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Meetings of the Royal Botanical Society of The Netherlands

MEETING OF THE SECTION FOR PLANT MORPHOLOGY, ANATOMY AND CYTOLOGY ON 30 OCTOBER 1987

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CELL WALL TEXTURE IN GROWING PLANT CELLS

The cell wall formed during growth of a cell is generally called the primary cell wall. Different textural and functional requirements mean that this wall may be subdivided into the "initial" wall and the "growth" wall. The initial wall may be defined as the wall formed after cytokinesis. An alternative term could be "meristematic" wall. The growth wall, laid down during extension of the cell, could also be called the "extension" wall.

Initial walls show a random texture of interwoven microfibrils, while the textures of growth walls are composed of lamellae, i.e. monolayers of parallel-oriented microfibrils, or of layers, a pack of parallel-oriented microfibrils.

Two processes determine the primary wall texture during growth: (1) passive reorientation of the microfibrils of the initial wall in axial direction and (2) deposition of parallel-oriented microfibrils and reorientation in the axial direction of whole lamellae or layers in older parts of the growth wall. This shift of microfibrils can be determined quantitatively by examining both the outer and inner surface of the growing cell wall.

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THE CORTICAL CYTOSKELETON IN THE GREEN ALGA *APHANOCHAETE MAGNA* DURING CELL DIVISION

The structure and dynamic behaviour of the cortical cytoskeleton during cell division in the branched filamentous green alga, *Aphanochaete magna*, was investigated using integrated studies including transmission electron microscopy of thin sections and immunofluorescence microscopy of whole cells. Interphase cortical microtubules (MTs) are usually arranged in an irregular reticulate pattern. However, in the base of apical and side-branch initiating cells a longitudinal arrangement is found which correlates with the presence of a large basal vacuole. At pre-prophase, the cortical cytoskeleton starts to disassemble from the base towards the apex of the cell. At metaphase, some persistent cortical MTs may still be found in the apex of some cells but at anaphase the cortical cytoskeleton completely disappears. It starts to reassemble in the apex at early telophase, repolymerizes progressively towards the base at late telophase and becomes fully re-established in both daughter cells when the new cross wall is formed. Additionally, extensive reorganization of microtubular structures occurs within the cell during division, including formation of MT-asters from nucleus-associated centrioles at pre-prophase and at late telophase, development of the spindle, and formation, maturation and depolymerization of the phycoplast at the transition of mitosis and cytokinesis (Segaar & Lokhorst 1988). However, a structural relation (e.g. reorientation of MTs) between the cortical cytoskeleton and other MT-structures within the cell (asters, spindle, phycoplast) has not been found. Thus, it seems that the cortical cytoskeleton

is an autonomous MT-structure showing cell-cycle related dynamic behaviour which is independent of that of the aster/spindle/phycoplast complex. The behaviour of the cortical cytoskeleton in *Aphanochaete magna* is compared with that reported in other green algae and in several higher plant cell systems.

Segaar, P.J. & Lokhorst, G.M. (1988): *Protoplasma.* **142:** 176–187.

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CELL DIVISION IN THE GREEN ALGA *TREBOUXIA* STUDIED WITH RAPID FREEZE FIXATION AND FREEZE SUBSTITUTION

The formation of the new septum plasma membrane (PM) during cell division (autosporogenesis) in *Trebouxia aggregata* was studied in thin sections after freeze fixation by immersion in liquid propane, substitution in 1% (w/v) OsO_4 in acetone at *c.* −70°C, and embedding in Epon at ambient temperature. Many attempts to fix cells using aqueous solutions of aldehydes gave unsatisfactory results, presumably because of the low permeability of the (sporopollenin containing?) outer cell wall.

After division of the chloroplast and nucleus the new septum develops as a finger-like invagination from the PM at a site of the cortical cytoplasm marked by the duplicated set of centrioles. This area contains an extensive system of irregularly branched tubular membranes which are locally coated at their cytoplasmic faces. Analysis of serial sections indicates that this partially coated reticulum (PCR), first described by Pesacreta & Lucas (1984), is part of a continuum of membranes that extend from the advancing septum to the terminal cisternae at the *trans*-side of Golgi bodies in more distant parts of the cell. Additional evidence of physical membrane transfer from the PCR to the septum comes from the observation that membrane coats identical in size and structure to those that mark the PCR appear in the septum PM. The PCR, which is not associated with ribosomes, differs from Golgi bodies because (1) it consists of branching tubules and fenestrated lamellae, (2) its membranes are not stacked, (3) its cisternae have a larger diameter than those of dictyosomes, (4) portions of its membrane are coated and (5) its membranes are involved with an extensive array of microtubules. It is suggested that in dividing cells of *Trebouxia*, membrane flow occurs from the Golgi apparatus, via the PCR and its coated regions, to the septum PM. Given the fact that a large portion of the PCR-associated microtubules converge towards the septum initiation site, a microtubular involvement in the orientation and positioning of PCR membranes with respect to the area of membrane growth seems possible.

Pesacreta, T.C. & Lucas, W.J. (1984): *J. Cell. Biol.* **98:** 1537–1545.

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NODULIN GENE EXPRESSION IN *VICIA SATIVA* STUDIED WITH ENGINEERED *RHIZOBIUM* AND *AGROBACTERIUM* STRAINS

