

Genomic Instabilities in Tissue Culture – a Physiological Normality?

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Abstract

In the present paper, the general role of *in vitro*-culture systems for fundamental research in plant sciences is stressed. As an example, studies on the effect of physiological stimuli on genome variation are presented. The results of systematic investigations on 37 primary callus culture variants derived from secondary phloem of carrot roots indicated a drastic diminution in repetitive DNA fractions as a predominant change at the total genome level during high cell division growth. Also, RAPD fingerprint analyses confirmed reproducible quantitative polymorphism in primary cultures due to the effects of tissue culture and growth regulators. The general implication of these results could be confirmed by *in vivo* - studies on different tissues and tissues of different ages.

Introduction

From the historical point of view, *in vitro*-culture techniques for plants were developed primarily to facilitate basic physiological research (Haberlandt, 1902). However, the search for commercial applications of these techniques started very early in the sixties and tied up personal and funding capacities in this area, so that fundamental research on *in vitro* cultures, as a complementing system to research on intact plants, became a stepchild in physiological investigations (Neumann, 1999). Nevertheless, *in vitro*-cultures of plant tissues proved to be especially worthwhile in revealing basic aspects of metabolic pathways (e.g. Steward et al., 1952; Gross et al., 1993), of plant growth and development (e.g. Grieb et al., 1994) and of differential genome variability (e.g. Nagl 1976, 1979, 1992; Schaefer et al., 1978; Duehrssen and Neumann, 1980; Cullis, 1987; Bassi, 1990; Arnholdt-Schmitt,

1995; Arnholdt-Schmitt et al., 1995; Bogani et al., 1996; Schaefer et al., 2000). In the present paper, recent results on physiological and differential genome variation obtained with the help of well-defined primary tissue cultures and analytical procedures using restriction enzymes and RAPD fingerprinting strategies will be given. Additionally, in vivo investigations on different tissues and tissues of different ages will be presented to demonstrate, as an example, the general relevance of results achieved by the integration of in vitro – cultures as model systems in basic research.

Material and Methods

Plant material and primary callus culture

Carrot plants from the local market and of the cultivars Rote Riesen, Rotin, Lobbericher and Pariser Markt were used. For in vivo analyses, parts of the xylem and secondary phloem tissue of individual plants were isolated from the tap root. Blades of young and older leaves were harvested separately. For in vitro experiments, primary callus cultures were initiated from the secondary phloem of carrot roots taken from plants of different ages. 5 explants of 2 to 4 mg isolated from the secondary phloem next to the cambial ring were cultured in liquid medium (Neumann, 1966) in T-tubes (Steward et al., 1952) which were continuously rotated at 1 rpm under permanent illumination (approximately 4000 lux, osmium lumilux white) at 21°C and 28°C.

DNA isolation and restriction analysis

High-molecular weight DNA was extracted employing the method described by Murray and Thompson (1980) which was later simplified by Power et al. (1986). Removal of RNA was obtained by treatment with ribonuclease A and T1. Further purification of the extracts was achieved by using chloroform-isoamylalcohol. Quantification of the DNA was performed chemically employing the diphenylamine reaction (Richard, 1974). Genomic DNA was digested completely by the methylation-insensitive restriction enzyme *Bst*NI (Biozym diagnostic) using 3 to 4 U/ μ g DNA for at least 4 hours. The recognition sequence is 5'-CC(AT)GG. Electrophoresis was performed with 2 to 3 μ g of DNA fragments in 1 % agarose (Bio-Rad Standard Low-*Mr*) for 15 hours (50 V at 15 °C) and results were documented photographically (Kodak Tri-X Pan, 400 ASA) using a transilluminator and light of 302 nm. Scanning of the restriction fragments was carried out on the negatives. To level out differences between the amount of DNA in the different variants with the amount actually loaded onto the gel, the integral of the sum of all fragments distributed as bands and background in the gel of one variant was corrected to the total integral of the DNA fragments of the compared variant.

