

4.4.1 An example for the calculation procedure of MW of four protein spots on 2-DE

a) Calculation of MW of the marker proteins based on their RF values and the RF value of BPB (RF = b / a).

Mw of Marker	Spot (b)	BPB (a)	RF (b/a)	Calculated value for MW	Residue	Residue²
KD	mm	mm		KD	KD	KD
94	13.0	189.1	0.0687	93.942456	0.057544	0.00331131
67	28.3	189.1	0.1496	67.139901	-0.139901	0.01957229
43	52.8	189.1	0.2792	42.878934	0.121066	0.01465698
30	98.8	189.1	0.5224	30.054551	-0.054551	0.00297581
20,1	152.8	189.1	0.8080	20.078305	0.021695	0.00047067
					Σ Residues²	**0.04098706**
					Standard Error of Estimate	**0.11688607**

B) Calculation of MW of four selected protein spots based on their RF values, the RF value of the BPB, using polynomal regression of the 3rd grade.

Parameters of the polynomal regression of the 3rd grade

a0	126.240701
a1	-543.108643
a2	1135.5212
a3	-1010.28454

Calculation of RF value and MW of four selected protein spots

Protein Spot	Spot (b)	BPB (a)	RF Value	Calculated value for MW
Number	mm	mm		Dalton
8	27.5	189.1	0.1454257	68.29676236
44	39.5	189.1	0.20888419	53.68713517
80	56.0	189.1	0.29613961	40.99395678
136	70.5	189.1	0.37281861	34.87356958

4.4.2 List of Homologue Protein Spots Synthesized During the Induction Phase of Somatic Embryogenesis in Carrot Petiole Culture using Protein Identification Database Swiss-Prot

CBB stained (CBB) ^{14}C-leucin labelled (^{14}C)   CBB stained and ^{14}C-leucin labeled (CBB + ^{14}C)

Spot	Protein	EC Number	t0	t7	t14
2	PHENYLALANINE AMMONIA-LYASE	EC 4.3.1.5		CBB + ^{14}C	CBB + ^{14}C
8	α-GLUCOSIDASE α-AMYLASE FAMILY baker`s yeast P53341	EC 3.2.1.20	^{14}C	CBB + ^{14}C	^{14}C
13	IDENTICAL TO E2 GLYCOPROTEINS				CBB
22	PYRUVATE DECARBOXYLASE ALPHA-CARBOXYLASE PYRUVIC DECARBOXYLASE ALPHA-KETOACID CARBOXYLASE	EC 4.1.1.1		CBB + ^{14}C	CBB + ^{14}C
23	CYTOCHROM D UBIQUINOL OXIDASE SUB UNIT I	EC 1.10.3.-		CBB + ^{14}C	CBB + ^{14}C
24	CYTOCHROM P450	EC 1.14.14.1		CBB	CBB
26	SIMILARITY TO DEHYDROPYRIMIDINASE FAMILY			CBB	CBB
30	β-FRUCHTOFURANOSIDASE / INVERTASE / INVERTASE INV3-DAUCA glycosyl hydrolysis Q39693	EC 3.2.1.26		^{14}C	^{14}C
42	NADH DEHYDROGENASE (UBIQUINONE) UBIQUINONE REDUCTASE TYPE I DEHYDROGENASE present in mitochondria, can be degraded to form EC 1.6.99.3 NADH DEHYDROGENASE CYTOCHROM C REDUCTASE	EC 1.6.5.3 EC 1.6.99.3		^{14}C	^{14}C
43	CYTOCHROM-C OXIDASE	EC 1.9.3.1		CBB + ^{14}C	^{14}C
44	α-AMYLASE	EC 3.2.1.1	CBB	CBB	^{14}C
45	PYRUVATE KINASE	EC 2.7.1.40	CBB	CBB	CBB
46	RUBISCO P25826 Alluaudia procera chloroplast Q42903 Lopezia riesenbachia chloroplast	EC 4.1.1.39		CBB + ^{14}C	CBB + ^{14}C
47	RUBISCO P48690 Castania sativa chloroplast P00874 Zea mays chloroplast	EC 4.1.1.39		CBB + ^{14}C	CBB + ^{14}C
55	RUBISCO	EC 4.1.1.39	^{14}C	^{14}C	^{14}C
71	LONG-CHAIN ACYL-COA DEHYDROGENASE	EC 1.3.99.13		CBB	CBB

Spot	Protein	EC Number	t0	t7	t14
77	CELL DIVISION PROTEIN				
79	ACETYL-COA C- ACETYLTRANSFERASE ACETYL-COA THIOLASE	EC 2.3.1.9			
80	ALCOHOL DEHYDROGENASE	EC 1.1.1.1			
85	PUTATIVE HYDROGENASE EXPRESSION/FORMATION PROTEIN Q58400				
86	TOBACCO A-TYPE CYCLIN cell-cycle regulated transcription of A- & B-type plant cycline genes O04399				
89	LYSOPHOSPHOLIPASE	EC 3.1.1.5			
90	Glycerol-3-PHOSPHATE DEHYDROGENASE Q27634	EC 1.1.1.8			
97	DOMAIN-LEUCINE ZIPPER TRANSCRIPTION FACTOR P54841				
109	ADP-HEPTOSE SYNTHASE Q48046				
124	GLYOXAL OXIDASE (FRAGMENT) Q01845				
128	PHYTOENE SYNTHASE	EC 2.5.1.-			
130	PHOSPHOFRUCTOKINASE	EC 2.7.1.11			
132	ACETALDEHYDE DEHYDROGENASE P97091				
133	PHYTOENE SYNTHASE	EC 2.5.1.-			
134	CARROT DNA BINDING PROTEIN transcriptional regulator DCKUROD Q43428				
136	DNA-BINDING PROTEIN (HOMEOBOX GENES) Q43426 somatic embryos (Komamine)				
137	GERANYL TRANSFERASE FARNESYL-DIPHOSPHATE SYNTHASE terpenoid and sterol biosynthese	EC 2.5.1.10			
138	FRUCTOKINASE	EC 2.7.1.4			
143	PUTATIVE GLUCAN ENDO-1,3- BETA-GLUCOSIDASE GVI PRECURSOR starch and sucrose metabolism	EC 3.2.1.39			

Spot	Protein	EC Number	t0	t7	t14
146	GLUCAN ENDO-1,3-BETA-GLUCOSIDASE PRECURSOR P79062				
148	ORNITHINE CYCLODIAMINASE	EC 4.3.1.12			
151	METHIONINE AMINOPEPTIDASE	EC 3.4.11.18			
154	50 S RIBOSOMAL PROTEIN (Mitochondria) P91353				
160	GUANINE NUCLEOTIDE REGULATORY PROTEIN (fragment)				
162	HYPOTHETICAL PROTEIN (involved in the biosynthesis of polysaccharides)				
186	CYTOCHROM -C OXIDASE	EC 1.9.3.1			
190	HYPOTHETICAL PROTEIN Q57662 related to energy production, cell division, metabolism, transcription, translation and replication				
192	METHYL COENZYM M REDUCTASE GAMMA SUBUNIT terminal step methanogenese, in anaerobic degradation of biomass PYRROLINE-5-CARBOXYLATE REDUCTASE terminal step in prolin biosynthesis	EC 1.8.-.- EC 1.5.1.2			
199	CYTOCHROM -C OXIDASE mitochondrial inner membrane protein P27168	EC 1.9.3.1			
222	PUTATIVE GLUTAMATE RACEMASE	EC 5.1.1.3			
230	PROTEIN TYROSIN PHOSPHATASE (FRAGMENT)				
232	CYTOCHROM B (FRAGMENT)				
237	ATP-BINDING TRANSPORT PROTEIN Q56005				
245	NADH DEHYDROGENASE F (FRAGMENT)	EC 1.6.5.3			
267	RNA-BINDING PROTEIN Q44555 NADH DEHYDROGENASE F (FRAGMENT) Q33305				
276	RNA-BINDING PROTEIN Q44555				

4.4.3 Control Mechanisms During Somatic Embryogenesis

Analyses of the protein population of 2-DE demonstrated differences in the gene expression of carrot petiole explants during different culture periods. Using the global protein analysis information resource search program in a database, we were able to identify homologues of 52 protein spots from the total number of 281 spots synthesized during the entire induction period of carrot petiole explants during t0, t7 and t14. Given the fact that our orientation point was the MW and the pI of the spots on the 2-DE, without sequencing of the 281 spots, some fundamental criteria for the identification of these spots had to be established. One of the first criteria was to search for the proteins in the database matching the molecular weight and pI of the spots from our 2-DE experiment, considering the ± deviation from the multiple standard error of estimate of spot and residue of the nearest marker protein. Another criterion was trying to find homologue spots from carrot 2-DE reported by other researchers. Furthermore, we had to choose the spots that could be relevant to our experiment, considering the biochemical pathways using the on-line service of Boehringer Mannheim and as far as possible trying to establish a connection between different spots. Attention was drawn in the first instance to the spots, which were merely labelled at t7 and t14 or only at t14, as the possible candidates for the proteins, which play a cardinal role in the induction of somatic embryogenesis.

As the first homologue, spot No. 136 can be mentioned, which is only labelled at t7 and t14 but absent at t0. This pattern suggests that the synthesis of the spot can be part of the induction program. The homologue of this spot has been reported by Komamine, registered as carrot DNA-binding protein as a transcriptional regulator, homeobox gene (Trembl: Q43426) and the other homologue (Trembl: O04079) which is reported as being responsible for the changes in the activity and mRNA of cinnamyl alcohol dehydrogenase during tracheary element differentiation in *Zinnia elegans*. Many observers have reported genes that appear a few days after culture as being possibly responsible for the organ

differentiation in cultured explants. In maize it seems that homeotic genes determine the differentiation of cells to leaf or hypocotyls (Strasburger, 1998). Spot No. 134 is homologue to a protein, which is a transcriptional regulator in carrot (Trembl: Q43428) synthesized from the beginning of culture from t0 till t7 and t14. Spot No. 86, however, was newly synthesized for the first time at t7 and appeared at t14 too. This spot was homologoue with the (Trembl: O 04399) protein responsible for the cell cycle regulation of the transcription of plant cyclin genes of tobacco. Spot No. 199 is a homologue (Swiss-Prot: P27168) to carrot cytochrome C oxidase polypeptide II (EC 1.9.3.1), a component of the respiratory chain and an integral membrane protein in the inner mitochondrial membrane, which catalyzes the reduction of oxygen to water, having copper as its co-factor.

Protein spots which were identified as homologue but not merely originating from carrot, can be classified in three groups:

First, the spots, which were labelled at t0, t7 and t14 will be discussed. Spots labelled at t0 indicating a metabolic response by the cell to the application of the auxin 2,4-D. These spots were homologues, for example spot 55, to Rubisco (EC 4.1.1.39), although there were many rubisco homologues which were merely stained with CBB, e.g. spot 134 carrot mRNA transcriptional regulator (Trembl: Q 43428). Spot 154 50s ribosomal protein (Trembl: P91353) originated from mitochondria. Spot 190 hypothetical protein (Trembl: Q57662). Spot 230 fragment of protein is a homologue to tyrosin phosphatase (Trembl: Q16128) and spot 245 to NADH Dehydrogenase (Trembl: Q50183), spot 267 and 276 homologues to RNA-binding proteins (Trembl: Q44555)).

Second, a group of spots whose new synthesis started at t7 and were also synthesized at t14.

Spot 2 phenylalanin ammonia-lyase (PAL, EC 4.3.1.5), an enzyme which is probably located in cytoplasm, a key enzyme of plant metabolism catalyzing the first reaction in the biosynthesis of L-Phenylalanin, a source of a wide variety of natural products based on the phenylpropane skeleton catalytically activates L-Phenylalanin to be converted to trans cinnamate and NH_3, having dihydroalanin (DHA) as its co-factor. This class of enzymes causes the formation or breakage of C-N bounds. The mode of activity of this enzyme in plants regulates either the protein to be synthesized, or phenols like anthocyanines. Being a key enzyme, its mode of action is controlled by different factors, such as light, temperature, ethylene and carbohydrate synthesis of the cell (Richter, 1996).

Spot 22: pyruvate decarboxylase (EC 4.1.1.1) is also called alpha-carboxylase. The reaction catalyzed by this enzyme causes a 2-oxo acid to catalyze to an aldehyde and release CO_2. This class of enzymes causes the formation and breakage of C-C bonds.

Spot 43 and 186 (cytochrom-C oxidase, EC 1.9.3.1) and 232 (cytochrom b) were labelled at t7 and t14. Spot 24 (EC 1.14.14.1), 186 (EC 1.9.3.1), which was merely stained, also identified as homologue for cytochrom. The NADH and $FADH_2$ molecules formed during the first three stages of aerobic respiration each contain a pair of electrons that were gained when NAD^+ and FAD^+ were reduced. The NADH molecules carry their electrons to the inner mitochondrial membrane, where they transfer the electrons to a series of membrane-associated proteins collectively called the electron transport chain. The first of the proteins to receive the electrons is a complex membrane-embedded enzyme called NADH dehydrogenase (Spot 245: EC 1.6.5.3). A carrier called ubiquinone (Spot 23: EC 1.10.3.-) then passes the electrons to a protein-cytochrome complex called the bc_1 complex. This complex, along with others in the chain, operates as a proton pump, driving a proton out across the membrane. Cytochromes are respiratory proteins that contain heme groups, complex carbon rings with many alternating single and double bonds and an iron atom in the center. The electron is then carried by another carrier, *cytochrome c*, to the cytochrome oxidase complex. This complex uses four such electrons to reduce a molecule of oxygen, each oxygen then combines with *2* hydrogen ions to form water: $O_2 + 4 H^+ + 4e^- ==> 2 H_2O$

This series of membrane-associated electron carriers is collectively called the electron transport chain. NADH contributes its electrons to the first protein of the electron transport chain, NADH dehydrogenase (spot 42, 245: EC 1.6.5.3, EC 1.6.99.3 and 267: Q44555, Q33305). $FADH_2$, which is always attached to the inner mitochondrial membrane, feeds its electrons into the electron transport chain later, to ubiquinone (Spot 23: EC 1.10.3.- and 42 EC 1.6.5.3). It is the availability of plentiful electron acceptor (often oxygen) that makes oxidative respiration possible. The electron transport chain used in aerobic respiration is similar to, and may well have evolved from, the chain employed in aerobic photosynthesis (Häder, 1999; Michal, 1999; Neumann, 1995; Richter, 1996).

Rubisco (Spot 46 LC Rubisco precursor, Spot 55 and Spot 47 EC 4.1.1.39) is necessary for carbon fixation and is located in chloroplasts. It catalyzes two reactions: the carboxylation of D-Ribulose 1,5-Biphosphate, the primary event in the photosynthetic CO_2 fixation, as well as the oxidative fragmentation of the pentose substrate in the photorespiration process. Both reactions occur simultaneously and in competition at the same active sight. Substrates of this enzyme are CO_2, H_2O and O_2, having Cu as co-factor producing two metabolites, 3-Phospho-D-Glycerate and 2-Phosphoglycolate (Haeder, 1999; Michal, 1999; Neumann, 1995; Richter, 1996).

Spot133: EC 2.5.1.- and Spot 128: EC 2.5.1.- homologue to phytoen synthase of the carotinoid and isoprenoid biosynthetic pathway, in which 2 Geranyl Diphosphate (see Spot 137: EC 2.5.1.10), an open chain monoterpen, is catalyzed to pyrophosphate and phytoene. Phytoene is located in the plastid and thylakoid membrane. In plants the enzyme complex of phytoene synthase (Spot 133: EC 2.5.1.-) produced first cis-Phytoene, then trans-Phytoen. The membrane associated Phytoene-Desaturase oxidizes phytoene through different steps to all-trans- α- or β-Carotin (Haeder, 1999; Michal, 1999; Richter, 1996).

Spot 136 (Q43426) is a homologue to DNA binding protein homeobox genes. In homeotic genes, the region of homology, usually 180 bp in length, located within the coding sequence of the gene is called homeobox gene. It is a gene that plays a role in determining a tissue´s identity during development (Hartwell, 1999). Spot No. 136 is labelled at t7 and t14, but absent at t0. This pattern suggests that the synthesis of the protein spot can be part of the induction program. The homologue of this spot has been reported by Komamine (1995) registered as carrot mRNA for DNA-binding protein a transcriptional regulator, homeobox genes (Trembl: Q43426) and the other homologue (Trembel: O04079) which is reported to be responsible for the changes in the activity and mRNA of cinnamly alcohol dehydrogenase during tracheary element differentiation in *Zinnia elegans*. There are a few more candidate spots, which presumably are involved in similar developmental events (Haeder, 1999; Michal, 1999; Richter, 1996).

Third group of protein homologues are those spots, which were labelled at t14, meaning a new synthesis of protein at late stages in induction, which probably could play a decisive role in the induction of somatic embryo development.

Spot 44 is a homologue to α-Amylase (EC 3.2.1.1). Spot 143 (EC 3.2.1.39) has been identified as homologue to α-Amylase and Spot 146 (P79062) and 162, suggesting that there is a continuous activity of this enzyme during t0, t7 and t14, but at 14th day of culture the new synthesis of this enzyme can be an indication of the stimulation of transcription and a high demand of the cell for energy to support its increased demand for the metabolic functions of the developing meristems. Spot 138: EC 2.7.1.4, fructokinase is precursor of Spot 130: EC 2.7.1.11, phosphofructokinase, Spot 45: EC 2.7.1.40, is analog to Pyruvate Kinase.

On the basis of homologue spots identified in these experiments, it can be concluded that during the induction stage of carrot petiole culture and after application of the auxin 2,4-D, there are changes leading to an increase in energy production, cell division and many other cell metabolic activities, transcription, translation and replication, leading to an increase in secondary metabolic compounds such as anthocyanin and a higher

synthesis of phytoen as carotinoids. An increase in anaerobic glycolysis due to low oxygen partial pressure of the culture, leads to changes in energy household of the cultured cell, an increase in synthesis or break down of alcohol or photorespiration in response to the increased metabolic demand of the induced cells and changes in carbohydrates household, such as starch and sugar synthesis and degradation.

Carbohydrate metabolisms plays an important role in the induction of somatic embryogenesis. Carbohydrates, particularly glucose are essential for the realization of somatic embryogenesis in autotroph cultures. Glucose in itself has rather a regulatory effect on the induction and realization of somatic embryogenesis (Pleschka, 1995). Substances with low molecular weight can also affect somatic embryogenesis due to compartmentalization of the cell.

5 DISCUSSION

5.1 Histology of Somatic Embryogenesis

The first step in this study was to examine the histomorphological changes in the cultivated petiole explants of *Daucus carota*.

The histological observation of the petiole explants showed that different parenchymatic cells e.g. cells with different positions in the tissue, show different competence, which is characterized for a special morphogenic process. Moreover, this morphological processes occurs at different intervals of time. First, parenchematic cells around the vascular bundles are induced to rhizogenesis, and later, sub-epidermal cells induced to somatic embryogenesis. An aggregate of parenchematic cells in the vicinity of the vascular bundles is transformed to cytoplasm-rich cells 24–48 hours after culture in an auxin-containing medium. The cell aggregates can be distinguished microscopically from the adjacent cells in the petiole transection (Schäfer, 1985). On the second day of the culture, these cells start to divide, on the fourth day of the culture, meristemic zones can be observed with the growth direction toward the epidermis. These meristemic zones of petiole explants can give rise to adventive roots after transfer to an auxin-free medium or a medium with low concentration of auxin. The histological observations indicate that the rhizogenic competent cells are originally vacuolized, appearing in the vicinity of vascular bundles. According to our observation, cambial cells do not participate in this process, it is, however, not clear if the cells originate from phloem or xylem parenchyma. There are reports about pericycles as the origin of the adventitious roots; others (Esau 1969) reported the primary phloem as the space between the phloem strands and intervascular parenchyma, which can be the origin of the adventive roots. Zee et al., (1979) observed this phenomenon more towards phloem rather than xylem. Morphogenic structures of the original rhizogenic zones are different from those of adventive root primordias.

Application of 2,4-D leads to the emergence of competence in the rhizogenic meristems growing towards the periphery. At this stage there is no connection between the appearing meristems and the vascular bundles. The growth of these meristems leads to the rupture of the neighboring tissue. Guiderdoni and Demarly (1988) observed the same trend in sugar cane. They reported a meristemic active zone around the vascular bundles. These meristems ruptured the tissue and the epidermis so that they came in direct contact with the nutrient medium. Afterwards, all the meristems formed a primordium, which later took the function of root meristems. De Vries et al. (1988) are of the opinion that 15 days after the culture of hypocotyle explants of carrot the emerging cells or callus are not embryogenic. They are still connected to the explants, which upon transfer to a hormone-free medium can give rise to root. Some reports claim on the content of the reduced nitrogen, which determines the nature of the meristems (Halperin 1966; Jones 1974). Jones (1974) and Konar et al, (19072a) propose two possible ways for the formation of roots in cell suspension culture, the first from PEMs or meristemic zones on the outer periphery of the callus, the second possible way is the formation of the root from the meristem cells located at the very center of callus, which resembles the development of the secondary roots. Kato and Takeuchi (1963) reported the development of embryos and the formation of root and shoot as non-polar organs from carrot root callus. The so called neomorphs have been reported by many observers using different media and culture conditions (Krikorian and Kann, 1981). These structures do not always show a clear structural variation from embryos. Embryos show a distinct polarity accompanied by a defined root pole, neomorphs are more or less callus-like structures and can even possess a smooth surface. This phenomenon has been observed by Krikorian and Kann (1981) in *Hemerocallis* suspension culture, Trolinder and Goodin (1988) using *Gossypium hirsutum* explants, and Konar et al, (1972a) in *Atropa belladona* suspension culture. There were no signs of root formation when carrot petiole explants were cultured 14 days in a modified B5+ with 0.5 ppm 2,4-D (Grieb et al., 1992). Trolinder and Goodin (1988) using a low concentration of the auxin 2,4-D or short exposition of explants to auxin resulted in the formation of roots. This could lead to the conclusion that the development of adventive roots is dependent on the concentration, duration of contact and the type of auxin used (Grieb et al., 1992; Kamada and Harada, 1979a; Trolinder and Goodin 1988). Li and Neumann (1984) used the unstable auxin IAA and the stable auxin 2,4-D to observe the transformation of the vacuolized parenchematic

cells located around the vascular bundle into cytoplasm rich rhizogenic competent cells. In case of unstable IAA, the parenchematic cells could differentiate to adventive roots. In a 2,4-D treatment, however, parenchaymatic cells could not differentiate to form advantive roots. Neumann (l995) is of the opinion that for the differentiation of the cells a so-called quiescent period is needed. This quiescent period is not afforded when the cells are subjected to an intensive and permanent cell division, which is the case when auxin 2,4-D is used (Linser and Neumann, 1968; Fellenberg, 1978). When IAA is supplemented to the culture, it degrades in 4-6 days and the cells have the so-called, quiescent period, and 14 days after the culture root formation can be observed. Kinetin induced high cell division in the IAA system, and the formation of meristems only around the vascular bundles was observed without any root formation (Neumann, 1972; Schäfer, 1985). In the same $B5^{+}$ petiole culture (0.5 ppm 2,4-D) 10 days after, vacuolized cells, which tended to be nearer the epidermis than the vascular bundles were transformed to cytoplasm rich cells. After 14 days these cells were scattered along the long axis of the 1 cm long petiole explants (Grieb et al., 1992; Zee and Wu, 1979; Schäfer, 1985; Diettrich et al., 1986). This phenomenon is observed in other plant species too like clover (Cui 1986) and coriander (Schäfer, 1985; Zee, 1981). It seems there is a varying induction in time and subsequently varying differentiation of cells in different parts of the tissue, so that by using the auxin 2,4-D, first the vacuolized cells around the vascular bundle differentiate to rhizogenic meristems, and secondly the vacuolized sub-epidermal cells differentiate to embryogenic meristem. This trend has been observed in other *Daucus spp* (Le, 1996) and other members of umbeliferae e.g. parsley (*Peteroselinum crispum*) and dill (*Anethum graveolens*, Schäfer 1985). According to Grieb et al., (1997) at the beginning of this process cells near the glandular channels differentiate into cytoplasm-rich cells, later on other cells which are located between the glandular channel and the epidermis transform to cytoplasm-rich cells. The vacuolized parenchematic cells are smaller in size as compared to the cells of ground cambium tissue. The cytoplasm-rich cells undergo cell division resulting an intensive protein synthesis (Nomura and Komamine, 1986b; Grieb et al., 1992) resulting in a polarization of embryogenic competent cells (Handro et al., 1973, Halperin and Jensen, 1967). Different morphogenic reactions according to Grieb et al., 1992, are related to position effects within the explants or the location of the cell in the tissue. Steward et al, 1965, argue that a prerequisite for the induction of somatic embryogenesis is the isolation of embryogenic

cells from their neighboring cells. Sussex 1983 proposed that a minimum specific numbers of cells is required in the culture, so that they become able to respond to an induction signal (Reinert, 1970; Wetherell, 1984; Nomura and Komamine, 1985; Halperin, 1966). Embryogenic cells undergo many cell divisions and form embryogenic meristems or PEMs. These structures can be observed microscopically using a cytoplasm dyeing agent. Embryogenic and rhizogenic meristems rupture the epidermis, detach themselves from the rest of the tissue, and go over to the nutrient medium (Halperein and Jansen, 1967; Torrey and Reinert, 1961; Grieb et al., 1992). 14-16 days after the culture in an auxin-containing medium, induction of somatic cells is complete (Grieb et al., 1992). Petiole culture using the stable auxin 2,4-D leades to indirect embryogenesis (Grieb, 1992) using IAA, however, results in direct embryogenesis (Schäfer, 1985). It seems that the type of embryo development is related to the kind, concentration, and duration of culture in auxin containing medium (Grieb, 1992). Grieb (1992) reported the existence of different cell types in embryogenic clusters or PEMs. According to Grieb (1992) cells on the periphery of embryogenic PEMs / clumps undergo an embryogenic development. The cells in the center of PEMs / clumps do not undergo the process of embryogenic competence. McWilliam et al. (1976) proposed that the cells which have entered embryogenic development inhibit the neighboring cells, through which these cells lose their starch content, absorb water, become enlarged, and become apparent as microscopic visible vacuoalized cells, as if the cells located in the center of meristem serve as an energy source for the cells on the periphery, which have attained the embryogenic competence.