The symbiotic interaction between certain leguminous plant species and bacteria of the genus *Rhizobium* leads to the formation of root nodules in which nitrogen fixation takes place. Plant genes have been identified that are expressed exclusively in these nodules. These genes encode the so-called nodulins. They have been classified into late nodulin genes, which are first expressed around the onset of nitrogen fixation, and early nodulin genes, which are first expressed at earlier stages of nodule development. Rhizobial genes essential for nodule induction, the *nod* genes, have been

localized on a very large plasmid, the so-called sym plasmid. In order to determine which genes of the sym plasmid are responsible for the induction of nodulin gene expression, a 12-kb *nod* region of the sym plasmid of *Rhizobium leguminosarum* was introduced into a sym plasmid-cured strain of *Rhizobium leguminosarum* and *Rhizobium trifolii*. *Rhizobium leguminosarum* is normally restricted to *Pisum, Lens* and *Vicia* species as host plants, *Rhizobium trifolii* to *Trifolium* species. Both newly formed strains gained the ability to induce nodules on *Vicia sativa*, in which all known nodulins could be detected. Subsequently, the role of the rhizobial chromosome in the induction of nodulin gene expression was examined by introduction of the same 12 kb *nod* region into *Agrobacterium tumefaciens*. This *Agrobacterium* transconjugant also gains the ability to induce nodules on *Vicia*. In these nodules only early and no late nodulin genes could be detected. An electron microscopical study shows that these nodules have a normal organization with an apical meristem and peripheral vascular bundles but that the infection process is severely disturbed. Shortly after release of the bacteria from the infection thread, the infected host cells disintegrate. It is not possible to determine from our observations whether this disturbance is due to the lack of a signal provided by the rhizobial chromosome or to some sort of host defence response elicited by the *Agrobacterium*. The study of such nodules, blocked at defined stages in development, may, however, provide clues to the functions of the nodulins.

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AN AUTORADIOGRAPHICAL STUDY OF CALLUS INITIATION IN CULTURED EMBRYOS OF *ZEA MAYS* (L.)

When immature (1·5–2·0 mm) embryos of *Zea mays* (imbred line A188) were cultured on a modified Murashige & Skoog medium, supplemented with 2 mg l^{-1} 2,4-dichlorophenoxy acetic acid, over 90% of the embryos produced white compact callus with embryogenic potencies. These cultures were used to examine cell cycle events during the early period of callus initiation. The objective was to find a possible relationship between cell cycle behaviour and callus initiation.

Embryos were therefore incubated for 8 h on nutrient agar containing 5 μCi ml^{-1} [^{3}H]thymidine after various periods of culture on a cold nutrient medium. Subsequently, the embryos were fixed in 4% glutardialdehyde and embedded in 2-hydroxy-ethyl metacrylat. Sections (3 μm) were further processed for autoradiography. The fractions of labelled nuclei (*FLN*) as well as the mitotic index (*MI*) were studied in the abaxial and adaxial subregions of the scutellar top, middle and base.

It was found, that during the first day all regions showed a similar response to [^{3}H]thymidine uptake and mitotic activity. Subsequently, regions that do not produce callus (top region and adaxial base) slowed down in DNA replicating activity, while cell division ceased. The abaxial middle and basal scutellum, however, showed an increase in *FLN* and *MI* in the period between 48 and 56 h of culture. A zone with meristematic cells became visible in these regions and most division planes were periclinal. After a 72-h culture period these divisions gave rise to a protuberance which indicates callus development.

The results further suggest that (i) the cell cycle in the whole scutellum is disturbed during the first 8 h of culture and the cells probably arrest in G_1, (ii) after recovery of the proliferation activity (8–24 h) cells in the top and the adaxial base become arrested in G_2, and (iii) some cell cycle synchronization has taken place in the abaxial middle and base during the period of callus initiation.

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EFFECTS OF SOIL AND ACIDIFICATION ON NEEDLES OF *PSEUDOTSUGA MENZIESII* (MIRB.) *FRANCO*

Needles of the Douglas fir, *Pseudotsuga menziesii* (Mirb.) *Franco* (family Pinaceae), of plants cultivated on garden mould or sterile sand, were investigated. The plants were supplied either with tap water (pH 7·5), or with nitric (65%) and sulphuric (96%) acid (1:1) acidified water (pH 4·0). The young plants were cultivated in a greenhouse at a temperature of 24°C and harvested after 5 months.

Cell contents, formation of extracellular calcium oxalate crystals, and formation of thick cell walls were studied.

The number and surface of calcium oxalate crystals were analysed by a morphometric method, using a MOP-30 (Zeiss) image analyser. There was a significant difference in the covering of mesophyll cell walls with crystals between plants cultivated on garden mould and plants cultivated on sterile sand. On sterile sand there was a higher percentage of covering both under tap water and acidified water conditions. Using garden mould there was a better chance of a crystallization point on a certain surface of a mesophyll cell wall under acidified water conditions than under tap water conditions.

Starch grains were only abundant in needles, stems and roots of plants cultivated on sterile sand with acidified water. Under these conditions cells of mesophyll and transfusion parenchyma of the needle, and also of the cortex and the pith of the stem and pericycle of the root, contain many starch grains with a large variation in size. Other cells of the aforementioned tissues had a brown, graniferous cell contents with a certain degree of degeneration.

The cell walls of the central phloem ray cells and the periclinal walls of the adaxial and abaxial epidermal cells were thicker in the needles of plants cultivated on sterile sand than on garden mould. There was no difference in cell wall thickness between plants grown under tap water or acidified water conditions that were cultivated on the same substrate.

In this investigation the presence of calcium oxalate crystals, starch accumulation and the degeneration of cell contents might serve as an indication of soil condition and acidification. The thickness of the cell wall might only serve as an indication of soil condition.

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ECOLOGICAL TRENDS IN THE WOOD ANATOMY OF EUROPEAN TREES AND SHRUBS

Ecological trends for the occurrence of qualitative, wood anatomical, character states have been analysed for 505 species from Europe, Cyprus and Madeira. Macroclimatic gradients from boreal, via temperate to Mediterranean are closely related with a decreasing incidence of scalariform vessel perforations, exclusively solitary vessels, and fibre-tracheids. In this sequence the incidence of vessels of different size classes and vascular tracheids increases. Ring-porous tendencies and spiral vessel wall thickenings have their peaks in the temperate zone. The subtropical woody flora of Madeira shows a low percentage of species with any of the above attributes.

In the graded series from dry, via normal, to mesic, the incidence of scalariform perforations strongly increases and that of vascular tracheids decreases. Other attributes show a weaker or ambiguous relationship with moisture availability. There is also a relationship between some wood anatomical characters with plant habit: the incidence of species with scalariform perforations,

ring-porosity, and solitary vessels gradually decreases in the series from trees, via shrubs to dwarf shrubs, while the reverse holds true for species with different vessel size classes and vascular tracheids. Climbers score high for ring-porous tendencies, different vessel size classes and spiral thickenings.

