Acta Bot. Neerl. 40(4), December 1991, p. 363–376

Meetings of the Royal Botanical Society of The Netherlands

ABSTRACTS OF THE ANNUAL MEETING OF THE ROYAL SOCIETY ON 31 MAY 1991

The Cloned Avirulence Gene *avr9* of the Fungal Tomato Pathogen *Cladosporium fulvum* is Responsible for Cultivar Specific Resistance

G.F.J.M. van den Ackerveken, J.A.L. van Kan and P.J.G.M. de Wit. Department of Phytopathology, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands

Cladosporium fulvum is the causal agent of tomato leaf mold. The interaction between *C. fulvum* and tomato is used as a model system to study the gene-for-gene hypothesis in which fungal avirulence gene products interact with resistance gene products of the plant.

Different races of *C. fulvum* have arisen as a result of adaptation of the fungus to resistance genes present in cultivated tomato. These new races have overcome their initial avirulence by evading recognition by the plant. In this way, several fungal races have overcome resistance gene Cf9 of tomato by a deletion of the avirulence gene *avr9* encoding the corresponding race-specific elicitor (van Kan *et al.*, 1991, *Mol. Plant-Microbe Interact.* **4**: 52–59).

Genomic clones carrying the *avr9* gene were isolated from a genomic library (λEMBL3) of race 5 of *C. fulvum* using a cDNA-probe encoding the race-specific elicitor. Sequence analysis revealed a 59 bp intron, a possible TATA-box and several repeats in the promoter- and terminator-region. Stable transformants were obtained after co-transformation of the *avr9* gene with pAN7-1 (Hygromycine resistance) to race 2.4.5.9.11, which is virulent on tomato cultivar Cf9. Cultivar specificity of these transformants was converted phenotypically from virulent to avirulent on tomato cultivar Cf9, indicating that the introduced *avr9* gene has changed the genotype of race 2.4.5.9.11. into race 2.4.5.11.

This is the first report on the cloning of a fungal avirulence gene of which the processed gene product (28 aa.) is the primary inducer of the hypersensitive response (HR) in a resistant cultivar. Many avirulence genes of plantpathogenic bacteria have been cloned until now, but for none of them the gene product itself induces HR.

Sexual Adhesion in *Chlamydomonas eugametos* Is Mediated by a Single Pair of Sulphated Glycoproteins

M. Versluis, F.M. Klis, P. van Egmond and H. van den Ende. Department of Molecular Cell Biology, University of Amsterdam, Kruislaan 318, NL-1098 SM Amsterdam, The Netherlands

Sexual adhesion between gametes of plus and minus mating type of the unicellular green alga *Chlamydomonas eugametos* is mediated by mating type-specific cell adhesion molecules or agglutinins on the flagellar surface. After mild fixation with glutaraldehyde, gametes of opposite mating type were still capable of specifically agglutinating with each other. This allowed studying the initial recognition steps without interference by cellular responses that accompany in-vivo cell adhesion. In-vitro and in-vivo adhesion varied in the same way with the pH. Isolated agglutinins of both mating type inhibited sexual adhesion at similar concentrations. This suggest that mt^+ and mt^- agglutinins interact with each other. This was confirmed by demonstrating that charcoal particles adsorbed with purified agglutinins aggregate with each other.

Acid hydrolysis of ^{35}S-labelled agglutinins released essentially all radioactive label as free sulphate. Short periods of acid hydrolysis, and β-elimination released sulphated oligosaccharides. No evidence was found for sulphated tyrosine or sulphated oligosaccharides that are N-linked, indicating that the sulphate groups are attached to O-linked sugars.

In-vitro adhesion was sensitive to salts, sulphated monosaccharides, and free amino acids at concentrations from 10 mM upwards. Sulphated polysaccharides caused inhibition in the micromolar range (50–275 μg/ml). This indicates that the interaction between the agglutinins is ionic in nature. Oligosaccharides derived from purified mt^- agglutinin inhibited in-vitro adhesion at concentrations between 1–3 μg/ml. Considering the presumably ionic nature of the agglutinin–agglutinin interaction, we propose that sulphated sugar side-chains of the mt^- agglutinin are directly involved in sexual adhesion.

The investigations were supported by the Foundation for Biological Research (BION), which is subsidized

by the Dutch Organization for Scientific Research (NWO).

Mass Spectrometric Studies of Plant Cell Wall Lignin

E.R.E. van der Hage, R.J.J.M. Steenvoorden, J.B.M. Pureveen, M.M. Mulder and J.J. Boon. FOM Institute for Atomic and Molecular Physics, Unit for Mass Spectrometry of Macromolecular Systems, Kruislaan 407, 1098 SJ Amsterdam, The Netherlands

Lignin, a major cell-wall component of all vascular plants, has been the subject of many investigations but a full understanding of its structural scheme has not yet been established. Unlike most biopolymers, lignin is a three-dimensional polymer network with several different types of linkages between its monomeric units. The study of these interunit linkages which form the key to a full understanding of its structural scheme, has been hampered by the fact that it is not possible to isolate native lignin in an intact state and depolymerize it quantitatively into oligomeric structural units.

Analytical pyrolysis techniques offer a rapid and effective method to degrade lignins into cleavage products in which information about interunit linkages is preserved. In-source pyrolysis mass spectrometry (PyMS) can be used to characterize and fingerprint lignin samples. Electron impact (16 eV) spectra of isolated lignins, obtained on a JEOL DX-303 mass spectrometer, will be presented which show mainly phenolic structural units reflecting the biomass from which they are derived.

Interfacing a gas chromatograph to a Finnigan INCOS 50 quadrupole mass spectrometer allows a detailed structural study of all separated lignin pyrolysis fragments. Electron impact (70 eV) mass spectra of various lignin samples will be presented.

Laser desorption of large biomolecules proves to be an exciting and promising new technique for volatizing large molecular structures intact. Results will be presented in which lignin model compounds are desorbed with a CO_2-laser, ionized with VUV-radiation generated from a Nd : YAG laser and subsequently analysed with a reflectron time-of-flight mass spectrometer.