LoSchiavo et al. (1989), using *Daucus carota* petiole explants in B5+ medium, have suggested a hypothetical schema for somatic embryogenesis at cellular level. According to this schema, the very first prerequisite for somatic embryogenesis is the embryogenic potential of the cell. This prerequisite, according to Steward et al. (1970), is nothing but the totipotency of the cell and genotype (Li and Neumann, 1985; Brown and Atanassov, 1985). Another parameter is the competence of the cell by itself, or in other words the ability to react according to the inducing stimulus. Furthermore this competence depends on the position and type of the cell in the plant tissue (Grieb et al., 1992). Developmental stages of the mother plant and the explants type e.g. leaf, petiole or root can also be a determining factor. According to Grieb et al. (1992) the hypocotyl cells of *Daucus carota* possess a greater embryogenic capacity than the root cells. The induction stage of somatic embryo-

genesis in carrot petiole explants starts with their culture in an auxin-containing medium, accompanied probably by DNA methylation and dedifferentiation (LoSchiavo et al., 1989). From the transformation stage, the cells progress into the determination stage induced by a stimulus indicated by cytoplasm accumulation in the cells, which are microscopically visible. The transformation stage is followed by an initiation stage in which the first cell division occurs. This stage is the birth-place of embryogenic meristems. There are some reports about the necessity of carbohydrates and other energy sources for the induction of somatic embryogenesis in carrot petiole cultures (Grieb et al., 1992). Neumann, de Garcia (1974) and Pleschka (1995) suggested that realization of embryogenesis necessarily needs an exogenous source of carbohydrate. The subculture of petiole explants in an auxin-free medium leads to the realization of somatic embryogenesis, which goes through globular, heart, torpedo stages, and finally ripe embryos form.

5.2 Protein Synthesis During Induction of Somatic Embryogenesis

The induction of embryogenic competence in carrot petiole culture is characterized by cytoplasm and organelle protein synthesis (Grieb, et al., 1997). Inhibition of 70S ribosomal protein synthesis in cell organelles using chloramphenicol (CAP) leads to inhibition of the cell translation process, resulting in a slowing down of the log phase period and a prolongation of the stationery phase (Grieb, 1992). Bahr (1988), using carrot root explants and tobacco petiole explants, reported 5 days delay in the exponential developmental stage using 6 ppm CAP.

CAP caused an increase in abnormal embryonic structures and the number of embryos in the culture (Grieb, 1992). Nuti Ronchi et al. (1984) reported a similar trend when amino acids, specially prolin, were supplemented to the auxin-containing culture. It has been reasoned that through CAP inhibition amino acids become more readily available (Grieb, 1992), followed by an increase in the mitotic activity of the cell (Nuti Ronchi et al., 1984).

Application of the translation inhibitor CAP causes a reduction in cell division activity (Bahr, 1988; Dadkhahi, 1978; Dadkhahi et al., 1982) and determines if the cells undergo the division or differentiation process (Grieb, 1992). Sethi et al. (1990), using transcription inhibitors cordycepin and actinomycin D, observed a similar trend leading to shoot differentiation in tobacco and Datura cell cultures. Organel proteins can have a regulatory function and control growth and development (Grieb, 1992). When these regulatory proteins are lacking, the cell cycle loops, causing a continuous cell division and increasing possibly embryonic material. The regulatory effect of the differentiation status depends on the embryonic cell and pre-embryonic stage (Grieb et al., 1997). Probably after sub-culturing the plant material in an auxin-free medium, the inhibition of some specific proteins of 70S organelles is terminated so that the embryonal development can be realized (Grieb et al., 1997). It is also reported that the embryogenic competence is not directly related to the division rate of embryonic material. What is principally important is that in the first instance the cytoplasm protein synthesis is vital for cell metabolism. Cytoplasm protein synthesis alone can bring the carrot embryogenic cell to the early torpedo stage, and probably organelle protein synthesis is not essential up to this stage, however, essential before the cotoledonary stage (Grieb, 1992). The transition of globular to heart and torpedo is an embryonic development process, the torpedo stage by itself is a control point, acting in the way zygotic embryo behaves. Giuliano et al. (1981) suggested that there are two ways of genetically controlling embryogenesis: one, up to globular form, and two, heart and torpedo form. He argues this hypothesis based on the result of an experiment in which the synchron cultures could only be established consisting of globular stage (Cyr et al., 1987; Scheibner et al., 1989). According to Cyr et al. (1987) the protein content of PEMs and globular are the same. The transition of globular to heart stage is coupled with the polarization of embryo and the differentiation of procambium; the transition of torpedo to mature embryo is coupled with intensive growth, increase in cell size and the formation of vascular elements (Cyr et al., 1987; Sciavone and Cooke, 1985). The protein content of cells at the torpedo stage is two times more than that of the globular stage, and the pattern of soluble proteins is associated with an increase in starch content of the cells (Zee and Wu, 1979; Cyr et al., 1987). Induction of somatic embryogenesis can be described using the regulation model of gene activation, proposed by Davidson and Britten (1979). They proposed that each structural gene possesses several receptor genes, so that a

structural gene can be regulated by different effectors; on the one hand, one receptor gene decides on the regulation of different structural genes so that through a single signal different gene batteries can be activated (Kleinig and Sitte, 1984), on the other hand, a single receptor gene decides on the transcription of many structure genes so that through a single signal different gene batteries can be activated. Hierarchical activation of protein synthesis has been reported in induced carrot callus root phloem explants (Gartenbach-Scharrer, 1988; Gartenbach-Scharre et al., 1990). Cremer et al. (1991) observed activation and termination of gene activity at the mRNA level in *Sinapsis alba* leaves. The physiological and differentiation status of petiole explants can be correlated, quantitatively and qualitatively, to the synthesized protein spots of a particular developmental stage. Whether the newly synthesized protein can be correlated to the differentiation process, i.e. correlated to embryogenesis or rhizogenesis, is not quite clear. McGee et al. (1989), compared the pattern of protein synthesis of an embryogenic line with a non-embryogenic line. He found two protein spots, which were present in the embryogenic line but absent in the non-embryogenic line, and postulated that these two spots play a role in carrot somatic embryogenesis. Ramgopal (1989) and many other researchers used this model to describe somatic embryogenesis at the molecular level. It is not clear if a single protein or a group of proteins plays a key role in the induction of somatic embryogenesis. Choi and Sung (1984) suggested that in an interaction with other non-embryogenic proteins the embryogenic proteins could exert an embryogenic function, leading to the induction of somatic embryogenesis. The patter of protein synthesis is not merely a qualitative parameter, meaning that not only the presence or absence of a protein in a specific period of a developmental stage is the cause of a specific biochemical change leading to a specific behavior of the cell, but it is also a quantitative parameter, meaning the intensity of the protein synthesis can also cause specific biochemical changes in growing cells and meristems, resulting in growth and developmental changes and behavior. Quantitative changes in protein pattern can trigger different differentiation modes (Grieb et al., 1992). This phenomenon has been observed in the formation of homeobox gene proteins in *Xenopus laevis*, in which a protein and its concentration triggered a specific differentiation mode. This specific differentiation mode leads to the formation of the small finger of the frog (Robertis et al., 1990). It is not very rational, however, to presume that a specific protein must be synthesized for the formation of each finger of the frog. It is almost

certain that the induction of somatic embryogenesis in petiole explants of carrot is regulated by auxin. A cell is induced when the indigenous balance of auxin and cytokinin in the cell is changed (Li et al., 1985; Schaefer et al., 1988; de Vries et al., 1988; Wilde et al., 1988). There are other reports arguing that the nutritional status of the cell, especially nitrogen nutrition, causes an increase in indigenous auxin in the cell, and other reports stating that the sugar metabolism of the cultured cells, which is the energy source for the metabolic activities of the cell, triggers the induction of embryogenesis and obviously has a regulatory function (Pleschka, 1995).

5.2.1 Protein Pattern of the Cultured Petioles

The program of histological and cytological differentiation is supposed to be accompanied by a program of molecular differentiation (Grieb et al., 1997). To describe the physiological status of the entire explants, which permits the induction of some cells in petiole tissue to perform somatic embryogenesis, soluble protein patterns at different stages during the induction phase were determined. These investigations were conducted at t0, t7 and t14, which characterized the protein complement and the formation of rhizogenic centers in the vicinity of vascular bundles and embryogenic centers underneath the epidermis. The evaluation of the different developmental stages provided a chance to illustrate proteins characteristic for a specific embryogenic status of the explants. The evaluation of the electropherograms and the obtained data demonstrate a sharp increase in total protein and in the number of single proteins, which occurs during the first seven days of culture. In the first labelling period (t0), 91 proteins could be detected, a sharp increase in the number of protein spots was observed at the second labelling period (t7). At this stage, 250 protein spots could be detected, 15 spots were common with the first labelling period and 156 spots were present as additional spots. The specific radioactivity of the total soluble protein at t7 increased after the application of ^{14}C-leucine, as compared to t0, similarly, the protein concentration increased. This indicates that at t7 the rate of protein synthesis is higher as compared to the previous period. No more than slight changes can be seen in the protein pattern at the third labelling period (tl4), with 17 newly synthesized protein spots from the

whole of the 257 detected, and 12 absent as compared to the former period (t7). The number of identical protein spots at both stages (t7 and t14) of the cultural cycle counts to 143. The protein concentration remains nearly at the equal level as at t7. On the other hand, the specific radioactivity of soluble protein was significantly reduced as compared to the earlier stage. Apparently, at this time the overall protein synthesis activity is reduced, but some metabolically relatively stable proteins synthesized during a previous period accumulated. During the first period of culture, a new protein-synthesizing program is apparently initiated in the cultured petioles, which merely undergoes some slight modifications afterward from t7 to t14. Endogenous concentrations of IAA in cultured carrot petiole explants show a similar pattern to the pattern of protein synthesis observed during this experiment (Grieb et al., 1997). Endogenous IAA concentrations in petiole explants were determined at t0, t7, t10 and t12 (Grieb et al., 1997; Imani, 1999). Results on the distribution of endogenous IAA within the explants, (obtained by using petiole explants of transgenic carrot plantlets at t0 and t5, containing the auxin sensitive MAS- promoter coupled to the GUS reporter gene indicating its activity with X-Gluc as a substrate to give a blue color), indicated that the presence of IAA is more or less evenly distributed throughout the petiole cross-section at t0. At t5, however, the X-Gluc response is mostly restricted to the cells around the vascular bundles destined to become root primordia. Some IAA also seems to occur in some areas of the section near the epidermis, where the development of somatic embryos can be observed after 10-12 days of culture. The remaining areas of the petiole, the originally occurring IAA is either inactivated by breakdown (or conjugation or both), or translocated to these morphogenic centers (Grieb et al., 1997).

If a protein spot is only stained, in that case its synthesis should have occurred prior to the labelling period and this should have ceased at the time of investigation. On the other hand, protein spots only labelled seem to be distinctively synthesized at the time of ^{14}C-leucine application. Proteins labelled and stained could have been synthesized prior to the ^{14}C-leucine application and the synthesis sustained during the three-hour labelling period.

A detailed description of all data obtained in the study is given in appendix E. For interpreting and grouping the proteins detected according to the occurrence at different stages of the growth phase and the stainability with CBB and/or labelling with ^{14}C-leucine,

the protein group classification of Grieb (1992) and McGee et al. (1989) is used for orientation.

Group 1: Proteins present only in the petiole explants during the first labelling period (4 proteins = 1.4 %)

Group 2: Proteins present at t0 and t7 (4 proteins = 1,4 %)

Group 3: Proteins present at all stages investigated either stained with CBB and / or labelled (28 proteins = 9,9 %)

Group 4: Proteins only labelled during the first 5 hours after inoculation, which are missing at later stages (9 proteins = 3.2%)

Group 5: Proteins labelled during the first 5 hours after inoculation, which however, are present throughout the experiment (44 proteins = 15.6 %)

Group 6: Proteins detectable only at t7 and t14 (166 proteins = 58.9 %)

6.1: Proteins stained at t7 and t14 (29 proteins = 10.3 %)

6.2: Proteins labelled and stained at t7 (78 proteins = 27.7%)

6.3: Proteins newly synthesized at t7 (59 proteins = 20.9 %)

Group 7: Proteins present only at t7 (8 proteins = 2.8 %)

Group 8: Proteins present only at t14 (17 proteins = 6 %)

Group 9: Proteins present at t0 and t14 (2 proteins = 0.7 %)

The changes in the protein pattern were associated with the various stages in the cultural cycle investigated (Grieb et al., 1997). After 5 hours of culture, proteins of the groups 1 - 5 and of group 9 were detected, at t7 the proteins of groups 2,3 and 5 – 7, and at t14 proteins of groups 3, 5, 6, 8, and 9 appeared at the same time. Apparently, these changes in the protein complement are supposed to be part of a program of molecular differentiation, as the histological investigations illustrate. Apparently, alteration in differentiation of the

petiole explants during the cultural cycle of 14 days consists of a sequential, ordered activation and termination of genetic sub-programs. Group 1 is the termination of the first sub-program of the petiole, being representative for the original tissue. At the mean time, a sub-program linked with explantsation and the inoculation procedure is activated (group 4) and terminated before t7. A different sub-program, which is already active in the petiole, on the other hand, continues during culture (group 3). Proteins of this sub-program are supposed to have housekeeping function for the carrot petiole. The proteins of group 2 are already synthesized in the petioles at t0, but at t7 of culture their concentration is reduced. They were only labelled and at t14 they are no longer detectable. Furthermore, a sub-program represented by 44 proteins starts at t0 (group 5) and continues throughout the culture period. It seems that, these proteins are typical of cultured petiole explants. Formation of the rhizogenic centers in the vicinity of the vascular bundles (t7), could be an indication that the proteins of some other sub-programs are synthesized (groups 6 and 7). Still, a large group of these proteins (166) is also present at t14 (group 6). The 8 proteins of group 7 could only be detected at t7 and should be specific for this physiological stage. This is an indication that inside the differentiation program in the cultured petiole, sub-programs are constantly switched on and off at distinct stages in the cultural period. It is important to know, which function each individual sub-program accomplishes, for the continuous progress of the differentiation program of the petiole all-together. Even if the extracts used for 2 D-electrophoresis contain proteins from different cells of the petiole explants, as historadioautograms specify e.g. at t7 most of the ^{14}C-leucin is assimilated and concentrated adjacent to vascular bundles, and to a lesser degree in the epidermis area. So the largest part of the proteins labelled are supposed to have been synthesized in these morphogenic areas of the cultured explants. At t14 only rather small changes in the protein pattern can be observed as compared to t7, coinciding with the appearance of the cytoplasm-rich, sub-epidermal cells with latent embryogenic potential and embryogenic centers. At t14, proteins of group 8 not present at t7 are merely stainable and should have been synthesized between 7 and 14 days of the cultural period (spot No. 13, 74, 76, 97, 106, 142, 144, 145, 146, appendix E). Remaining spots, however, are traceable by the stain and label both, and therefore the synthesis of these should have happened between t7 and t14 and should continue at t14 (4 proteins: No. 85, 87, 88, 130, appendix E). Four proteins of this group are merely labelled at this period (No. 117, 118, 119, 192, appendix E). The

interesting question is, which protein or group of proteins at t14 are explicitly related to somatic embryogenesis.

5.2.2 Elucidation of Inductive and Control Mechanisms During Somatic Embryogenesis

One important aspect of tissue culture is the production of plant secondary products, one of these products is anthocyanine. Cultivated carrot root explants are capable of producing high amounts of anthocyanine (Neumann, 1996). Iron increases anthocyanin production and molybdenum reduces production of this secondary product. Production of anthocyanin is related to the interaction between nitrogen and carbohydrate metabolism of the cell. Iron increases sugar uptake from the medium resulting in carbohydrate accumulation in the cell, molybdenum as a cofactor of the enzyme nitrate reductase increases the activity of this enzyme, resulting in higher amino acid synthesis and a higher sugar demand (Neumann, 1995). The influence of ethylene on anthocyanin, anthocyanidin and carotinoid accumulation in *Vaccinium pahalae* suspension culture showed that an exogenous application of ethanol significantly reduced growth and secondary metabolites production, whereas incorporation of 5.0-10.0 mg l^{-1} $NiCl_2$ effectively reduced ethylene accumulation and improved product accumulation (Shibli et al., 1997). The effect of light and light quality on *Vitis vinifera* L. embryogenic culture suggests that phytochrome appeared to be inductive, although this effect was adversely influenced by the blue absorbing photoreceptor e.g., anthocyanin

During oxygen limitation in higher plants, energy metabolism switches from respiration to fermentation. As part of this anaerobic response, the expression of genes encoding pyruvate decarboxylase (PDC) and alcoholdehydrogenase (ADH) is strongly induced followed by changes in post translational regulation (Bucher, 1994). Bucher et al. reported ethanolic fermentation in transgenic tobacco expressing *Zymonoonas mobilis* pyruvate decarobxylase, stating that aerobic fermentation takes place when the respiratory system is inhibited, without increasing ADH transcriptional level. Expression of enzyme ADH, ethanol and acetaldehyd production is practically demonstrated in all tissue culture

experiments (Neumann, 1995; Yatazawa and Furhashi, 1982). GC experiments showed that the retention period of peak of a sample of carrot cell suspension culture liquid solution during induction phase (0.5 ppm 2,4-D) corresponded with retention period of peak of ethanol. Enzyme analysis of ADH in carrot cell suspension culture also indicating presence of alcohol in culture liquid solution (unpublished results of our institute). Now the question arises, if and up to what extent in culture liquid solution dissolved alcohol influences the permeability of the cell wall and cell membrane and their selectivity. NO_3^- and NH_4^+ nutrition influence fermentation, so nitrate may serve as an alternative electron acceptor in anaerobic plant metabolism and the nitrate-supported energy charge may be due to an accelerated glycolytic flux resulting from a more effective NADH reoxidation capacity by nitrate reduction plus fermentation than by fermentation alone (Muller et al., 1994).

Higher plants need high concentration of oxygen for their metabolism and growth, however, some plants like rice have developed a mechanism to encounter low assimilation of oxygen. In this case, saccharose as the product of starch mobilization produces fructose-6-phosphate through starch depletion, and alcohol in the form of ethanol will be formed. During the low partial pressure of oxygen (anoxia) practically ATP synthesis via oxidative phosphorylation is drastically slowed down but important processes of dissimilation like oxidative decarboxylation and the citrus cycle still function. For the production of ethanol from pyruvate as the end product of glycolysis the enzyme pyruvat Decarboxylase (spot22: EC 4.1.1.1) catalyzes pyruvate to acetaldehyde (spot 132: P97091) and CO_2. Activity of this enzyme depends on the concentration of partial oxygen pressure and rises with the intensity of anoxia. In maize its activity becomes 5-9 times more than the control. In the next step, alcohol dehydrogenase (spot 80: EC 1.1.1.1) catalyzes the NAD bound hydrogen, which was formed during glycolysis leading to ethanol formation and reoxidation of specific metabolites causing glycolysis remain active, despite partial pressure of oxygen (Bucher et al., 1994; Richter, 1996; Haeder, 1999; Michal, 1999).
Rubisco enzyme activity of carrot secondary phloem explants showed a continuous increase. ^{14}C labelled Rubisco already showed a new synthesis of this enzyme a few hours after inoculation in an auxin-containing medium. By transition of explants from log phase (5-7 days) of cell division to stationary phase as autotrophic nutritional mode, the activity

of this enzyme increased drastically (Kumar et al., 1982). Rival et al. (1997) mentioned a 38.8 % increase in Rubisco activity in maize somatic embryos.

α-Amylase hydrolyses starch, a polymer reserve substance, which affects osmotic pressure of the cell and related poly- and oligosaccharides producing eventually glucose. This enzyme is located in the starch sucrose pathway. Changes in α-Amylase activity during plant regeneration from rice calli showed an increase in α-Amylase activity in regenerative calli after transfer to the regeneration medium, while the calli transferred to callus maintenance medium did not increase and maintained a stable state (Abe et al., 1996;Sreedhar and Brewley, 1998). As it was discussed before, there are many reports concerning the accumulation of starch in tissue culture cells and in different meristem cells (Grieb et al., 1992, Matsumoto, 1996, Cailloux, 1996, Gram, 1996, Canhoto, 1996, Pedroso, 1995 and still unpublished new results from our institute). There are also reports stating that the mineral nutrition, especially nitrogen- and sulfur-containing compounds, enhances the synthesis of storage reserves and the accumulation of starch in developing somatic embryos of alfaalfa (*Medicago sativa*). Some other reports suggest that a proper combination of carbohydrate and osmoticum e.g. polyethylene glycol enhances somatic embryo maturation in loblolly pine (*Pinus taeda* L.) and even improves the morphology of zygotic embryos (Li et al., 1998). Observations suggest that phytohormones possess a gene activating potential and have a stimulatory effect on the activity of the enzyme. The most prominent example is the mobilization of reserve carbohydrates in the aleuron layer of seeds through GA hormone signals leading to embryo mobilization of a homologue to Spot 137 (EC 2.5.1.10) as a precursor of GA and ABA synthesis (Richter, 1996). Carman et al., (1996) reported that concentrations of carbohydrates and sugars, like maltose and sucrose, in the endosperm of *Triticum aestivum* L. during early embryony increased to high levels, which is evidence that stored fructans and amylopectines in the endosperm are hydrolyzed and used as nutrients by the growing embryo. Lou et al., (1995) reported the beneficial effect of sugar as a carbohydrate source and affector of osmotic potential and its concentration in initiation media for inducing somatic embryogenesis in cucumber (*Cucumis sativus* L.). Carbohydrates seem to be a critical factor. Embryogenic efficiency and embryo development are promoted by high carbohydrate concentration (Loiseau et al., 1995). Although petiole cells are incubated in an auxin-containing medium, it is not probable that the new synthesis of α-Amylase at this stage is signaled by auxin, although

presence of some forms of α-Amylase has been observed during all periods in these series of experiments. Starch is a mixture of amylose and amlyopectin. Amyloplast and chloroplast are the location of synthesis of starch in plant cells. So fructose 6-P and glucose 6-P produced during photosynthesis will be converted to glucose 1-P and subsequently to ADP-D-glucose, which is acting on primary sugars and leads to starch synthesis. High concentrations of saccharose in the cytosol lead to a decrease in Pi- and an increase in 3-phosphoglycerate concentration, resulting in the coordination of both photosynthetic products, and ultimately, a higher rate of starch and sucrose synthesis. The activity of starch synthesis later leads to a linear production of amylose, which branches later by a glucan branching enzyme to amylopectin. The degradation of starch can be realized through three classes of enzymes, exo-enzymes like β-Amylase, endo-enzyme like α-Amylase, or degradation by phosphorylation. Different enzymes degrade starch, Amylase and amylopectin, leading to the production of different end products. Once through hydrolyzes of polysaccharide glucose bound by specific glycosidase like α- and β-Amylase, leading to the production of disaccharide maltose, which later, through catalytic activity of maltase, produces free glucose, or through phosphorylase which catalyses starch G-1-phosphate to high-energy monosaccharide. In this process, in chloroplast localized phosphoglucomutase converts G-1-phosphate to G-6-phosphate. Glucosephosphat-Isomerase catalyses G-6-phosphate to F-1-phosphate, which later through ATP dependent phosphofructokinase will be converted to Fru-1,6-P2. Cleavage of this product by fructosebiphosphat-Aldolase delivers 2 molecules of a triosephosphate, which can readily be exported from the chloroplasts (Haeder, 1999; Lehninger, 1994;Michal, 1999;Neumann, 1995;Richter, 1996;Strasburger, 1998). Regulation of glycolytic metabolism in fresh-cut carrots under low oxygen atmosphere suggests that phosphofructokinase may be involved in the regulation of glycolysis under low oxygen atmosphere (KatoNoguchi, 1996), but differential transcript levels of genes associated with glycolysis and alcohol fermentation in rice plants (*Oryza sativa L.*) suggest that the mRNA levels of genes engaged in glycolysis and alcohol fermentation may be regulated differently under submerged stress (Umeda, 1994). The activities of sucrose metabolism enzymes in glycerol-grown suspension cultures of sweet orange (*Citrus sinensis L Osbeck*) show high activities of sucrose phosphat synthase, sucrose synthase and invertase (Spot 30: EC 3.2.1.26), and an appreciable accumulation of sucrose, reducing sugars and starch in glycerol-grown tissues (Vu et al., 1995).

5.3 Role of Nitrogen In Somatic Embryogenesis

5.3.1 Effect of the Nitrogen Source on Medium pH

The uptake of nitrate ions by cultured plant cells leads to the extrusion of OH^- ions into the substrate which thus drifts towards alkalinity, while NH_4^- uptake results in the excretion of H^+ ions causing the substrate to become more acidic (Neumann, 1995). The final pH of media containing both $N0_3^-$ and NH_4^- depends on the relative proportion of each kind of ion. The presence of both ions provides a partial buffering mechanism for culture media that persists until the concentration of either becomes depleted. The intake of both forms of nitrogen by plants is adversely affected by the pH changes that the ions individually induce: ammonium ion uptake is less efficient in acid solutions, while nitrate ion uptake is lowered when the solution tends towards alkalinity (Mengel 1991). Effective nitrogen uptake *in vitro* can therefore depend on a balance between both nitrate and ammonium ions. The nitrate ion is not readily absorbed into plant cells from solutions which have a pH greater than 7 (Martin and Rose, 1976). Whereas it is usually satisfactory to add extra ammonium to a medium in the form of NH_4Cl, the use of an equivalent amount of $(NH_4)_2S0_4$ can lead to a marked decrease in the pH of the medium during the culture period. This is probably because the SO_4^{2-} ion is less well absorbed than Cl^-. Plants grown on $N0_3^-$ produce organic acids as a means of neutralizing the excess OH that results from $N0_3^-$ assimilation. Smaller amounts of organic acids are produced when NH_4^- is the nitrogen source, so that NO_3^- grown plants usually have a higher ion content and internal osmolarity than NH_4^- grown plants. In media lacking nitrate, glutamine was superior to other amino acids added singly, and ammonium could serve as the sole nitrogen source if the proper pH was maintained (Dougall and Verma, 1978).