Within ecologically diverse genera or families, very little of these salient trends, established on a floristic basis, can be traced back. The ecological trends seem to be the result of selection for specialized wood anatomical features in the Mediterranean region and other dry and/or warm regions in general. Primitive features, such as scalariform perforations, solitary vessels, and fibre-tracheids, are not selected against in cool boreal and mesic habitats. For some characters, macroclimatic factors (chiefly temperature) seem more important than moisture availability, for others moisture availability is equally important.

The biological significance of the reported trends should be viewed in relation to hydraulic safety and efficiency of the secondary xylem and with evolutionary correlative constraints on individual xylem characters. For a full report see Baas & Schweingruber (1987).

Baas, P. & Schweingruber, F.H. (1987): *IAWA Bull.* **8:** 245–274.

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AIRBORNE AND ALLERGENIC GRASS POLLEN GRAIN DETERMINATION BY LIGHT- AND UV-MICROSCOPY

In The Netherlands, daily pollen counts have been in progress in Leiden since 1970 and in Helmond since 1975. The counts are registered per species, per genus or per family as the number of pollen grains per cubic metre of air, averaged over 24 h. The entries in the pollen lists are ordered either by species (3 ×) or by genus (29 ×) or by family (13 ×). The grasses (Gramineae) are ordered at family level (Spieksma *et al.* 1985). Determination down to genus level, and certainly down to species level, is very difficult within the family of grasses. Should this be possible, then determination at these levels would be a valuable supplement to the pollen counts.

The first successful attempt at morphological identification of grass pollen grains was made by Liem (1968). She developed a method involving marked dehydration of the pollen and studied them, thereafter, by a light microscope.

Attempts have been made by us to establish whether Liem's method would also be appropriate to other grass species, and whether it might be used in the daily pollen counts now in progress in The Netherlands in Leiden and Helmond.

Pollen walls, and sometimes the cytoplasm, show autofluorescence by UV excitation, due to the presence of molecules with rings and with unsaturated bonds. During excitation, however, a photochemical reaction occurs resulting in a colour shift and in fading. The autofluorescence emission spectra have been measured for several types of pollen and have shown some specificity (Willemse, 1972). In this study the pollen wall autofluorescence of some grass pollen was measured to discriminate between the different grass species.

Pollen from 20 different grass species was prepared by a dehydration method and then examined with a light microscope (800 ×). Although differences were found between the grass pollen species, these were insufficiently marked to provide a key to identification of all the 20 species. Two grass species were found to show typical dehydration features which permitted their identification.

Pollen from 21 grass species was studied using incident UV light microscopy. By this autofluorescence technique, 15 of the 21 grass pollen species could be identified. A key for 21 airborne and allergenic grass pollen species is presented.

Liem, A.S.N., Groot, J. & Kuilman, L.W. (1968): *Rev. Paleobotan. Palynol.* **7:** 213–231.
Spieksma, F.T.M., Assem van den, A. & Collette, B.J.A. (1985): *Grana* **24:** 99–108.
Willemse, M.T.M. (1972): *Acta Bot. Neerl.* **21:** 1–16.

MEETING OF THE SECTION FOR VEGETATION RESEARCH ON 21 JANUARY 1988

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'OOIBOSSEN' ALONG THE RIVER WAAL

Over 100 vegetation relevés were made in forest communities in 1986 in the area of the river Waal (a Rhine branch) between Zaltbommel and Millingen aan de Rijn. They were classified by the Research Institute for Nature Management (RIN) on the basis of an improved syntaxonomic system for forests in The Netherlands. Application of this system, with the given characteristic species based on the Braun–Blanquet approach, leads to a clear classification, except for the relevés of very disturbed sites. The data on soil, water-balance, management and history for the relevés were also classified. There is a clear relation between these habitat factors and the communities.

The following (sub)communities were distinguished:

Salicetum albo-fragilia, willow forests mostly in river forelands; a 'wet' subassociation with *Myosotis palustris* was distinguished on wet young river clay soils recently (1950) dug-out, and a 'dryer' subassociation typicum without differential species.

Fraxino-Ulmetum alnetosum, mainly found in poplar plantations on young dry river-clay soils; only a few forests on the inland side of the dike are slightly better developed.

Fraxino-Ulmetum typicum, well developed in manor forests situated on the land-side of the dike, on sandy to loamy soils with a low mean water level.

Stellario-Carpinetum, found in an old forest area (Personnenbos) on an old developed river clay soil with a fluctuating water level.

Violo odoratae-Ulmetum, also found in one particular forest area (Colenbrandersbos) situated in the river forelands on a calcareous riversand soil which is seldom flooded.

In a floristic comparison between forest sites in the river forelands and the inland side of the dike, 65% of the species showed a preference for one of the two environments. As a result of this comparison, a new subcommunity is proposed between Salicion and Fraxino-Ulmetum as part of the second one, named after the genus *Salix*.

The development of some willow woodlands of the Groenlanden/Tiengeboden during the last 10 years (1976–1986) has also been studied, e.g. an open willow shrub-like woodland changing into a high closed willow woodland and leading to a shift in the spectrum of species in the herb layer. Annuals and pioneer species, which require a high light intensity and a moist soil, disappeared. Only a few new species appeared and some of those already present showed a substantial increase.

A start was made to study the forest dynamics by making permanent transect plots and drawings of the horizontal and vertical forest structure. The development of several species can be seen from their relative place in different layers.

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THE IMPACT OF FIVE MOWING REGIMES ON THE ECOLOGICAL QUALITY OF ROAD VERGE VEGETATION

Road verges may have an important ecological value. In order to maintain or even to enhance the ecological quality of road verge vegetation, a study was implemented in 1982 to test the effects of five management regimes, i.e. mowing twice a year, annual mowing in June, annual mowing in September, mowing every second year and no mowing. These effects were studied on road verges on clay and sandy soil, in full sun and under a tree canopy. Before 1982 the vegetation was mown twice a year. Ecological quality was expressed in various parameters, e.g. species diversity, species rareness, abundance of species characteristic for haylands and/or wood edges, absence of species characteristic for disturbance; all parameters, unweighed as well as weighed, by abundance values.