Molecular Signals in the *Rhizobium*-Legume Symbiosis

G. Bloemberg, H. Spaink, B. Zaat, C. Wijffelman and B. Lugtenberg. Department of Plant Molecular Biology, Leiden University, Leiden, The Netherlands

Bacteria of the genus *Rhizobium* are characterized by the ability to induce nodules on roots of leguminous plants. The bacteria invade plant cells in these specialized organs and subsequently differentiate to bacteroids. These bacteroids fix nitrogen, which can be used by the plant. The nodulation process can be divided in recognizable stages: (i) Root colonization; (ii) Root adhesion; (iii) Root hair branching and root hair curling; (iv) Infection thread formation; (v) Nodule initiation; (vi) Infection thread branching and nodule development; (vii) Bacterial release from the infection threads; (viii) Bacteroid development and start of the nitrogen fixation; (ix) Nodule persistence. One of the most intriguing aspects of this complex plant-bacterium interaction is the specificity of each *Rhizobium* strain for its own set of plants that it can infect (host-specificity).

It has been shown that a number of *Rhizobium* genes are required for a good nodulation (*nod* genes). Some of these nodulation genes are interchangeable between the different *Rhizobium* strains without affecting the host-specificity (common genes), whereas other genes determine the host-specificity.

As the nodulation process is very complex, specific communication between the plant and bacterium is necessary. At present considerable progress has been made in the identification of signal molecules. In free-living bacteria the *nod*-genes are not expressed, except for the *nodD* gene. The expression of the other *nod* genes was found to be regulated by molecules present in the root exudate of the host plants. Extensive analysis of these exudates showed that the responsible signal molecules were from flavonoid origin. These compounds activate in concert with the *nodD* protein the promoters of the other *nod* genes.

In response to these plant signals the bacteria produce also signal compounds. A number of *nod* proteins is involved in the synthesis of these compounds, which are excreted by the bacterium. These compounds are able to induce several responses on the roots of leguminous plants. The structures of these signal molecules are being investigated. Until now all these compounds have a sugar-backbone as a basic structure, consisting of four or five *N*-acetylglucosamines, with different modifications including the presence of fatty acids.

Nuclear DNA Sequence Polymorphisms as Tools for Evolutionary Genetics

W. van Houten. Hugo de Vries Laboratory, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands

In recent years, sequence polymorphisms in chloroplast DNA (cpDNA) have become a standard tool for the cladistic analysis of plant genera. The considerable advantages of cpDNA (small genome size, no recombination, maternal inheritance, slow evolution) are also limitations. Direct analysis of the nuclear DNA gives a realistic picture of the reticulate evolutionary patterns of recombining diploid genomes and parallels the evolution of classic taxonomic characters. I am developing a set of cloned probes of nuclear DNA of

the lactucean species *Microseris pygmaea* (Asteraceae) useful as markers both for the evolutionary history of Lactuceae and for the genetic analysis of morphological character differences. These probes are used to detect restriction fragment length polymorphisms (RFLPs) within and among populations and species of *Microseris*. The inheritance of RFLPs is studied in segregating offspring of a hybrid between the two most divergent biotypes of *M. pygmaea*. One of our goals is to construct a genetic map of *Microseris pygmaea* based on RFLPs and to use this map for the analysis of (polygenically determined) morphological and physiological differences that have arisen in the evolution of the species from a founder individual.

Nuclear DNA contains various fractions with widely varying rates of sequence evolution. Probes can be found for each level of variation. A synthetic simple-sequence probe $(GATA)_4$, detects hypervariable 'fingerprint' loci, probably due to variation in the number of tandem repeats of the simple sequence at various places in the genome (VNTRs). The fragment pattern obtained with each restriction enzyme differs among plants in one population and even between parent and offspring in inbred strains. This variation makes fingerprinting useless for cladistic analysis in *M. pygmaea* and limits its use in genetic analysis. Three fingerprint loci can be identified in the model hybrid.

Specific genomic probes were isolated by cloning fragments cut with PstI or EcoRI from genomic DNA. Low-level repetitive and single-copy clones are selected. The sequences of two repetitive DNAs have been determined. Specific stable multiband patterns can be detected with some repetitive DNA probes. The level of variability detected by each genomic clone has to be characterized individually. Two approaches are used: digests with restriction enzymes recognizing a 6-basepair site ('six-cutters') are separated by agarose gel electrophoresis. Digests with '4-cutters' are separated on polyacrylamide. The latter method is more sensitive, and, as 4 bp-sites are about 16 times as frequent as 6 bp sites, a larger part of the genome is assayed for variation.

A Postulated Physical Model for the Kinetic Behaviour of Ion Channels Derived from the Three-Dimensional Architecture of Ion Channels

S.A. Vogelzang and H.B.A. Prins. Ecotrans Department of Plant Physiology, University of Groningen, 9750 AA Haren, The Netherlands

The kinetics of outward rectifying ion channels have been studied in the plasmalemma of *Plantago* root protoplasts using the Hodgkin and Huxley (HH) model (1952, *Loligo. J. Phys.* (Lond.) **116**, 473–496). It describes the behaviour of four independent membrane-bound particles. Each has a probability n of being in the correct position to set up an open channel. Thus the probability for an open channel is n^4. The hypothetical particles are assumed to bear an electrical charge which makes their distribution in the membrane voltage dependent. Changes in membrane potential cause the particles to move between their permissive and non-permissive position with first-order kinetics, hence n relaxes exponentially towards a new value. Activation curves of the outward rectifying *Plantago* ion channel could partly be characterized with the n^4 HH-model. One extra particle with a different probability (m) distribution is required to describe the kinetic behaviour of the ion channel.

A three-dimensional (3-D) architecture of the calcium channel/foot structure of sarcoplasmic reticulum was determined from electron micrographs by Wagenknecht (1989, *Nature* **338**, 167–170). This structure can be used as a basis for understanding the physical behaviour of ion channels.

Our postulation is that the ion channel is composed of five moving particles. Four lever-units on the lateral sides and one central unit. The central unit can rotate round a central core from a non-permissive to a permissive position if the four lever-units are in an outer position (open state). If one or more levers are in an inner position the central unit cannot rotate (closed state).

This physical model matches the kinetical n^4m model and predicts that voltage dependent conformational alterations of the central unit can change the probability distribution of the lever units. Consequently only the central unit needs to be voltage dependent.