DNA isolation and RAPD fingerprinting

DNA isolation for RAPD analyses was performed by employing the DNeasy Plant Mini Kit from Qiagen (Hilden, Germany). Extracted DNA was checked for quality and quantity with the help of a video densitometer in comparison to lambda DNA in 1 % agarose. 5 to 10 ng DNA was used for RAPD analysis in 25 reaction volumes. The PCR was performed with RAPD analysis kits (Ready-to-go RAPD beads, Amersham Pharmacia Biotech, Freiburg, Germany) in a Techne thermocycler (model Progene). 10mer random primers were obtained from Roth (Karlsruhe, Germany) or from Amersham Pharmacia Biotech (Freiburg, Germany). Conditions for the PCR reaction were as follows: 5 min at 95 °C, 45 cycles at 95 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min; for final extension 5 min at 72 °C. RAPD fragments were separated in 1.5 % agarose (Gibco-BRL). The method applied was carefully checked for good repeatability which included repeated extractions, PCRs and electrophoreses, the use of various thermocyclers and the effect of different personnel working with the method.

Results

Digestion of the DNA of the secondary phloem of carrot roots by the restriction enzyme *Bst*NI characterizes the *Daucus carota* genome by a distinctive pattern of fragments. As shown by the video densitometric scan of the DNA of cv. Rote Riesen in Fig. 1A, at least 19 bands of repetitive fragments can be distinguished. Analysis of thirty-one individual plant genomes, including plants of cv. Rote Riesen, cv. Rotin, cv. Lobbericher, cv. Pariser Markt and an unknown genotype from the local market displayed the same pattern of restriction fragments, indicating a general characteristic of the *Daucus carota* genome (data not shown, see Arnholdt-Schmitt, 1993). Initiation of primary cultures from explants of the secondary root phloem of various carrot genotypes at different ages in the presence of myo-inositol (0.28 mM), IAA (11.42 µM) and kinetin (0.47 µM) at 21°C and 28°C induced callus growth, that was mainly due to cell division activity ($r = 0.81$). The mass of actually produced callus varied significantly between 14.2 mg and 161.3 mg, which, in a complex manner, was due to different factors that were included in the study (see Arnholdt-Schmitt, 1999). Two weeks after inoculation, a drastic diminution in the intensity of the bands of repetitive fragments was observed, as is shown as an example for cv. Rote Riesen in Fig. 1B. This change in genome organisation was found to take place in all of the 37 investigated growth variants and, as though, seems to occur independently of the carrot genotype, the age of plants used for the experiments, the growth temperature (21°C or 28 °C), and the actual callus growth intensity. This phenomenon, which indicates a transient elimination of repetitive DNA fractions during callus growth induction (for detailed discussion see Arnholdt-Schmitt, 1995), was checked for confirmation by applying RAPD analysis on comparable primary cultures. Fig. 2 shows the RAPD pattern of the genomic DNA of explants isolated from the secondary root phloem of 3 individual plants of an unknown carrot genotype at t_0 and three weeks after initiation of a primary culture. A putative loss of a band of 1570 bp was observed in cultured explants taken from all three

plants. By varying the relationship between primer and template DNA, it could be demonstrated, that the band of 1570 bp was existent at a minor level also in the primary culture, but was below the detection limit, when standard conditions were applied (data not shown; for method see Schaffer and Arnholdt-Schmitt, 2001; Schaffer, 1999; Schäfer et al., 2000). Fig. 3 indicates, that growth regulators may be involved in the induction of polymorphism in genomic DNA. At the 14th day of primary culture, a distinctive, amplified fragment of 976 bp was clearly visible, if secondary root phloem explants were cultured without any growth regulators or with myo-inositol and IAA only, but was not evident any longer, when kinetin was supplied in addition to myo-inositol and IAA. By varying the reaction conditions for primer and template DNA concentration, this putative loss of a fragment could again be deduced to a merely quantitative effect on the number of fragments produced by the chosen primer (data not shown, see Schäfer et al., 2000).

