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# Meetings of the Royal Botanical Society of The Netherlands

## MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE, LEIDEN, ON 10 NOVEMBER 1995

### *In Vitro* Regeneration Systems of Cereals

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*In vitro* culture is an experimental tool for fundamental studies as well as for applied purposes such as plant breeding or genetic transformation.

Plant tissues can be maintained in culture and under defined conditions it is possible to demonstrate that individual cells are totipotent and have the capacity to regenerate new plants. Applications of *in vitro* techniques are now well established for many dicotyledonous species, but monocotyledonous species are more difficult to culture *in vitro* and the development of efficient regeneration systems has been slower. However, in recent years considerable progress has been made and meanwhile grass cell cultures are being used as experimental and practical tools.

Plant regeneration from *in vitro* cultures is possible via somatic embryogenesis and organogenesis. Somatic embryos show a bipolar morphology similar to zygotic embryos with coleorhizal and coleoptilar regions. They germinate to plantlets in the same way as do seed-derived embryos. In the organogenic pathway a meristem is induced on callus tissue, and this meristem grows out and forms shoots. Whole plants can be regenerated when these shoots form adventitious roots at their base.

Generally, the embryogenic pathway is preferred for the production of plants as the regeneration process is more similar to normal plant development. Furthermore, somatic embryogenesis offers more experimental opportunities as it is, for example, possible to use the system for *in vitro* selection or for transformation purposes.

Different explant sources have been used for the induction of embryogenic cereal cultures: apical meristems, seedling mesocotyls, immature embryos, mature embryos, immature ovary tissue, immature inflorescences, anthers and isolated microspores. Depending on the type of explant one can distinguish somatic, zygotic and androgenetic embryogenesis. Progress achieved in establishing embryogenic cultures as well as applied aspects have been presented.



### Variation in Early Morphogenesis of Zygotic Embryos in Some Angiosperms

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Embryos of Angiosperms are formed through a series of developmental stages starting with zygote formation and leading to an organism consisting of a suspensor, an embryo axis with root and shoot meristem, and one or two cotyledons. The pathways along which development proceeds vary among plant species. In some cases, embryos remain relatively small and storage products are accumulated in the surrounding endosperm and/or perisperm. In other cases, the endosperm is ephemeral and storage occurs in the embryo itself, either in the cotyledons or in the hypocotyl. The various parts of the embryo might function differently in various species and, depending upon the function, the parts are adapted to that function by cell proliferation and/or cell differentiation.

Comparison of embryogenesis in *Zea mays* (Van Lammeren 1986, *Acta Bot. Neerl.* **35**, 169–188), *Phaseolus vulgaris* (XuHan 1995, doctoral thesis, WAU, Wageningen), *Ranunculus sceleratus* (XuHan 1995, doctoral thesis, WAU, Wageningen) and *Brassica napus* (Van Lammeren *et al.* 1996, *Acta Soc. Bot. Pol.*, in press) with light microscopy, fluorescence microscopy, confocal laser scanning microscopy and scanning and transmission electron microscopy, clearly revealed the species-specific differentiation of the cells of the suspensor and embryo, resulting in divergent morphology and function. Additionally, the organization of the microtubular cytoskeleton was determined in developing embryos and endosperm. It was established that microtubules in the endosperm functioned in the positioning of the nuclei during the nuclear phase of endosperm formation. They influenced the formation of anticlinal walls during alveolus formation, and are of importance during cell formation and shaping in both embryos and the cellular endosperm.

## *In Vitro* Techniques to Overcome Interspecific Crossing Barriers in Lily

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In order to introduce new characters (resistance, flower shape, etc.) from wild species into the cultivar assortment of lily, it is necessary to overcome interspecific crossing barriers. Using the integrated *in vitro* pollination, embryo rescue and polyploidization methods, it is possible to control pollination, fertilization and embryological and meiotic processes. Therefore, various techniques, e.g. cut style, grafted style and *in vitro* isolated ovule pollination, have been developed to overcome prefertilization barriers. Postfertilization barriers can be circumvented by *in vitro* pollination and/or rescue methods as embryo, ovary-slice and ovule culture. The influence of several medium components (sucrose and hormone concentrations, phytagel) was demonstrated.

Using these methods, wide interspecific crosses between species and cultivars from the different sections of the genus *Lilium* have been produced, e.g. *L. longiflorum* × *L. concolor*, *L. longiflorum* × *L. dauricum*, *L. longiflorum* × *L. henryi*, *L. longiflorum* × *L. martagon*, *L. longiflorum* × *L. candidum*, *L. longiflorum* × Asiatic hybrids (LA), *L. longiflorum* × oriental hybrids (LO), *L. longiflorum* × *L. rubellum*, *L. longiflorum* × *L. canadense*, oriental × asiatic hybrids (OA) and *L. henryi* × *L. candidum*. In particular, the OA-hybrids are a breakthrough and hold promise for the future.