Although some parameters have improved during the last 5 years, while others have not been changed at all or have even deteriorated within the same condition, it can be shown, thus far, that in most situations maintainance of the regime of mowing twice a year produced the highest ecological value. In shaded situations other mowing regimes, i.e. no mowing (sandy soil) and annual mowing in September appeared to result in about the same ecological quality.

Apart from the regime of no mowing in the shaded plots on sandy soil, all regimes with a lower mowing frequency than once a year entailed a general deterioration of almost all (botanical) ecological values. So far there are no reasons to propose drastic changes in the current mowing regimes in road verges in comparable situations. This research will be continued to gain a better knowledge of the ecological values as described over a longer period.

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EFFECTS OF FOREST FERTILIZATION ON THE MYCOFLORA

Forest fertilization is intended to mitigate the harmful effects of air pollution on the vitality of forests. It is, therefore, only a temporary measure until an acceptable level of air pollution is achieved. In order to give a balanced judgement on the acceptability of corrective fertilization, it is necessary to study the effects on many different forest components.

Recently, a fertilization experiment was executed at the municipal forest of Harderwijk (central part of The Netherlands). After 2 years of studying the effects on the mycoflora, the following (temporary) conclusions can be drawn:

1. Fertilization with liquid manure (amended with potassium, magnesium, calcium, and phosphorus) leads to a decrease in species diversity of both ectomycorrhizal and saprotrophic macrofungi. Concomitant with the decrease in diversity of saprotrophic species, a dramatic increase in litter thickness was observed.

2. Fertilization with calcium (as calcium carbonate) also leads to a significant decrease in both the diversity and abundance of ectomycorrhizal and saprotrophic species. Here again an increase in litter thickness was observed. It is probable that this negative effect on litter decomposition is not a direct effect of liming *per se*, but reflects the increased availability of nitrogen as an indirect effect of an increased pH. Species composition of the manured and limed plots is (almost) identical and a few species of saprotrophic fungi are exclusively confined to both treatments.

Investigations in the northern part of The Netherlands indicate that application of lime after the removal of the litter layers has a positive effect on ectomycorrhizal species and only a weak, negative, effect on saprotrophic species.

3. Fertilization with potassium, magnesium and/or phosphorus has no effect on ectomycorrhizal and saprotrophic fungi.

It is not clear, however, whether this type of fertilization would make our forests more uniform.

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SEED BANK, GERMINATION AND ESTABLISHMENT IN HEATHLANDS AND THE IMPLICATIONS FOR MANAGEMENT

Two key factors are involved in the process of replacement of heather by grasses, i.e. the presence of grass caryopses (subsequently referred to as grass seeds) in the seed bank and the reduction of the canopy of the *Calluna* vegetation. A set of experiments has been carried out in heathlands dominated by *Calluna vulgaris* and *Erica tetralix* and in former heathlands now dominated by *Molinia caerulea* or *Deschampsia flexuosa*. Estimates have been made of the amount of viable seed in the soil and of the emergence and establishment of seedlings in these vegetation types, which were experimentally treated by removing litter, mowing and sod cutting, respectively.

In both heath and grass vegetation large amounts of heather seeds were persistently present mostly in the organic layers of the soil. *Molinia* seeds were also persistently present, although in much smaller amounts and exclusively in the organic layers of the soil. *Deschampsia* seeds germinated or disappeared by predation or rotting from the soil soon after dispersal.

No severe inhibition of germination of heather seeds occurred in litter of the heather species and under the intact canopy in the different vegetation types. However, germination was strongly inhibited under a thick layer of grass litter probably because of the strong reduction in received radiation.

The ability of the top layer of the soil to maintain sufficient humidity seems to be a determining factor for germination and establishment of heather seeds. In this repect, a litter layer does inhibit germination because it dries out quickly due to its loose structure. In addition, the mineral sand is unable to maintain sufficient humidity under dry soil conditions. Both *Erica* and *Calluna* seedlings were best established in the mown vegetation where litter was removed. Contrary to *Calluna* seedlings. *Erica* seedlings were established well in the sod cut plots. Seedlings of *Molinia* emerged in considerable amounts in the mown vegetation where litter was removed. The number of *Molinia* seedlings in the sod cut plots was small, but is still of importance because these seedlings were well established.

To re-establish heathland, the most favourable depth of sod cutting in wet vegetation is down to the mineral soil layer. In dry vegetation, however, the most favourable depth is in or down to the humus layer. The most favourable time for mowing and sod cutting is just before seed setting of the grass species (Bruggink 1987).

Bruggink, M. (1987): Nutrientenbalans van droge zandgrondvegetaties in verband met eutrofiëring via de lucht. Stichting Milieubeleid en Ekologie, Nijmegen.

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MANAGEMENT PRACTICES AS A REMEDY TO DIMINISH THE IMPACT OF ACID RAIN

Air pollutants (acid rain) affect the plant species composition of all types of ecosystem. Semi-natural ecosystems are originally often characterized by their relative species richness. Until recently, the

effect of acid rain on the species composition of ecosystems was mainly considered to result from acidification. Acid rain, however, also causes eutrophication. In the Dutch situation, additional eutrophication largely results from atmospheric ammonia (NH_3/NH_4^+) deposition volatilized from the manure used in intensive agricultural systems. Increased ammonia availability results in changed competitive relations between plant species and this leads, ultimately, to changes in species composition (Heil & Bruggink 1987).

Deposition of acid rain is not equally distributed but depends on the structure of the intercepting surface. It has been suggested that forests trap higher amounts of acid deposition compared with treeless vegetation types because of their rough canopy structure. It is often assumed that the interceptive properties of short vegetation are smaller. Our measurements show that these canopies can capture as much wet and dry deposition as forests (Heil *et al.* 1988). The importance of spatial and temporal differences in the canopy structure of short vegetation for interception of acid rain is discussed.