To analyse, whether this kind of quantitative polymorphism in the genome, which was induced by the defined *in vitro*-culture conditions and which was accompanied by a change of the physiological and differentiatonal state of the tissue, would be significant also under *in vivo* conditions, different tissues of carrot plants as well as leaves of different age were analysed by RAPD fingerprinting. Fig. 4 demonstrates the pattern of amplified fragments produced by primer OP-G06 of the DNA of xylem tissue (lane 1) and of the secondary phloem (lane 2) of carrot roots, the blade of young leaves (lane 3), the blade of older leaves (lane 4) and a mixture of petioles from younger and older leaves (lane 5). The RAPD profiles of the root tissues (xylem and secondary phloem) were identical regarding the occurrence as well as the intensity of bands. This could also be confirmed by additional primers (data not shown, see Schäfer, 1999). By comparing these patterns of the root material with that of the leaves, a fragment of 1050 bp (white arrow) is visibly lost in older leaves and remarkably diminished in its amount in young leaves as compared to the root tissues. In repetitive experiments it became clear that, in fact, the putative loss of the 1050 bp fragment in older leaves was based again on a quantitative polymorphism, which obviously kept the amplified fragment from older leaves around the detection limit. Additionally, 5 bands of the DNA of older leaves display a significantly reduced intensity (black arrows) also in comparison to younger leaves, whereas two fragments show a higher rate of amplification (white circles) in older leaves. Band intensities in the pattern of the petioles may serve as a control variant for the method, since this sample was a mixture of younger and older tissues which is also mirrored by intermediate band intensities in comparison to younger and older leaves. Without the necessity of screening a great number of primers, this tendency of decreased band intensities in the fingerprints of older leaves was easily confirmed by the use of only some additional primers (data not shown, see Schäfer, 1999).