In general, the hybrids show $F_1$-sterility. Using chromosome doubling techniques, tetraploids with restored fertility were produced from these diploid hybrids. With these hybrids a crossing programme at polyploid level is now being carried out.

## Cell Biological Aspects of Somatic Embryo Development of Maize

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For regeneration of maize via somatic embryogenesis, the formation of a storage cotyledon, the scutellum, is a prerequisite. The zygotic scutellum accumulates starch, lipids and proteins, but hardly any lignin (Emons *et al.* 1993, *Acta Bot. Neerl.* **42**, 319–339). In somatic embryos scutellum formation is suppressed by the synthetic plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) by which embryogenic callus can be kept in a developmental stage, comparable to the globular stage of the zygotic embryo (Emons & Kieft 1991, *Plant Cell Rep.* **10**, 485–488). In this environment cells enlarge and walls split at the side of the middle lamella. The cells that eventually give rise to somatic embryos are those that remain attached in small clusters.

Suppression by 2,4-D can be overcome by adding ABA to the culture medium. However, extra sucrose, while omitting the mannitol that is needed in the proliferation medium, also initiates scutellum formation, even at high 2,4-D concentration (Emons *et al.* 1993, *J. Plant Physiol.* **142**, 597–604).

Histological analysis shows that for scutellum formation a functional exogenic shoot meristem is essential, which is from protodermal origin (Emons *et al.* 1993, *J. Plant Physiol.* **142**, 597–604). In maize genotype 4C1 and the line A188 proper scutellum formation occurs only if somatic embryos have been attached to callus until shoot meristem formation (Emons & Kieft, 1991, *Plant Cell Rep.* **10**, 485–488). If scutellum formation takes place, a coleoptile develops opposite to it, as in zygotic embryos (Emons & De Does 1993, *Can. J. Bot.* **71**, 1349–1356).

Pyrolysis mass spectrometry reveals that the scutellum cells in properly developing somatic embryos contain much starch and that phenol metabolism leading to lignin is suppressed, while in non-regenerable structures grown from suspension cells phenolic acids, mainly ferulic acid and lignin are abundant. A further conspicuous feature, seen only in somatic embryos with development comparable to zygotic embryos, is the accumulation of lipids (Mulder & Emons 1993, *J. Anal. Appl. Pyr.* **25**, 255–264).

During embryo development, zygotic embryos accumulate the storage protein globulin in the scutellum, root cortex, coleoptile and leaf primordia. Western blotting and immunocytochemistry demonstrates that both embryogenic callus and developing somatic embryos accumulate one of these globulins and that they are more abundant when the embryo maturation medium contains ABA (Thijssen *et al.* in prep.).

## Induction and Characterization of Desiccation Tolerance of Somatic Embryoids

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Somatic embryoids of carrot (*Daucus carota*) and alfalfa (*Medicago sativa*) can survive almost complete dehydration to 5% moisture content, which is

 

comparable to seeds. Embryoids only acquire this desiccation tolerance when they are treated with abscisic acid (ABA) at the proper stage of their development (torpedo stage). Subsequently they are slowly dried and prior to imbibition they are prehydrated in moist air to prevent imbibitional damage.

Leakage measurements of carbohydrates and potassium, together with freeze-fracture analysis of plasma membranes with TEM, have demonstrated that membranes of intolerant embryoids have lost their function as semipermeable barrier. Preservation of membrane function is essential for desiccation tolerance. With model systems it has been demonstrated that phospholipid bilayers are protected in the dry state through di- and oligosaccharides by the so-called 'water replacement theory'. Carbohydrate measurements with ion exchange HPLC (Dionex) of developing and slowly dehydrating carrot embryoids did not reveal a clear correlation between germination and carbohydrate content. However, it has been clearly shown by combining all data that carrot embryoids need a minimal level of sucrose and umbelliferose in order to survive desiccation. Thus these two carbohydrates are essential for desiccation tolerance. However, they are not the only limiting parameter, because there are embryoids that contain enough sugars but do not germinate. Sucrose and umbelliferose function as protectors of membranes and proteins, as was demonstrated with FTIR, but they might also be involved in the formation of a stable glassy state.

ABA is the essential growth regulator during the development of desiccation tolerance. Respiration as well as cell cycle activity are reduced by ABA. Both processes are more or less correlated with desiccation tolerance, but again they are not the only parameters involved.

It can be concluded that somatic embryoids can acquire complete desiccation tolerance, which might be a general phenomenon. We have investigated and distinguished several fundamental processes of desiccation tolerance that may contribute to the development of a commercially applicable, dry artificial seed.