Our results also show that management of the canopy structure is essential to prevent eutrophication of the habitat by acid deposition. Management, by mowing the vegetation before maximum standing crop, leads to smoother canopy surfaces and thus to substantially smaller amounts of acid deposition in these canopies.

Heil G.W. & Bruggink, M. (1987): *Oecologia* (Berlin) **73:** 105–108.
Heil G.W., Werger, M.J.A., de Mol, W., van Dam, D. & Heijne, B. (1988): *Science*, **238:** 764–765.

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VEGETATION DEVELOPMENT IN THE NATURE RESERVE OEVERLANDENRESERVAAT AMSTELVEENSE POEL

A management regime was applied in 1960 to restore open fen vegetation which had turned into fen woodland in the fens bordering the Amstelveense Poel. From 1977 onwards, the University of Amsterdam assisted in botanical investigations in the area to evaluate the results of a mowing regime that aimed at the development of a diverse and botanically interesting landscape that would reflect the natural possibilities of this fenland.

In 1975 sods were cut down to the groundwater level at sites in the fens that were formerly occupied by small nurseries, in order to remove enriched garden soil. In 1977 four permanent plots were laid out on cut parts of the fens and four on uncut parts.

Over the area where sods had been cut, a new *Sphagnum*-layer developed following an initial period with ruderal species. In the first 5 years of its development the vegetation showed many resemblances to the Calthion palustris. Now it seems to be developing towards a Pallavicinio–Sphagnetum in other parts of the reserve where trees and shrubs were cut. The *Sphagnum* layer here has gradually turned into a *Polytrichum* carpet, partly due to heavy damage caused by mowing and harvesting in a very wet season. The vegetation here still belongs to the Thelypterido-Phragmitetum but is locally very poor in species ($< 8\ m^2$).

The species that have disappeared since 1980 are partly ruderals and species of mineral rich soils and include acrocarpous and pleurocarpous mosses that became suppressed in the closing *Sphagnum*/*Polytrichum*-carpet.

Comparison of groundwater samples taken in 1977 and 1978 and in 1982 and 1983 indicates that the influence of slightly brackish water from the adjacent lake has diminished. Available amounts of phosphorus and nitrogen also show a tendency to be lower than in 1977 and 1978. The largest changes in overall groundwater composition were found at sites where sods had been cut.

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EFFECTS OF GROUNDWATER WITHDRAWAL ON MARSH VEGETATION IN THE 'ZUIDLAARDERMEER' AREA

In the polder area, 15 km south of the city of Groningen in the northern part of The Netherlands, vegetation types belonging to the alliances of Calthion palustris, Caricion lasiocarpae occur in some nature reserves and point towards seepage of Ca^{2+}-rich groundwater.

Hydro-ecological investigations were carried out to assess the impact of groundwater withdrawal from the main aquifer on the nature reserves in the polder area and along the lake 'Zuidlaardermeer'.

Hydrological research shows that most of the study area had changed from seepage to an infiltration area, due to pumping activities and drainage. Replacement of Ca^{2+}-rich groundwater by Ca^{2+}-poor water was also observed in various parts of the study area. An analysis of former distribution patterns of calciphilous marsh plants revealed that before the start of the water extraction, Ca^{2+}-rich groundwater must have been present in the top soil of most of the polder area.

A rough sketch of the former vegetation zonation was made, with special reference to the original peat-forming plant communities. This reconstruction has been partly derived from the literature and partly from macro-rest analysis of the peat deposits. Additional information has been obtained from the present distribution patterns of characteristic marsh-plant species.

Now only small relics of these vegetation types have been preserved. Practically the whole polder area is in agricultural use and the few remaining nature reserves suffer from drainage and associated acidification. This is particularly true for nature reserves that lie in the vicinity of the main infiltration area ('Hondsrug'). The situation along 'Zuidlaardermeer' appears to be slightly better. Ca^{2+}-rich groundwater and associated vegetation types were still found here. Detailed analysis of the groundwater composition in deeper layers seems to indicate, however, that replenishment of Ca^{2+}-rich water from the main aquifer has stopped. Every year considerable amounts of the remaining Ca^{2+}-rich groundwater are lost to the surrounding drainage channels in the polder area. Under such conditions the preservation of the present marsh plant communities is no longer safeguarded.

MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE ON 24 SEPTEMBER 1987. Organized in co-operation with The Section for Tissue Culture of The Netherlands Society for Cell Biology

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THE ROLE OF POLYPEPTIDE GROWTH FACTORS IN CONTROL OF MAMMALIAN CELL PROLIFERATION

Mammalian cells require a variety of nutrient components for optimal *in vitro* proliferation. In addition, however, a set of hormones, the so-called polypeptide growth factors, is required to stimulate these cells to enter the cell cycle, as a prerequisite for DNA duplication and mitosis. Mammalian cells *in vitro* are routinely cultured in media supplemented with fetal calf serum, which serves both as a source for various nutrients and for polypeptide growth factors. In order to investigate the role of growth factors in cell proliferation in more detail, it is therefore essential to work under serum-free (Barnes & Sato 1980) or growth factor-defined assay conditions (Van Zoelen

et al. 1985). This latter approach involves the fact that polypeptide growth factor activity in serum is inactivated by a chemical treatment (see details in Van Zoelen *et al.* 1985). As a consequence, defined polypeptide growth factors like epidermal growth factor (EGF), insulin or platelet-derived growth factor (PDGF) should be added to stimulate cell proliferation. The mechanism by which these growth factors exert their growth stimulating activity is currently under investigation.

Using the above approach, we have shown that non-tumorigenic cells, like the normal rat kidney (NRK) cell line, become quiescent when they are cultured in the absence of polypeptide growth factors. In contrast, NRK cells transformed by the simian sarcoma virus (SSV) proliferate as rapidly in the presence as in the absence of externally added growth factors. This is mainly caused by the fact that SSV-NRK cells are able to produce growth factors themselves, not only a PDGF-like growth factor, which parallels expression of the *v-sis* oncogene (Waterfield *et al.* 1983), but in addition both transforming growth factor (TGF) type alpha and beta (Van Zoelen *et al.* 1987). As a consequence such transformed cells can stimulate their own proliferation by the production of growth stimulating hormones, a process referred to as autocrine growth stimulation.

