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Meetings of the Royal Botanical Society of The Netherlands

MEETING OF THE SECTION FOR FERTILIZATION RESEARCH IN PLANTS ON 12 FEBRUARY 1993

Changes in Cytoskeletal Patterning and DNA Synthesis During the Induction of Embryogenesis in Microspores and Pollen of *Brassica napus* L.

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Analysing molecular processes and cytological changes coinciding with induction of embryogenesis in microspores and pollen of *Brassica napus* c.v. Topas, attention was paid to the induction and dynamics of DNA synthesis in nuclei of microspores and pollen and to changes in the microtubular and microfilamental arrangements during the first days of culture. The patterning of the microfilamental network, observed after rhodamine–phalloidin staining of whole cells, does not show prominent changes. The microtubular network, visualized immunocytochemically on sectioned material, however, exhibited a number of changes that directly relate to changed division patterns: (i) symmetrical divisions in microspores are preceded by a change of spindle orientation; (ii) symmetrical divisions in microspores are induced after a shift in nuclear position caused by the disruption or disturbed formation of cytoplasmic microtubules; and (iii) symmetrical divisions are induced in vegetative cells when generative cells remain attached to the pollen wall which is probably caused by the disappearance of cytoplasmic microtubules. (See Hause, B. *et al.* (1993): *Cell Biol Intern.* **17:** 153–168.)

DNA synthesis was visualized immunocytochemically on sectioned material after pulse or continuous labelling of isolated microspores and pollen cultured under embryogenic (32°C) and non-embryogenic (18°C) conditions. DNA contents of nuclei were determined microspectrophotometrically. (i) Microspores do show DNA synthesis at 18 and 32°C within 1 h of culture. (ii) The G2 phase was observed to be less than 1 h. (iii) Both G1 and G2 microspores were induced to embryogenesis. (iv) Vegetative nuclei were always at G1 at the onset of culture and became labelled only at 32°C condition, sometimes within 4 h of culture. The data obtained will be published and discussed in more detail (see Binarova, P. *et al.* (1993) *TAG* (in press)).

An Auxin Test for Quantifying the Degree of Apomixis in *Poa pratensis L.*

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A quick and reliable method to determine the fractions of apomictic versus sexual embryo sacs in a single plant would be very useful for selecting genotypes of the facultatively apomictic species Kentucky bluegrass (*Poa pratensis* L.) for breeding highly apomictic elite cultivars. Classical histology of sections is very laborious, homogeneity tests of progenies are time-consuming, and the species has proved to be not amenable to clearing techniques.

Because of the pseudogamous nature of *Poa pratensis*, examination of dissected embryo sacs a few days after fertilization permits the classification of aposporic and sexually originated embryos, both accompanied by fertilized endosperm, of different ploidy level (Naumova *et al.* (1993): *Acta Bot. Neerl.* **42:** 299–312). A single application of the inflorescences a few days before anthesis with 80 mg l^{-1} 2, 4-D could substitute for endosperm development, and without fertilization full-sized seeds are recovered about 3 weeks after anthesis (Matzk (1991): *Sex Plant Reprod.* **4:** 88–94). Auxin-induced seeds are either empty, or contain one or more embryos, and never endosperm.

The embryos are supposed to derive from aposporic embryo sacs, whereas the empty seeds represent aborted sexually originated embryo sacs. A minimum of 100 seeds was examined of eight genotypes and the percentage of apomixis was calculated as the ratio of the number of seeds with at least one embryo and the total number of induced (non-fertilized) seeds. The percentage of apomixis ranged from 61 to 90% whereas two parents proved to be completely sterile. The calculated percentages of apomixis was generally higher than the estimates from the dissected embryo sac analysis (Naumova *et al.* (1993) ibid.) but the ranking of genotypes was the same. Preliminary comparisons of the apomictic tendencies with the estimates of realized apomixis as evidenced by the degree of homogeneity of offspring revealed a similar ranking.