Localization of Ca^{2+} in Embryogenic Plant Cells

A.C.J. Timmers. Department of Plant Cytology and Morphology, Agricultural University, Wageningen, Aboretumlaan 4, 6703 BD, Wageningen, The Netherlands

A number of basic cellular processes in plants are controlled by the concentration of cytosolic Ca^{2+}, such as secretion, cell polarity, growth, division and gene expression. Knowledge of the distribution of Ca^{2+} in plant cells can inform one about the regulation of growth and differentiation of plant cells. From the known techniques to visualize the distribution of Ca^{2+} in plant cells, we picked out two to study the distribution of Ca^{2+} in embryogenic plant cells of *Daucus carota* L. Somatic embryos of carrot can easily be obtained from a liquid callus culture by transferring pro-embryogenic masses (pems) from auxin-containing to auxin-free medium. Somatic embryos arise from pems and proceed through the subsequent stages of embryogenesis. For the localization at the EM level, Ca^{2+} was fixed by precipitation with fluoride and visualized as electron

dense precipitates by antimonate (Poenie, M., Epel, D., 1987, *J. Histochem. Cytochem.* **35**: 939–956). Precipitates were mainly found in cell walls, vacuoles, nuclei, nucleoli and sometimes in amyloplasts. For a detailed analysis of the distribution of free cytosolic Ca^{2+} in living, intact plant cells in multicellular tissue, confocal scanning laser microscopy (CSLM) is the method of choice.

We developed a technique to load embryogenic cells of carrot with fluo-3, a fluorescent Ca^{2+} indicator with an excitation maximum close near the excitation line of the argon laser. Initial attempts to load fluo-3 as its acetoxymethyl ester or at low pH were without success. We loaded fluo-3, in its membrane impermeable form, with the aid of digitonin, which permeabilizes selectively the plasma membrane. Then, a bright fluorescence was observed in the epidermal layer of heart and torpedo shaped somatic embryos of carrot. Vacuoles and cell walls were always negative, while a very prominent staining of the nucleus and nucleolus was observed. Early stages of embrogenesis were characterized by a strong fluorescent signal which was already present in the first embryogenic cell.

Variation for Mycelial Morphology in *Puccinia brachypodii*

C.A. Swertz. Department of Plant Breeding, Agricultural University Wageningen, P.O. Box 386, 6700 AJ Wageningen, The Netherlands

Puccinia brachypodii Otth (Uredinales) covers a complex of grass rust fungi characterized by clavate-capitate uredial paraphyses, urediospores with scattered germ pores, and long-covered telia. Aecia are found on *Berberis* and *Mahonia*. The complex consists of at least three varieties or closely related species depending on the view of the taxonomist. The taxa are delimited on the basis of host range, arrangement of uredia and telia, and length of urediospores and teliospores, and of paraphyses. These morphological features show a wide overlap, making identification often arbitrary.

A useful tool in identification of rust fungi belonging to this complex, and probably also for other rusts, can be found in the morphology of the primary infection structures of the urediospores. When freshly collected urediospores are applied onto segments of the adaxial side of primary leaves of barley and are incubated overnight in a moist chamber, urediospores will germinate and develop primary infection structures. Morphology of infection structures can be studied with phase contrast microscopy.

The infection structures appear to show large differences among samples from different host plants (e.g. *Poa, Anthoxanthum, Arrhenatherum, Brachypodium*) in length of penetration peg, shape of the substomatal vesicle, and orientation of the primary infection hyphae.

It is suggested that the method of observing the morphology of the primary infection structures of urediospores can be useful in identification and classification of rust fungi.

Cloning and Characterization of the Avirulence Gene *avr9* of the Tomato Pathogen *Cladosporium fulvum*

G.F.J.M. van den Ackerveken, J.A.L. van Kan and P.J.G.M. de Wit. Department of Phytopathology, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands

Cladosporium fulvum is the causal agent of tomato leaf mold. The interaction between *C. fulvum* and tomato is used as a model system to study the gene-for-gene hypothesis in which fungal avirulence genes interact with resistance genes of the plant and vice versa.

The product of avirulence gene *avr9*, a peptide specifically inducing necrosis on tomato genotypes containing the Cf9 resistance gene, was isolated and sequenced. By using an oligonucleotide probe the cDNA clone was isolated and the structure of the *avr9* mRNA was determined. cDNA analysis indicated that a 63 amino-acid precursor is produced of which the C-terminal 28 amino acids are cleaved off to produce the necrosis-inducing peptide.

Southern analysis of different races of *C. fulvum* releaved that races which are virulent on tomato genotypes carrying the Cf9 gene lack the *avr9* gene. Genomic clones carrying the avirulence gene *avr9* were isolated from a λEMBL3 library of race 5. The structure and sequence of the gene will be presented.

Flow Cytometric Analysis of Phytoplankton in Water Samples

H.W. Balfoort. Aquatic Ecology Department, University of Amsterdam, Kruislaan 320, 1098 SM Amsterdam, The Netherlands

In flow cytometry, light scatter and fluorescence of individual particles in suspension is measured at high speed. When applied to phytoplankton cells, the light scattering and autofluorescence properties of the cells can be used for the identification and counting. The optical properties may also provide information on the size and the physiological state of individual cells. Commercially available flow cytometers are not suited for the analysis of the wide size range of algal cells and colonies, usually present in eutrophic coastal and inland waters. Therefore a flow cytometer has been specially developed for the analysis of algae from 1 μm to 1000 μm: the optical plankton analyser (OPA). The first prototype is used for the identification and characterization of algal mixtures in bioassays and in monitoring studies of cyanobacteria blooms. The

second prototype is used for phytoplankton monitoring studies in the North Sea. A data analysis system, based on artificial neural networks, will be realized in the near future for the further automation of flow cytometric identification and counting of algae in water samples.

Flow Cytometric Measurements on Plant Cell Population Dynamics

F.T. Nicoloso and J. Val. Department of Plant Molecular Biology, Leiden University, Nonnensteeg 3, 2311 VJ Leiden, The Netherlands

In order to measure the number of cells for population dynamical studies on plant cell suspension cultures, a flow cytometric counting method was developed. As each cell contains a nucleus, counting cells is equivalent to counting nuclei. The bisbenzimidazole dye Hoechst 33258 specifically binds to DNA and can be used for staining of the nuclei. After fixation, maceration and staining of the cells, nuclei are counted with a flow cytometer. Along with these countings, DNA distributions are obtained as an extra population-dynamical parameter. This method was tested on samples taken from batch fermenter cultures of a *Nicotiana tabacum* cell suspension by means of a specially constructed sampling device, suited for relatively large particles. It was found that the variance among countings of the same sample and among different samples of the same time of culture was less for flow cytometric countings than for manual countings. The time needed for performing flow cytometric countings is about 25% of that needed for manual countings.