The role of nitrogen is connected with the chemical changes in which that specific nitrogen form exposes to the culture and its effect on the pH of the culture medium. In diammonium sulfate treatments, the pH of the culture is reduced and because of the release of OH^- in KNO_3 treatments the pH of the medium increases and casein hydrolysate as nitrogen source acts as a buffer. The buffering capacity of the diammonium sulfate and potassium nitrate is within a range of 2. Casein hydrolysate, showed only a

narrow difference between its pH and pK values. It is indeed a suitable buffer and it stabilizes the pH of the culture significantly. A mixture of three different nitrogen forms in the solution had a pH capacity being between the organic and non-organic nitrogen containing solution. So the solution makes use of different characteristics of each nitrogen form. Diammonium sulfate reduces the pH of the solution, potassium nitrate exerts an opposite effect by increasing pH, and finally casein hydrolysate acts as a buffer to stabilize the pH of the system. Experiments with petiole culture ($B5^-$) under different pH conditions showed that in the induction phase the pH of the solution had a maximum fluctuation of one pH range 12 days after the culture. In general the pH tended to reach a value around 6. After subculture in an auxin-free medium, the pH value decreased for the next two weeks. This can be a result of the release of H^+ (protons) in exchange for readily available and absorbable cations like Ca and K. After 14 days, however, the pH of the culture solution rose so that 30 days after sub-culture in an auxin-free medium the pH of the nutrient solution tended to again reach 6 on the pH scale. Considering the realization of somatic embryogenesis on the base of the occurrence time and the number of embryonal bodies, cultures with higher adjusted pH values showed a better performance. In one experiment, petioles were incubated in stock solutions with an initial pH of 4.5, 5.0, 5.8, 6.5 and 7.2. Petioles with a pH of 4.5 failed to produce any embryo. Petioles cultured in stock solution with a pH of 5.0 showed restricted embryogenesis. Other treatments with a higher pH went through all stages of realization of somatic embryogenesis and produced young plants.

The same experiment was conducted using cell suspension as the source material. The increase in the pH of the solution was one pH scale more than that of the petiole culture, however, showing the same trend. Treatment with low solution pH failed to give rise to advanced stages of embryogenesis. This is evident because the optimum pH of most of the enzymes is in the pH range of 6. To find out the role of pH and its relation to the kind of nitrogen, three stock solutions with initial pH ranges of 4.2, 5.8 and 7.2 were prepared. With a pH of 4.2 there were no sign of realization of somatic embryogenesis regardless of the type of nitrogen. With a pH of 5.8 and 7.2, respectively, casein hydrolysate or KNO_3 treatments showed normal embryo development. The development was better under the pH of 5.8, indicating that an optimum pH for somatic embryogenesis is around 6. As far as

$(NH_4)_2SO_4$ is concerned it showed a slight improvement below a pH of 7.2, suggesting a relation between pH and the occurrence and degree of realization during carrot somatic embryogenesis. As a result firstly, a pH readjustment can be forced to the system when the pH reducing nature of diammonium sulfate encounters the adjusted pH of 7.2, secondly, under a higher pH range plant cells can more readily use nitrogen of diammonium sulfate for its vital metabolic activities, growth and development. A parallel experiment conducted with different pH ranges showed a similar trend indicating an optimum pH of 5.8 - 6.8 as a general rule. The optimum pH range of control B5 medium is between 5.8 and 6.8. Casein hydrolysate treatment follows the same trend. In $(NH_4)_2SO_4$ treatment, only under a high pH of 8, the cells divided more rapidly compared to a culture with a lower pH. The vitality of the cells was determined by using neutral red. The cells were vital even under a pH of 4.2. Nitrate treatment showed a retardation trend as the pH reached the scale of 8.0.

In regard to the pH of the suspension solution, as a general rule, diammonium sulfate lowers the pH of the solution to 3.5, nitrate by contrast increases it up to around 7.0, and casein hydrolysate keeps the pH of the solution nearly constant, around 6.0. Treatments with a low concentration of $(NH_4)_2SO_4$ led to embryo formation. This can be interpreted as follows: by decreasing the concentration of the amount of $(NH_4)_2SO_4$ supplied, the pH is less affected. Diammonium treatment decreases the pH to a critical level by stagnating physiological and biochemical processes through irreversible damaging and deactivation of proton pump. Many higher plants suffer toxic effects when ammonia is the exclusive nitrogen source. The toxicity of NH_4^+ causing low pH is particularly severe (Yan et al., 1992). According to Felle (1987), Membrane transport, metabolism, and cytoplasmic buffer are the main factors involved in pH control. Cytoplasmic pH is not a function of external pH, the cell does not react to pH changes caused by week acids or bases, and the plasmalemma pump reacts to cytoplasmic pH, but if turned off or stimulated the pH change is small. According to him pH is mainly determind by H^+ producing and consuming processes and pH control is more based on the pH changes produced inside the cell than on changes in external pH (Felle, H. 1987). Kosegarten et al. (1999) suggest that under NO_3^- nutrition an increased apoplastic pH depresses Fe^{3+} reductase activity and in this way influence Fe^{2+} transport across the plasma membrane negatively, resulting in Fe chlorosis. According to him, the significant increase in apoplastic pH of the NO_3^-

treatment might derive from NO_3^-/proton cotransport. These results were not found in NH_4^+ or $NH_4\ NO_3$ sole nutrition treatments.

The question is, which is the dominating factor, the nitrogen form or the pH. It is understandable that different nitrogen forms affect the pH of medium differently or a specific nitrogen form has a specific pH effect on the medium and it is obvious too that pH in itself is important for the induction and realization of somatic embryogenesis and embryo development. It seems, however that the pH factor is a "more co-dominant factor" than the nitrogen form.

5.3.2 IN-VITRO **Cell Division and Cell Growth**

Nitrogen is a vital constituent of many key biomolecules. Nitrogen, its forms and proportion, and pH can influence the cell division, differentiation, growth and development of somatic embryos *in vitro.* Nevertheless, the metabolic aspects and physiological and molecular properties of nitrogen metabolism during cell differentiation and morphogenesis are not well understood. A number of fundamental questions have still to be dealt with: cell differentiation and morphogenic responses and the role of nitrogen in the regulation of morphogenesis through the evaluation of the uptake and primary assimilation of inorganic nitrogen in plant morphogenesis *in vitro* and *in vivo,* the explicit role of certain metabolites, such as amino acids and polyamines etc., the contribution of nitrogenous metabolites in the modulation of plant growth regulators, and the assessment of the current condition of the widely studied tissue culture systems, *Daucus carota, Nicotiana tabacum* and *Pinus radiata* as model systems for understanding the common principles of nitrogen metabolism (Singh, 1995).

Nitrate and ammonia are the most common inorganic nitrogen compounds used as nutrient salts in tissue culture media for *in vitro* plant cultures (Murashige and Skoog, 1962, Mantell and Hugo, 1989, Mordhorst and Lörz, 1993, Niedz, 1994). The pathways for the assimilation and utilization of nitrogen from the environment are clear for the *in vivo* growth of plants. On the other hand, information on plant tissues grown in culture is very limited. The most widespread inorganic nitrogen form acquired by plants grown *in vivo* is nitrate. Intact plants are generally set to obtain optimal quantities of nitrate when the

exogenously presented nitrate concentrations fluctuate from 10 μM to 100 mM (Crawford, 1995; Glass and Siddiqui, 1995; Neumann, 1995 and Mengel, 1991). In plant cells, nitrate can either be transformed to nitrite and afterward to ammonia via the enzymes nitrate reductase (NR, EC 1.6.6.l) (Crawford, 1995; Mengel, 1991; Srivastava, 1995) and nitrite reductase (NiR, EC 1.6.6.4) (Sawhney, 1995) correspondingly, or the nitrate may possibly be stored in the vacuoles. Ammonia, produced by these reactions, is further utilized in combination with carbon skeletons to produce glutamine and glutamic acid. This reaction is catalyzed by glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (GOGAT, EC 1.4.1.14) (Lam et al., 1995, Mengel, 1991, Neumann, 1995, Singh, 1995). A minute fraction may be utilized via glutamate dehydrogenase (GDR, E.C.l.4.1.24) (Bhadula and Shargool, 1995, Singh, 1995). Once it has entered into the organic cycle, nitrogen can be builtin new amino acids, amides, proteins, nucleic acids, chlorophylls, alkaloids, polyarni-nes, vitamins, plant growth regulators etc. Singh et al., (1995) have proposed that nitrate may possibly function as a signal molecule of plant growth via amplified gene expression for enzymes in charge of the uptake and utilization of nitrate e.g. NR, NiR, GS, and GOGAT (Crawford and Arst, 1993, Hoff et al., 1994, Crawford, 1995). Despite the fact that nitrate and ammonium salts have been universally used as nutrients in tissue culture media, numerous reports specify that reduced nitrogen forms, particularly amides and amino acids, e.g. glutamine, glutamic acid, proline and alanine, etc., can improve cell prolif-eration as well as regeneration in specific genotypes (Neumann, 1995, Jullien et al., 1979, Stuart and Strickland, 1984a7 b, Olsen, F. L. 1987, Shetty and Asano, 1991a, b, Shetty et al., 1992a, b, Gill et al., 1993, Thorpe, 1993, Murthy et al., 1996a, b), but the function of these compounds in the induction and expression of morphogenesis is explicit.

5.3.2.1 Inorganic Nitrogen

The requirement for nutrient salts such as nitrate, ammonium and some forms of reduced organic nitrogen in plant cell culture media has long been realized. Many of the early tissue culture media contained only nitrate (Gautheret, 1937;White, 1939) and the significance of reduced nitrogen forms for an improved growth and regeneration emerged only later

(Murashige and Skoog, 1962, White, P. R. 1963, Gamborg et al., 1968). Preece (1995) emphasized the inevitability of optimizing both, nutrient requirements and plant growth regulators for morphogenic responses. Different forms of nitrogen in the culture media alter the endogenous levels of cell metabolites as well as proteins, nucleic acids, plant growth regulators (PGRs) and other explicit regulatory molecules (Preece, 1995). This nitrogen requirement may or may not be met by both inorganic or organic forms of nitrogen, depending on the species and culture conditions (Preece, 1995). The primary events of cell division, cell size increase and entry into S-phase of DNA synthesis were improved and the rate of cell mortality deceased when *Asparagus officinalis* mesophyll cells were cultured on MS medium with 30 mM L-glutamine as the only nitrogen source (Jullien et al., 1979). In this culture system an organic form of nitrogen is most suitable, since cultured asparagus cells, suspectfully lack NR activity and consequently are not capable using nitrate ions (Jullien et al., 1979). Seelve et al. (1995) have publicized that exogenously supplied ammonium improved GS activity, ammonium content and the growth of asparagus callus as compared to those with no ammonium addition. However, high ammonium supplementation reduced GS activity and the growth of the calli. Furthermore, a study of nitrate utilization in tobacco suspension cultured cells during a culture cycle indicated that the patterns of utilization of nitrate ions for cell growth and the expression of nitrate uptake proteins and reducing enzymes were comparable to those of plant seedlings (Heimer and Filner, 1971;Behrend and Mateles, 1975;Deane-Drummond, 1990;Zhang and Mackown, 1992;Glass and Siddiqui, 1995).

Ammonia and nitrate each can be employed as the only source of nitrogen in cell culture. Experiments with tobacco cell culture showed that cells proliferate better in a medium containing nitrate as the only nitrogen source, through which there is a marked increase in cell dry weight, cell proliferation is higher, and there is an increase in the pH value compared with ammonium treatment. This is an indication of lower cell division activity in an ammonium treatment; cell development in both treatments was nearly the same (Neumann, 1995). The fall in the pH of the culture medium can be an indication that ammonium was taken up preferentially to nitrate. An additional factor, which may influence nitrogen modulated cell division and growth is the pH of the growth medium. Ammonium uptake in suspension cultures of *Ipomoea* cells (Martin and Rose, 1976), tomato

roots (Sheat et al., 1959), maize roots and leaves (Singh et al., 1984) and soybean plants (Tolley-Henry and Raper, 1986) was reliant on the pH of the medium. Ammonium uptake rates in ammonium partially continuous cultures of carrot were 25% higher when the pH of the medium was 4.5 compared to a pH of 5.5 or 6.5 (Steiner and Dougall, 1995). It is suggested that this alteration in culture medium pH possibly caused the cells to aggregate or disaggregate, which consequently increased the rate of ammonium uptake from the medium. In a number of liquid media both forms of inorganic nitrogen are used e.g. MS medium, and it seems there is a timely preferential selectivity for the ammonium form of nitrogen (Neumann, 1995).

5.3.2.2 Organic Nitrogen

The influence of reduced organic nitrogen in cell cultures on cell division and cell growth is not altogether comprehensive and not always positive. Filner (1966) monitored the L forms of alanine, asparagine, aspartic acid, glutamic acid, proline, valine, histidine, and leucine, which inhibited cell growth and repressed NR activity in short-term cultivation of tobacco cells in culture media containing one of these amino acids. As products of nitrate assimilation, exogenous and/or endogenous ammonium and amino acids accumulated and in general reduced NR activity in plants; on the other hand, an ammonium-induced NR activity has been made known in some plants inclucing tissue culture and cell suspension cultures (Srivastava, 1992, 1995). Hence, amino acids can adjust the nitrogen utilization of *in-vitro* cultures by regulating primary nitrogen assimilation. Concurrently, many amino acids can be readily transformed into other amino acids and integrated into proteins in the cell culture (Dougall, 1965, 1966;Thorpe, 1993).

5.3.3 Somatic Embryogenesis

5.3.3.1 Inorganic Nitrogen

Cellular totipotency and flexibility of differentiation programs leads to potential differentiation of somatic cells to the specific types of regenerants, i.e., shoots, roots, microtuber or somatic embryos, in defined culture conditions (Singh, 1995). Equilibrium of nitrogenous compounds in addition to phytohormones, predominantly auxin and cytokinins, in the media and in the tissues or cell masses is essential for morphogenesis to take place (Olsen, 1987, Thorpe, 1980, 1982, 1983). The amount and form of the supplemented nitrogen in the culture media influences the growth and metabolic activity of the cultured cells as well as their morphology and regenerative potentials. Reprogramming of the entire gene expression pattern with explicit signals for genes is necessary to initiate the regeneration process. Dudits et al. (1995) studied the contemporary molecular understanding of the procedures concerned with the induction of regeneration and indicated the fundamental role of hormones or stress-induced activation of signal transduction systems which may modify DNA structure transcription, or induce the events that lead to the formation of either dedifferentiated callus tissues or somatic embryos. Nitrogenous compounds may be involved in this process either as carriers, catalysts, transporters or other regulatory molecules (Dudits et al., 1995).

It is necessary to look back at the first reports dealing with two fundamental dogma of somatic embryogenesis since the earliest successes were achieved in media supplemented with coconut milk or coconut water. Attention was focused on the role of complex, naturally occurring liquid endosperms that normally bathe zygotic embryos in nourishing young somatic embryos (Steward and Shantz, 1959;Steward et al., 1969). Subsequent investigations showed that both the induction of embryogenic growth and the promotion of maturation in carrot cultures could be achieved in the totally defined media in the absence of CW (e.g. Kato and Takeuchi, 1963). However, it was during this early

period of research that the basic requirements for somatic embryogenesis in carrot were demonstrated:

(1) An auxin or auxin-like substance was critical for embryo initiation, and the lowering of the auxin concentration or its complete absence fostered embryo maturation (Halperin and Wetherell, 1964;Halperin, 1966;Steward et al., 1967).
(2) Reduced nitrogen was reported to be a prerequisite and of outmost importance for both initiation (Halperin and Wetherell, 1964b;Halperin, 1966) and maturation of somatic embryogenesis (Ammirato and Steward, 1971)

The initial observations of somatic embryos by Steward and Reinert were with cultures containing complex media, including CW and casein hydrolysate, both of which serve as sources of reduced nitrogen. The specific requirement for ammonium in carrot somatic embryogenesis was reported by Halperin and Wetherell (1964b). Most culture media used for somatic embryogenesis contain ammonium nitrate (Ammirato, 1984). The source of reduced nitrogen may vary and is a complex agenda (e.g., CW). Mixtures of amino acids (Kato and Takeuchi, 1966) and single amino acids have all been employed (Wetherell and Dougall, 1976). Many studies have claimed that inorganic nitrogen in the form of ammonium is required for the initiation of embryogenesis in carrot cell cultures, stating that nitrate alone is insufficient, but that supplementation of the culture medium with ammonium chloride induced embryo formation (Halperin and Wetherell, 1965; Wetherell and Dougall, 1976). Some claimed that the content of reduced nitrogen determines the nature of the meristems (Halperin 1966;Jones 1974).

In the present study using KNO_3, an oxidized nitrogen form, during the realization phase as the sole source of nitrogen, contrary to the hypothesis that “somatic embryogenesis needs necessarily a reduced form of nitrogen” (Halperin et al., 1965;Kamada et al., 1979, 1984b, Wetherell et al., 1976), the globular stage was formed already 5-6 days after initiation of the culture and 10-12 days thereafter, heart structures appeared. Torpedoes emerged 13-16 days later and led to the formation of plantlets 15-18 days from the beginning of the subculture in auxin-free medium. To verify this phenomenon, different parallel experiments were conducted using modified and original Gamborg B5 media

differing in their nitrogen form and using different cell material. An independent experiment was conducted in our institute in which the same results were obtained. So it is clear by now, that the realization of somatic embryogenesis can be fostered by using only oxidized form of nitrogen. The assimilation of ammonium is against the exchange of H^+ ions in the culture medium. This aspect made it interesting to investigate how important the pH value is and what role the pH plays in the process of ammonium assimilation and somatic embryogenesis. In the meantime, one must not forget the competitive process of ammonium against other cations present in the nutrition solution. In some liquid solutions both forms of inorganic nitrogen are used e.g. in the MS medium. In this medium it seems there is a timely preferential selectivity for the ammonium form of nitrogen. Later on, cells use nitrate for performing their metabolic activities (Neumann, 1995). Ammonium is a reduced form of nitrogen, hence it can be readily used for the synthesis of amino acids. Nitrate, as an oxidized form of nitrogen, must first be reduced. This process needs some energy source from the metabolism of the cell.

Adequate nitrogen in the medium is important for somatic embryogenesis as no embryos are produced from explants cultured with very low levels of nitrogen or inadequate forms of nitrogen in the medium (Reinert et al., 1967;Tazawa and Reinert, 1969;Reinert and Tazawa, 1969;Wetherell and Dougall, 1976;Nomura and Komamine, 1995). Using ^{15}N NMR analysis, Thorpe (1993) confirmed that ammonium is the preferred source of nitrogen in carrot and white spruce embryogenic tissues, as it is taken up from the medium early and utilized more rapidly than nitrate. Dougall and Verma (1978) reported that carrot suspension cultures could grow and produce somatic embryos in the presence of ammonium as the only nitrogen source, if the pH of the medium is controlled by permanent titration. According to Kamada and Harada (1984 a, b) the induction phase of somatic embryogenesis in carrot requires no nitrogenous compounds, if the appropriate level of 2,4-D exists, nevertheless, reduced nitrogen is required for advanced embryo development. Low levels of ammonium (1-5 mM) as the single nitrogen source and low pH have been reported to induce somatic embryogenesis in carrot, even in the absence of the auxin (Smith and Krikorian, 1990, Merkle et al., 1995).

It seems that if programming of somatic cells is induced just once by the induction factor, the repetitive cycles of embryogenesis follow the same programs and looping. Tazawa and Reinert (1969) reported that the existence of ammonium in the medium is not indispensa-

ble for embryo formation *in vitro,* but a certain level of intracellular ammonium is a must for this process. A threshold level of tissue ammonium and its correlation to the embryogenic response of the cells in all the cultures, however, were not apparent (Singh, 1995). Mordhorst and Lörz (1993) reported that for the duration of embryogenesis and the development of isolated barley microspores, the level of total nitrogen content in the medium, the nitrate : ammonium ratio, and the ratio of inorganic : organic nitrogen were not correlated to the frequency of initial divisions, and had only moderate effects on planting efficiency, although they had significant effects on embryogenesis and plant regeneration.

When potassium nitrate at the level corresponding to MS nitrate, or ammonium sulfate at the level corresponding to MS ammonium or glutamine (10 mM), was added in the culture medium as the single nitrogen source, the somatic embryos produced were at the cotyledonary notch of peanut seed cultures *in vitro,* even if they were considerably fewer in number compared with cultures raised in standard MS nitrogen having all nitrogen forms (Singh, 1995). However, glutamic acid (10 mM) as the only nitrogen source in the medium was found to cover the requirement for nitrogen (Singh, 1995). Khanna and Raina (1997) have in recent times publicized that the nitrogen content of the callusing medium and the composition of NO_3^- and NH_4^+ nitrogen considerably influence the shoot regeneration from the calli in Basmati rice cultivars.

The differences observed in diverse studies may be linked to genotypic dissimilarity, dissimilarity in the source tissues, and the interaction of nitrogen with other supplementary components of the media and to the endogenous condition of various metabolites and plant growth regulators. Numerous other aspects may be involved in nitrogen-mediated regeneration inducing recycling and metabolism of nitrogen compounds in the plant cells during the culture.

The reasoning, interpretations and reports by others on why nitrate is not the preferential source of nitrogen for cell growth and regeneration in *in-vitro* cultures remain unresolved, rather left over, particularly in view of its utilization *in vivo* (Crawford, 1995). Various reports dispute the possibility that *in-vitro* cultured cells do not have adequate physiological resources to promote nitrate uptake, transport and assimilation during the early culture

phase. When a whole seedling system was used to investigate the effect of nitrate on regeneration, the reaction induced differed from that observed for the explants culture systems (Wetherell and Dougall, 1976;Singh, 1995). This suggests that the failure of the cellular ability to take up and assimilate the nitrate may be related to the physical isolation of the explants and distorted growth of the root system in the presence of high levels of cytokinins. In carrot and white spruce embryogenic tissues, the assimilation of inorganic nitrogen into glutamine, glutamate and alanine during embryo development, and the conversion into arginine and aliphatic amines are confirmed using ^{15}N tracer techniques (Thorpe, 1993).

Low activity of NR in the early stages of carrot somatic embryogenesis has been associated with poor embryogenic potential in the absence of reduced nitrogen (Kamada and Harada, 1984a). The activity of the ammonia assimilating enzyme glutamine synthetase was also reduced during somatic embryogenesis in carrot, following an initial activation (Higashi et al., 1996). It is thus probable that exogenously supplied amino nitrogen in the form of glutamine and/or other amino acids may be crucial to provide the adequate nitrogen for the synthesis of metabolites for embryogenesis. It is perceptible that during the embryo development the assimilation of nitrogen occurred via the GS-GOGAT cycle of ammonia assimilation, causing its incorporation into ornithine and eventually polyamines (Singh, 1995).

5.3.3.2 Organic Nitrogen

There is increasing evidence of the efficacy of the supplemented amino compounds in the culture medium in the presence or absence of nutrient nitrogen salts. Many amino acids are efficient in improving somatic embryogenesis at different stages of development and transition. It seems that young embryos do not have the vigorous enzyme systems to assimilate nitrate and ammonium. Furthermore, the addition of certain amino compounds including glutamine, glutamic acid and alanine, improve the production, development and transition rate of somatic embryos.

The casein hydrolysate in our treatment produced the highest number of embryonal bodies, but the formation of plantlets failed in this treatment. This phenomenon has its

own importance, because in this way a synchronized culture will be established which proceeds only till the late torpedo stage, but no further. It is comparable to the dormant state of a zygotic embryo. The existing hypothesis proclaiming that all torpedoes will be automatically transferred to plantlets must be revised subsequently. The biochemical pathway behind the synchronal development would make us able to understand more about the "induced somatic dormancy" which, most likely, is not too far away from zygotic dormancy.

The synchronized development up to the torpedo stage in the casein hydrolysate treatment opens ways to produce "artificial seeds" by automation of such process, rough calculation indicates yielding up to approximately 15.7 million torpedoes of greater than 800 μ in size in a 1000 lit bioreactor in a period of just 90 days (unpublished, Institute for Plant Nutrition, Department of Tissue Culture JLU, Giessen)..

The application of the principles of plant cell division and regeneration to practical plant propagation is the result of continuous studies in many laboratories worldwide, on the standardisation of explants sources, media composition and physical state, environmental conditions and adaptation of in vitro plants. Particularly important are the studies on the molecular causes of organogenesis and somatic embryogenesis. However, further practical applications of micro propagation, which is also commercially viable, depends on reducing the production costs such that it can compete with seed production or traditional vegetative propagation methods (e.g., cuttings, tubers and bulbs, grafting etc). There is a need for the development of an automated system for mass micro propagation of commercially important crop plants. A bioreactor system could be used for precise control over the physiochemical environment at each stage of the process. Following embryo development, an automated imaging system could determine embryo quality and then the embryos could be coated with a polymer for storage creating a synthetic seed. This would allow for the propagation of elite clones and make more efficient use of the limited natural resources.