The auxin test may be developed into a routine text for estimating aposporic tendency after the

correlation of the results with this realized degree of apomixis has been more fully established.

Molecular Analysis of Exine Proteins from *Brassica oleracea* Pollen

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Proteins in the outer layer of the pollen grain, the exine, may have a function in the process of pollination, i.e. pollen capture, pollen hydration, pollen acceptance and pollen-tube growth. The function of exine proteins will be dependent on the site of synthesis, the sporophytic tapetum or the gametophytic pollen grain. For instance, the pollen component involved in sporophytic self-incompatibility is expected to be synthesized in the tapetal tissue, and deposited in the exine. So an anther-specific λZAP II expression library has been screened with an antiserum raised against proteins isolated from the exine-held tryphine. This resulted in the isolation of a number of sequences, of which clones 3, 15 and 25 were anther-specific (Ruiter, R.K., *et al.* (1993): *Acta Bot. Neerl.* **(42:** 379).

Clone 3 represents a highly expressed single copy gene. The 1·5 kb transcript is only present in the anther tissue(s) surrounding the pollen grains, during the binucleate pollen developmental stage. Partial sequence analysis learned that we are dealing with a glycine-rich gene. Of the 38 isolated cDNA clones, 32 were homologous to this clone 3.

Clone 15 is a 0·6 kb long cDNA clone. The corresponding transcript was found in both complete anthers (including pollen) and mature pollen grains. The strong signal in complete anthers compared to only pollen grains suggests that this sequence is expressed in both the pollen and the surrounding tissues. The gene is present in two or three copies per genome and its sequence codes for a protein with about 50% amino acid identity with two cold-regulated *Arabidopsis thaliana* genes (Kurkela, S. & Franck, M. (1990): *Plant Molecular Biology* **15:** 137–144; Gilmour, S.J. *et al.* (1992): *Plant Molecular Biology* **18:** 13–21).

Clone 25 is a 0·6 kb long cDNA clone probably expressed in both the pollen and the surrounding anther tissues. The gene is present in two or three copies per genome. The cDNA clone is coding for a protein with approximately 85% amino acid similarity with BetVII (Valenta, R. *et al.* (1991): *Science* **253:** 557–560), a putative profilin. Profilin is an actin-binding protein that probably regulates actin-polymerization. Therefore, it may play a role in formation of the actin skeleton in developing pollen tubes.

Experiments to establish the role of these proteins in pollen and anther development, especially in relation to their position in the exine, are in progress.

MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE ON 26 MARCH 1993

Identification of Single Embryo-forming Cells in Carrot Suspension Cultures

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In carrot (*Daucus carota* L.), somatic embryos can develop from clusters of embryogenic cells, pro-embryogenic masses, or from single embryogenic cells. Two types of single embryogenic cells have been described. The first of these, designated as type 1, is a small round cytoplasmic cell (Nomura, K. & Komamine A. (1985): *Plant Physiol.* **79:** 988–991) and the second type is a more elongated and vacuolated cell (Backs-Hüsemann, D. & Reinert, J. (1970): *Protoplasma* **70:** 49–60). The formation of small meristematic cell clusters from both types of cells is dependent on the presence of the auxin 2,4 dichlorophenoxyacetic acid (2,4-D). After subsequent removal of auxin these clusters will develop into somatic embryos.

To provide statistically reliable data on the type and number of single embryogenic cells, a culture system by which large numbers of immobilized cells can be tracked has been developed. Using this system, more than 30 000 individual cells, obtained after sieving through a 22 μm sieve, were followed. After 6 days of culture in the presence of 2,4-D and another 9 days in the absence of hormones, 0·5% of these cells

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had developed into somatic embryos. Several cell types could be distinguished at the start of the experiments by inspection of the recorded images. Small dark cells (resembling type 1 cells) and oval cells formed somatic embryos with a frequency of 1·0%. Other cell types (e.g. elongated cells) were capable of forming somatic embryos with a frequency between 0·2 and 0·6%.