Studies on NRK cells have shown that besides growth stimulation polypeptide growth factors are also able to induce phenotypic transformation of non-tumorigenic cells. This process involves the fact that in the presence of specific combinations of growth factors, these cells assume various characteristics of transformed NRK cells, including an altered morphology, loss of density-dependent inhibition of growth and induction of anchorage-independent proliferation. This process is referred to as phenotypic transformation, since it is reversible upon removal of the growth factors added. Under serum-free and growth factor-defined assay conditions, we have shown (Van Zoelen *et al.* 1986) that only specific combinations of growth factors are able to induce this process. The molecular mechanisms underlying this process are still unclear.

Many tumour cell lines have a strongly reduced requirement for exogenous polypeptide growth factors, since they are able to produce these growth factors autonomously. In the case of the oestrogen-dependent human breast tumour line MCF-7, however, no cellular proliferation is observed in the absence of growth factors. By using combinations of insulin and oestrogens, it has been shown that in these cells the autocrine production of growth factors is controlled by the presence of this steroid hormone (Dickson & Lippman 1987).

The above data show that polypeptide growth factors play a central role in the control of proliferation of both normal and transformed mammalian cells.

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DIFFERENTIATION OF HUMAN MESENCHYMAL CELLS

Differentiation can be considered to be the appearance of new structures and functions. The newly formed products are characteristic of a certain type of cell or tissue. *In vitro* studies of the various stages of cell differentiation are possible but studies with normal cells are hampered, mainly due to their limited cell growth, in contrast to studies with tumour cell lines where growth is not limited. Studies with tumour cell lines have an additional advantage, namely that these cells can be classified according to their stage of differentiation. Furthermore, induction to other stages of differentiation

is possible by adding chemical inductors into the culture fluid. For example, under the influence of phorbolesters a promyelocytic leukaemic cell line (HL-60) will obtain monocytic characteristics, but under the influence of dimethyl sulphoxide (DMSO) this HL-60 cell line will express myelocytic characteristics. DMSO is also able to induce mature differentiation features of primitive cells derived from a rhabdomyosarcoma. Thus, as a model system for differentiation pathways of mesenchymal cells, tumour cell lines can be used combined with differentiation inductors.

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DIFFERENTIATION IN PLANTS

Differentiation in an intact plant primarily takes place in and near the meristems that are located at the apex of the shoots and the roots. These meristems are the centres of organization of the primary structure of the main organs of the plant. Unlimited growth and a high degree of autonomy are characteristic for meristem function. These characteristics lead to the ultimate size and shape of the plant which are much more flexible than in animals.

Another important differentiation process is induced by wounding. Plants possess a remarkable regeneration capacity. Regeneration also occurs in tissue culture on isolated explants. In principle, roots and shoots may be regenerated on such explants. This property enables the investigator to study *in vitro* differentiation in depth. Organ regeneration can be manipulated: either roots or shoots are formed dependent on the conditions imposed on the tissue.

A special case of induced differentiation is the development of flower buds on explants of stem tissue of tobacco. Two hormones are involved in the induction: an auxin and a cytokinin. The different aspects of the process can be traced back to one of these hormones or to an interaction of both. One example is the arrangement of the flower buds on the surface of the tissue. This spacial pattern of development is controlled by the auxin concentration. Observations like this throw doubt on the popular concept of the 'hormone balance'. This concept implies that the ratios between the hormone concentrations rather than the concentrations themselves determine the direction of regeneration processes.

A differentiation process at the biochemical level is the induction of biosynthetic pathways that lead to production and accumulation of secondary metabolites. The structure of these compounds may be very complex. A number of them have economic value. In many cases the secondary metabolism is confined to specialized cells. This means that biochemical differentiation is coupled to morphological differentiation and, therefore, would be inducible in tissue culture.

Transformation by certain strains of *Agrobacterium* can also be used as a means to induce differentiation. Sometimes patterns of growth and differentiation are obtained which cannot be induced in any other way. Application of the transformation technique will certainly broaden our insights into the fundamentals of differentiation.

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TRANSFORMATION OF PLANT CELLS

Transformation of plant cells and the regeneration of transgenic plants has become an important tool for research concerning an increasing number of fundamental and applicable problems in plant research. Two topics form the base of this development. Both have been unravelled at the

fundamental level resulting, finally, in the development of tools for application in plant cell biotechnological research. Those topics are:

1. The totipotency of plant cells; a property that allows the regeneration of complete plants from isolated somatic plant cells (protoplasts).

2. The crown gall system; a naturally occurring system that allows the transfer of foreign DNA from *Agrobacterium tumefaciens* to plant cells.

The development of transformation methods, based on these two topics, is one of the key evolvements in modern molecular and cellular biology. Methods to obtain single somatic plant cells (protoplasts) have been defined and can now be used for a wide range of species; from dicot to monocot, from sugar beet to poplar.

The most important transformation systems have been developed from the crown gall system; in the first instance mediated by the bacterium *Agrobacterium tumefaciens* via the co-cultivation method (Marton *et al.* 1979), followed by the development of DNA transformation methods such as have been developed for animal systems, based on direct DNA transfer (Krens *et al.* 1982), electroporation (Fromm *et al.* 1986), fusion with liposomes (Des Hayes *et al.* 1985), micro-injection (Miki *et al.* 1987). Recently other methods have been developed to apply DNA directly to certain tissues by microinjection (De La Pena *et al.* 1987) or by incubation of seeds with DNA (Graves *et al.* 1986). All the methods mentioned here finally result in integration and expression of foreign DNA. This application of DNA to tissues brings us back to the situation in 1965 when Ledoux described similar kinds of experiments (Ledoux *et al,* 1965). Unfortunately Ledoux did not have the disposal of probes and reporter genes to prove the transfer and expression of the DNA unequivocally.