Techniques that have the potential to further increase the efficiency of micro propagation, but still need further improvements, include: simplified large scale bioreactors, cheaper automatization facilities, efficient somatic embryogenesis and synthetic seed production, greater utilisation of the autotrophic growth potential of cultures, and good repeatability

and quality assurance of the micropropagated plants. Plant biotechnology includes the technique tissue culture to clonally produce large numbers of identical individuals at minimal cost. Somatic embryogenesis is the best method for high frequency somatic embryo production in most of the Umbelliferae members (Stephen and Jayabalan 1999). Somatic embryogenesis as a propagation system is still experimental and is not used commonly to propagate commercially important species. However, the potential for clonal propagation of traditionally seed propagated plants like forestry species and vegetables are enormous. This technology will probably require synthetic seeds to deliver these plants to the field (Brown, D.C.W. 1994). Artificial seed is a seed, which has been manufactured rather than naturally formed. Artificial seeds usually comprise plant-derived cells such as a somatic embryo encapsulated in an artificial seed coating. An artificial seed will usually give rise to a clonal plant i.e. one, which is genetically identical to the plant from which it has been derived (Bouton, J. 1998). The concept of artificial seed, also commonly referred to as synthetic seed or encapsulated embryos is potentially more efficient method compared to conventional micropropagation. An embryo formed from a somatic cell and not from gametes is genetically identical to the plant from which it has been derived, unlike a seed, which inherits genes from both the male parent via pollen and female parent via ovule. Somatic embryos may be used for clonal propagation and in the formation of artificial seeds of woody plants. Synthetic seed technology can capture the genetic superiority of plants that are selected for tissue culturing. Only vigorous, superior ecotypes will be selected for culture. Selection of superior plants for culture increases the likelihood that synthetic seed will grow into superior plants. This is an advantage over the collection and planting of seeds from natural populations, because at least some of those seeds will be genetically defective or sterile. Synthetic seeds offer an advantage over commercial seed selection and cultivar development, which generally takes from 10 to 20 years. In contrast, synthetic seeds are produced within a few years. Another benefit of this technique is that one will, at the very least, have protocols for micropropagating superior ecotypes of these plants, which could result in rapid production of large numbers of seedlings available for direct transplanting or for use in commercial seed orchards (Bouton, J. 1998). These synthetic seeds are essentially germinated embryos that are enclosed in a gel-like substance that contains fertilizers, fungicides and insecticides. These seeds have the advantage of breaking their seed coats almost immediately without the worry of uncertain climactic

conditions. It would dramatically reduce the need for fertilizers, pesticides and herbicides. The first schemes of direct fluid drilling of somatic embryos, developed into techniques of encapsulating embryos in hydrated coatings and, recently, into the idea of drying in vitro-derived embryos. The next stage of technique under development would be the development of an effective coating to provide protection during storage and rehydration and the improvement in bioreactor production of embryos (Stephen, R. and Jayabalan, N. 1999). The paradigm that must be pursued in research is a dry somatic embryo with a synthetic endosperm, which would contain additives such as protein or lipid reserves, fungicides and/or Rhizobium, all of which would be protected with a synthetic coating to control dehydration and protect against physical damage during handling. An automated production of a synthetic seed system for genetically improved crops for an accurate evaluation of the vigor of seed to evaluate quickly and accurately dormant and hard seed, accurately predict germination of seed, rapid detection and effective control of pathogens that are seed borne, treating seed internally and externally with fungicides and insecticides for more optimum plant populations and higher yield, substitution of bio-engineered organisms for chemicals to control seed and soil borne pathogens, verification of the genetic purity of your breeding lines and varieties prior to distribution and planting, protection of proprietary products from infringement by use of genetic markers, seed coating to promote precision planting and eliminate thinning and the potential field performance of the seed and its longevity.

The maturation phase is the period of embryo development, cell division and histo-differentiation, in which cell expansion and reserve deposition occurs (Verhagen and Wann, 1989). A wide range of diverse media containing various forms and levels of nitrogen and combinations of inorganic and organic nitrogen have been shown to influence embryo development and maturation. It may be concluded, here, that nitrogen has a specific function in the development and maturation of somatic embryos, and in addition to an adequate amount of the inorganic nitrogen forms nitrate and ammonium, and certain amino acids, especially glutamine, proline, alanine and serine, etc., can improve embryo maturation in a defined time phase. Consequently a better embryo conversion rate can be achieved.

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7 APPENDICES

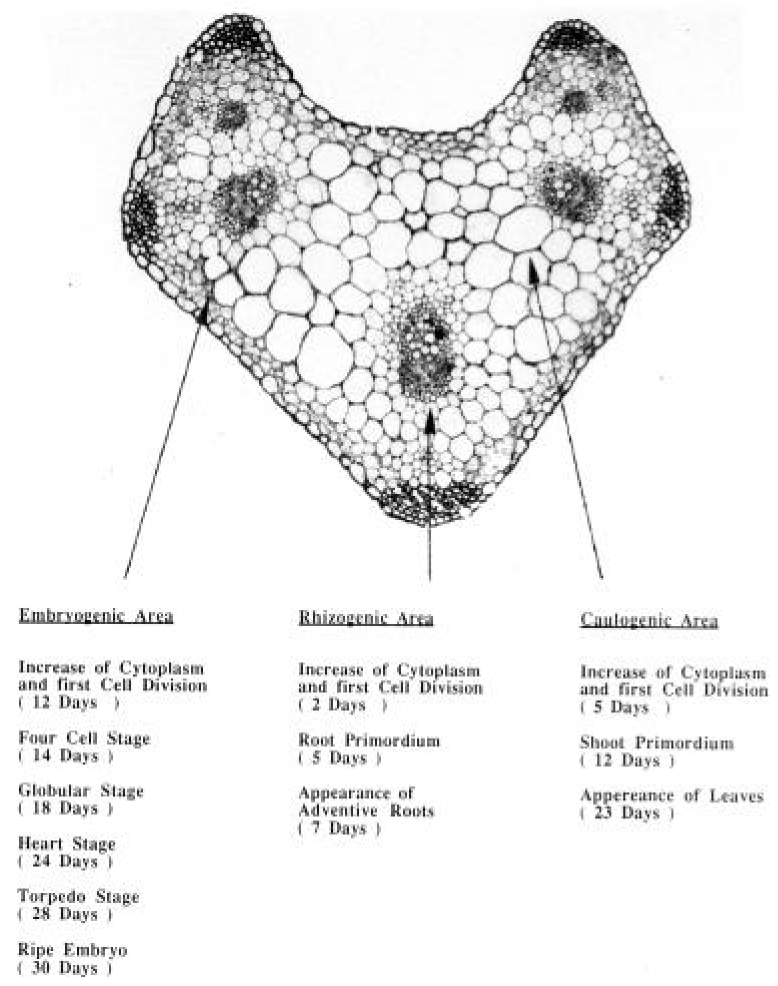


Fig 1. Section of t0 carrot petiole explant showing embryogenic, rhizogenic and caulogenic areas (Schäfer et al., 1988).

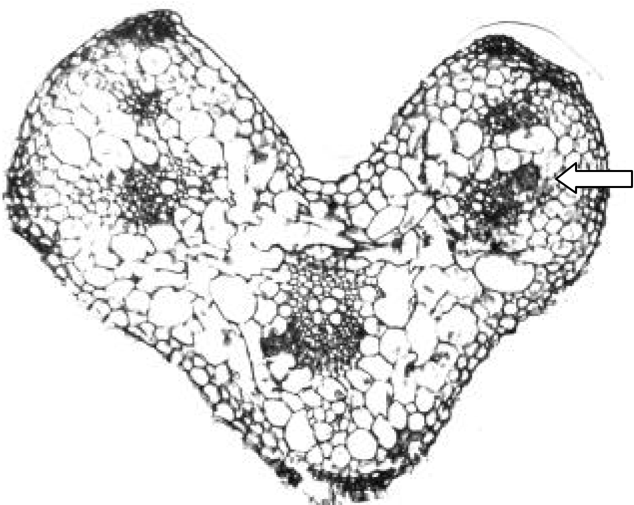


Fig 2. Section of t7 carrot petiole explant during induction phase cultured in an auxin-containing medium ($B5^+$) stained with hematoxylin showing rhizogenic meristem (arrow).

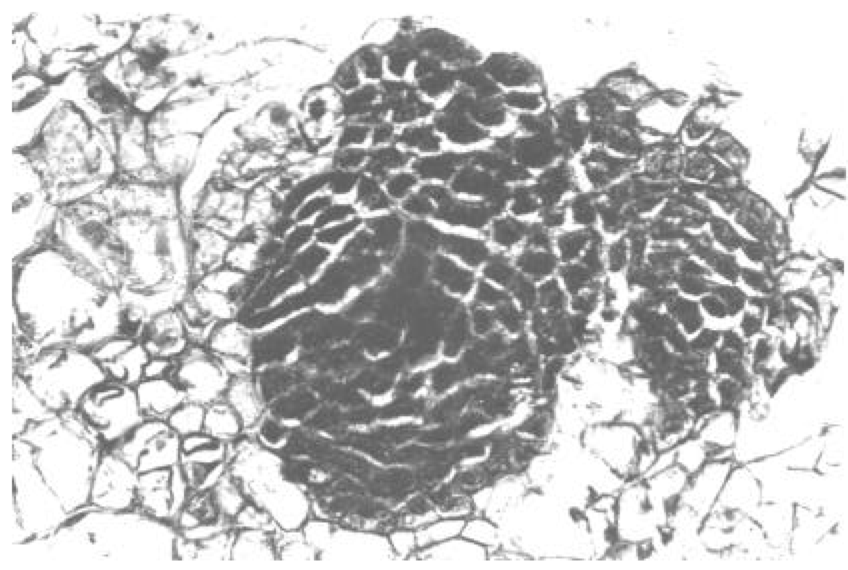


Fig 3. Section of t7 carrot petiole explant during induction phase cultured in an auxin-containing medium ($B5^{+}$) stained with hematoxylin showing rhizogenic meristem.

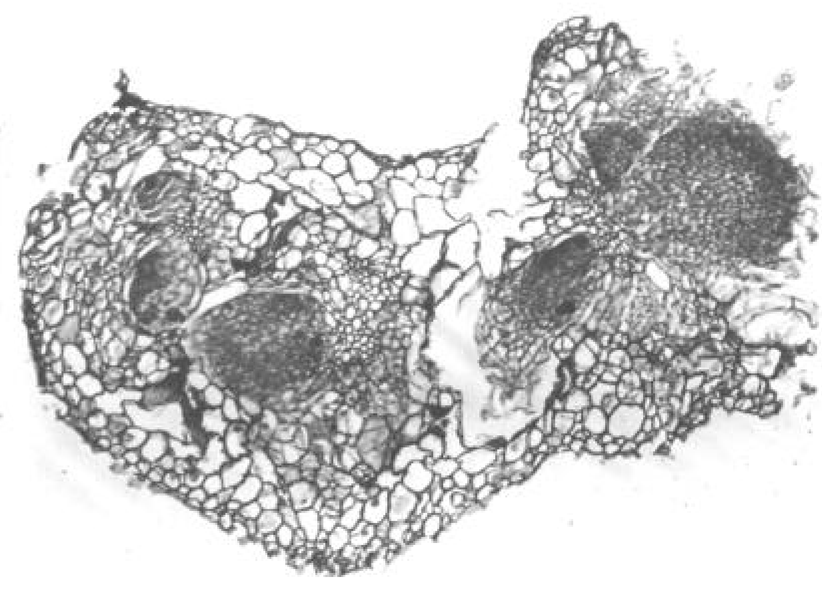


Fig 4. Section of t14 carrot petiole explant during induction phase cultured in an auxin-containing medium ($B5^{+}$) stained with hematoxylin showing rhizogenic meristems.

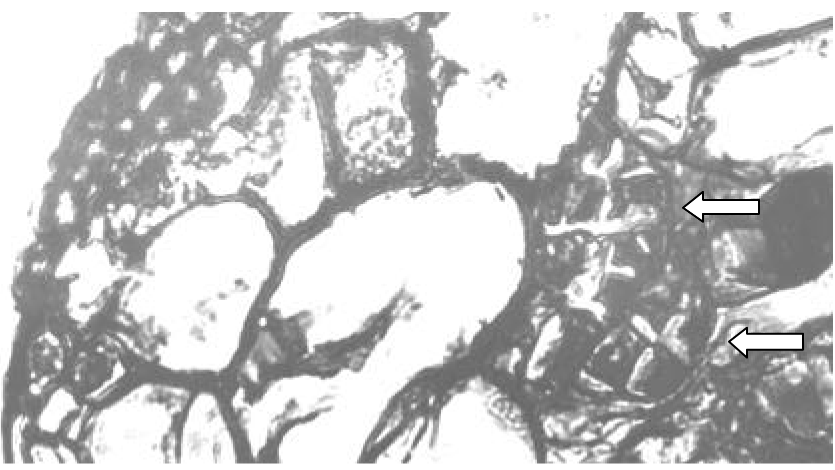


Fig 5. Section of t14 carrot petiole explant during induction phase cultured in an auxin-containing medium ($B5^{+}$) stained with hematoxylin showing dividing embryogenic cells (arrows).

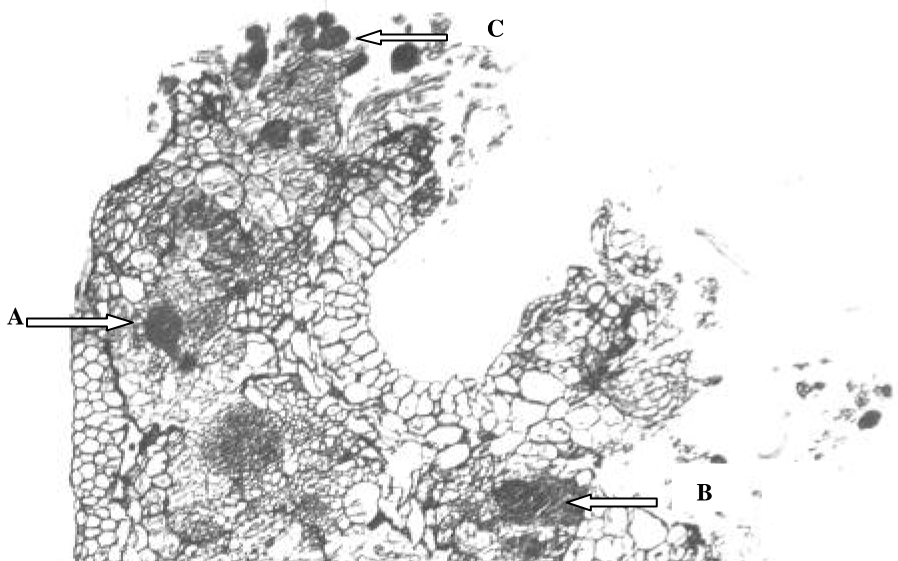


Fig 6. Section of t21 carrot petiole explant during realization phase cultured in a medium with out auxin 2,4-D ($B5^{-}$) stained with hematoxylin showing embryogenic (A), rhizogenic (B) meristem and globular stage (C).

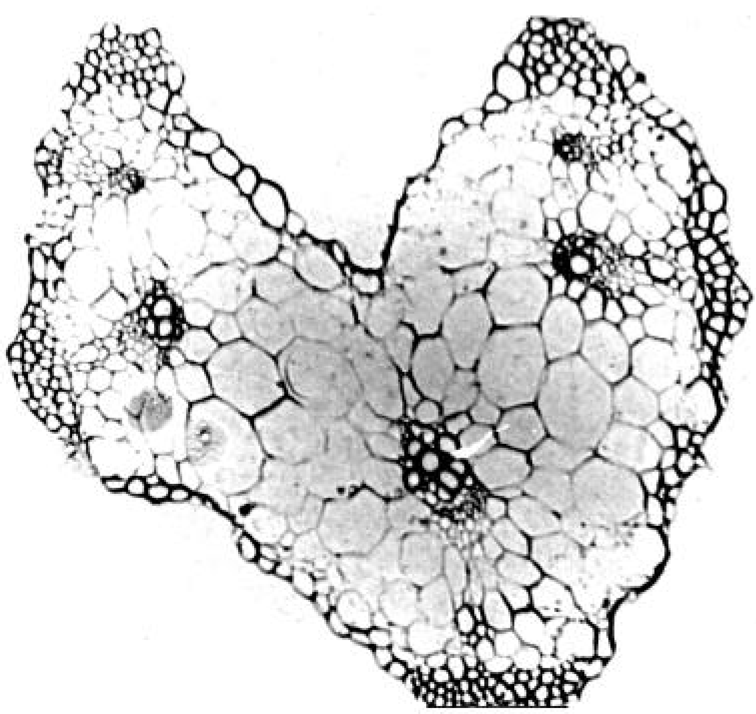


Fig 7. Section of t0 carrot petiole explant during induction phase cultured in an auxin-containing medium ($B5^{+}$) labelled with ^{14}C-Leucine. The cell membrane is labelled with ^{14}C-Leucine suggesting a possible assimilation of ^{14}C-Leucine by membrane-bound-proteins.

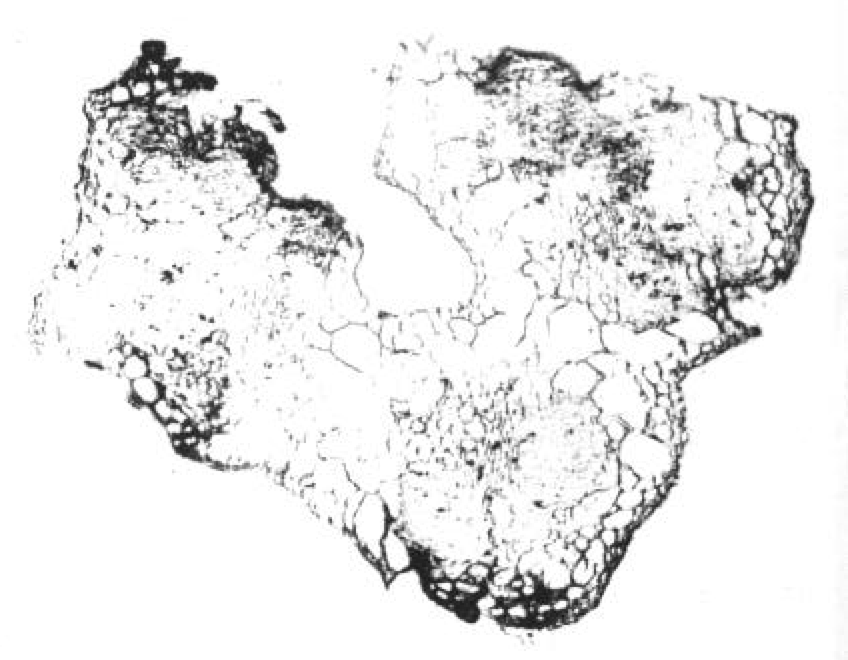


Fig 8. Section of t7 carrot petiole explant during induction phase cultured in an auxin-containing medium ($B5^+$) labelled with ^{14}C-Leucine suggesting preferential assimilation of ^{14}C-Leucine.

Fig 9. Section of t7 carrot petiole explant during induction phase cultured in an auxin-containing medium ($B5^+$) stained with hematoxylin and labelled with ^{14}C-Leucine suggesting preferential assimilation of ^{14}C-Leucine in comparison to only labeled sample in fig, 8.

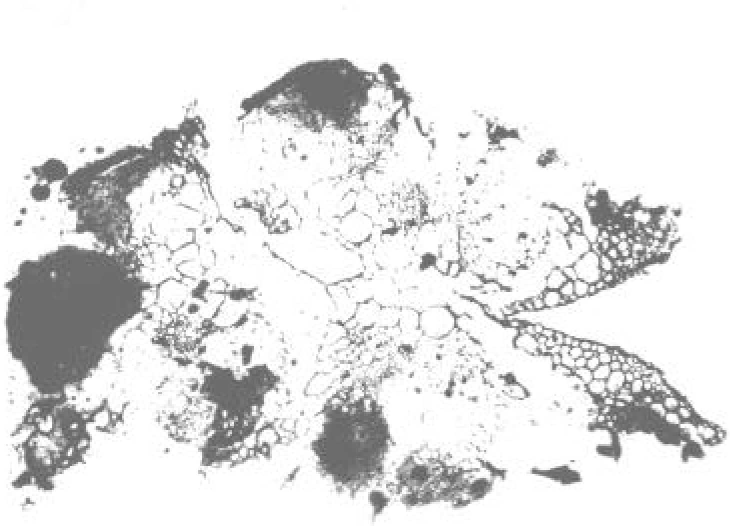


Fig 10. Section of t14 carrot petiole explant during induction phase cultured in an auxin-containing medium ($B5^+$) labelled with ^{14}C-Leucine suggesting preferential assimilation of ^{14}C-Leucine.

Fig 11. Section of t14 carrot petiole explant during induction phase cultured in an auxin-containing medium ($B5^+$) stained with hematoxylin and labelled with ^{14}C-Leucine suggesting preferential assimilation of ^{14}C-Leucine in comparison to only labeled sample in fig, 10.

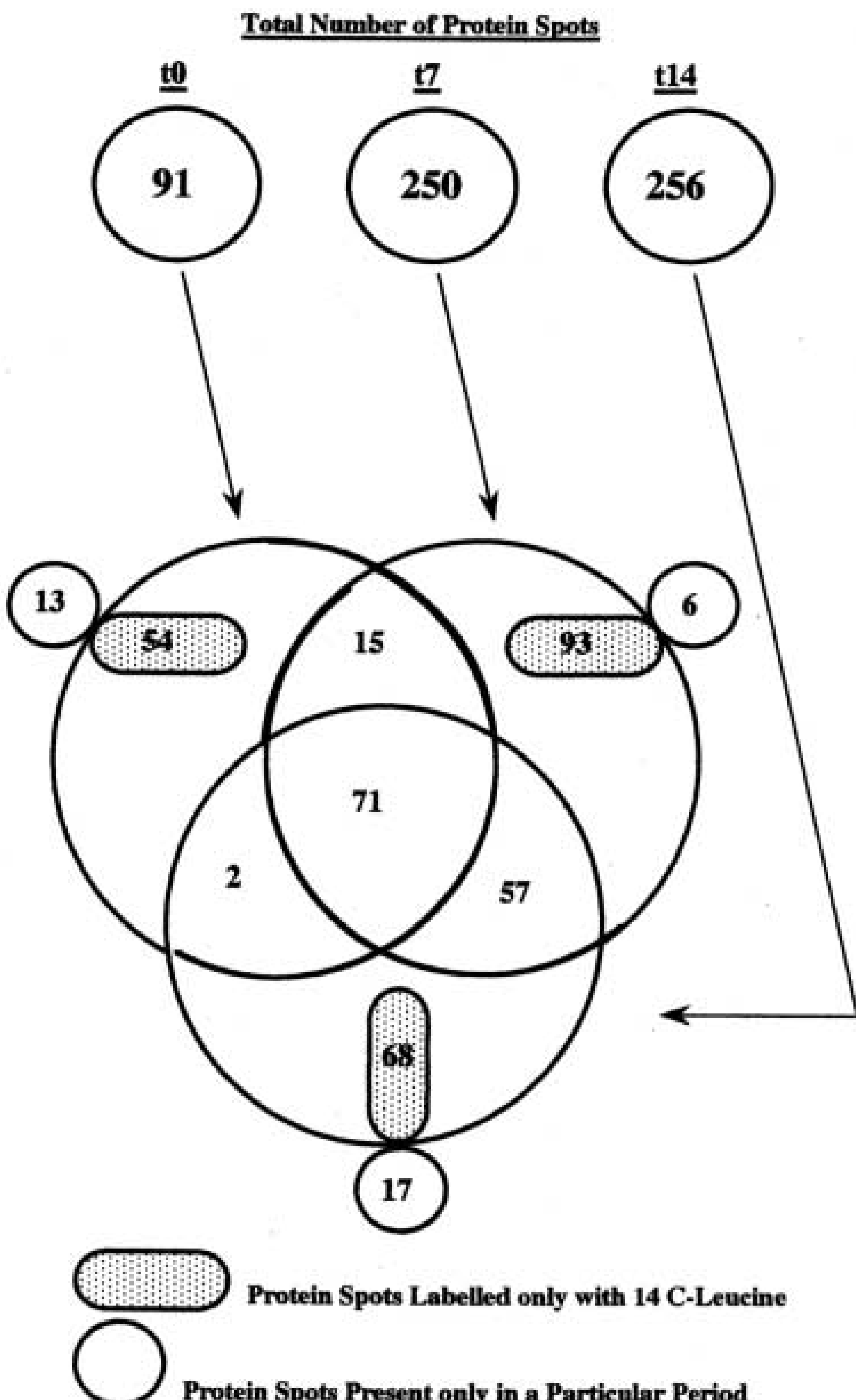
Table 1. Fresh weight (FW), weight of acetone powder (AP), protein concentration and specific activity of soluble protein after application of ^{14}C-leucine in carrot petiole explants after 5 hours (t0), 7 days (t7) and 14 days (t14) culture in an auxin-containing media (modified B5 with 0.5 ppm 2,4-D). Adapted and extended from Grieb, 1992.

	t0	t7	t14
FW mg/10 explants	41	113	256
AP %	4.48	10.67	11.44
AP : FW	1 : 22.4	1 : 9.3	1 : 8.6
mg soluble protein/g FW	108.76	415.06	475.65
mg soluble protein/100 mg AP	242.78	389.36	415.58
dpm . 10^4 / g FW	13.99	89.23	69.87
dpm . 10^4/100 mg AP	31.48	83.21	61.02
dpm . 10^4/100 g protein	13.06	21.42	14.67

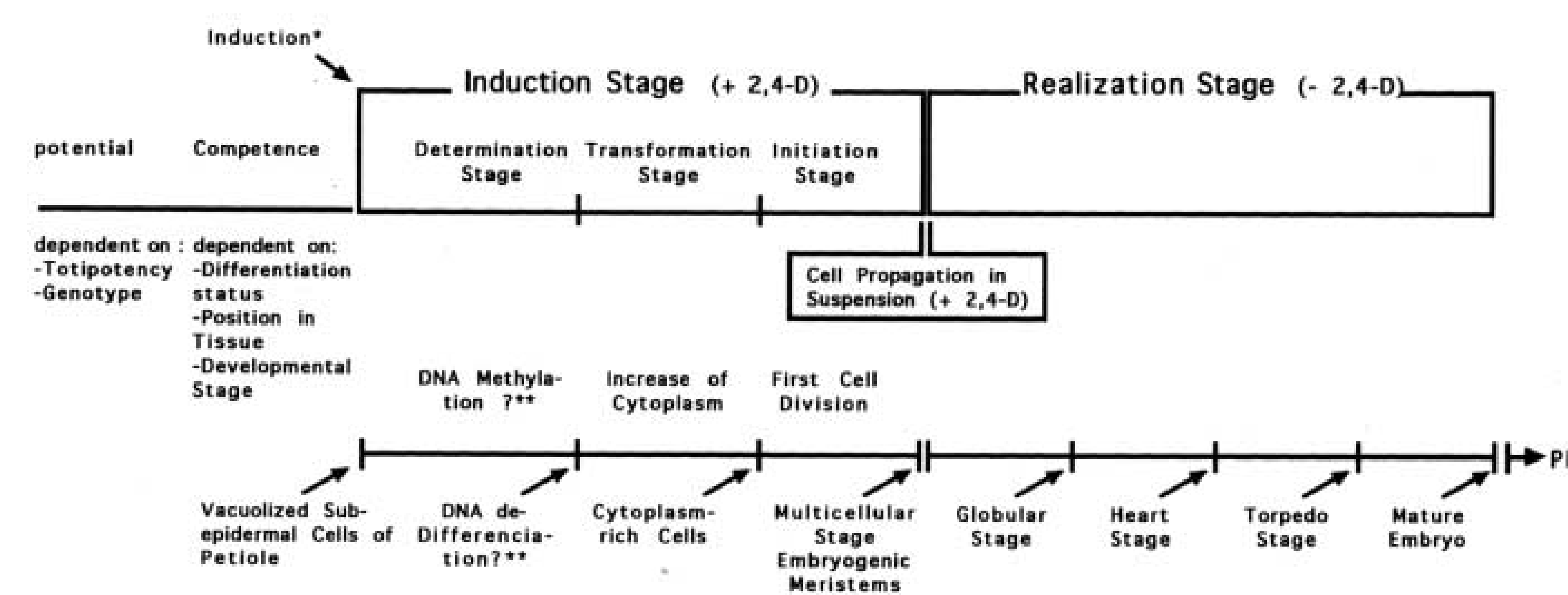
Table 2. Distribution of Coomassie brilliant blue R-250 stained and ^{14}C-leucine labelled 2-DE of protein spots in a 5 hour (t0), 7 day (t7) and 14 day (t14) old carrot petiole culture (*Daucus carota* L. var Rotin) in an auxin-containing media (modified B5 with 0.5 ppm 2,4-D). Adapted and extended from Grieb, 1992.

	t0	t7	t14
Protein spots present in a particular time period	91	250	256
Protein spots present only in that very particular time period	13	6	17
Protein spots present in all time periods (House Keeping Proteins)	71	71	71
Protein spots present in a time period but absent in the other time periods	13	174	17
Protein spots stained only with CBB	12 (13.2 %)	35 (14.0 %)	54 (21.0 %)
Protein spots stained with CBB and labelled with ^{14}C-leucine	25 (27.5 %)	122 (48.8 %)	134 (52.5 %)
Protein spots labelled only with ^{14}C-leucine	54 (59.3 %)	93 (37.2 %)	68 (26.5 %)

Appendix C: Distribution and Comparison of Protein Spots during Different Induction Periods in Auxin Containing (0.5 ppm 2,4-D) Carrot Petiole Culture (*Daucus carota* L.).