Isolation and Characterization of Mutants of *Tagetes erecta* with Altered Thiophene Content

J.J.M.R. Jacobs, P. Huijs, A.M.W. Engelberts, A.F. Croes and G.J. Wullems. Novaplant Cell Biotechnology Group, Department of Experimental Botany, University of Nijmegen, The Netherlands

Seeds of *Tagetes erecta* were treated with different concentrations of the chemical mutagen ethyl-methanesulphonate (E.M.S.). M1-plants were grown in the greenhouse and were allowed to self-pollinate. M2-plants were grown in vermiculite. After 3 weeks, samples were taken from the roots of 316 M2-plants for thiophene extraction and analysis by HPLC.

In the roots from untreated control plants, BBT and BBTOAc were the predominant thiophenes. Several M2-plants displayed an altered HPLC pattern, with a very prominent new peak at a retention time that was slightly longer than that of BBT. The new trait did not segregate in the M3-generation, which indicates that the M2-plants were homozygous for the mutation that accounts for the new peak.

Mutant plantlets of the M3-generation were further characterized in a feeding experiment with [^{35}S]-sulphate. This experiment proved that sulphur is being incorporated in the unidentified new component, as is the case with thiophenes.

The result supports our hypothesis that the new compound is a thiophene intermediate. The feeding experiment revealed some other differences between mutant and wild-type plantlets. The most spectacular observation was that in hypocotyls of mutant plantlets 10–30 times less counts of [^{35}S]-sulphur were incorporated into BBT-OAc compared to the wild-type plantlets, which had a very large peak of ^{35}S-labelled BBTOAc in their hypocotyls.

Further analysis showed that the new compound was also produced in sterile root cultures which were initiated from mutant plantlets in erlenmeyer shake flasks.

The fact that the new peak is also present in the sterily grown root cultures encourages us to characterize these root cultures, which are stable and can be propagated indefinitely, in further experiments. We intend to compare in-vivo synthesized proteins and in-vitro translation products from the mutant and a wild-type root culture by two-dimensional gel analysis.

Thiophene Production in *Tagetes* Root Cultures

R.R.J. Arroo, E. van de Westerlo, M. de Waard, A.F. Croes and G.J. Wullems. NOVAPLANT Cell Biotechnology Group, Department of Experimental Botany, University, Toernooiveld, 6525 ED Nijmegen, The Netherlands

In the roots of *Tagetes* species (Marigolds) thiophenes are formed. These are heterocyclic sulphur-containing compounds with a strong nematicidal activity. Transformation of *Tagetes patula* with *Agrobacterium rhizogenes* resulted in hairy root clones which grow much faster than untransformed roots and are easy to manipulate. Thus, these hairy roots present an attractive model to study the production of thiophenes in *Tagetes*.

High performance liquid chromatography (HPLC) was used to quantify thiophenes in root extracts. It was shown that the amount of thiophene accumulation is highest during the exponential growth phase of the root cultures.

Sulphur as [^{35}S]-sulphate is taken up by the roots and subsequently incorporated into thiophenes. So by feeding [^{35}S]-sulphate to exponentially growing roots we could quantify thiophene synthesis. Under standard conditions thiophene biosynthetic capacity of the roots is 12 nm hr^{-1} g FW^{-1}. At present we study bioconversion of thiophenes, using several ^{35}S-labelled precursors in feeding experiments.

Continuous Cultures as a Tool for the Study of Coniferin Production in Cell Cultures of *Linum flavum*

A. Oostdam and L.H.W. van der Plas*. Vrije Universiteit, Department of Plant Physiology, De Boelelaan 1087, 1081HV Amsterdam and *Agricultural University, Department of Plant Physiology, Arboretumlaan 4, 6703BD Wageningen, The Netherlands

To study the physiological factors and metabolic processes that influence the amount of coniferin (coniferyl alcohol-β-D-glucoside) produced by cell suspension cultures of *Linum flavum*, continuous cultures have been established.

Coniferin is the major secondary metabolite produced by cell cultures of *L. flavum*. Other metabolites that can be synthesized include lignin, flavonoids and various lignans, all products of the phenyl propanoid pathway. Coniferin may accumulate up to 12% on a dry weight basis (W.v. Uden, 1990, *Planta* **183**: 25–30) in batch cultures.

In general, the advantage of continuous cultures over batch cultures is that one isolated factor can be altered and its effect on the cell culture studied, while all other parameters e.g. medium composition, growth rate and cell density, remain unchanged. This is in contrast to batch cultures, where all these parameters change with time.

Experiments have been carried out under phosphate and glucose limited conditions with a growth rate (μ) of 0·11 day^{-1}. Coniferin production is high (approximately 9% DW) under phosphate limited conditions. As expected, the coniferin content decreased when the culture was switched to glucose limitation, but a significant amount (approximately 4% DW) was still present under C-limitation.

Data collected on the respiration of phosphate and glucose limited cells show a slightly higher respiration in the latter on a dry weight base. This is possibly due to a relative decrease of cell-wall material such as cellulose. In the phosphate limited situation, there was a significant use of the alternative oxidase that disappeared in the glucose limited cells, which is in accordance with the overflow hypothesis of H. Lambers (*Physiol. Plant.* **55**: 478–485).

Isolation of Mitochondria from *Petunia hybrida* Cell Suspensions. The Flexibility of Plant Cells to Adapt their Respiration to Changing Growth Conditions

W.A.M. van Emmerik, A.M. Wagner, J. Zwiers and L.H.W. van de Plas*. Department of Biochemistry and Physiology of Plants, Vrije Universiteit, De Boelelaan 1087, 1081HV Amsterdam, and *Department of Plant Physiology, Agricultural University, Arboretumlaan 4, 6703BD Wageningen, The Netherlands

Mitochondria were isolated from small amounts (2–10 g FW) of *Petunia hybrida* cell suspensions. A high yield was obtained (about 32%) as measured by fumarase activity.

The respiration (total, cytochrome and CN-resistant) of cells and isolated mitochondria followed the same course in time during a batch cycle: a rapid increase in the early logarithmic phase followed by a decline.

Respiration of cells and isolated mitochondria was compared by correcting the latter for yield. In the late logarithmic phase, the respiration rate of the cytochrome pathway of mitochondria (in state 3) appeared to be higher than the cellular rate. Thus cellular respiration might be limited by ADP or substrate. Stimulation of cellular respiration with the uncoupler FCCP showed that the substrate is not limiting respiration during the whole batch cycle. ADP is only limiting the in-vivo respiration during the late logarithmic phase.

The large increase of in-vivo respiration after inoculation of a batch culture in fresh medium could be explained by: (i) the new formation of (components of the) electron transport chains by new-synthesis or rearrangement; (ii) increase of activity of already present mitochondria.