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SOMATIC CELL HYBRIDIZATION OF ANIMAL CELLS

Through fusion of somatic cells, the nuclei and cytoplasms of two or more diploid cells are combined within one plasma membrane. In the living animal this process is restricted to cells with similar characteristics and occurs only in special cases of tissue differentiation, e.g. development of muscle cells, or as a symptom of a variety of diseases. In culture, spontaneous fusion of animal cells can be observed between dissimilar cell types, although it may be at a very low frequency. In the last decades, several techniques incorporating biological, chemical or physical agents, have been designed to increase the hybridization of cells *in vitro*. A common element of all these methods is the establishment of a close contact between the cells, in combination with a local structural disorganization of the plasma membrane. Furthermore, the development of a variety of animal cell mutants has enabled the selection for true cell hybrids from a cell fusion mixture.

In a heterokaryon, the activities of the parental constituents mutually influence each other and generally equilibrate to the level of the most active one. Heterokaryons have a limited life span. For proliferation, additional fusion of their genomes is required. This occurs only when both nuclei enter

mitosis simultaneously. Thus, two truly hybrid daughter cells are produced, both with a single nucleus incorporating the chromosomal complements of the two parental cell types (synkarion). In most heterokaryons, however, the onset of mitosis in a single nucleus results in destruction of the interphasic chromosomes of the remaining partner because of their precocious attempt to participate in the same process (premature chromosome condensation).

Most synkaryons suffer from chromosome loss during subsequent proliferation, particularly when they are derived from interspecific hybridizations. There exists a tendency to lose most of the chromosomes from the parental cell type which shows less favourable *in vitro* growth characteristics. This phenomenon can be used to an advantage, however, since it creates the possibility to designate structural genes for proteins to specific chromosomes by simultaneously examining the hybrids for gene products and karyotype. The remaining genes in a cell hybrid operate in a new regulatory environment, with the result that their activities can be preserved (co-expression), abolished (extinction), or induced (activation). Thus, cell hybrids can be used to study gene regulation and cell differentiation in eukaryotic cells. The contribution of specific cellular compartments to these processes can be analysed by fusion with cell fragments, resulting in reconstituted cells (cybrids).

Though differentiated characteristics tend to become extinguished in a somatic cell hybrid, certain combinations of parental cells have been found to preserve facultative gene expression. The most famous example of this phenomenon is the continuous expression of antigen-specific immunoglobulin synthesis by cell hybrids (hybridomas) derived from fusions between mouse or rat myeloma cell lines, and B-lymphocytes obtained from antigen-stimulated animals. In many aspects, the hybridoma technique illustrates the potential of somatic cell hybridization of animal cells for fundamental and applied research.

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CELL FUSION; PLANT ASPECTS

A plant is a differentiated organism, i.e. it is composed of cells with a specific function, organized into organs such as roots, stems, leaves and flowers. A constant growth and supply of differentiating cells is provided by the meristems, areas of undifferentiated, continuously dividing cells. Cell division also takes place in the production of male and female gametes, which form the zygotes after subsequent fusion. In this way new combinations or different properties within a species are made. Sexual crossing is the main instrument in plant breeding. However, due to crossing barriers not every desired trait can be transferred to a crop from another species.

An alternative to sexual crosses is somatic hybridization, in which somatic cells of different parents are artificially fused. The ability of free, isolated plant cells to regenerate into complete, intact plants is important in this process and is called the totipotency of plant cells. Different organs of the plant may serve as starting material for the production of free cells during which dedifferentiation occurs. For the actual fusion of the membranes it is necessary to remove the cell walls from the isolated cells, i.e. to make protoplasts. This is the first technique involved in the process of somatic hybridization: protoplast isolation.

Other techniques include: the induction of protoplast (membrane) fusion, recognition and selection of hybrid fusion products (heterokaryons), culture of heterokaryons (often at low densities), regeneration of plants from hybrid callus, characterization of hybrids/cybrids and, if necessary, a limited back-crossing programme.

The general goals in somatic hybridization are:

1. New combinations of genetic material, which cannot be obtained by sexual crosses or require a long and tedious crossing-programme.

2. New combinations of nuclei with cytoplasms which cannot be made in any other way. This includes the asymmetric fusions and cybridizations.

3. Gene mapping; localization of genes on chromosomes by studying chromosome elimination or complementation of mutants after fusion. Eventually the aim is to transfer from one species to another traits such as: resistance to pathogens, tolerance to stress conditions, yield improvement, quality improvement and cytoplasmic male sterility. Programmes have also been initiated to circumvent long crossing programmes, to enlarge the genetic variability within a crop, to prepare fast growing, high producing cell lines (plant hybridomas) used for the production of secondary metabolites.

At this moment we must realize that many goals are not readily feasible in all instances; undesired side-effects are known to occur, e.g. polyploidization and hybrid sterility. However, cell fusion remains a powerful tool in the genetic manipulation of plants especially since the precise biochemical and genetic mechanisms of many traits are still obscure.

MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE ON 13 NOVEMBER 1987

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PERCEPTION AND TRANSDUCTION OF AUXIN IN THE REGULATION OF MEMBRANE-BOUND PROCESSES

The current knowledge concerning the molecular action of auxin on membranes of higher plant cells is still too fragmentary to recognize a consistent picture of the hormone action at this level. In general, it is assumed that the competence of a plant cell to respond to auxin is caused by the association of the hormone with a specific membrane-located receptor, which initiates a sequence of events resulting ultimately in the well-known macroscopic phenomena of plant growth and development. In contrast to the numerous descriptions of high affinity binding sites for auxin detected in various plant species (Rubery 1981; Venis 1985), the reports concerning the coupling of these auxin binding sites with defined biochemical events, others than those connected with auxin transport, are surprisingly rare. However, it is clear that two types of auxin binding sites (localized at the plasma membrane) act as specific influx and efflux carriers of the hormone (Hertel 1983). The auxin transport is suggested to be correlated with its action, but not to be identical to its primary action (Hertel, 1983). Auxin binding sites detected on endomembranes were assumed to represent related carriers in a mirror image orientation in the membrane, though their transport capacity has not yet been proved. Otherwise, the auxin binding sites of the endoplasmic reticulum may be precursors of the plasma membrane-located auxin transport carriers or of auxin receptors that possibly exist. Until now there are only two reports to demonstrate first evidence for the occurrence of an obviously plasma membrane-located auxin binding site with receptor function. An auxin-binding protein was extracted and purified from membranes of maize coleoptiles, and polyclonal antibodies against this protein were able to block the auxin-promoted reaction in the pea split assay (Löbler & Klämbt 1985). However, since the biochemical action of the auxin binding protein is as yet unclarified a carrier function cannot be excluded.