From these results is can be concluded that morphologically different cell types, present in a carrot suspension culture, are able to develop into somatic embryos.

Cyclic Somatic Embryogenesis in Cassava

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A cyclic system of embryogenesis has been developed in cassava. Primary embryos were initiated from leaf explants. The embryogenic response of leaf explants from glasshouse-grown plants was dependent on the time of the year. Leaf explants from *in vitro* grown plants gave a more stable response. However, the production of somatic embryos from leaf explants of donor plants, grown under standard conditions, was no higher than 2·0 germinated embryos per initial explant (GE/IE) which is 10 times lower than that of leaf explants from glasshouse-grown plants. The embryogenic capacity was increased to 6·6 GE/IE by growing *in vitro* donor plants at low light intensity. Leaf explants isolated from plants which were pretreated with 2,4-D gave the highest production (9·4 GE/IE).

Somatic embryos, cultured on the same medium as used for primary embryogenesis, form new somatic embryos. In succeeding cycles the production was between 6·8 and 9·6 GE/IE. Using liquid instead of solid media increased the production to 16·2 GE/IE. Fragmentation of starting embryos increased the production in both solid and liquid media to 12·5 and 32·1 GE/IE, respectively.

In both primary and cyclic embryogenesis the embryos were formed directly from the explants. Only the cotyledons of somatic embryos initiated new embryos. The first embryogenic divisions occurred in the cells near the vascular tissue. Embryos younger than the torpedo-shaped stage and embryos which have the first true leaf did not initiate somatic embryos. Mature germinated embryos with well-developed green cotyledons (GE) were at the optimal stage to start a new cycle.

Culture of embryos on BA-supplemented medium supported the conversion of embryos to shoots. As for the induction of new embryos, mature germinated embryos were optimal for shoot conversion. Only one of the in total 485 *in vitro* evaluated regenerants had a visible deviation, i.e. variegated leaves. More than 100 regenerants were evaluated in the glasshouse. Various differences were found between regenerants and control plants. Regenerants were larger, their leaves were longer and the virus symptoms were less severe than in control plants. Furthermore, regenerants formed a higher number of tuberized roots with a higher total fresh weight. The form, position and texture of the roots was more variable. Most of these differences seemed to be caused by physiologically induced variation. In using the cyclic culture of embryos in cassava, no laborious procedures are needed to select true-to-type plants. Furthermore, the procedure may be useful for practical applications such as meristem culture and plant propagation.

Pyrolysis Mass Spectrometry of Cell Wall Development in Somatic Embryos of Maize

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Two types of somatic embryos of *Zea mays* were studied with pyrolysis mass spectrometry (PY-MS) before and after subjection to chemical and enzymic treatments to solubilize lipids, proteins and polysaccharides. The first type of somatic embryo grows attached to callus tissue and obtains all of the histological features that are present in zygotic embryos. The second type grows in suspension culture, from cell clusters, as single structures, which histologically represent the embryo axis (without a scutellum) and which have a shoot meristem that is blocked early in development. Comparison of the PY-MS results of the first type of embryo with that of zygotic embryos showed a close resemblance. Neither had mass peaks for phenolic acids and lignin. The mass spectra of the embryos blocked in development, however, showed mass peaks both for phenolic acids and for lignin. Because the lignin in untreated material can be obscured by the abundance of lipids, proteins and starch, the somatic embryos were treated with dichloromethane to remove most of the lipids, and subsequently with pronase, alpha-amylose and cellulase. After these treatments the mass spectra of the first type of embryo showed no mass peaks for lignin, but some ferulic acid was present.

Localization of Intracellular Calcium and Calmodulin Distribution in Cultured Carrot Cells

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The presence and distribution of calcium and calmodulin was investigated during somatic embryogenesis of carrot. Activated calmodulin was observed by fluorescence microscopy, using fluphenazin.