Appendix D: Hypothetical Scheme of Somatic Embryogenesis at the Cellular Level in Carrot Petiole Explants (adapted and extended from Grieb, 1992).



* Transfer in an auxin-containing medium

** LoSCHIAVO et al. 1989

Appendix E: List of Protein Spots during Different Induction Periods in an Auxin-Containing Carrot Petiole Culture. Adapted and extended from Grieb, 1992.

Spot	t0	t7	t14	pI	MW (KD)
1.	– –	○ –	○ –	6.25	112.65
2.		○ ●	○ ●	6.20	76.88
3.	– –	○ ●	○ ●	5.75	77.21
4.	– –	○ ●	○ -	6.50	68.28
5.	– –	○ –	○ –	6.40	68.28
6.	– –	○ ●	– –	5.94	76.65
7.	– –	○ –	○ ●	5.80	70.50
8.	– ●	○ ●	○ –	5.65	68.28
9.	– ●	○ ●	– ●	5.10	76.88
10.	– –	○ ●	○ –	5.05	69.75
11.	– ●	– ●	– ●	4.96	75.22
12.	– –	– ●	– ●	4.80	72.03
13.	– –	– –	○ –	4.95	66.16
14.	– –	– ●	– ●	4.90	61.55
15.	– –	– ●	– –	4.80	67.16
16.	– –	○ ●	○ ●	5.05	57.62
17.	– –	○ ●	○ –	5.20	57.62
18.	– –	– ●	– –	5.20	63.33
19.	– –	– ●	– ●	5.60	58.54
20.	– –	– ●	– ●	5.40	61.55
21.	– –	○ ●	○ ●	5.70	64.13
22.	– –	○ ●	○ ●	5.70	60.93
23.	– –	○ ●	○ ●	6.10	59.72
24.	– –	○ –	○ –	6.20	58.31
25.	– –	○ –	○ ●	6.40	60.08
26.	– –	○ –	○ –	6.50	62.18
27.	– –	○ –	○ –	6.75	63.47
28.	– –	○ –	○ –	6.90	63.47
29.	– –	○ –	○ –	7.05	63.47
30.	– –	– ●	– ●	6.95	66.79
31.	– –	– ●	– ●	7.25	57.40
32.	– –	– ●	– ●	7.20	54.69
33.	– –	– ●	– ●	7.10	54.48
34.	– –	– ●	○ ●	7.10	57.40
35.	– –	– ●	– ●	7.10	60.32
36.	– –	– ●	– ●	6.95	60.08
37.	– –	○ ●	○ ●	6.95	57.62
38.	– –	– ●	– ●	6.95	55.22
39.	– –	– ●	– ●	6.70	55.22
40.	– –	– ●	– ●	6.70	57.62
41.	– –	– ●	– ●	6.35	56.07
42.	– –	– ●	– ●	6.25	55.75
43.	– –	○ ●	○ –	6.10	56.84
44.	○ –	○ –	– ●	5.75	53.67
45.	○ –	○ –	○ –	6.05	54.18
46.	– –	○ ●	○ ●	6.20	52.87
47.	– –	○ ●	○ ●	6.35	52.48
48.	– –	○ ●	○ ●	6.65	52.19
49.	– –	○ ●	○ ●	6.90	52.19
50.	– –	○ ●	○ ●	6.85	50.34
51.	– –	○ –	○ –	7.10	50.79

Protein spots appeared on historadioautograms at t0, t7 and t14 of carrot petiole culture explants. (– no spot at that stage, ○ stained with CBB, ● labelled with ^{14}C-leucine).

Appendix E: List of Protein Spots during Different Induction Periods in an Auxin-Containing Carrot Petiole Culture. Adapted and extended from Grieb, 1992.

Spot	t0	t7	t14	pI	MW (KD)
52.	– –	○ –	○ –	7.25	50.79
53.	– –	○ –	○ –	7.45	50.79
54.	– ●	– ●	– ●	5.60	50.34
55.	– ●	– ●	– ●	5.50	49.89
56.	– –	– ●	– ●	5.40	49.98
57.	– –	○ –	○ –	6.00	44.06
58.	– –	○ ●	○ –	6.70	48.60
59.	– –	○ ●	○ –	6.85	47.37
60.	– –	○ ●	○ –	7.00	47.37
61.	– –	○ –	– –	6.83	53.63
62.	– –	○ ●	– ●	7.60	47.78
63.	– –	– ●	– ●	7.75	48.60
64.	– –	– ●	– ●	7.90	48.18
65.	– –	– ●	○ ●	7.40	43.39
66.	– –	– ●	– ●	7.20	43.72
67.	– –	– ●	– ●	7.05	43.72
68.	– –	– ●	– ●	7.00	44.06
69.	– –	○ ●	○ ●	6.85	44.40
70.	– –	○ ●	○ ●	6.65	44.75
71.	– –	○ –	○ –	6.40	44.75
72.	– –	○ ●	○ ●	6.20	42.75
73.	○ –	○ –	○ ●	5.55	43.72
74.	– –	– –	○ –	5.35	43.39
75.	– –	○ –	○ –	4.95	44.06
76.	– –	– –	○ –	4.80	42.13
77.	– –	○ –	○ –	4.90	41.54
78.	○ –	– –	– –	5.58	46.90
79.	– –	○ –	○ –	5.70	41.54
80.	– –	○ –	○ ●	5.95	40.97
81.	○ –	○ –	○ –	6.40	40.15
82.	– –	– ●	○ ●	6.85	40.15
83.	– –	○ ●	○ ●	6.95	39.63
84.	– –	○ ●	○ ●	7.15	40.69
85.	– –	– –	○ ●	7.80	40.15
86.	– –	– ●	○ ●	7.80	42.13
87.	– –	– –	○ ●	7.95	40.97
88.	– –	– –	○ ●	8.00	39.13
89.	– –	○ –	○ –	6.90	38.89
90.	– –	○ –	○ –	6.75	38.65
91.	– –	○ ●	○ ●	7.75	36.17
92.	– –	○ ●	○ ●	7.40	36.73
93.	– ●	○ ●	○ ●	7.25	37.13
94.	– ●	○ ●	○ ●	7.15	37.33
95.	– ●	○ ●	○ ●	7.00	37.54
96.	– ●	○ ●	○ ●	6.90	37.97
97.	– –	– –	○ –	7.20	35.81
98.	– –	○ ●	○ ●	7.10	36.35
99.	– –	– ●	– ●	7.00	36.73
100.	– –	– ●	– ●	6.90	36.73
101.	– –	○ ●	– ●	6.55	36.73
102.	○ –	○ ●	○ ●	6.30	37.75

Protein spots appeared on historadioautograms at t0, t7 and t14 of carrot petiole culture explants. (– no spot at that stage, ○ stained with CBB, ● labelled with ^{14}C-leucine).

Appendix E: List of Protein Spots during Different Induction Periods in an Auxin-Containing Carro t Petiole Culture. Adapted and extended from Grieb, 1992.

Spot	t0	t7	t14	pI	MW (KD)
103.	– –	○ ●	○ ●	6.15	38.19
104.	○ –	○ ●	○ ●	6.00	39.13
105.	○ ●	○ ●	○ ●	5.75	39.38
106.	– –	– –	○ –	4.50	37.33
107.	– –	– ●	– –	5.10	39.61
108.	– –	– ●	– ●	5.45	36.54
109.	○ –	○ –	○ –	5.70	37.54
110.	– –	○ ●	○ ●	5.95	37.13
111.	– ●	– ●	– ●	6.25	35.81
112.	– –	○ ●	○ ●	7.10	34.99
113.	– –	– ●	○ ●	7.45	34.54
114.	– –	– ●	○ ●	7.60	34.40
115.	– –	– ●	○ ●	7.80	34.26
116.	– –	○ ●	○ ●	8.00	34.12
117.	– –	– –	– ●	7.85	35.31
118.	– –	– –	– ●	8.05	35.21
119.	– –	– –	– ●	8.15	32.42
120.	– ●	○ ●	○ ●	7.80	32.72
121.	– –	○ ●	○ ●	7.55	32.72
122.	– –	○ ●	○ ●	7.30	32.97
123.	– –	○ ●	○ ●	7.10	33.73
124.	– –	○ ●	○ ●	7.05	34.26
125.	– –	○ ●	○ ●	6.90	34.69
126.	– –	○ ●	○ ●	7.00	33.73
127.	– –	○ ●	○ ●	6.95	32.62
128.	– –	○ ●	○ ●	6.85	33.14
129.	– –	○ ●	○ ●	6.85	33.99
130.	– –	– –	○ ●	6.55	34.12
131.	– –	○ ●	○ ○	6.55	33.49
132.	– –	– ●	○ –	6.55	32.62
133.	– –	– ●	○ ●	6.25	34.12
134.	○ ●	○ ●	○ ●	6.10	35.15
135.	– –	– ●	– ●	6.10	36.17
136.	– –	– ●	– ●	5.90	34.84
137.	– –	– ●	– ●	5.75	34.69
138.	– –	– ●	– ●	5.60	35.47
139.	– –	– ●	– –	5.12	36.61
140.	– –	○ –	○ –	4.60	33.73
141.	– –	○ –	○ –	4.60	33.25
142.	– –	– –	○ –	4.50	33.30
143.	– –	○ –	○ –	4.50	32.82
144.	– –	– –	○ –	4.20	34.69
145.	– –	– –	○ –	4.20	33.99
146.	– –	– –	○ –	4.20	33.37
147.	– –	○ –	○ –	4.15	33.03
148.	– –	– ●	– ●	5.50	35.21
149.	– ●	○ –	○ ●	5.75	33.73
150.	– –	○ ●	○ ●	6.00	33.73
151.	– –	○ ●	○ ●	6.25	33.25
152.	– –	○ ●	○ ●	6.25	32.46
153.	– –	○ ●	○ ●	6.20	31.95

Protein spots appeared on historadioautograms at t0, t7 and t14 of carrot petiole culture explants. (– no spot at that stage, ○ stained with CBB, ● labelled with ^{14}C-leucine).

Appendix E: List of Protein Spots during Different Induction Periods in an Auxin-Containing Carro t Petiole Culture. Adapted and extended from Grieb, 1992.

Spot	t0	t7	t14	pI	MW (KD)
154.	– ●	○ ●	○ ●	6.10	32.42
155.	– ●	– –	– –	5.85	36.04
156.	– ●	– ●	– ●	5.65	32.06
157.	– ●	– ●	○ ●	5.35	32.15
158.	– –	– ●	– ●	4.90	31.86
159.	– –	– ●	– ●	7.05	31.85
160.	– –	– ●	– ●	6.70	31.51
161.	– –	– ●	○ ●	7.55	31.71
162.	– –	○ ●	○ ●	7.80	31.51
163.	– –	– ●	○ ●	7.45	30.92
164.	– –	○ ●	○ –	7.20	30.89
165.	– ●	○ ●	○ ●	6.25	30.86
166.	○ ●	○ ●	○ ●	6.10	30.98
167.	– ●	○ ●	○ ●	5.95	31.17
168.	– ●	○ ●	○ ●	5.70	31.55
169.	– ●	○ ●	○ ●	5.40	30.92
170.	– ●	○ ●	○ ●	5.40	30.47
171.	○ ●	○ ●	○ ●	5.75	30.86
172.	– ●	○ ●	○ ●	6.05	30.42
173.	– –	○ ●	○ ●	6.25	30.32
174.	– –	○ ●	○ ●	6.30	29.94
175.	○ ●	○ ●	○ ●	6.85	30.42
176.	○ ●	○ ●	○ ●	6.80	30.22
177.	– –	○ ●	○ ●	7.35	30.42
178.	– –	○ ●	○ ●	7.50	30.37
179.	– –	○ ●	○ ●	7.65	29.84
180.	– –	○ ●	○ ●	7.45	29.85
181.	– ●	○ ●	○ ●	7.35	29.61
182.	– –	○ ●	○ ●	7.25	29.76
183.	– –	○ ●	○ ●	6.95	29.11
184.	– –	○ ●	○ ●	7.00	29.38
185.	– –	○ ●	○ ●	7.00	29.76
186.	– –	○ ●	○ ●	6.60	29.90
187.	○ ●	– ●	– ●	6.40	29.79
188.	– –	○ ●	○ ●	5.90	30.13
189.	– –	○ ●	○ ●	5.80	29.81
190.	○ ●	○ ●	○ ●	5.65	29.85
191.	– –	– ●	– ●	5.45	29.79
192.	– –	– –	– ●	5.30	30.13
193.	– –	– ●	– ●	5.30	29.72
194.	– –	– ●	○ ●	5.00	29.720
195.	○ –	– ●	– –	5.10	27.81
196.	○ –	– ●	○ –	5.45	29.54
197.	○ ●	– ●	– –	5.42	28.56
198.	○ –	– ●	○ ●	5.55	29.23
199.	– –	○ ●	○ ●	5.60	29.34
200.	– ●	○ ●	○ ●	5.95	29.76
201.	– ●	○ ●	○ ●	6.05	29.63
202.	– ●	○ ●	○ ●	6.15	29.60
203.	– ●	○ ●	○ ●	6.10	29.30
204.	– –	– ●	○ ●	6.25	29.21

Protein spots appeared on historadioautograms at t0, t7 and t14 of carrot petiole culture explants. (– no spot at that stage, ○ stained with CBB, ● labelled with ^{14}C-leucine).

Appendix E: List of Protein Spots during Different Induction Periods in an Auxin-Containing Carrot Petiole Culture. Adapted and extended from Grieb, 1992.

Spot	t0	t7	t14	pI	MW (KD)
205.	– –	– ●	– –	6.73	27.71
206.	– –	○ ●	○ –	7.10	29.56
207.	○ –	○ ●	○ ●	7.00	29.04
208.	– –	○ ●	○ ●	7.25	28.64
209.	– –	○ ●	– ●	7.10	29.04
210.	– –	– ●	– –	7.35	27.88
211.	– ●	○ ●	○ ●	7.50	28.92
212.	– ●	○ ●	○ ●	7.75	28.87
213.	– –	○ –	○ –	8.20	28.48
214.	– –	○ –	○ –	8.00	28.33
215.	– ●	– ●	○ ●	7.75	28.27
216.	– –	○ ●	○ ●	7.50	28.31
217.	– ●	– ●	– ●	7.15	28.40
218.	○ ●	○ ●	○ ●	6.95	28.55
219.	– ●	○ ●	○ ●	6.30	28.74
220.	– ●	○ ●	○ ●	6.10	28.72
221.	– ●	– –	– –	5.90	27.49
222.	– ●	– ●	– ●	5.55	28.64
223.	– ●	– ●	– ●	5.15	28.07
224.	○ ●	– ●	– ●	6.55	28.07
225.	○ ●	○ ●	○ ●	5.80	28.07
226.	○ ●	○ ●	○ ●	5.95	27.94
227.	– ●	– –	– –	6.18	25.81
228.	– ●	– –	– –	6.30	25.96
229.	○ ●	○ ●	○ ●	6.25	27.94
230.	– ●	– ●	– ●	6.60	27.81
231.	– ●	○ ●	○ ●	6.95	27.67
232.	– ●	○ ●	– ●	7.10	27.81
233.	– –	○ ●	○ ●	7.50	27.60
234.	– –	○ ●	○ ●	7.80	27.31
235.	– ●	– –	– –	8.18	26.32
236.	– –	○ ●	○ –	6.45	27.39
237.	– –	○ –	○ –	6.50	26.60
238.	– –	○ ●	○ ●	6.25	27.34
239.	– –	○ ●	○ ●	6.25	26.74
240.	○ ●	– –	– –	6.16	24.55
241.	– –	○ ●	○ ●	5.90	27.23
242.	– –	○ ●	○ –	5.75	27.46
243.	– –	○ ●	– ●	5.50	27.56
244.	– –	– ●	– ●	5.45	25.25
245.	○ ●	– ●	– ●	5.90	25.09
246.	– –	– ●	– ●	6.30	25.47
247.	– –	– ●	– ●	6.95	26.29
248.	– ●	○ ●	○ ●	7.10	25.18
249.	– –	○ ●	○ ●	7.70	25.63
250.	– –	○ –	○ –	7.70	25.14
251.	○ ●	– ●	– –	6.88	21.41
252.	○ ●	– –	– –	7.04	20.13
253.	– –	– ●	– ●	6.55	24.02
254.	– –	– ●	○ ●	6.20	24.10
255.	– –	– ●	– ●	6.20	21.85

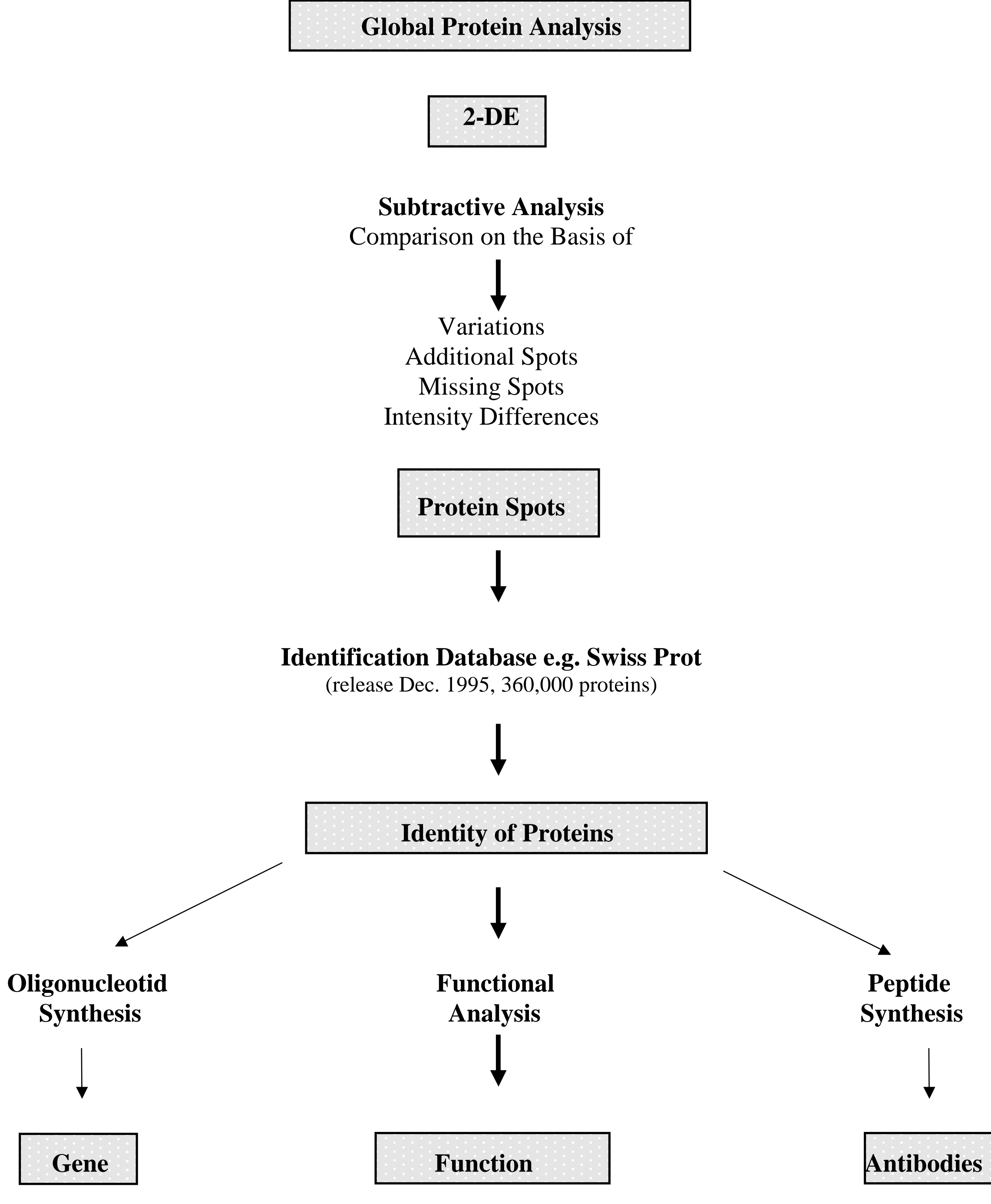
Protein spots appeared on historadioautograms at t0, t7 and t14 of carrot petiole culture explants. (– no spot at that stage, ○ stained with CBB, ● labelled with ^{14}C-leucine).

Appendix E: List of Protein Spots during Different Induction Periods in an Auxin-Containing Carrot Petiole Culture. Adapted and extended from Grieb, 1992.

Spot	t0	t7	t14	pI	MW (KD)
256.	– –	○ ●	○ ●	7.10	21.44
257.	– ●	– ●	– ●	6.05	19.02
258.	– ●	– ●	– ●	5.85	19.10
259.	– ●	○ ●	○ ●	5.40	19.70
260.	– ●	– ●	– ●	5.20	19.42
261.	– ●	– –	– –	5.21	23.29
262.	○ ●	– –	○ –	4.30	18.20
263.	○ –	– –	– –	4.68	18.55
264.	○ ●	○ ●	○ ●	5.45	13.99
265.	○ ●	○ ●	○ ●	5.65	13.99
266.	– –	– ●	○ ●	5.80	14.15
267.	– ●	○ ●	○ ●	6.25	17.64
268.	– ●	○ ●	○ ●	6.30	16.43
269.	– –	– ●	○ ●	7.25	18.41
270.	○ ●	– ●	○ ●	6.95	18.41
271.	○ ●	– ●	– –	7.10	11.08
272.	○ ●	– ●	○ ●	7.40	13.48
273.	– –	– ●	○ –	6.90	11.03
274.	– –	– ●	– ●	7.50	4.90
275.	– –	– ●	○ ●	6.90	4.90
276.	– ●	– –	○ –	6.25	12.03
277.	– –	– ●	○ ●	6.25	4.71
278.	○ ●	– ●	– ●	5.60	4.90
279.	– ●	– ●	○ ●	5.45	12.26
280.	– ●	– –	– –	5.30	15.25
281.	– ●	– –	– –	4.64	10.01
282.	– ●	– –	– –	4.28	13.35

Protein spots appeared on historadioautograms at t0, t7 and t14 of carrot petiole culture explants. (– no spot at that stage, ○ stained with CBB, ● labelled with ^{14}C-leucine).