The second possibility could be rejected, because the respiration of isolate mitochondria showed the same rise as the in-vivo respiration (carrier capacity was measured in the presence of three substrates and ADP). It is not clear if new formation of chain components is accomplished by new synthesis or rearrangement.

New synthesis of the terminal oxidase of the cytochrome pathway (cytochrome oxidase) can be prevented by chloramphenical (CAP). Cells growing in the presence of CAP showed the same respiration peak of the cytochrome pathway as untreated cells. However, after 1 day in batch culture cytochrome oxidase activity of CAP-treated cells already showed a decline, whereas it increased in untreated cells.

The Effect of Cold Storage and Gibberellins on the Development of Axillary Buds of Tulip Bulbs Grown *In Vitro*

H.A. Dekker, P.C.G. v.d. Linde* and C. Kollöffel. Department of Plant Ecology and Evolutionary Biology, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht and *Bulb Research Centre, Vennestraat 22, 2160 AB Lisse, The Netherlands

In tulip, in-vitro induction of buds has been obtained frequently, but attempts to develop these adventitious buds *in vitro* to normal bulbs have not yet been very successful. As a first step for a better understanding of bulb growth, we cultured *in vitro* the axillary bud attached to the shoot.

Tulip bulbs (*Tulipa gesneriana*, cv. 'Apeldoorn') were dry stored at 5°C for 0, 6 and 12 weeks and at 17°C for 12 weeks. Subsequently explants of the shoot,

together with a small piece of the basal plate carrying the innermost axillary bud, were excised. The explants were cultured *in vitro* in 2·5 ml liquid medium (Murashige and Skoog) supplied with 120 g/l sucrose and seven different amounts of GA4+7 (0, 0·001, 0·003, 0·01, 0·03, 0·1 and 1 mg per explant).

Axillary buds from 0 weeks 5°C or 12 weeks 17°C bulbs had a dry weight of 50 and 70 mg respectively at the end of the culture period (8 weeks). The final dry weight of buds excised from 6 weeks 5°C bulbs was 300 mg, that of buds from 12 weeks 5°C bulbs was more than 1 g. Their water content was about 80%. It can be concluded that a cold treatment of the mother bulb enhances the growth of the axillary buds *in vitro*. Addition of Ga4+7 had no effect on buds from 0 weeks 5°C of 12 weeks 17°C bulbs. The dry weight of buds collected from 6 and 12 weeks 5°C bulbs increased with increasing amounts of GA4+7; the optimum being 0·1 mg GA4+7. Under these in-vitro conditions, growth of the axillary buds was comparable to the in-vivo growth of daughter bulbs in planted flowering bulbs.

The growth rate of the shoot was significantly higher when the shoots were excised from 12 weeks 5°C bulbs as compared with bulbs that received other treatments (0 and 6 weeks 5°C, 12 weeks 17°C). GA4+7 had no clear effect on shoot growth rate. The shoots appeared to stop growing and dried out as soon as the axillary buds started growing.

Mathematical Modelling in Plant Physiology: Respiration in Plant Cells

J. Val and M.H.N. Hoefnagel. Department of Plant Molecular Biology, Leiden University, Nonnensteeg 3, 2311 VJ Leiden, The Netherlands

From oxygen exhaustion experiments with plant cells in a biological oxygen monitor, it is inferred that respiration is well below the capacity of the respiratory system of these cells. Furthermore, oxygen uptake in such experiments cannot be described by a simple Michaelis-Menten equation. The oxygen uptake rate is supposed to be regulated by changes in the ATP/ADP levels in the cell. Two possible regulation mechanisms are analysed mathematically. In the first ADP has direct influence through coupling on the cytochromal chain. The ADP influx in the mitochondria over carrier enzymes is regulated by concentration differences of ATP and ADP in and outside the mitochondrion. The second is an indirect effect of ATP. ATP has a negative feedback on the glycolysis, which is the main source for electrons used during cytochromal respiration. Mathematical simulations of these models show that both models describe the experimental behaviour of the oxygen uptake rate. From the observed very small changes in the ATP level during oxygen exhaustion, the latter regulating system appears to be the most important. Parameter estimations on the models from the data have also been performed.

Isolation of Inside-Out Submitochondrial Particles (SMP) from Plant Mitochondria

C.W.M. van den Bergen, M.J. Wagner, K. Krab, A.M. Wagner and L.H.W. van der Plas*. Biological Laboratory, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam and *Department of Plant Physiology, Landbouw Universiteit Wageningen, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

Submitochondrial particles (SMP) were isolated from potato (*Solanum tuberosum* cv. Bintje) tuber mitochondria by sonication followed by differential centrifugation. The polarity of the SMP was assayed by the activity of cytochrome-c oxidase with or without 0·02% Triton X-100. Minimally, the sonication medium has to contain 10 mM $MgCl_2$ to obtain SMP that were more than 85% inside-out.

It is likely that cytochrome-c is lost during SMP isolation. Therefore cytochrome-c was added before sonication. This resulted in an enhancement of respiration with NADH or succinate as substrate. It is concluded that cytochrome-c was enclosed during sonication.

Compared to mitochondria, SMP were enriched in cytochrome-c oxidase activity. Also, oxidation by SMP of added NADH is faster than oxidation of malate by mitochondria. The rate of NADH oxidation was about 50% sensitive to rotenone.

About 10% of the mitochondrial protein was recovered in the SMP preparation.

Use of Polarized Fluorescence Spectroscopy to Measure Fluidity of Mitochondrial Membranes from Cold-Treated Tulip Bulbs

W.A. Kanneworff and L.H.W. van der Plas*. Department of Plant Physiology, Free University, de Boelelaan, Amsterdam and *Department of Plant Physiology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

Many plants are able to withstand the large fluctuations in temperatures that accompany the seasonal changes normally occurring in temperate climate zones. Many species are even dependent on such seasonal temperature changes for the realization of their developmental program. They will only germinate or produce flowers after they have undergone a cold period for several weeks or months. This resistance to long periods of low temperatures requires adaptations of the plant cell membranes. To prevent a

decline of the correct functioning of membrane-bound enzyme systems the fluidity of the membranes has to be changed: at lower ambient temperatures the cells need membranes with an increased fluidity. Such fluidity changes can be realized by increasing the percentage of unsaturated fatty acids but also by changes in the lipid type or in the lipid/protein ratio.