A polarized distribution of activated calmodulin was present, even when no morphological evidence for embryo polarity was apparent (Timmers, A.C.J., De Vries, S.C. & Schel, J.H.N. (1989): *Protoplasma* **153:** 24–29). Overall distribution of activated and inactivated calmodulin was analysed by immunolabelling with anti-calmodulin.

The calcium distribution was followed by confocal laser scanning microscopy using fluo-3 after pretreatment of the cells with 0·1% digitonin (Timmers, A.C.J., Reiss, H.D. & Schel, J.H.N. (1991): *Cell Calcium* **12:** 515–521). A high concentration of free cytosolic calcium coincided with the development of proembryogenic masses to somatic embryos.

MEETING OF THE SECTION FOR VEGETATION RESEARCH ON 23 APRIL 1993

Dispersal: Key-factor in the Restoration of Species-rich Grasslands

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Restoration of species-rich grasslands on the Veenkampen, a set-aside grassland site on clay-on-peat soils in the Gelderse Vallei (The Netherlands), was attempted by ameliorating the habitat quality for absent plant species. This involved raising the water table and decreasing the soil fertility by removal of nutrients. However, secondary succession towards a plant community richer in plant species proceeded slowly.

Seed addition trials were performed for 10 perennial grassland species absent from the target site, but present on ditch banks in the surrounding landscape. These species could successfully establish on bare soil, but much less so on intact sods. This indicated that the availability of seeds limited the distribution of these plant species on the target site.

It is well-documented that most grassland species have limited seed-dispersal capacities. Such species could benefit from linear landscape elements functioning as corridors in order to recolonize restored sites. A corridor model was developed to assess the importance of four factors on the migration speed of plants: seed-dispersal capacity, probability of establishing new populations (habitat quality), and composition and width of corridors. The model was parameterized for grassland species.

Simulations indicated slow overall migration rates, less than 5 m year^{-1}, and significant main effects and interactions between factors. Considering the costs of increasing the quality and widths of present corridors and the large distances between seed sources and target sites in the landscape, the usefulness of corridors for plant migration was questioned. Instead, reintroduction of seeds by professional managers is considered a valid method for the conservation of plant species with limited seed-dispersal capacities.

Restoration of Species-rich Grasslands on Reconstructed River Embankments

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In the last few decades the area and number of botanically valuable, species-rich grasslands on river dykes have decreased dramatically. The main causes are the intensification of agricultural activities and large-scale reconstructions of the river embankments.

Two factors are important for the restoration of species-rich grasslands after reconstruction: (i) the method of reconstruction, and (ii) management practice after reconstruction.

Most plant species can be saved by conserving a strip of the former vegetation while reconstructing a river embankment. Consequently, they can serve as a source of seeds for the recolonization of the reconstructed parts of the dyke. This process stimulates the redevelopment of the vegetation, and is especially apparent close to the conserved zone. When it is not possible to save part of the original vegetation, the upper soil layer can be put aside and replaced after the reconstruction. Replacement of the top-soil also allows the reappearance and accelerated

redevelopment of species-rich grasslands. From the propagules incorporated into the top-soil almost all the species which were originally present reappeared. The use of the former under-layer as the new upper-layer after reconstruction also stimulates redevelopment of the vegetation. Redevelopment on imported clay without propagules appears to be considerably retarded and the proximity of a conserved zone is again important.

Species-rich vegetation only develops with the right management. In the early years after reconstruction the methods used for reconstruction appear to have the most influence. After this period the influence of the management increases.

Good management practices, e.g. mowing twice a year and removing the hay, grazing twice a year, and a combination of mowing and grazing, stimulate the redevelopment of a species-rich vegetation with good erosion prevention. Bad management practices, e.g. mowing without removing the hay and mowing only every other year, prevent rapid restoration of botanically valuable species-rich grasslands and result in poor erosion prevention.