Appendix F: Schema of Global Protein Analysis Information Resource Search Program in Database.



Appendix G: Emergence of Different Groups of Proteins in Carrot Petiole Explants after 5 Hours, 7 Days and 14 Days Cultured in an Auxin-Containing Medium (0.5 ppm 2,4-D). Adapted from Grieb, 1992.

Time	0	5 Hours	1-7 Days	7 Days	7-14 Days	14 Days
Explant	Petiole	Adaptation		Intensive growth of rhizogenic zones around vascular bundles		Embryogenic meristems
Cell	Parenchyma cells		Cell division in meristemic zones around vascular bundles		Increase of cytoplasm of sub-epidermal cells and first cell division	Increase of cytoplasm rich cells
	■■ **1** ■■	■■ **1** ■■				
	■■ **2** ■■	■■ **2** ■■	■■■■	■■ **2** ■■		
	■■ **3** ■■	■■ **3** ■■	■■■■	■■ **3** ■■	■■■■	■■ **3** ■■
	■■ **9** ■■	■■ **9** ■■			■	■■ **9** ■■
		■■ **4** ■■				
		■■ **5** ■■	■■■■	■■ **5** ■■	■■■■	■■ **5** ■■
			■	■■ **6.1** ■■	■■■■	■■ **6.1** ■■
			■	■■ **6.2** ■■	■■■■	■■ **6.2** ■■
				■■ **6.3** ■■	■■■■	■■ **6.3** ■■
				■■ **7** ■■		
					■	■■ **8** ■■

Appendix H: Effect of Different Nitrogen Forms During the Realization of Somatic Embryogenesis in Carrot Petiole and Suspension Culture.

Table 1. Role and Effect of Different Nitrogen Sources During the Realization Phase of Somatic Embryogenesis in a 63-Day-Old Carrot Petiole Culture (Modified Gamborg B5 without 2,4-D) using 415.6 ppm N as KNO_3, 28.4 ppm N as $(NH_4)_2SO_4$ and 32.5 ppm N as Caseine Hydrolysat (CH: Caseine Hydrolysat, θ: No Nitrogen supplemented).

	KNO_3 + $(NH_4)_2SO_4$ + CH	-------	KNO_3 + $(NH_4)_2SO_4$	KNO_3	$(NH_4)_2SO_4$	CH	KNO_3 + CH	$(NH_4)_2SO_4$ + CH
Color	Green	Brown	Green	Green	Dirty Beige	Pale Green	Green	Dirty Beige
Initial pH of Sol.	5.21	4.95	4.94	4.92	4.92	5.35	5.30	5.38
Adjusted pH of Sol.	5.70	5.70	5.70	5.70	5.70	5.70	5.70	5.70
pH of sol. after 63 Days	5.87	3.79	5.94	6.02	3.72	4.35	6.27	4.23
Globular	189	0	134	39	0	82	84	0
Heart	88	0	67	24	0	59	110	0
Torpedo	195	0	56	20	0	27	74	0
Plantlet	137	0	112	72	0	0	47	0

Table 2. Role and Effect of Different Nitrogen Sources During the Realization Phase of Somatic Embryogenesis in a 42-Day-Old Carrot Cell Suspension Culture (Modified Gamborg B5 without 2,4-D) using 476.5 ppm N as KNO_3, $(NH_4)_2SO_4$ and Caseine Hydrolysat respectively (CH: Caseine Hydrolysat, Control: Using 415.6 ppm N as KNO_3, 28.4 ppm N as $(NH_4)_2SO_4$ and 32.5 ppm N as Caseine Hydrolysat).

	Control	CH	$(NH_4)_2SO_4$	KNO_3
FW g	2.61	3.15	0.41	2.29
Dry Weight %	5.15	2.22	not determinable	5.04
Chlorophyll a µg/g	603	200	177	518
Chlorophyll b µg/g	301	277	161	585
Ratio a : b	2 : 1	1 : 1.4	1.1 : 1	1 : 1.1
Chlorophyll a+b µg/g	904	477	338	1103
Protein µg/g	401	490	not determinable	285
Osmotic Potential of Cell Sap (ml Osmol)	149	198	not determinable	402

Table 3. Effect of Different Nitrogen Sources and Concentrations on Growth and pH of Solution During the Realization Phase of Somatic Embryogenesis in a 93-Day-Old Carrot Petiole Culture (Modified Gamborg B5 without 2,4-D) using 176, 276 and 476 ppm N as KNO_3, $(NH_4)_2SO_4$ and Caseine Hydrolysat (CH: Caseine Hydrolysat).

	CH 176 ppm	CH 276 ppm	CH 476 ppm	$(NH_4)_2SO_4$ 176 ppm	$(NH_4)_2SO_4$ 276 ppm	$(NH_4)_2SO_4$ 476 ppm	KNO_3 176 ppm	KNO_3 276 ppm	KNO_3 476 ppm
FW g	18.1	30.5	32.9	1.2	1.3	1.6	38.1	49.0	56.1
Initial pH of Sol.	5.9	6.0	6.2	4.8	4.9	4.9	4.9	4.9	4.9
pH of Sol. after 15 days	6.1	6.2	6.3	4.9	4.9	5.0	5.0	5.0	5.0
pH after 78 days	5.5	6.2	6.2	3.9	3.8	3.9	5.6	5.8	6.2
Occurrence of SE	YES	YES	YES	NO	NO	NO	YES	YES	YES

Table 4. Role and Effect of Different Nitrogen Sources and Concentrations During the Realization Phase of Somatic Embryogenesis in a 42-Day-Old Carrot Cell Suspension Culture (Modified Gamborg B5 without 2,4-D) using 176, 276 and 476 ppm N as KNO_3, $(NH_4)_2\ SO_4$ and Caseine Hydrolysat (CH: Caseine Hydrolysat, -: not determinable).

	CH 176 ppm	CH 276 ppm	CH 476 ppm	$(NH_4)_2\ SO_4$ 176 ppm	$(NH_4)_2\ SO_4$ 276 ppm	$(NH_4)_2\ SO_4$ 476 ppm	KNO_3 176 ppm	KNO_3 276 ppm	KNO_3 476 ppm
Chlorophyll a μg/g	364	401	823	θ	θ	θ	234	482	638
Chlorophyll b μg/g	697	545	1069	θ	θ	θ	211	477	386
Ratio a : b	1:1.9	1:1.4	1.3	θ	θ	θ	1.1:1	1:1	1.6:1
Chlorophyll a+b μg/g	1061	946	1892	θ	θ	θ	445	959	1024

Table 5. Effect of Different Concentrations of Organic Nitrogen Sources on Growth During the Realization Phase of Somatic Embryogenesis in a 30-Day-Old Carrot Cell Suspension Culture (Modified Gamborg B5 without 2,4-D) using 0, 20, 40, 60, 80 and 100 ppm N as Caseine Hydrolysat (CH: Caseine Hydrolysat).

	0 ppm CH	20 ppm CH	40 ppm CH	60 ppm CH	80 ppm CH	100 ppm CH
FW g	0.11	0.25	0.39	0.45	0.52	0.69
DW g	0.01	0.02	0.04	0.05	0.06	0.07
DW g %	9.09	8.00	10.25	11.11	11.54	10.14

Table 6. Effect of Different Concentrations of Reduced Nitrogen Sources on Development of Somatic Embryos in a 20-Day-Old Carrot Cell Suspension Culture (Modified Gamborg B5 without 2,4-D) Using Different Concentrations of $(NH_4)_2\ SO_4$.

$(NH_4)_2\ SO_4$	1.4 ppm	2.8 ppm	5.6 ppm	14.0 ppm	28.4 ppm
mM	0.1	0.2	0.4	1	2
pH of Sol.	4.62	5.00	4.91	4.93	4.92
PEMs	+	++	++	++	+
Globular	+	++	+	++	+
Heart	θ	+	+	+	θ
Torpedo	θ	+	+	+	θ
Plantlet	θ	θ	θ	θ	θ

Table 7. Nitrogen Dependent Changes in pH Value and Occurrence of Somatic Embryogenesis Using Different Nitrogen Sources in 12 and 28-Day-Old Carrot Cell Suspension Culture (Modified Gamborg B5 without 2,4-D) using 476.5 ppm N as KNO_3, $(NH_4)_2SO_4$ and Caseine Hydrolysat respectively (CH: Caseine Hydrolysat, Control: Using 415.6 ppm N as KNO_3, 28.4 ppm N as $(NH_4)_2SO_4$ and 32.5 ppm N as Caseine Hydrolysat).

	Control	CH	$(NH_4)_2SO_4$	KNO_3
Initial pH	5.47	6.24	4.97	4.93
pK Value	6.75	7.03	7.18	6.96
pH after 12 days	5.84	6.34	4.93	4.96
pH after 28 days	6.14	6.22	3.89	6.18
Occurrence of SE	YES	YES	NO	YES

Table 8. Changes and Effects of pH Value During Induction and Realization of Somatic Embryogenesis in 12, 30 and 43-Day-Old Petiole Culture with Different Initial pH (Modified Gamborg, $B5^+$: with 2,4-D, $B5^-$: without 2,4-D) using 415.6 ppm N as KNO_3, 28.4 ppm N as $(NH_4)_2SO_4$ and 32.5 ppm N as Caseine Hydrolysat.

Initial pH $B5^+$	**4.5**	**5.0**	**5.8**	**6.5**	**7.2**
pH after 12 days ($B5^+$)	5.55	5.60	5.84	5.99	6.29
pH after 30 days ($B5^-$)	5.89	5.84	6.14	6.42	6.50
pH after 43 days ($B5^-$)	6.15	6.10	6.28	6.45	6.60
DW %	2.06	5.02	5.15	6.54	4.83
Occurrence of SE	+	++	+++	++++	+++

Table 9. Changes and Effects of the pH Value During Realization of Somatic Embryogenesis in 30 and 82-Day-Old Cell Suspension Culture with Different Initial pH (Modified Gamborg B5 without 2,4-D) using 415.6 ppm N as KNO_3, 28.4 ppm N as $(NH_4)_2SO_4$ and 32.5 ppm N as Caseine Hydrolysat.

Initial pH $B5^-$	**4.5**	**5.0**	**5.8**	**6.5**	**7.2**
pH after 30 days ($B5^-$)	7.06	7.03	6.96	6.76	6.83
pH after 82 days ($B5^-$)	7.75	8.13	6.87	7.67	6.78
PEMs	+	++	+++	++++	+
Globular	+	++	+++	++++	+
Heart	θ	++	+++	++++	+
Torpedo	θ	θ	+++	++++	θ
Plantlet	θ	θ	+++	++++	θ

Table 10(a). Effect of pH Value on the Realization of Somatic Embryogenesis in Cell Suspension Culture

after 29 Days Using Different Sources of Nitrogen (476 ppm N) with an Initial pH of **5.8** (Modified Gamborg B5 without 2,4-D, CH: Caseine Hydrolysat, Control: Using 415.6 ppm N as KNO_3, 28.4 ppm N as $(NH_4)_2SO_4$ and 32.5 ppm N as Caseine Hydrolysat).

Initial pH B5⁻ (5.8)	Control	CH	$(NH_4)_2SO_4$	KNO_3
PEMs	++	+++	+	++
Globular	++	+++	+	++
Heart	++	++	θ	++
Torpedo	++	+++	θ	++
Plantlet	+++	θ	θ	+++
FW g	6.65	2.44	θ	10.97
DW %	3.46	12.30	θ	4.28

Table 10(b). Effect of pH Value on the Realization of Somatic Embryogenesis in Cell Suspension Culture after 29 Days Using Different Sources of Nitrogen (476 ppm N) with an Initial pH of **6.8** (Modified Gamborg B5 without 2,4-D, CH: Caseine Hydrolysat, Control: Using 415.6 ppm N as KNO_3, 28.4 ppm N as $(NH_4)_2SO_4$ and 32.5 ppm N as Caseine Hydrolysat).

Initial pH B5⁻ (6.8)	Control	CH	$(NH_4)_2SO_4$	KNO_3
PEMs	+++	+++	+	++
Globular	+++	+++	+	++
Heart	+++	++	θ	++
Torpedo	+++	+++	θ	++
Plantlet	++++	θ	θ	+
FW g	7.05	2.75	θ	5.91
DW %	3.97	12.36	θ	6.60

Table 10(c). Effect of pH Value on the Realization of Somatic Embryogenesis in Cell Suspension Culture after 29 Days Using Different Sources of Nitrogen (476 ppm N) with an Initial pH of **7.2** (Modified Gamborg B5 without 2,4-D, CH: Caseine Hydrolysat, Control: Using 415.6 ppm N as KNO_3, 28.4 ppm N as $(NH_4)_2SO_4$ and 32.5 ppm N as Caseine Hydrolysat).

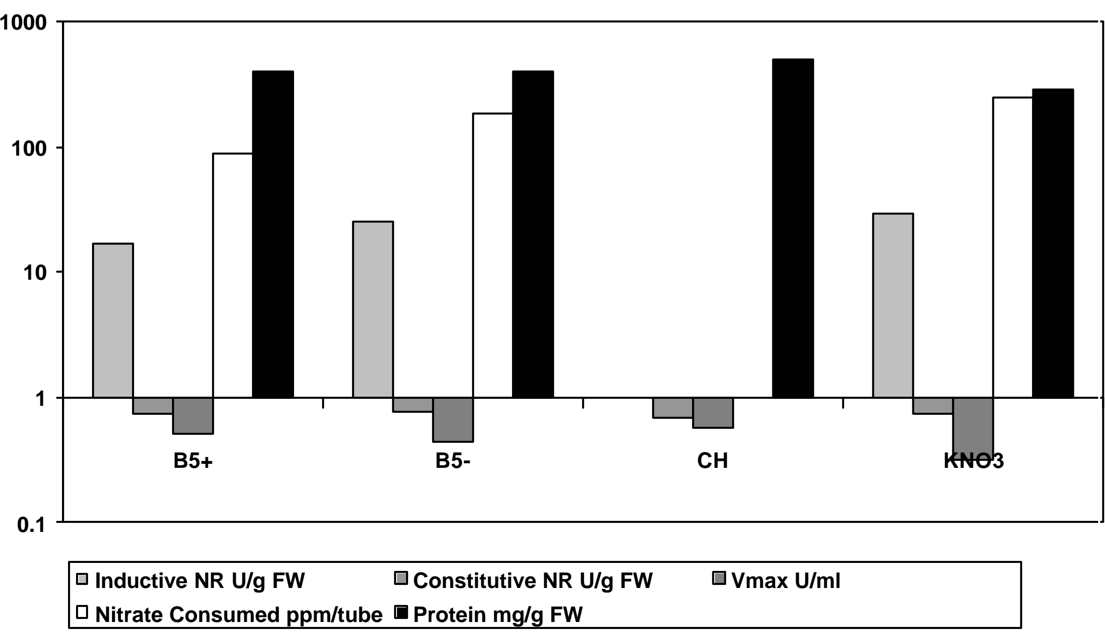
Initial pH B5⁻ (7.2)	Control	CH	$(NH_4)_2SO_4$	KNO_3
PEMs	++	+++	+	+
Globular	++	+++	+	+
Heart	++	+++	θ	+
Torpedo	++	+++	θ	+
Plantlet	+	θ	θ	θ
FW g	3.67	4.02	θ	1.91
DW %	6.54	8.71	θ	10.99

Table 10(d). Effect of pH Value on the Realization of Somatic Embryogenesis in Cell Suspension Culture after 29 Days Using Different Sources of Nitrogen (476 ppm N) with an Initial pH of **8.0** (Modified Gamborg B5 without 2,4-D, CH: Caseine Hydrolysat, Control: Using 415.6 ppm N as KNO_3, 28.4 ppm N as $(NH_4)_2SO_4$ and 32.5 ppm N as Caseine Hydrolysat).

Initial pH B5⁻ (8.0)	Control	CH	$(NH_4)_2SO_4$	KNO_3
PEMs	++	+++	+	+
Globular	++	+++	+	+
Heart	++	+++	+	θ
Torpedo	++	+++	θ	θ
Plantlet	++	θ	θ	θ
FW g	9.22	4.26	0.11	0.66
DW %	3.25	8.97	(54.54)	8.18

Appendix I: Activity of Inductive, Constitutive, Km Value, Vmax and pH Optimum of Enzyme NR. Soluble protein Content, FW and DW of a 35-Day-Old Carrot Cell Suspension Culture During the Realization Phase ($B5^-$: without 2,4-D, $B5^+$: with 2,4-D, using 476.5 ppm N as KNO_3, $(NH_4)_2SO_4$ and Caseine Hydrolysat respectively (CH: Caseine Hydrolysat, Control: Using 415.6 ppm N as KNO_3, 28.4 ppm N as $(NH_4)_2SO_4$ and 32.5 ppm N as Caseine Hydrolysat).

	$B5^+$	$B5^-$	CH	KNO_3
Inductive NR U/g FW	16.89	24.85	0.00	28.93
Constitutive NR U/g FW	0.7353	0.7556	0.6959	0.7360
K_m mMOL	3103×10^{-5}	1616×10^{-5}	3157×10^{-4}	7536×10^{-6}
V_{max} U/ml	0.5086	0.4437	0.5738	0.3139
Nitrate Consumed ppm/tube	88.67	186.18	θ	247.14
Soluble Protein mg/g FW	401.72	395.25	490.29	285.84
pH Optimum	6.5	6.5	6.5	6.5
FW g/tube	4.78	20.40	3.18	7.81
DW g/tube	0.41	0.98	0.50	0.54
DW %	8.57	4.80	15.72	6.91



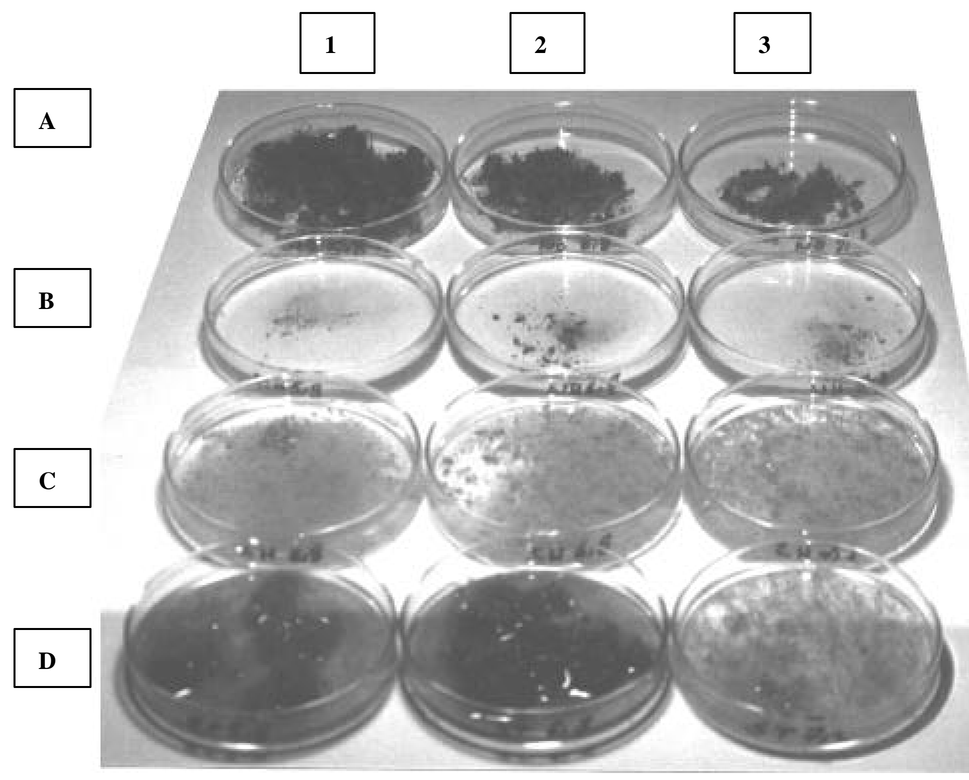


Fig.1 Effect of different pH and nitrogen forms during the realization phase of carrot culture ($B5^-$). Under pH of 5.8 (1), 6.8 (2) and 7.2 (3), using only KNO_3 (A1-A3), $(NH_4)_2SO_4$ (B1-B3), casein hydrolysate (C1-C3) and control (D1-D3).

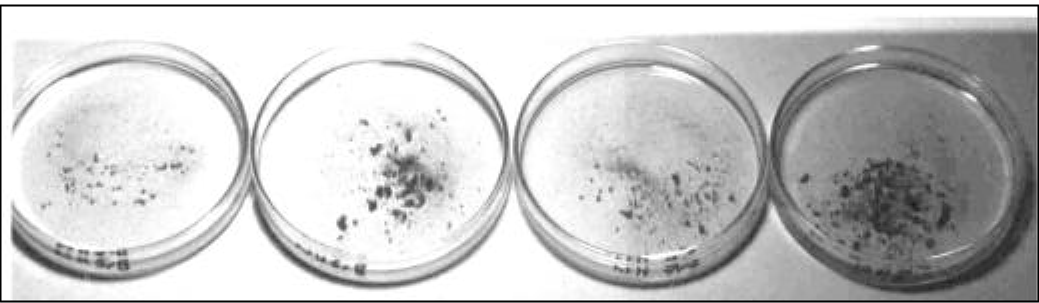
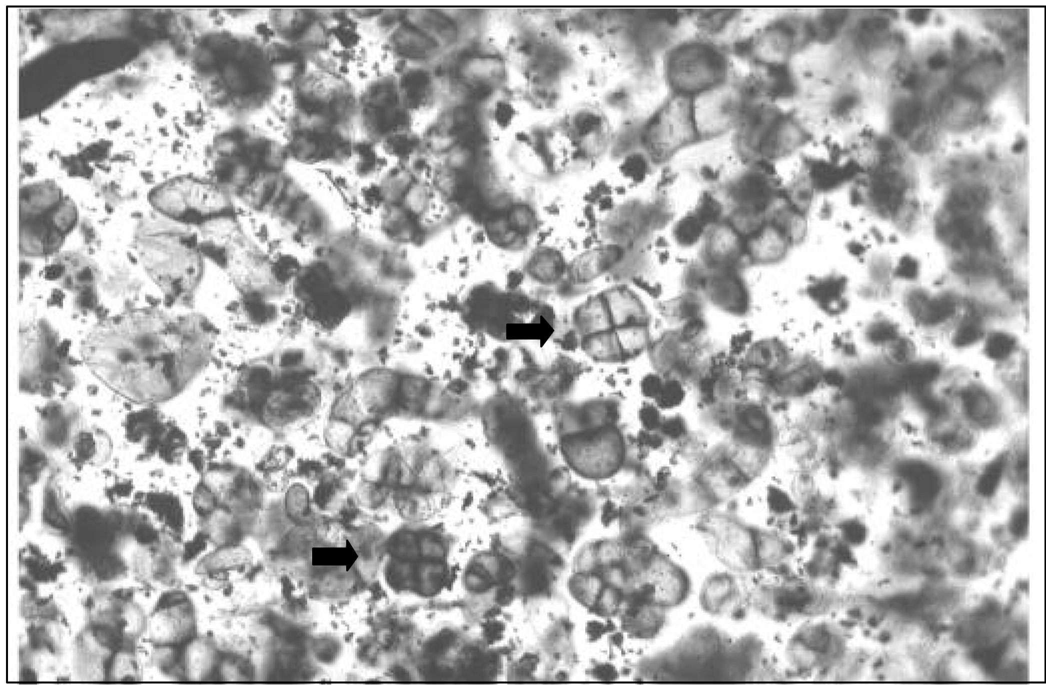


Fig. 2 Effect of different pH (from left to right 5.8, 6.8, 7.2 and 8.0) using 476 ppm N as $(NH_4)_2SO_4$ (33.52 mM) as only source of nitrogen on the realization phase of carrot somatic embryogenesis ($B5^-$).