### Signal Molecules in Plant Embryogenesis

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The observation that the carrot somatic embryo mutant ts11, arrested in the globular stage, can be rescued by addition to the medium of a secreted endochitinase (De Jong *et al.* 1992, *Plant Cell* **4**, 425–433; De Jong *et al.* 1993, *Plant Cell* **5**, 615–620) revealed an unexpected function of this class of plant hydrolytic enzymes in somatic embryo development. The arrested phenotype of ts11 is the result of a transient decrease in the amount of the EP3 chitinase present (De Jong *et al.* 1995, *Devel. Genet.* **16**, 332–343). The EP3 chitinase is a class IV chitinase and is one of the five members of a gene family. Two of the five encoded EP3 chitinase proteins were purified and found to be active in ts11 rescue. Unexpectedly, a related class I chitinase was also found to be able to rescue ts11, underscoring the fact that the original ts11 phenotype is not the result of a mutation that inactivates one particular chitinase. The carrot EP3 chitinase gene for which class IV-specific probes could be designed is expressed in a certain subset of suspension cells in both wild-type ts11 suspension cultures, but not in somatic embryos themselves. This suggests a 'cross-feeding' mechanism of action by which secreted proteins produced by one cell type influence the development of embryos from another cell type. Using in situ mRNA localization, the EP3 endochitinase gene was also found to be expressed in the carrot endosperm between 3 days and 14 days after pollination in a layer of endosperm cells that lined a central cavity filled with liquid endosperm that surrounds the developing embryo. Consequently, a search for substrates of the EP3 endochitinase is now focused on the developing endosperm. Recent results that deal with the expression of *Arabidopsis* homologues as well as progress towards identifying natural substrates for chitinases have been presented.

## MEETING OF THE SECTION FOR PLANT MORPHOLOGY, ANATOMY AND CYTOLOGY ON 24 NOVEMBER 1995

### What is the Cell Wall Polymer Phytomelan? A chemical approach

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The outer layer of the seed coat of several taxa of the Asparagales (monocotyledons) is encrusted with a characteristic carbon-rich compound, phytomelan. The presence or absence of this compound is a useful taxonomic feature. Phytomelan is amongst others also known in the fruit wall of several Compositae.

In the literature phytomelan is described as a black crust, covering the epidermal cells like a cuticula. Wittich and Graven studied the histochemistry and

 

development of the phytomelan layer in the seed coat of *Gasteria verrucosa* in more detail. (Wittich, P.E. & Graven, P., 1995, *Protoplasma* **187**, 72–78). In *Gasteria verrucosa* phytomelan is deposited as a tertiary wall in the exotestal cells, against the outer tangential cell wall and between the thickened radial walls, whereas in the fruit of *Helianthus annuus* the phytomelan is deposited in the intercellulars as one single layer against the sclerenchymatic fibres of the pericarp.

Chemical degradation of phytomelan containing plant tissues by alkali fusion led to catechol as degradation product (Nicolaus, R.A., 1968, *Melanins*, Hermann, Paris). Histochemical tests on catechol in developing seed coat of *Gasteria verrucosa* indicate that this component is one of the precursors in the phytomelan formation. Specific stains on other plant polyphenols were all negative.

The opaque, brittle, coal-like phytomelan is chemically inert. Until recently the main factor of the 'complex' chemical composition of phytomelan was the C:H:O ratio of 3·7:2·1:1 and the lack of nitrogen. Our results show that it is extremely difficult to isolate the pure blocks of phytomelan. The insolubility of the phytomelan makes it also extremely difficult to study its chemistry.

In our study with pyrolysis mass spectrometry we did not obtain any information on the chemical structure of phytomelan. The small samples of phytomelan were not pyrolysable and stuck to the insertion probe. This indicates that phytomelan is a carbon-rich and very condensed, probably ether-bond polymer. FTIR and $^{13}$C-NMR indicate that phytomelan is a small amorphous polymer with ether bonds and, probably, some ester and methyl bonds. It is an extremely condensed aromatic system with organic substituents. The NMR spectra show no peaks for hydroxy groups. The absence of these groups is a cause of the chemical inertness of phytomelan. The cell wall polymer phytomelan contains no specific anorganic elements. The absence of these elements is also confirmed by SEM in combination with energy dispersive X-ray analysis. Röntgen diffraction confirmed the indication of the NMR spectra that the phytomelan is not crystalline.

Additional $^{13}$C-NMR experiments are in progress and will lead us to the final pieces of the puzzle of the chemical structure of this cell wall polymer.