Polarized fluorescence spectroscopy using diphenylhexatriene as a probe can be used as a method to assess the overall fluidity changes of cell membranes. To demonstrate the use of this method, changes in membrane fluidity of cold-treated tulip bulbs are studied. Cold treatment of tulip bulbs is common practice. For the production of good quality flowers a cooling period is necessary: for the cv. Apeldoorn a 12-week period at 5°C is optimal. The adaptation of the cell membranes to this cold treatment was studied with membrane preparations from isolated mitochondria. A rapid increase of the membrane fluidity was observed after transfer of the bulbs from 17 to 5°C although clear phase transitions were not observed during measurement of the fluidity at temperatures between 4 and 30°C. When, after cooling, bulbs were transferred to 17°C, there was a slight decrease in membrane fluidity. After 1 week 17°C the original precooling level of membrane fluidity still was not recovered. The changes in membrane fluidity were not reflected in a changed fatty acid composition of the mitochondrial membrane. Apparently, polarized fluorescence spectroscopy is a rapid method to detect overall changes in membrane fluidity.

A Novel Technique for the Identification of Root Regions, Tissues and Cell Types, Active in Proton Extrusion

R. Dorhout. Transport Physiology Research Group, University of Utrecht, Sorbonnelaan 16, 3508 TB Utrecht, The Netherlands, and Department of Nematology, Agricultural University, Binnenhaven 10, 6709 PD Wageningen, The Netherlands

Proton extrusion by root cells is important for a range of processes, such as cell elongation, uptake of solutes and mobilization of minerals at the root–soil interface. Various techniques have been used to determine proton extrusion by roots, as measuring the pH at the root surface with antimony microelectrodes or detecting colour changes of indicators around roots with the agar dye method. In this study, a new technique is developed which enables the localization of proton extrusion activity by roots at macroscopic and microscopic level.

Fluorescein, an acidic fluorescent dye with a pK of 4·9 is able to pass cell membranes in its undissociated form. Cells having a relatively high proton extrusion activity, resulting in a low pH in the walls, accumulate the dye. Young pea seedlings with a main root of about 3–4 cm were used to study the uptake of the dye. After an incubation period of 16 h in a 0.01% (w/v) fluorescein solution with a pH of 7·9, dye uptake was restricted to the apical root regions as was visible under a UV-lamp (emission wavelength with a peak at 366 nm). Under more acidic conditions (pH 5·0) the dye also accumulated in the root base. Dye accumulation in the root tip was prevented when the proton buffering strength of the incubation medium was increased. CCCP almost completely suppressed dye accumulation in the root apex at a concentration of 5×10^{-2} mM. Handcut cross-sections of the root apex of seedlings incubated in an unbuffered fluorescein solution (pH 7·9), viewed with an epifluorescence microscope equipped with suitable filter combination sets, showed that proton extrusion activity may vary with cell type and their location in the root. The method described can be used to identify root regions, tissues and cells inside the root, active in proton extrusion.

A Method for Determination of 2,4-Dichlorophenoxyacetic Acid in Cells and Medium of Plant Cell Suspension Cultures

K.B. Koens, P. Mulder*, T.B. van Vliet, F. van Iren and K.R. Libbenga. Department of Plant Molecular Biology, Leiden University, Nonnensteeg 3, 2311 VJ Leiden, and *Center for Chemistry and the Environment, Leiden University, Einsteinweg 5, 2333 CC Leiden, The Netherlands

Determination of the plant growth regulator 2,4-dichlorophenoxy-acetic acid (2,4-D) is commonly carried out by using the radioactively labelled compound. In order to avoid specific problems of working with radioactivity, a method was devised for determining non-labelled 2,4-D by using gas chromatography with electron capture detection (GC-ECD).

In short, the complete procedure consists of:

(i) Pottering of cells after addition of internal standard, i.e. 3·5-dichlorophenoxyacetic acid; (ii) cleaning of the sample by using chloroform extraction; (iii) extraction of 2,4-D with ethylacetate; (iv) methylation of the extract with diazomethane; (v) analysis by GC-ECD.

Methylation is necessary for a good separation of 2,4-D and the internal standard, and for reducing retention times.

For preparation of samples of the medium, pottering and cleaning steps can be omitted. In 1 day, 15 samples can be extracted and methylated.

The detection limit of this method is about 5×10^{-9} mol l^{-1}. Recovery is 60–100%. Some results of application of this method for determination of 2,4-D in batch cultures of tobacco will be shown.

Detection and Identification of Gibberellins from Tulip Bulbs by Combined Gas Chromatography-Mass Spectrometry

M. Rebers, E. Vermeer, E. Knegt and L. van der Plas. Department of Plant Physiology, Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

The involvement of gibberellins (GAs) in the regulation of stem elongation and flowering has been implicated in cold requiring plants, including tulip. To investigate the role of GAs in the cold requirement of tulip, research is directed towards quantitative analyses, metabolic studies and comparative biological activities of endogenous GAs. Using combined gas chromatography–mass spectrometry (GC–MS), eight different endogenous GAs were identified in purified extracts from sprouts of 6 and 12 weeks cooled tulip bulbs (*Tulipa gesneriana* L. cv. Apeldoorn). The presence of free GAs 4, 9, 14, 48/49 and 53 could be demonstrated, in estimated concentrations less than 100 ng g^{-1} fresh weight. Traces of GAs 16, 24 and 34 were indicated by GC–MS-single ion monitoring (GC–SIM). After hydrolysis of the conjugated GA fraction, GAs 14, 34 and 48/49 were detected. Consideration of the hydroxylation patterns of the ent-gibberellane ring structure indicates more than one family of GAs: one with a C-13 hydroxyl group (GA_{53}) and others whose members are either nonhydroxylated (GAs 9 and 24), or lack a C-13 hydroxyl group (GAs 4,14, 16, 34, 48/49). Current research is directed towards quantitative analyses with ^{2}H-GAs, in relation to the cold pretreatment of the bulbs.

Hormone Mutants as a Tool for Physiological Research

S.M. de Bruijn and M.P.M. Derkx. Department of Plant Physiology, Wageningen Agricultural University, Aboretumlaan 4, 6703 BD Wageningen, The Netherlands

Traditionally, effects of hormones on various physiological processes in plants were studied by either increasing their level (by application of the growth regulator in study) or decreasing their level (by application of inhibitors). The use of hormone mutants has several advantages in comparison with these conventional techniques: the endogenous hormone level can be manipulated without any experimental disturbance of the intact plant system. In addition, mutants are unique tools to study changes in sensitivity towards hormones. Such a study is hardly possible by classical techniques.