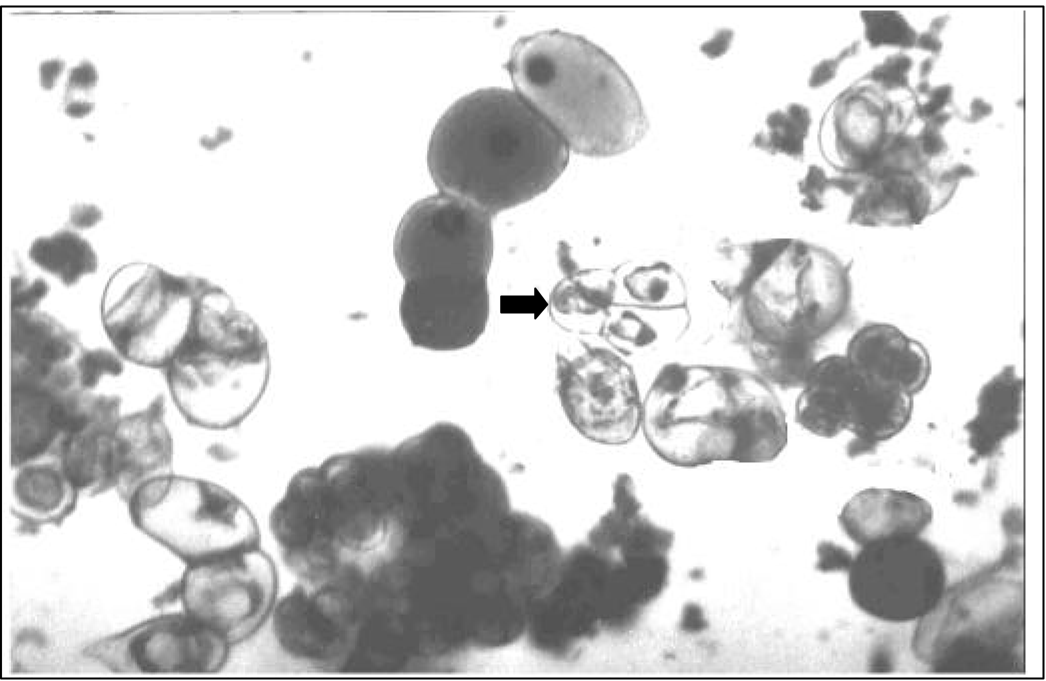
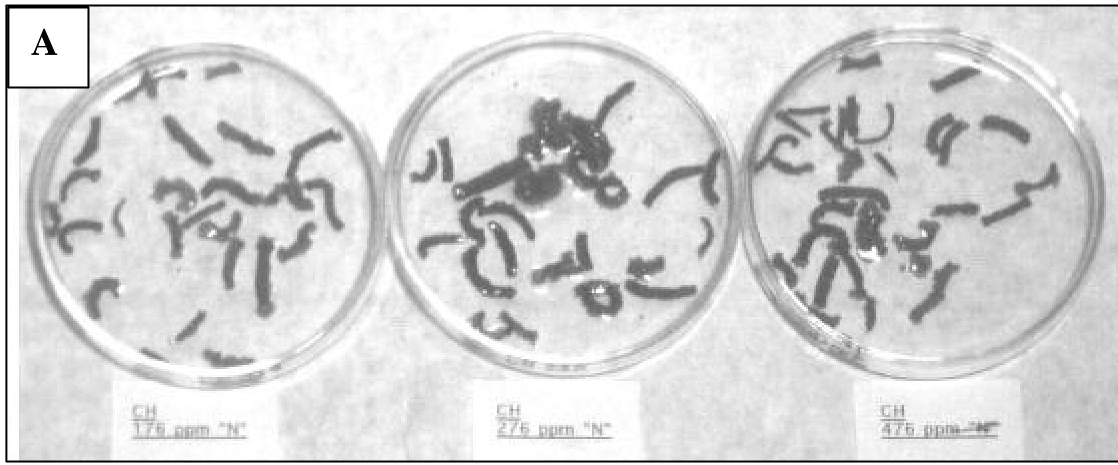
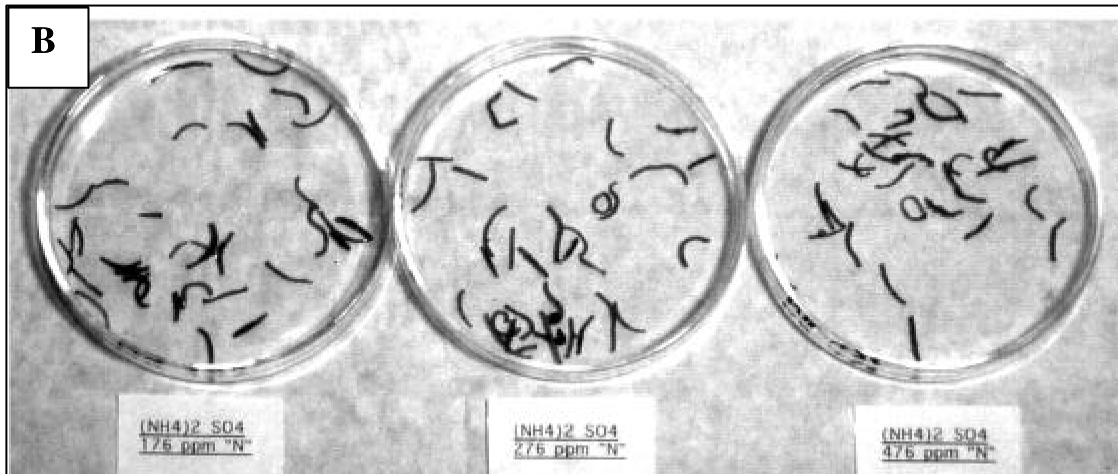


Fig. 3 With neutral red stained carrot suspension culture (B5$^-$) using 476.5 ppm N (33.52 mM) as $(NH_4)_2$ SO_4 as only source of nitrogen. Despite stoppage of embryogenesis some cells (dark cells) are still division active and vital showing tetraoidal stage and an unequal cell division, which is important for the development of the suspensor (arrow).





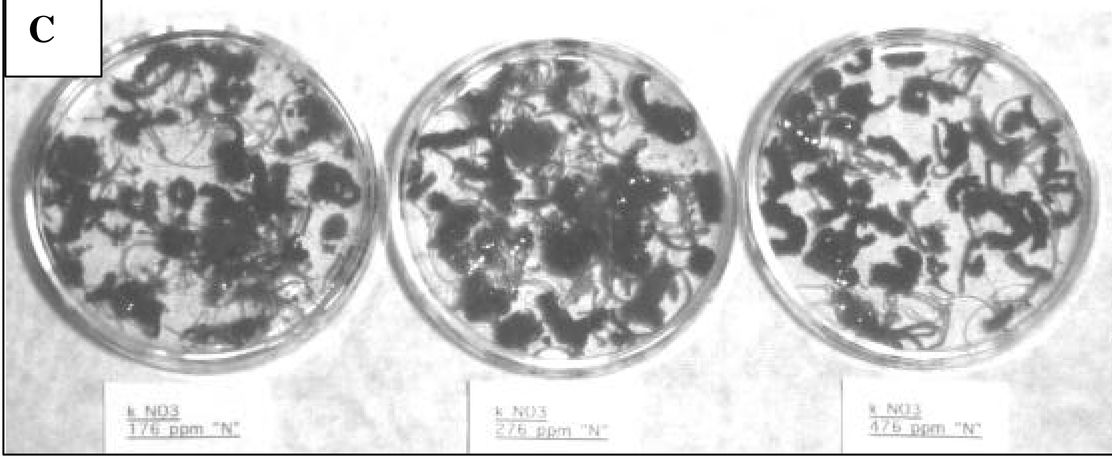
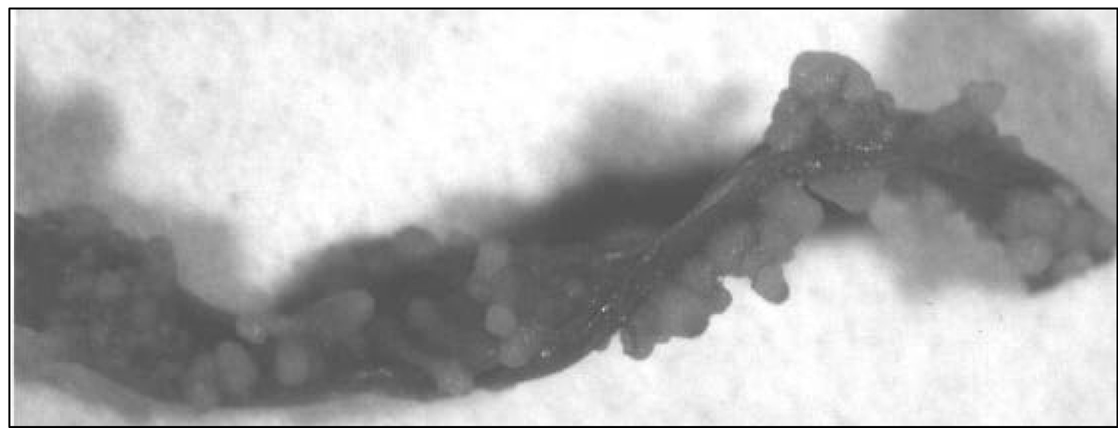
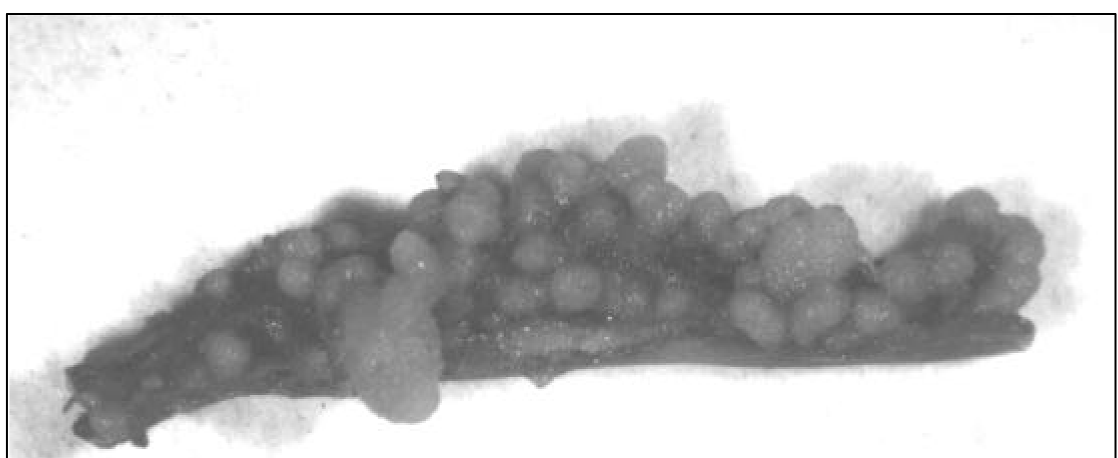


Fig. 1 Twenty one days old carrot petiole culture supplemented (from left to right) with 176, 276 and 476 ppm N as casein hydrolysate (A), $(NH_4)_2$ SO_4 (B) and KNO_3 (C) as only source of nitrogen ($B5^-$).





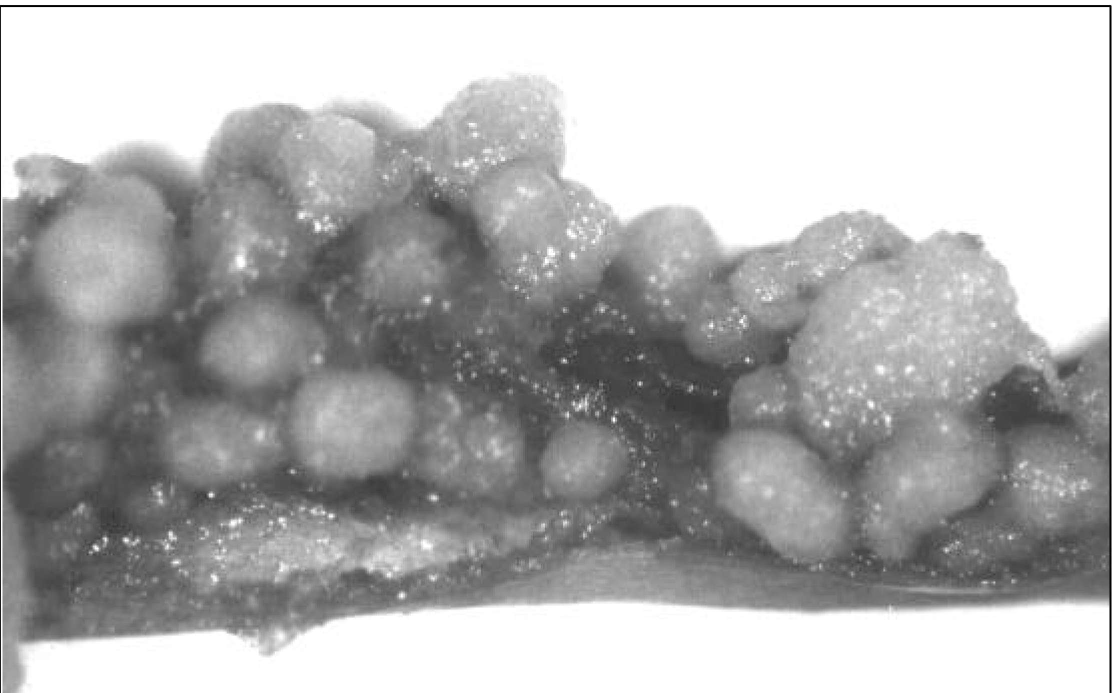


Fig. 2 Carrot petiole culture during the realization phase ($B5^{-}$), supplemented with casein hydrolysate as only source of nitrogen. The growing meristems, mostly globular, have ruptured the petiole epidermis.

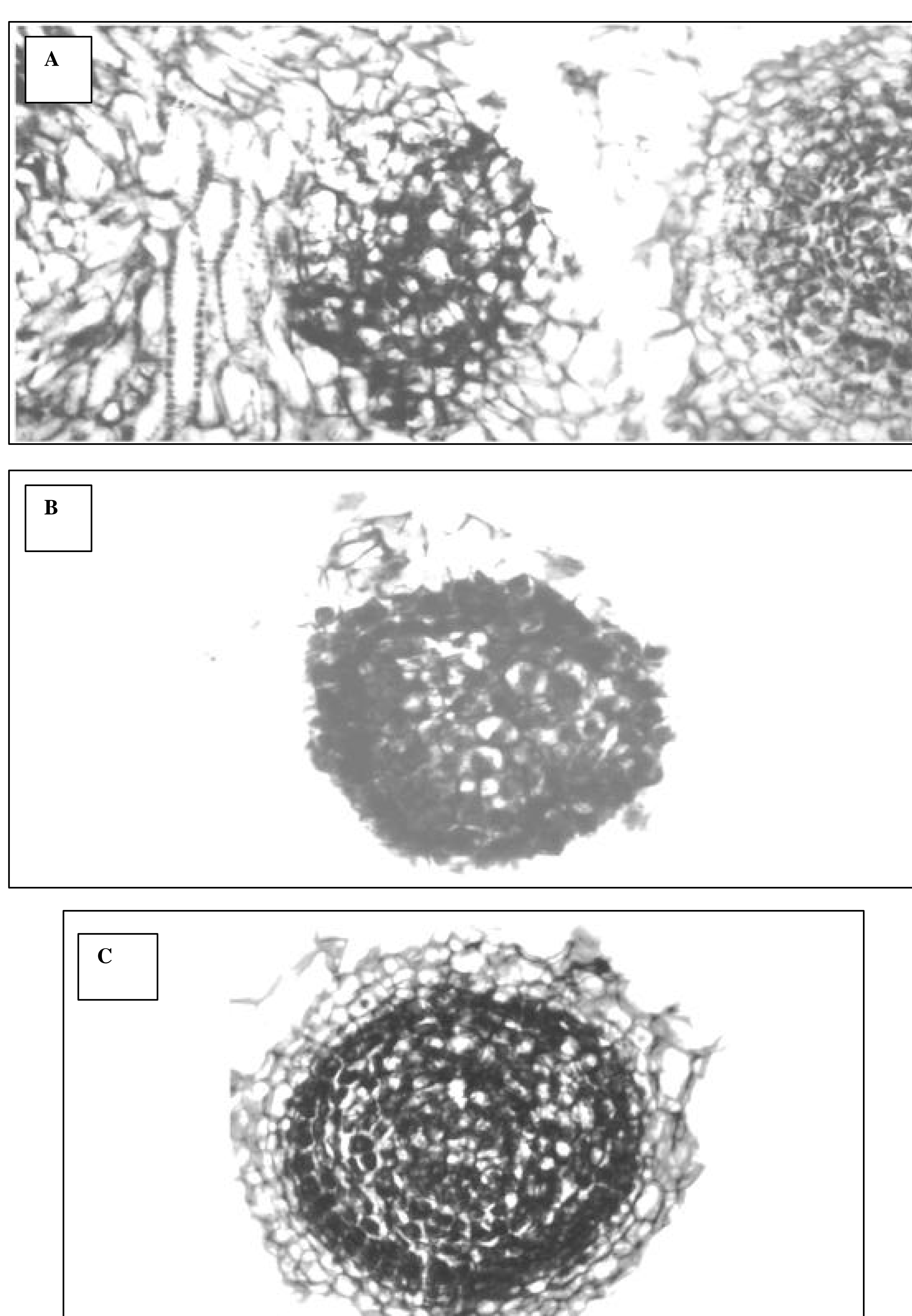


Fig. 3 Section of a rhizogenous meristem (upper left) showing a disarranged aggregate of cells near vascular bundles and globular form (upper right) (A). Section of embryogenic meristem with small cells and large nucleus (B). Section of globular stage showing circular arrangements of cells with large nucleus, surrounded by cytoplasm rich cells (C). Stained with eosin-fast green dye.

Appendix K: Different Nitrogen Concentrations and Forms During the Realization Phase of Somatic Embryogenesis in Carrot Culture.

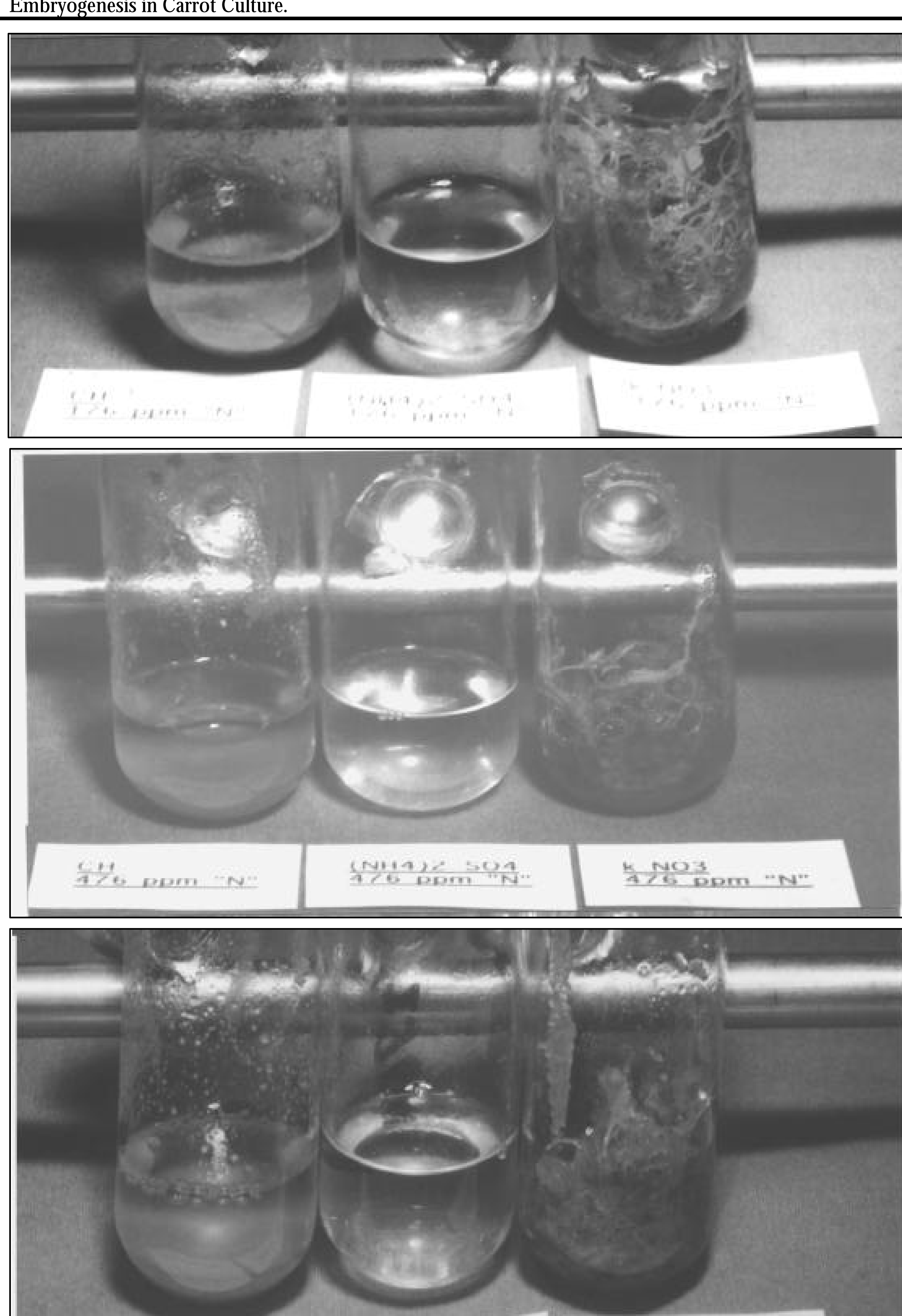


Fig. 4 Effect of nitrogen forms (from left to right, CH, $(NH_4)_2\ SO_4$ and KNO_3) and concentrations (from top to buttom, 476, 276 and 176 ppm) on the realization of carrot somatic embryogenesis.

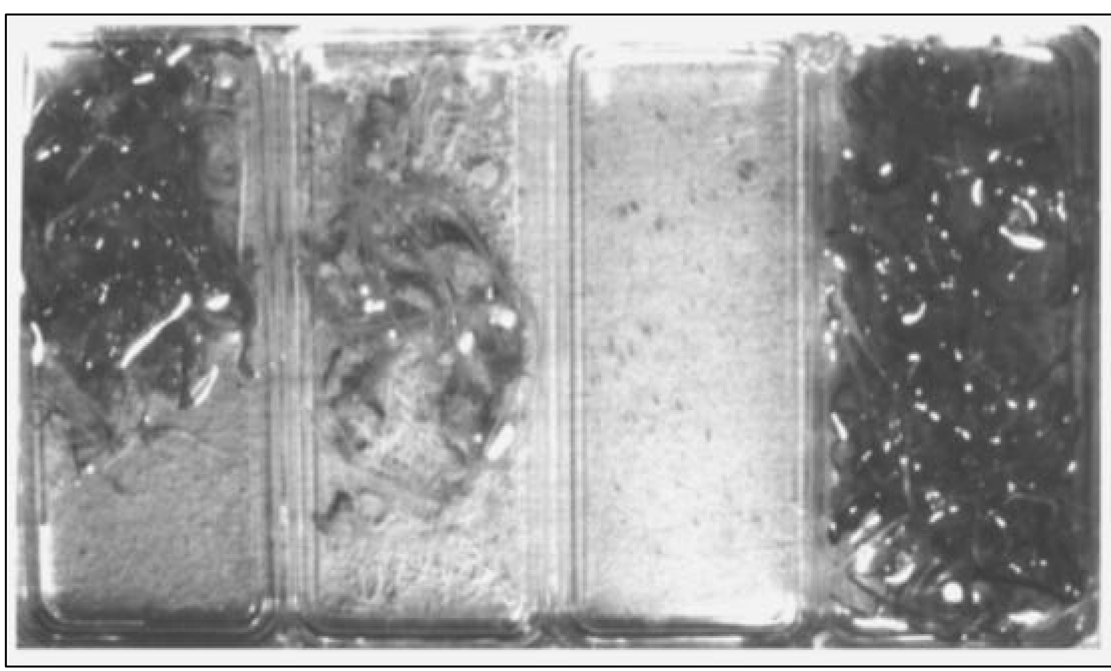


Fig. 5. Effect of nitrogen forms on the realization stage of carrot somatic embryogenesis. From left to right, control, CH, $(NH_4)_2\ SO_4$ and KNO_3.

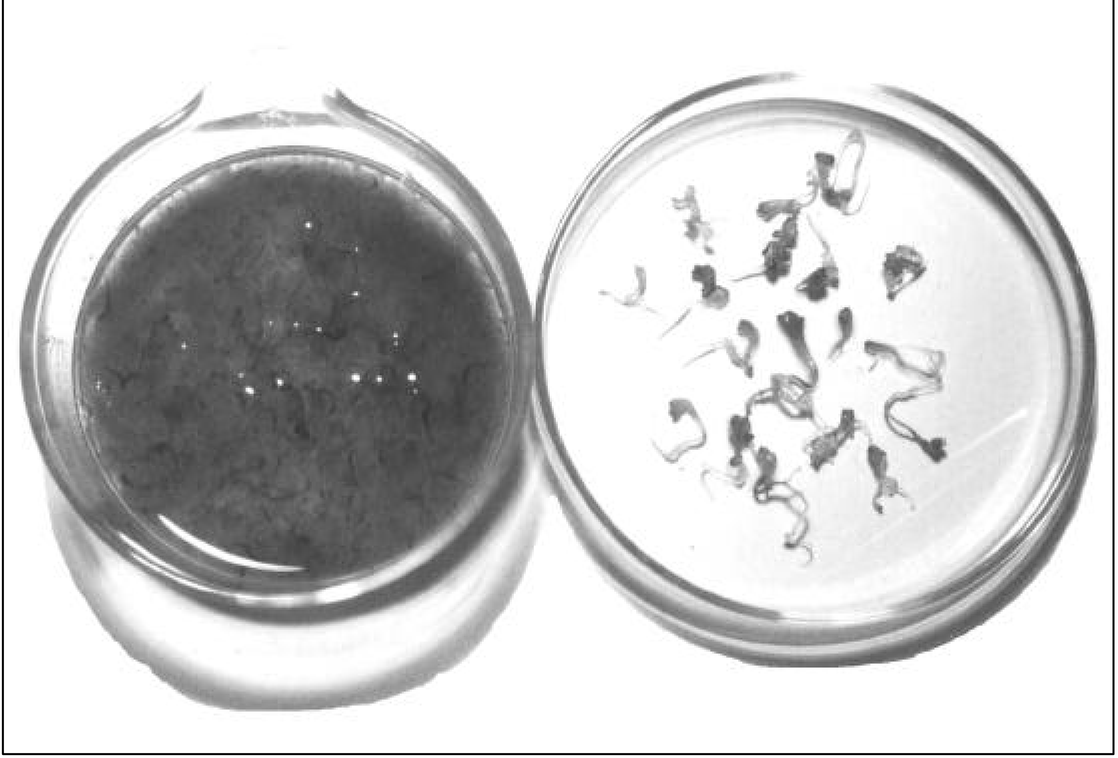


Fig. 6 Realization of somatic embryogenesis leading to formation of mature embryo and plantlet using 476 ppm KNO_3 as only source of nitrogen.

Appendix L: Pattern of Somatic Embryogenesis using 476 ppm Casein Hydrolysate as Only Source of Nitrogen Form During Realization phase of Carrot Cell Suspension Culture.