## Histochemistry and Ultrastructural Development of Phytomelan in the Seed Coat of *Gasteria verrucosa*

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In *Gasteria verrucosa* phytomelan is deposited as a tertiary wall in the exotesta, against the outer tangential cell wall and between the thickened radial walls.

We studied the development of the phytomelan layer using light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) in combination with cryo- and histochemical techniques. The development of phytomelan in the exotesta can be divided into three stages (Wittich, P.E. & Graven, P., 1995, *Protoplasma* **187**, 72–78).

(1) Callose deposition. Between the plasma membrane and the outer tangential secondary cell wall callose is deposited, filling approximately two-thirds of the original cell volume with callose. During the synthesis 'rod-like structures' connect the plasma membrane with the newly synthesized callose. The deposited block of callose seems to function as the template and carbohydrate source for the phytomelan synthesis.

(2) Callose conversion. The callose is broken down into a PATAg negative network and soluble sugars. These sugars might function as a substrate for the phytomelan synthesis and a cellulose wall that is synthesized by the plasma membrane against the converting callose. Just prior to the callose conversion an osmophilic product of the stacked endoplasmatic reticulum is excreted into the periplasmatic space.

(3) Phytomelan synthesis. The colourless suspension of converted callose slowly turns brown and darkens into black phytomelan. TEM and SEM show growing globules of 15 nm phytomelan units during the darkening of this layer. These globules have an electron dense border and will finally fuse together, resulting in a massive block of phytomelan. Some histochemicals which are used on the darkening phytomelan reveal that phenolics, probably catecholic residues, are involved in the phytomelan synthesis.

## Phylogenetic Aspects of the Development of the Ascus Apical Apparatus in Leotiales and Pezizales

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The order of the inoperculate discomycetes (Leotiales, Ascomycotina) currently contains over 2000 species. Families and genera are often ill-defined for want of stable morphological characters. Light microscopic and electron microscopic studies

have shown that the structure of the ascus apex is diverse and a rich, yet virtually unexploited resource of characters.

The ultrastructure of the ascus was studied with transmission electron microscopy (TEM) in four stages of development in 42 taxa of Leotiales. In the apical apparatus a central cylinder is surrounded by an apical thickening with an annulus, a rigid or more flexible ring of usually strongly PA-TCH-SP-reactive material. In most of the species the ascus opens by an eversion on the annulus. Other dehiscence mechanisms are related to the particular structure of the apical apparatus.

The structures seen in TEM need not correlate with the iodine reaction observed in light microscopy, and the latter seems to be less reliable taxonomically than the former.

Cladistic analyses of the whole group using 18 ascus characters resulted in numerous equally parsimonious cladograms (Hennig86). For subsamples of species phylogenetic hypotheses were obtained that need future testing with other morphological and molecular characters. The analyses were of value in delimiting the 14 proposed types of apical apparatus in Leotiales. Most of the characters are useful at generic level.

The family Leotiaceae comprises at least two separate evolutionary lineages. The family Sclerotiniaceae is monophyletic, and the Geoglossaceae probably paraphyletic (Verzkley, G.J.M., 1995, Ph.D. Thesis, Leiden).

These findings were compared with similar work done in the operculate discomycetes (Pezizales). It is concluded that there is no direct evidence for homology of the operculum in Pezizalean asci and the central cylinder in Leotialean asci.

## Localization of RNA synthesis in Nuclei of *Brassica napus*

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Nuclear activity and nuclear changes were studied during early plant embryogenesis. Microspores of *Brassica napus* were used as a model system since their developmental pathway changes from gametogenesis to embryogenesis if cultured under embryogenic conditions (heat shock, at least 8 h 32°C). We tried to relate the patterns of RNA synthesis and transport to different embryogenic stages by light and electron microscopy.

To understand more about the functional organization of the interphase nucleus we did experiments to visualize the sites of transcription by incorporating bromouridine into nascent RNA. Experiments with cells from microspore cultures only occasionally gave a faint labelling in the nucleolus and did not label the nucleoplasm. Therefore we used isolated nuclei from leaves of *Brassica napus*. After immunolabelling and visualization of the signal with confocal laser scanning microscopy, we found a typical punctate labelling pattern throughout the nucleus, in some cases including the nucleolus. These results are in agreement with observations made on mammalian cells by other groups. Until now nascent RNA has not been successfully labelled in whole cells.

Another approach to acquire an indication about the functional organization of the nuclei is high resolution *in situ* hybridization using a ribosomal 18S RNA probe. These experiments were done with LRWhite sections which allows quantification of the signal. Initial results from experiments with the ribosomal probe showed a specific label in the nucleolus and the cytoplasm. There are indications that the label intensity increases during normal pollen development. Experiments with embryogenic material are in progress.

 