A first example concerns the effect of abscisic acid (ABA) on assimilate partitioning. Several literature reports claim a stimulating effect of ABA on the transport of assimilates to seeds. ABA-deficient hormone mutants of *Arabidopsis thaliana* and *Pisum sativum* were used to test this hypothesis. Studies with transport of radiolabelled photosynthates to seeds of different genotype on the same plant, revealed that ABA has no significant influence on the growth rate of the seeds.

Hormone mutants of *Arabidopsis thaliana* have also been used to study the mechanisms of dormancy control in seeds. By pre-incubating wildtype and gibberellin (GA)-deficient seeds at various constant or alternating temperatures, dormancy could be broken or (re-)induced. These changes in dormancy were clearly correlated with changes in the sensitivity towards GAs. Moreover, the capacity to synthesize GAs was important: GAs were absolutely required for germination.

We conclude that hormone mutants are an interesting tool to solve physiological problems.

Anatomy and Pyrolysis Mass Spectrometry of Peat Forming and Peatified Plant Tissues

E. van der Heijden*‡, J.J. Boon*‡ and F. Bouman‡. *Unit for Mass Spectrometry of Macromolecular Systems, FOM Institute for Atomic and Molecular Physics, Kruislaan 407, 1098 SJ Amsterdam and ‡Hugo de Vries-Laboratory, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands

The taphonomy of plant material and the geochemical cycles leading to the decomposition and selective preservation of recognizable plant remains is of interest to geochemists. In our laboratory in-source pyrolysis mass spectrometry (m/z range of 20–1500) is used for chemical characterization of very small amounts of plant material, including peat and peatified plant tissues (Van der Heijden, E. *et al.*, 1990, *Proceedings Peat '90*, ed. Sopo, R., pp: 148–163, Jyskä). Detailed interpretation of the mass spectrometric data is difficult because the decomposition process during peat formation of the various plants in the peat is unknown. Knowledge of the peatification process on the anatomical and chemical level is of vital importance for a correct interpretation of fossilized organic matter.

Anatomical and chemical aspects of the decomposition process have been investigated by correlating microscopic observations of recognizable fossilized plant remains with physico-chemical characterizations of features salient to the decomposition process. In order to reveal the anatomical and chemical selectivity of the peatification process on a tissue level, strongly decomposed remains of *Calluna, Sphagnum*

and *Eriophorum* were anatomically and mass spectrometrically characterized and compared with native and less degraded plant tissues.

The different plant parts and tissues show a high specificity in anatomical and chemical characteristics. Bark of *Calluna* stems and roots show an excellent chemical and anatomical preservation. Selective removal of polysaccharides and alteration of the lignin macromolecule is observed in woody tissues of *Calluna*. Anatomical observations of the wood reveal a large variation in degree of decomposition between cell walls of identical tissues. The peatified *Eriophorum* stems are characterized by a selective removal of pentosans, whereas hexosans are readily well preserved. Details about chemical and anatomical aspects of this research will be presented.

How Evolutionarily Distant Are Amphipolar Seaweeds? Evidence from 18S rDNA and Internal Transcribed Spacer Sequences in *Acrosiphonia arcta*

M.J.H. van Oppen, J.L. Olsen, W.T. Stam and C. van der Hoek. Department of Marine Biology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

Some species of benthic marine algae occur only along polar and subpolar seashores of both hemispheres. These amphipolar disjunction patterns can be explained in two ways: (i) seaweeds occurring in the cold to cold-temperate zone of one hemisphere have managed to pass the equator; (ii) seaweeds that were formerly restricted to tropical latitudes have dispersed bidirectionally into the colder regions of both hemispheres.

Fragments of the 18S rDNA gene and the internal transcribed spacers are amplified *in vitro* by means of the PCR (polymerase chain reaction) method (Mullis, K.B. & Faloona, F., 1987. In *Meth. Enzymol.* **155:** 335). Amplified fragments can be sequenced directly (Sanger, F. *et al.*, 1977, *Proc. Natl Acad. Sci. USA* **74:** 5463).

Two sub-Artic and one sub-Antartic isolate of *A. arcta* show no sequence divergence within the 18S gene (expected) but also no difference in the two ITS regions (unexpected). This result will be confirmed by resequencing a new specimen of the present isolate as well as a second independent specimen from another sub-Antarctic locale. If this result is correct then we must either conclude that *A. arcta* has recently passed the equator (either naturally or artificially), or that the observed sequence variation in the ITS regions is insufficient for the level of this question. We reject the latter explanation because it is inconsistent with experimental results obtained in our laboratory for biogeographic isolates of *Cladophora albida*.

Genetic Analysis of Mitochondrial Genes for Two Types of Male Sterility in *Plantago lanceolata*

C.F.M. Groenendijk, J. van Brederode and J.M.M. van Damme*. Department of Plant Ecology and Evolutionary Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht and *Department of Plant Ecology, Institute for Ecological Research, Boterhoeksestraat 22, 6666 GA Heteren, The Netherlands

Populations of *Plantago lanceolata* (ribwort plantain) are gynodioecious i.e. they consist of hermaphrodite and male-sterile plants. Two types of male-sterile plants can be distinguished morphologically: plants with reduced stamen referred to as male-sterility type 1 (MS1) and plants without stamens but with an eight-lobbed corolla referred to as male-sterility type 2 (MS2). The two MS types in *P. lanceolata* have different cytoplasms. The MS1 cytoplasm is referred to as R (reduced stamens) and the MS2 type as P (petaloid stamens). In *P. lanceolata*, both cytoplasmic and nuclear genes are involved in the inheritance of male-sterility (Van Damme & Van Delden, 1982, *Heredity* **49:** 303–318). The cytoplasmic genes involved in male sterility are presumed to be located on the mitochondrial DNA (Pring & Leavings, 1978, *Genetics* **89:** 121–136). Evidence suggests that several restorer genes exist for each cytoplasm (Van Damme, 1983, *Heredity* **50**: 253–273).

Investigation of cytoplasm frequencies in populations is greatly facilitated if the different cytoplasm are distinguished at DNA level. With the help of RFLPs of mitochondrial genes we can discriminate between the P- and R-cytoplasm with several probes coding for maize mitochondrial genes {cob, cox1, cox2, (kindly provided by Professor C. Leaver)} and with an unknown *P. lanceolata* open reading frame pPL311 (Rouwendal *et al.*, 1987, *TAG* **75:** 59–65).