Restoration of Stoneworts (Characeae) in the Naardermeer by Supplying Dephosphatized Water

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The Naardermeer is a wetland located in the Vecht river plain area in the central part of The Netherlands. It consists of several shallow lakes, reedlands and marshy woodlands with a mesotrophic character. It is famous for its avifauna and the marshy and water vegetation, e.g. about 10 of the 20 stonewort-species occurring in The Netherlands.

Gradients in water quality are caused by fresh seepage-water from the sandy ice-pushed ridge (the Gooi) and the standing water. Pumping groundwater in the Gooi, combined with lowered water levels in the adjacent polders, has changed the hydrology and the water quality. As a consequence submerged water vegetation, including stoneworts and the angiosperm *Najas marina*, has diminished because of algal blooms. In 1984 a water-supply project was started to maintain a high water level in the nature reserve. In summer periods with water-shortages dephosphatized IJmeer-water is supplied. Investigation of the submerged vegetation is one of the monitoring projects.

For 5 years little or no improvement was seen in the submerged vegetation. Algal blooms continued and the tolerant *Potamogeton pectinatus* expanded. In 1989, stonewort vegetation recovered in a part of the lakes. In the last 3 years nearly the whole bottom of the lakes became covered with stoneworts on sandy substrates and *Najas marina* on muddy substrates. Water transparency is now very good. Even rare stonewort species like *Nitella hyalina* and *Tolypella glomerata* have reappeared. Naardermeer proved to be one of the best areas for stonewort vegetation in The Netherlands. The large amounts of *Potamogeton pectinatus* and *Myriophyllum spicatum*, epiphytical algal growth and the disappearance of some plant species from seepage-stands are problems yet to be solved.

Restoration of Acidified Rich-fen Ecosystems: An Impact Assessment Study

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Acidification and eutrophication cause great changes in rich-fen ecosystems in The Netherlands. In order to retain the rare species and species-rich vegetation types, restoration measures will be carried out to a large extent by nature conservation agencies, although there has been little research on which to base the measures. The research described here was carried out in the Vechtplassen area, where considerable research has been performed since 1980 which could be used as a framework for the study. A floating fen was selected where the pH of the surface water had dropped from 6·2 to *c.* 4·2 between 1980 and 1991. This was a very species-rich fen with many rare species such as *Eriophorum gracile, Scorpidium scorpioides, Carex lasiocarpa* and *C. diandra*. Nowadays the vegetation is dominated by *Sphagnum squarrosum, S. fallax* and *Polytrichum commune*. The main problem appeared to be the surplus of rain water, which could not discharge into a ditch and so remained in the fen forming a water lens. In autumn 1989 a ditch was excavated along the fen and connected with the main drainage system.

Four experimental sites of 16 m^2 were created, each managed differently: sod-cutting, drainage alone, drainage and sod-cutting, and a control. Water, soil and vegetation have been studied since 1989. No distinction could be made between control, drainage and sod-cutting concerning pH (pH 4–4·8) and EC (5–10 mS m^{-1}); only in the combined treatment was the pH significantly increased to *c.* 6·2 and the EC to 20 mS m^{-1}. The macro-ions in the water also showed no difference, except in the combination site, which had increased concentrations for Ca, Mg and HCO_3. High concentrations of phosphate were found in the control and in the drainage site; only the combination showed the 'natural' low concentrations. There were indications that *Polytrichum commune* is even favoured above the *Sphagnum* species. It can reach the more acid environment in the soil by

means of the internal transport in the long plants, e.g. in the control site, which contained high concentrations of P where the 'acid rain' supplied the nitrogen. The bryophytes have expanded enormously over the last 5 years, while outcompeting, e.g. *Scorpidium scorpioides* and diminishing the abundance and growth of phanerogams.

If restoration has to be carried out in a fen, the only option which provides sufficient abiotic 'buffered' conditions is a combined approach of sod-cutting and drainage of surplus water. Monitoring of the vegetation development showed some promising signs; some species recovered, but the rare ones have not yet appeared.