As a consequence of the difficulties involved in detecting auxin binding sites with receptor functions, a more recent experimental approach was to look for auxin-mediated effects in the time scale

of seconds on membrane-located processes (Zbell & Walter, 1986). The previous findings of auxin-mediated effects on the release of Ca^{2+} (Buckhout *et al.* 1980) and orthophosphate (Scherer & Morré 1978), from membranes as well as on protein phosphorylation (Zbell 1983; Morré *et al.* 1984), were interpreted as indications of an auxin action on polyphosphoinositide metabolism in plant cells (Zbell, 1983). Since polyphosphoinositides and the enzymes for their formation and hydrolysis are present in plant cells (Poovaiah *et al.* 1987), the question arises whether these constituents are functionally connected with a signal transduction pathway in plant cells. Indeed, using [γ^{32}P]ATP as a label for the polyphosphoinositides, a rapid decrease in radioactivity of the phospholipid fraction extracted from membranes of carrot suspension cells can be detected in the presence of auxin. The hormone effect was caused by a simultaneous release of inositol polyphosphates (Zbell & Walter 1986) and not by an inhibition of the lipid phosphorylation reactions, as was recently reported to be an effect of cytokinins (Falkenau *et al.* 1987). These findings point to an auxin-mediated control of a phospholipase C-like reaction, similar to the hormone-stimulated phosphoinositide response in animal cells (Boridge 1987). The inositol-1,4,5-trisphosphate which is liberated from the membrane-located polyphosphoinositides by the auxin-promoted enzymatic hydrolysis is known to act also in plant cells for the mobilization of Ca^{2+} from internal stores of the endoplasmic reticulum or vacuole (Drøbak & Ferguson 1985; Schumaker & Sze 1987). In contrast to the phosphoinositide response in animals, a protein kinase C-like enzyme stimulated by diacylglycerol, the other hydrolytic product of the reaction, has as yet not been clearly demonstrated to exist in plants (Poovaiah *et al.* 1987), though rapid auxin-promoted protein phosphorylations were found to be localized on membranes (Morré *et al.* 1984). It has been hypothesized that the release of Ca^{2+} from internal stores leads to a transient increase in cytoplasmic Ca^{2+} while the protein phosphorylation acts as a more persistent effect of the auxin transduction pathway. Both events could be connected to cellular processes such as exocytosis or the initiation of mitosis. The detection of the rapid auxin-stimulated phosphoinositide response, as well as the occurrence of high-affinity GTP binding (Hasunuma & Funadera 1987 unpublished) on isolated plant cell membranes, offers new experimental approaches for the analysis of the putative auxin receptor and its signal transduction pathway.

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HORMONES AND RESPIRATION IN PLANT TISSUE CULTURE: MODULATION OF THE CAPACITY AND THE ACTIVITY OF THE ALTERNATIVE RESPIRATORY PATHWAY BY PLANT HORMONES

During growth and development, the production of intermediates and ATP has to be adjusted to the needs of the growing plant tissue. Most plant tissue cultures grow heterotrophically and are dependent on the respiratory metabolism for these intermediates as well as for the ATP. Plant hormones may be an important factor in the adjustment between ATP-consuming processes (growth) and ATP-producing processes (respiration).

In plant mitochondria two pathways for the transfer of electrons to oxygen may occur: the energetically efficient cytochrome pathway and the energetically inefficient, CN-resistant alternative pathway. *In vivo*, the cytochrome pathway is preferentially used and the alternative pathway functions as an overflow: the excess of electrons is transferred to oxygen via the alternative pathway only when the cytochrome pathway is saturated.

Two problems can be distinguished:

1. Is there a modulation of the alternative pathway capacity by plant hormones?

The addition of the auxin, naphtyl-acetic-acid (NAA), gibberellic acid (GA), the ethylene-precursor amino-cyclopropane-carboxylic acid (ACC) and abscisic acid (ABA), in a concentration range of 10^{-7} to 10^{-3} M had no appreciable effects on alternative pathway-mediated respiration of isolated potato callus mitochondria. High concentrations ($> 10^{-4}$ M) of the cytokinin kinetin showed inhibitory effects. Apparently, the instantaneous effects of plant hormones on the alternative pathway capacity of isolated mitochondria is generally negligible. However, long-term incubation of potato tuber explants on hormone-containing media showed clear effects on alternative pathway induction: NAA (5×10^{-5} M) and ACC (10^{-5} M) increased this induction when present in the nutrient medium. ABA (4×10^{-5} M), on the contrary, showed inhibitory effects. The NAA-effect is most probably caused by a stimulation of the endogenous ethylene production: inhibitors of the ethylene biosynthesis (e.g. amino-ethoxy-vinylglycin) and of the ethylene action (e.g. 2,5-norbornadiene) inhibited the induction of the alternative pathway caused by NAA-addition.

Ethylene and abscisic acid apparently are the most important hormonal factors in the modulation of the capacity of the alternative pathway.

2. Is there a modulation of the in vivo *alternative pathway activity by plant hormones?*

The modulation of the activity of the alternative pathway is not directly connected to the modulation of its capacity: on the contrary, inhibition of the ethylene synthesis or ethylene action led to an increased employment of the (low) capacity present in this tissue. This *in vivo* activity is, in the first place, dependent on the amount of reduction equivalents that is left after saturating the cytochrome pathway: a decreased cytochrome pathway activity (as is observed with 2,5-norbornadiene) consequently results in an increased pathway activity (as long as this pathway is not already saturated itself).

In potato tuber explants, blocking of the ethylene synthesis or the ethylene action leads to an increased employment, while with abscisic acid a decreased employment is observed.

It is evident that modulation of the activity and of the capacity of the alternative pathway are two independent phenomena.