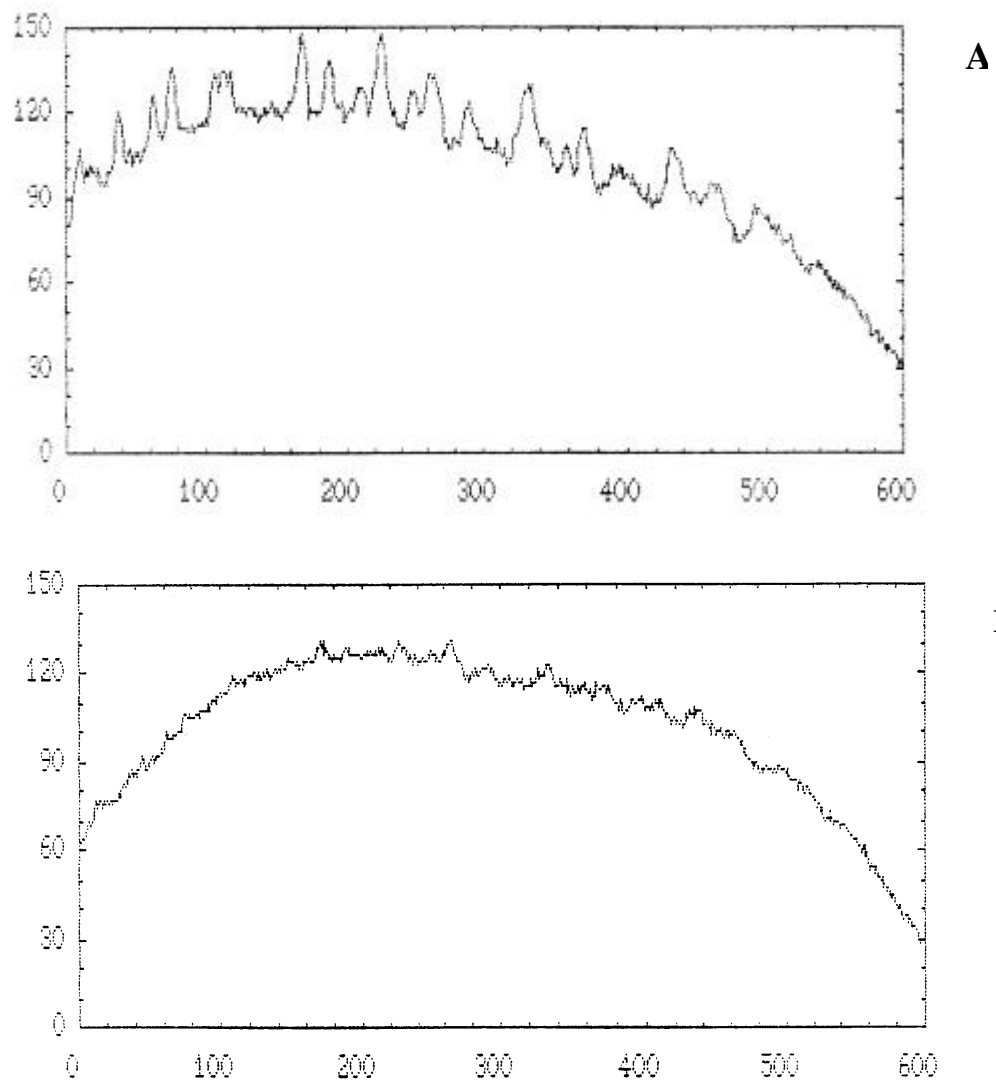


Figure 1: Restriction enzyme analysis with *Bst*NI of the genomic DNA of secondary root phloem explants of cv. Rote Riesen at t_0 (**A**) and 14 days after primary culture induction in the presence of myo-inositol, IAA and kinetin (**B**)

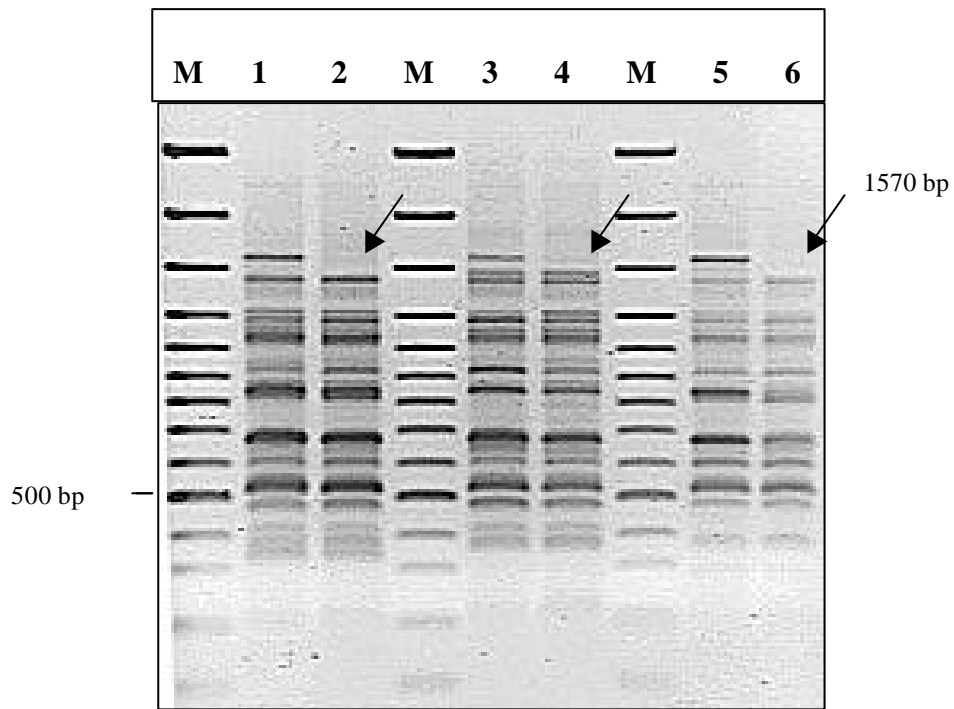


Figure 2: RAPD fingerprints of carrot secondary root phloem explants isolated from three individual plants at t₀ (lane 1, 3, 5) and 21 days after inoculation (lane 2, 4, 6). M = marker. Primer: OP-D08 5'-GTGTGCCCA-3' (see Schaffer, 1999 and Schäfer et al., 2000)