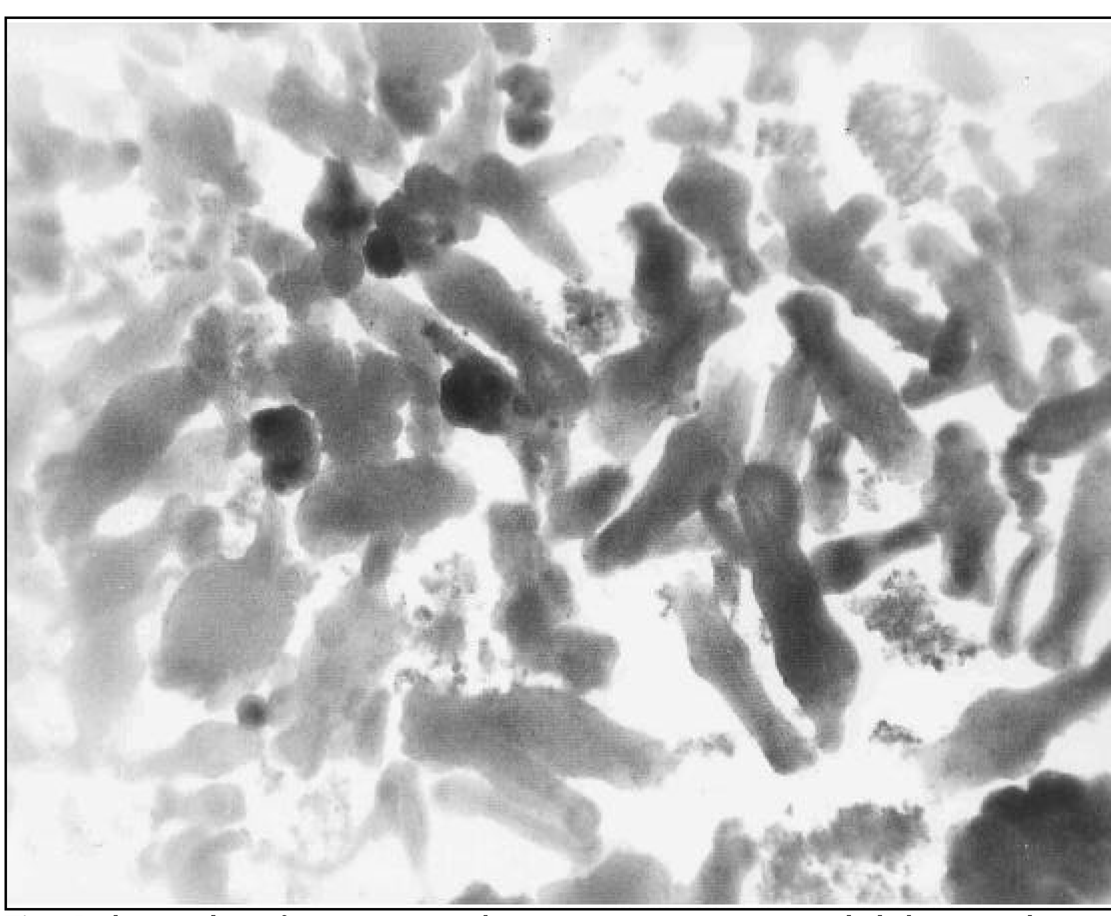


Fig. 1 Realization phase of carrot somatic embryogenesis using 476 ppm casein hydrolysate as only source of nitrogen. Culture proceeds only up to late torpedo stage (115 days in an auxin-free medium).

Fig. 2 Torpedoes formed during realization phase of carrot somatic embryogenesis using casein hydrolysate as only source of nitrogen (115 days in an auxin-free medium).

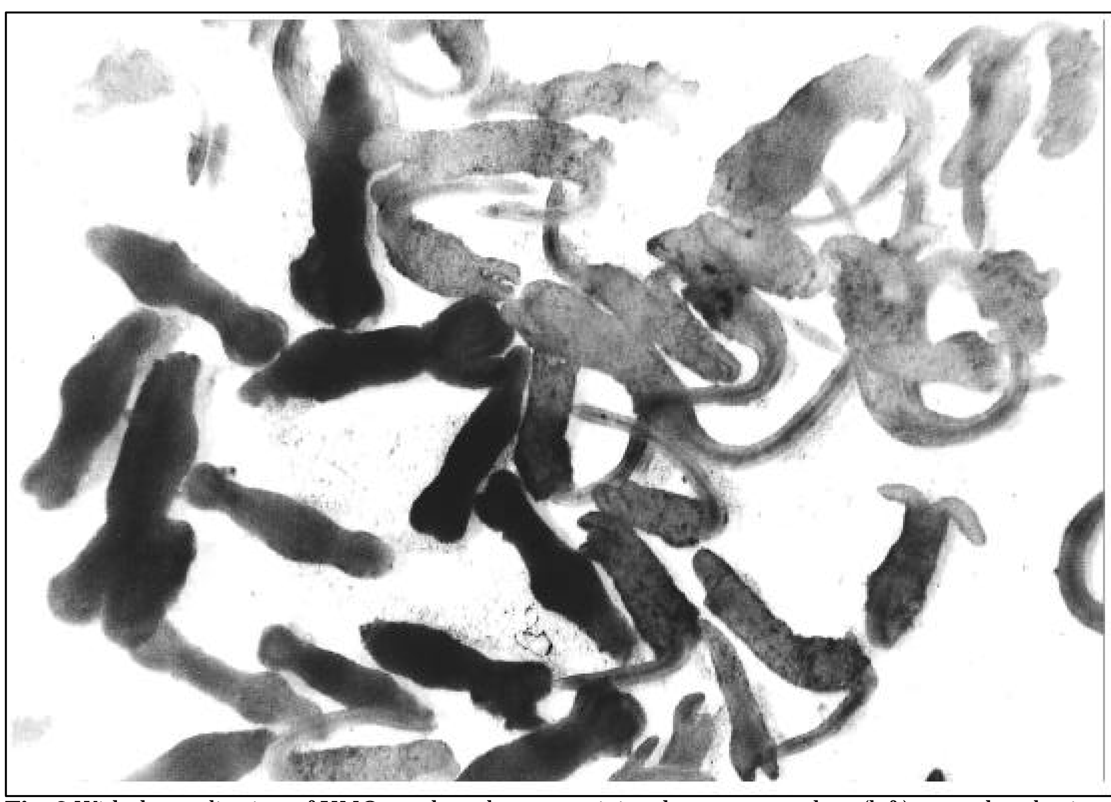


Fig. 3 With the application of KNO_3 to the culture containing dormant torpedoes (left), torpedoes begin to develop further after five days and give rise to plantlets (right).

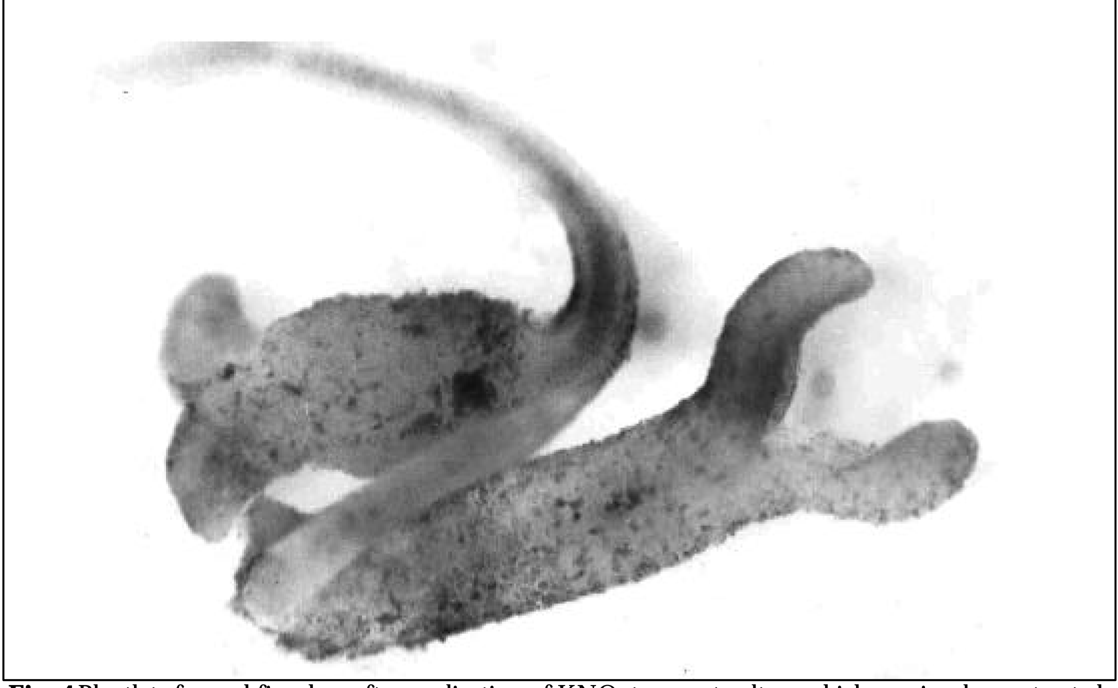


Fig. 4 Plantlets formed five days after application of KNO_3 to carrot culture which previously was treated with casein hydrolisate as only source of nitrogen.

Fig. 5 Young plants grown from a carrot culture which during the realization phase was supplemented with casein hydrolysate as only source of nitrogen (115 days in B5-), later treated for five days with KNO_3 to break the torpedo dormancy and finally transferred to agar (10 days on agar).

SWISS-2DPAGE RELEASE 11.0

24 November 1999

SWISS-2DPAGE

SWISS-2DPAGE is an annotated two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) database established in 1993 and maintained collaboratively by the Central Clinical Chemistry Laboratory of the Geneva University Hospital and the Swiss Institute of Bioinformatics (SIB).

The SWISS-2DPAGE database assembles data on proteins identified on various 2-D PAGE maps. Each SWISS-2DPAGE entry contains textual data on one protein, including mapping procedures, physiological and pathological information, experimental data (isoelectric point, molecular weight, amino acid composition) and bibliographical references. In addition to this textual data, SWISS-2DPAGE provides several 2-D PAGE images showing the experimentally determined location of the protein, as well as a theoretical region computed from the sequence protein, indicating where the protein might be found in the gel.
Cross-references are provided to Medline and other federated 2-DE databases (ECO2DBASE, HSC-2DPAGE, PHCI-2DPAGE, SIENA-2DPAGE, YEPD) and to SWISS-PROT, which provides many links to other molecular databases (EMBL, Genbank, PROSITE, OMIM, etc).

The protein entries in SWISS-2DPAGE are text files structured in a format similar to the one used in SWISS-PROT (for details see user manual).

For detailed information specific to the current SWISS-2DPAGE release, see the release notes.

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SWISS-PROT contains sequences translated from the EMBL Nucleotide Sequence Database, prepared by the European Bioinformatics Institute. For a recent reference see: Stoesser G., Tuli M.A., Lopez R. and Sterk P.; Nucleic Acids Res. 27:18-24(1999).

A small part of the information in SWISS-PROT was originally adapted from information contained in the Protein Sequence Database of the Protein Information Resource (PIR) supported by the Division of Research Resources of the NIH, National Biomedical Research Foundation, Georgetown University Medical Center, 3900 Reservoir road, N.W., Washington, D.C. 20007, U.S.A.
For a recent reference see: Barker W.C., Garavelli J.S., McGarvey P.B., Marzec C.R., Orcutt B.C., Srinivasarao G.Y., Yeh L.S.L, Ledley R.S., Mewes H.-W., Pfeiffer F., Tsugita A. and Wu C.; Nucleic Acids Res. 27:39-43(1999).

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HOW TO SUBMIT DATA OR UPDATES/CORRECTIONS TO SWISS-PROT

To submit new sequence data to SWISS-PROT and for all queries regarding the submission of SWISS-PROT one should contact:

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The EMBL Outstation - The European Bioinformatics Institute
Wellcome Trust Genome Campus
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Telephone: (+44 1223) 494 462
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E-mail: datasubs@ebi.ac.uk (for submission);
datalib@ebi.ac.uk (for enquiries)

To submit updates and/or corrections to SWISS-PROT you can either use the E-mail address: swiss-prot@expasy.ch or the WWW address:

http://www.expasy.ch/sprot/sp_update_form.html

CITATION

If you want to cite SWISS-PROT in a publication, please use the following reference:

Bairoch A. and Apweiler R.
The SWISS-PROT protein sequence data bank and its supplement TrEMBL in 1999.
Nucleic Acids Res. 27:49-54(1999).

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1) What is SWISS-PROT?

2) Conventions used in the database

2.1 General structure of the database
2.2 Classes of data
2.3 Structure of a sequence entry

3) The different line types

3.1 The ID line
3.2 The AC line
3.3 The DT line
3.4 The DE line
3.5 The GN line
3.6 The OS line
3.7 The OG line
3.8 The OC line
3.9 The reference (RN, RP, RC, RX, RA, RT, RL) lines
3.10 The CC line
3.11 The DR line
3.12 The KW line
3.13 The FT line
3.14 The SQ line
3.15 The sequence data line
3.16 The // line

(1). WHAT IS SWISS-PROT?

SWISS-PROT is an annotated protein sequence database. It was established in 1986 and maintained collaboratively, since 1987, by the group of Amos Bairoch first at the Department of Medical Biochemistry of the University of Geneva and now at the Swiss Institute of Bioinformatics (SIB) and the EMBL Data Library (now the EMBL Outstation - The European Bioinformatics Institute (EBI)). The SWISS-PROT protein sequence database consists of sequence entries. Sequence entries are composed of different line types, each with their own format. For

standardization purposes the format of SWISS-PROT follows as closely as possible that of the EMBL Nucleotide Sequence Database.

The SWISS-PROT database distinguishes itself from other protein sequence databases by four distinct criteria:

a) Annotation

In SWISS-PROT, as in most other sequence databases, two classes of data can be distinguished: the core data and the annotation.

For each sequence entry the core data consists of:

- *The sequence data;*
- *The citation information (bibliographical references);*
- *The taxonomic data (description of the biological source of the protein).*

The annotation consists of the description of the following items:

- *Function(s) of the protein;*
- *Post-translational modification(s). For example carbohydrates, phosphorylation, acetylation, GPI-anchor, etc.;*
- *Domains and sites. For example calcium binding regions, ATP-binding sites, zinc fingers, homeoboxes, SH2 and SH3 domains, kringle, etc.;*
- *Secondary structure. For example alpha helix, beta sheet, etc.;*
- *Quaternary structure. For example homodimer, heterotrimer, etc.;*
- *Similarities to other proteins;*
- *Disease(s) associated with deficiencie(s) in the protein;*
- *Sequence conflicts, variants, etc.*

We try to include as much annotation information as possible in SWISS-PROT. To obtain this information we use, in addition to the publications that report new sequence data, review articles to periodically update the annotations of families or groups of proteins. We also make use of external experts, who have been recruited to send us their comments and updates concerning specific groups of proteins.

We believe that our having systematic recourse both to publications other than those reporting the core data and to subject referees represents a unique and beneficial feature of SWISS-PROT.

In SWISS-PROT, annotation is mainly found in the comment lines (CC), in the feature table (FT) and in the keyword lines (KW). Most comments are classified by 'topics'; this approach permits the easy retrieval of specific categories of data from the database.

b) Minimal redundancy

Many sequence databases contain, for a given protein sequence, separate entries which correspond to different literature reports. In SWISS-PROT we try as much as possible to merge all these data so as to minimize the redundancy of the database. If conflicts exist between various sequencing reports, they are indicated in the feature table of the corresponding entry.

c) Integration with other databases

It is important to provide the users of biomolecular databases with a degree of integration between the three types of sequence-related atabases (nucleic acid sequences, protein sequences and protein tertiary structures) as well as with specialized data collections. SWISS-PROT is currently cross-referenced with about 30 different databases. Cross-references are provided in the form of pointers to information related to SWISS-PROT entries and found in data collections other than SWISS-PROT.
This extensive network of cross-references allows SWISS-PROT to play a major role as a focal point of biomolecular database interconnectivity.

d) Documentation

SWISS-PROT is distributed with a large number of index files and specialized documentation files. Some of these files have been available for a long time (this user manual, the release notes, the various indices for authors, citations, keywords, etc.), but many have been created recently and we are continuously adding new files. The release notes contain an up to date descriptive list of all distributed document files.

(2). CONVENTIONS USED IN THE DATABASE

The following sections describe the general conventions used in SWISS-PROT to achieve uniformity of presentation. Experienced users of the EMBL Database can skip these sections and directly refer to Appendix C, which lists the minor differences in format between the two data collections.

(2.1). General structure of the database

The SWISS-PROT protein sequence database is composed of sequence entries. Each entry corresponds to a single contiguous sequence as contributed to the bank or reported in the literature. In some cases, entries have been assembled from several papers that report overlapping sequence regions. Conversely, a single paper can provide data for several entries, e.g. when related sequences from different organisms are reported.

References to positions within a sequence are made using sequential numbering, beginning with 1 at the N-terminal end of the sequence.

Except for initiator N-terminal methionine residues, which are not included in a sequence when their absence from the mature sequence has been proven, the sequence data correspond to the precursor form of a protein before post-translational modifications and processing.

(2.2). Classes of data

In order to attempt to make data available to users as quickly as possible after publication, SWISS-PROT is now distributed with a supplement called TrEMBL, where entries are released before all their details are finalized. To distinguish between fully annotated entries and those in TrEMBL, the 'class' of each entry is indicated on the first (ID) line of the entry. The two defined classes are:

STANDARD Data which are complete to the standards laid down by the SWISS-PROT database.

PRELIMINARY Sequence entries which have not yet been annotated by the SWISS-PROT staff up to the standards laid down by SWISS-PROT. These entries are exclusively found in TrEMBL.

(2.3). Structure of a sequence entry

The entries in the SWISS-PROT database are structured so as to be usable by human readers as well as by computer programs. The explanations, descriptions, classifications and other comments are in ordinary English.
Wherever possible, symbols familiar to biochemists, protein chemists and molecular biologists are used.

Each sequence entry is composed of lines. Different types of lines, each with their own format, are used to record the various data that make up the entry. A sample sequence entry is shown below.

```
ID   GRAA_HUMAN     STANDARD;      PRT;   262 AA.
AC   P12544;
DT   01-OCT-1989 (Rel. 12, Created)
DT   01-OCT-1989 (Rel. 12, Last sequence update)
DT   15-DEC-1998 (Rel. 37, Last annotation update)
DE   GRANZYME A PRECURSOR (EC 3.4.21.78) (CYTOTOXIC T-
LYMPHOCYTE PROTEINASE
DE   1) (HANUKKAH FACTOR) (H FACTOR) (HF) (GRANZYME 1) (CTL
TRYPTASE)
DE   (FRAGMENTIN 1).
GN   GZMA OR CTLA3 OR HFSP.
OS   Homo sapiens (Human).
OC   Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia;
Eutheria;
OC   Primates; Catarrhini; Hominidae; Homo.
RN   [1]
RP   SEQUENCE FROM N.A.
RC   TISSUE=T-CELL;
RX   MEDLINE; 88125000.
```

RA   GERSHENFELD H.K., HERSHBERGER R.J., SHOWS T.B., WEISSMAN I.L.;
RT   "Cloning and chromosomal assignment of a human cDNA encoding a T
RT   cell- and natural killer cell-specific trypsin-like serine
RT   protease.";
RL   Proc. Natl. Acad. Sci. U.S.A. 85:1184-1188(1988).
RN   [2]
RP   SEQUENCE OF 29-53.
RX   MEDLINE; 88330824.
RA   POE M., BENNETT C.D., BIDDISON W.E., BLAKE J.T., NORTON G.P.,
RA   RODKEY J.A., SIGAL N.H., TURNER R.V., WU J.K., ZWEERINK H.J.;
RT   "Human cytotoxic lymphocyte tryptase. Its purification from granules
RT   and the characterization of inhibitor and substrate specificity.";
RL   J. Biol. Chem. 263:13215-13222(1988).
RN   [3]
RP   SEQUENCE OF 29-40, AND CHARACTERIZATION.
RX   MEDLINE; 89009866.
RA   HAMEED A., LOWREY D.M., LICHTENHELD M., PODACK E.R.;
RT   "Characterization of three serine esterases isolated from human IL-2
RT   activated killer cells.";
RL   J. Immunol. 141:3142-3147(1988).
RN   [4]
RP   SEQUENCE OF 29-39, AND CHARACTERIZATION.
RX   MEDLINE; 89035468.
RA   KRAEHENBUHL O., REY C., JENNE D.E., LANZAVECCHIA A., GROSCURTH P.,
RA   CARREL S., TSCHOPP J.;
RT   "Characterization of granzymes A and B isolated from granules of
RT   cloned human cytotoxic T lymphocytes.";
RL   J. Immunol. 141:3471-3477(1988).
RN   [5]
RP   3D-STRUCTURE MODELING.
RX   MEDLINE; 89184501.
RA   MURPHY M.E.P., MOULT J., BLEACKLEY R.C., GERSHENFELD H.,
RA   WEISSMAN I.L., JAMES M.N.G.;
RT   "Comparative molecular model building of two serine proteinases from
RT   cytotoxic T lymphocytes.";
RL   Proteins 4:190-204(1988).
CC   -!- FUNCTION: THIS ENZYME IS NECESSARY FOR TARGET CELL LYSIS IN CELL-
CC   MEDIATED IMMUNE RESPONSES. IT CLEAVES AFTER LYS OR ARG. MAY BE
CC   INVOLVED IN APOPTOSIS.
CC   -!- CATALYTIC ACTIVITY: HYDROLYSIS OF PROTEINS, INCLUDING FIBRONECTIN,
CC   TYPE IV COLLAGEN AND NUCLEOLIN. PREFERENTIAL CLEAVAGE: ARG-|-XAA,
CC   LYS-|-XAA >> PHE-|-XAA IN SMALL MOLECULE SUBSTRATES.
CC   -!- SUBUNIT: HOMODIMER, DISULFIDE-LINKED.
CC   -!- SUBCELLULAR LOCATION: CYTOPLASMIC GRANULES.

```
CC   -!- SIMILARITY: BELONGS TO PEPTIDASE FAMILY S1; ALSO KNOWN
CC   -!- AS THE TRYPSIN FAMILY. STRONGEST TO OTHER GRANZYMES
CC   -!- AND TO MAST CELL PROTEASES.
CC   -!- ------------------------------------------------------
CC   This SWISS-PROT entry is copyright. It is produced through
CC   a collaboration between  the Swiss Institute of
CC   Bioinformatics  and the  EMBL outstation - the European
CC   Bioinformatics Institute.  There are no  restrictions on
CC   its use  by  non-profit  institutions as long  as its
CC   content  is  in  no  way modified and this statement is
CC   not removed.  Usage  by  and for commercial entities CC
CC   requires a license agreement
CC   (See http://www.isb-sib.ch/announce/
CC   or send an email to license@isb-sib.ch).
CC   ---------------------------------------------------------
DR   EMBL; M18737; AAA52647.1; -.
DR   PIR; A28943; A28943.
DR   PIR; A30525; A30525.
DR   PIR; A30526; A30526.
DR   PIR; A31372; A31372.
DR   PDB; 1HF1; 15-OCT-94.
DR   MIM; 140050; -.
DR   PROSITE; PS00134; TRYPSIN_HIS; 1.
DR   PROSITE; PS00135; TRYPSIN_SER; 1.
DR   PFAM; PF00089; trypsin; 1.
KW   Hydrolase; Serine protease; Zymogen; Signal; T-cell;
Cytolysis;
KW   Apoptosis; 3D-structure.
FT   SIGNAL         1      26
FT   PROPEP        27      28        ACTIVATION PEPTIDE.
FT   CHAIN         29     262        GRANZYME A.
FT   ACT_SITE      69      69        CHARGE RELAY SYSTEM (BY
SIMILARITY).
FT   ACT_SITE     114     114        CHARGE RELAY SYSTEM (BY
SIMILARITY).
FT   ACT_SITE     212     212        CHARGE RELAY SYSTEM (BY
SIMILARITY).
FT   DISULFID      54      70        BY SIMILARITY.
FT   DISULFID     148     218        BY SIMILARITY.
FT   DISULFID     179     197        BY SIMILARITY.
FT   DISULFID     208     234        BY SIMILARITY.
FT   CARBOHYD     170     170        POTENTIAL.
SQ   SEQUENCE   262 AA;  28968 MW;  34E965D7 CRC32;
     MRNSYRFLAS SLSVVVSLLL IPEDVCEKII GGNEVTPHSR PYMVLLSLDR
KTICAGALIA
     KDWVLTAAHC NLNKRSQVIL GAHSITREEP TKQIMLVKKE FPYPCYDPAT
REGDLKLLQL
     TEKAKINKYV TILHLPKKGD DVKPGTMCQV AGWGRTHNSA SWSDTLREVN
ITIIDRKVCN
     DRNHYNFNPV IGMNMVCAGS LRGGRDSCNG DSGSPLLCEG VFRGVTSFGL
ENKCGDPRGP
     GVYILLSKKH LNWIIMTIKG AV
//
```

Each line begins with a two-character line code, which indicates the type of data contained in the line. The current line types and line codes and the order in which they appear in an entry, are shown in the table below.

```
---------  ----------------------------    --------------------
Line code  Content                         Occurrence in an
entry
---------  ----------------------------    --------------------
ID         Identification                  Once; starts the
entry
AC         Accession number(s)             One or more
DT         Date                            Three times
DE         Description                     One or more
GN         Gene name(s)                    Optional
OS         Organism species                One or more
OG         Organelle                       Optional
OC         Organism classification         One or more
RN         Reference number                One or more
RP         Reference position              One or more
RC         Reference comment(s)            Optional
RX         Reference cross-reference(s)    Optional
RA         Reference authors               One or more
RT         Reference title                 Optional
RL         Reference location              One or more
CC         Comments or notes               Optional
DR         Database cross-references       Optional
KW         Keywords                        Optional
FT         Feature table data              Optional
SQ         Sequence header                 Once
           (blanks) sequence data          One or more
//         Termination line                Once; ends the entry
---------  ----------------------------    --------------------

As  shown  in  the above table, some line types are found in
all  entries, others  are optional. Some line types occur many
times in a single  entry.
Each  entry  must begin with an identification line (ID) and
end  with a terminator line (//).
```

ACKNOWLEDGEMENTS

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