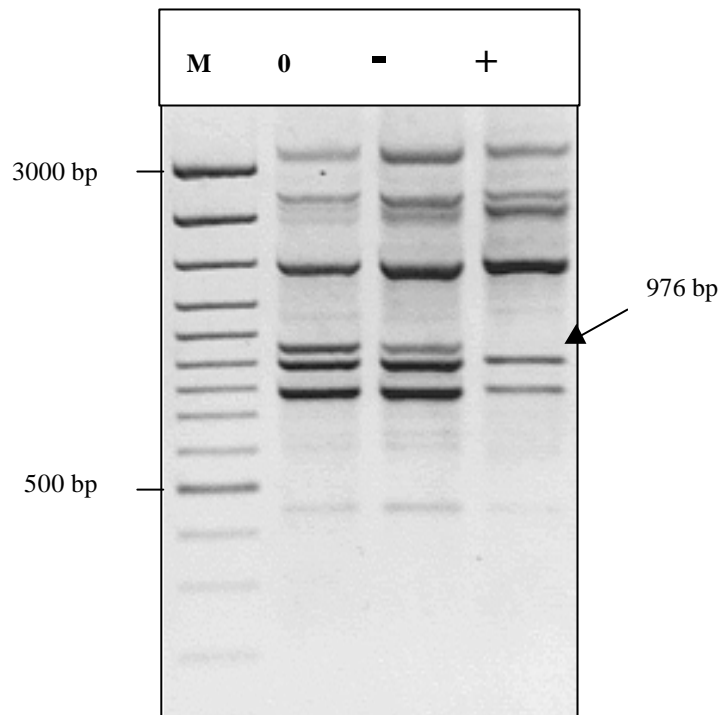


Figure 3: RAPD fingerprints of secondary phloem explants isolated from carrot roots 14 days after inoculation without hormones (0) or in the presence of myo-inositol and IAA without (-) or with (+) kinetin. Primer: P2 5'-GTTTCGCTCC-3' (see Schaefer et al., 2000)

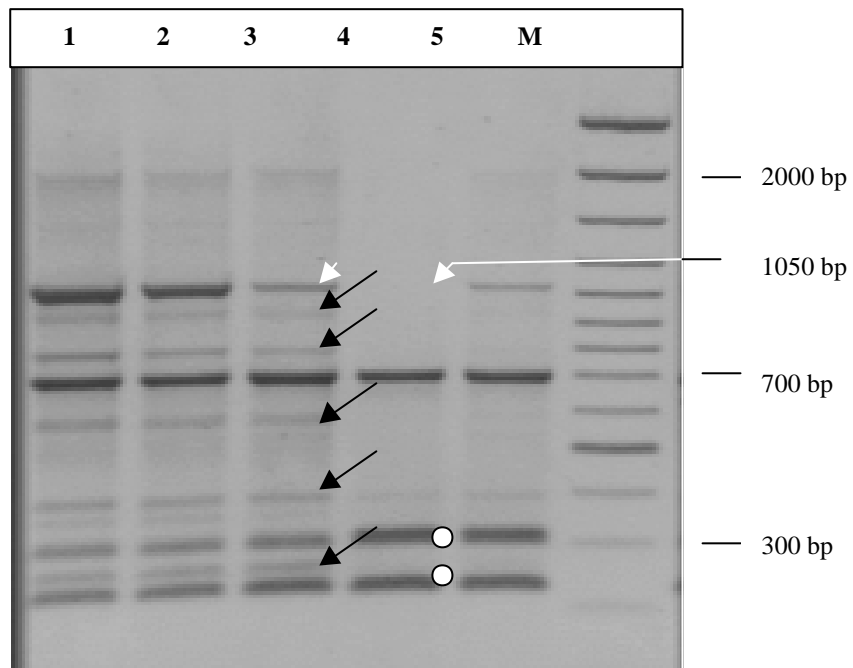


Figure 4: RAPD fingerprints produced by primer OP-G06 of various tissues of a carrot plant. (1) xylem of the tap root, (2) secondary phloem of the tap root, (3) young leaf, (4) older leaf, (5) petioles of young and old leaves. M = marker (see Schäfer, 1999)

Discussion

A great benefit of in vitro-culture techniques to basic physiological and biochemical research on plants is given by the possibility to induce dynamic adaptation of plant cells or tissues to well-defined chemical and physical conditions. Insights gained by this strategy are appropriate to reveal potential capabilities of plant cells and / or tissues to respond to physiological stimuli. Although, of course, the complexity of the whole plant organism in its environment has to be considered, this strategy in research helps to check objectively for the significance of potential pathways in a given situation. In the present paper, systematic investigations on a great number of short term in vitro-cultures of carrot root explants are reported, which demonstrate, that a change in the physiological and differentiatonal state of tissues is accompanied by non-random quantitative polymorphism at the total genome level. This kind of plasticity occurred independently of individual genotypic differences between single plants of various carrot cultivars and seems to be more associated with typical characteristics of the carrot genome per se interacting with physiological stimuli. To test the general relevance of the results of in vitro-culture experiments for whole plant organisms, the genome of different tissues and leaves of different ages of carrot plants were checked employing RAPD fingerprint analysis for the occurrence of quantitative polymorphism. In-vivo investigations verified, that differentiation as well as ageing were accompanied by quantitative polymorphism, suggesting a determining role of gross changes in DNA organisation for tissue development (for detailed discussion of methods and results see Arnholdt-Schmitt, 1995; Schäfer et al., 2000; Schaffer and Arnholdt-Schmitt, 2001). Experiments are running to characterize sequences of RAPD fragments which are differentially amplified or lost as a response to physiological stimuli, inclusively nutritional factors like phosphorus (Imani et al., in prep (a), Imani et al., in prep (b)). Sequence informations gained so far indicate, that gene sequences as well as non-gene sequences could be affected by this kind of quantitative polymorphism (Imani et al., in prep (b); Schaffer, 1999). In conclusion, in vitro culture experiments were shown to be helpful in revealing systematically occurring quantitative changes in DNA organisation, that seem to be involved in plant adaptation as a response to physiological stimuli. The general occurrence of quantitative polymorphisms in vivo in relationship to physiological events and differentiation could be confirmed with the applied fingerprint technique. Future experiments on ageing will reveal, whether the loss of band intensity shown by RAPD fingerprinting are related to the progressive loss of repeated sequences at the end of chromosomes (telomere shortening) observed during ageing (Shay and Wright, 1999). Since RAPD analysis enables screening for unknown sequences which are affected by quantitative polymorphism, future data will provide sequence information about differentially amplified or lost fragments related to physiological stimuli and, as though, are expected to contribute to the understanding of physiological and ecophysiological events.

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References

- Arnholdt-Schmitt, B. (1993): Hormonsensitivität und genomische DNS – Variabilität in ihrem Bezug zur Ertragsbildung bei *Daucus carota* L. ssp. sativus. Ein Beitrag zur Zellbiologie pflanzlichen Wachstums. Habilitation thesis, Justus-Liebig-University Giessen, Germany.
- Arnholdt-Schmitt, B. (1995): Physiological aspects of genome variability in tissue culture. II. Growth phase-dependent quantitative variability of repetitive BstN I fragments of primary cultures of *Daucus carota* L. Theor. Appl. Genet. 91, 816-823.
- Arnholdt-Schmitt B. (1999): On the physiology of yield production in carrots - Implications for breeding towards nutrient efficiency. Gartenbauwissenschaft 64 (1), 26-32.
- Arnholdt-Schmitt, B, S. Herterich and K.-H. Neumann (1995): Physiological aspects of genome variability in tissue culture. I. Growth phase-dependent differential DNA methylation of the carrot genome (*Daucus carota* L.) during primary culture. Theor. Appl. Genet. 91, 809-815.
- Bassi, P. (1990): Quantitative variations of nuclear DNA during plant development: A critical analysis. Biol. Rev. 65, 185-225.
- Bogani, P., A. Simoni, P. Lio, A. Scalpi, M. Buiatti (1996): Genome flux in tomato cell clones cultured in vitro in different physiological equilibria. II. A RAPD analysis of variability. Genome 39, 846-853.
- Chen, L.F.O., H.Y. Kuo, M.H. Chen, K.N. Lai, S.C.G. Chen (1997): Reproducibility of the differential amplification between leaf and root DNAs in soybean revealed by RAPD markers. Theor. Appl. Genet. 95, 1033-1043.
- Cullis. C.A. (1987): The generation of somatic and heritable variation in response to stress. Am. Nat. 130, 62-73.
- Dührssen, E. and K.-H. Neumann (1980): Characterization of Satellite-DNA of *Daucus carota* L. Z. Pflanzenphysiol. 100, 447-454.
- Grieb, B., U. Groß, E. Pleschka, B. Arnholdt-Schmitt and K.-H. Neumann (1994): Embryogenesis of photoautotrophic cell cultures of *Daucus carota* L.. Plant Cell Tiss. Org. Cult. 38, 115-122.
- Gross, U., F. Gilles, L. Bender, P. Berghöfer and K.-H. Neumann K. (1993): The influence of sucrose and an elevated CO₂ concentration on photosynthesis of photoautotrophic peanut (*Arachis hypogaea* L.) cell cultures. Plant Cell Tiss. Org. Cult. 33, 143-150.
- Haberlandt, G. (1902): Kulturversuche mit isolierten Pflanzenzellen. Sitzungsber. Mat. Nat. Kl. Kais. Akad. Wiss. Wien 111, 69-92.
- Imani, J., S. Schaffer, G. Langen and B. Arnholdt-Schmitt (a): Phosphorus depletion induces amplification of a sequence homologous to an intron of a leucine-rich repeat protein (LRR). (in prep). ACCESSION AJ311890 (<http://www.ncbi.nlm.nih.gov/>).
- Imani, J. S. Schaffer, G. Langen and B. Arnholdt-Schmitt (b): Phosphorus-independent loss of a RAPD fragment homologous to the photosystem I P700 chlorophyll A apoprotein A1 during cell division growth of a primary culture. (in prep). ACCESSION AJ311807 (<http://www.ncbi.nlm.nih.gov/>).

- Murray, M.G. and W.F. Thompson (1980): Rapid isolation of high-molecular-weight plant DNA. *Nucleic Acids Res.* 8, 4321-4325.
- Nagl, W. (1976): *Zellkern und Zellzyklen*. Ulmer, Stuttgart.
- Nagl, W. (1979): Differential DNA replication in plants: A critical review. *Z. Pflanzenphysiol.* 95, 283-314.
- Nagl, W. (1992): Replication. *Prog. Bot.* 53, 166-180.
- Neumann, K.-H. (1966): Wurzelbildung und Nucleinsäuregehalt bei Phloem-Gewebekulturen der Karottenwurzel auf synthetischem Nährmedium verschiedener Hormonkombinationen. *Les Phytohormones et l'Organogenèse* 38, 95 – 102.
- Neumann, K.-H. (1999): Some physiological Aspects in the development of plant cell and tissue culture. Symposium in Memory of the 100th Anniversary of the Foundation of Tissue Culture by Gottlieb Haberlandt. 8-9 October 1998, Vienna. *In Vitro Plant* 35(2), 159-161.
- Power, J.B., M.R. Davey, J.P. Freeman, B.J. Mulligan, E.C. Cocking (1986): Fusion and transformation of plant protoplasts. *Methods Enzymol.* 118, 578-595.
- Richards, G.M. (1974): Modification of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. *Anal. Biochem.* 57, 369-376.
- Schäfer, A., J.R. Blaschke and K.-H. Neumann (1978): On DNA metabolism of carrot tissue cultures. *Planta* 139, 97-101.
- Schäfer, C. (1999): Untersuchungen zum Einfluß ontogenetischer Faktoren auf die DNA Organisation (RAPD-Analysen). Undergraduate thesis (Diplomarbeit), Justus-Liebig- University Giessen, Germany.
- Schäfer, C., S. Schaffer and B. Arnholdt-Schmitt (2000): Differential RAPD fingerprints in carrot tissues. *Acta Hort.* 530, 437-445.
- Schaffer, S. (1999): Zum Einfluß von Wachstumsstatus und der Nährstoffversorgung mit Saccharose und Phosphat auf den RAPD-Fingerprint eines Gewebes. – Untersuchungen an einer Primärkultur von *Daucus carota* L. Undergraduate thesis (Diplomarbeit), Justus-Liebig-University Giessen, Germany.
- Schaffer, S. and B. Arnholdt-Schmitt (2001): Characterization of genome variation in tissue cultures by RAPD fingerprinting – a methodological comment. *Plant Biosystems* 135(1), 115-120.
- Shay, J.W. and W.E. Wright (1999): Telomeres and telomerase in the regulation of human cellular aging. In: *Molecular Biology of Ageing*, Alfred Benzon Symposium 44. V.A. Bohr, B.F. Clark and T. Stevnsner (eds.) Munksgaard, Copenhagen. pp 148-158.
- Steward, F.C., S.M. Caplin and F.K. Millar (1952): Investigations on growth and metabolism of plant cells. I. New techniques for the investigation of metabolism, nutrition and growth in undifferentiated cells. *Ann. Bot.* 16(1), 57-77.