To find out whether the observed RFLP differences between the P- and R- cytoplasms are consistent, we compared mitochondrial DNA RFLP patterns of MS1 and MS2 plants from 11 populations. We did not find any differences in the RFLP patterns for the R-cytoplasm, but we can distinguish at least five different RFLP patterns within the P-cytoplasm. These types are not correlated with the place of collection. MS2 plants with these different RFLP patterns will be used in crossing experiments. If the male sterility can not be restored by the set of nuclear restorer genes typical for the P-cytoplasm this could be a strong indication for the existance of more than the two male sterility cytoplasms distinguished so far.

A Method to Study Gapdynamics in Grasslands Using Infra-red Photography and Image Analysis

D. van Dorp, R.P. Keestra, M. Dannenburg and I. Raemakers. Department of Vegetation Science, Plant Ecology and Weed Science, Agricultural University, Bornsesteeg 69, 6708 PD Wageningen, The Netherlands

Gaps in the canopy of grasslands have a major influence upon the regeneration of plant species in natural vegetation. To record the spatial distribution of gaps a method was developed which consists of two parts: (i) infra-red photography showing live vegetation as light and bare soil as dark spots respectively and (ii) image analysis programme transforming the spatial information of the pictures into 256 grey levels. Specific thresholds for both vegetation and bare soil are used to obtain reproducible information on the size of individual gaps and the area and size distribution of gaps of permanent quadrats.The method will be illustrated by a data set of an experiment in which different management techniques (mowing, clipping and sod-cutting) were applied to manipulate the availability of gaps.

Ultrastructural Development of Oil and Mucilage Cells in *Cinnamomum burmanni* L. (Lauraceae)

M.E. Bakker. Rijksherbarium/Hortus Botanicus, P.O. Box 9514, 2300 RA Leiden, The Netherlands

The ultrastructural development of oil and mucilage cells in young apices of *Cinnamomum burmanni* L. was studied with transmission electron microscopy.

Future idioblasts of both cell types discern from the surrounding tissue by the absence of deposits in the vacuoles and the appearance of typical plastids lacking thylakoids. They deposit a suberized layer against the primary wall. At later stages, the two types of idioblasts can be recognized.

In mucilage cells, the mucilage is secreted by hypertrophied dictyosomes from which filled vesicles fuse with the plasmalemma and empty their contents into the space between the suberized layer and the plasmalemma. Initially the central vacuole is still present; later the cytoplasm is forced to move inward by the prolonged mucilage deposition and the central vacuole vanishes. Finally the cytoplasm degenerates and becomes completely embedded in the mucilage. In exceptional cases a cupule is present.

In oil cells, an inner wall layer is deposited against the suberized layer. Oil is formed in the plastids, transported through the cytoplasm accompanied by bundles of tubular ER and accumulated in an oil cavity. The latter is attached to the wall by a bell-like protrusion of the inner wall layer: the cupule. The inner wall layer increased in thickness throughout maturation of the oil cells. Oil accumulation progresses and later the oil penetrates the cytoplasm, which finally disintegrates.

The oil and mucilage cells both possess a suberized layer, an extraplasmatic space for accumulation of the secretory product, and specific plasmodesmata. Together with the presence of a cupule (sporadic in mucilage cells) and the similar appearance of the first deposited mucilage in mucilage cells and the inner wall layer in oil cells these features further strengthen a possible homology between oil and mucilage cells.

Development of the Inflorescence and Trunk Starch Distribution in the Sago Palm (*Metroxylon sagu* Rottb.)

D.L. Schuiling. Department of Tropical Crop Science, Wageningen Agricultural University, Wageningen, The Netherlands

Starch extracted from the trunk of the sago palm is a staple food for several million people, mainly in eastern Indonesia and Papua New Guinea. The palm builds up its starch reserves during 10–20 years of vegetative life. The reserves disappear again during the final generative phase in which the palm builds an enormous terminal inflorescence.

To monitor starch accumulation in time, palms from semi-wild stands on the island of Seram (Moluccas, Indonesia) were felled, their ages estimated, and the pith of their trunks was sampled at intervals of 0·5 to 1·0 m along the trunk.

The transition from vegetative to generative phase and the subsequent developmental stages of the inflorescence indicate physiological age stages of the palm. The hitherto unknown developmental morphology of the palm's inflorescence is described and illustrated.

A full-grown inflorescence consists of 18–27 first-order branches 2–4 m long, growing from the axils of bracts spirally placed in a 5/13 phyllotaxy on a 2 m long extension of the trunk. Each first-order branch has 7–25 distichously placed 30–60 cm long second-order branches; each second-order branch has 8–15 distichously placed 7–13 cm long third-order branches. The third-order branches are rachillae bearing spirally placed flower pairs.

The first morphological symptom of the transition to the generative phase is the shape of the apex when divested of all its leaves: conical, as opposed to concave in the vegetative phase. On a conical apex the first outwardly visible stages of the first-order inflorescence branches are found. Scanning electron micrographs show the increasing number of bracts on these in the course of their development.

Emergence of the consecutive orders of branches is phased: second-order branches do not emerge before all first-order branches are out, third-order branches

not before the second-order ones are. It takes 2–3 years from the first symptoms of transition in the apex to the emergence of the inflorescence at the top of the palm, and another three until fruits are ripe.

Information gathered so far suggests that starch is translocated upward towards the inflorescence when the fruits are growing, but not during the earlier stages of the generative period. This might indicate that starch reserves are not yet tapped to form the inflorescence, but only to form the fruits.

The research reported on here, was made possible by funds of the 'Netherlands Foundation for the Advancement of Tropical Science (WOTRO)'.

Influence of Photoperiod on Development of Bambara Groundnut (*Vigna subterranea* (L.) Verdc.)

A.R. Linnemann. Department of Tropical Crop Science, Wageningen Agricultural University, Wageningen, The Netherlands

Bambara groundnut (*Vigna subterranea* (L.) Verdc., syn. *Voandzeia subterranea* (L.) Thouars), a leguminous food crop, is cultivated by smallholders over much of semi-arid Africa, where in terms of production the crop ranks third among the pulses after groundnut (*Arachis hypogaea* L.) and cowpea (*Vigna unguiculata* (L.) Walp.). In 1986 the Department of Tropical Crop Science of Wageningen Agricultural University initiated a research project on the ecophysiological requirements of bambara groundnut, to yield information that could be used as a basis for crop improvement and further agronomic research.

Accessions from different locations in Africa were exposed to several day-length treatments of short days of 12 h or less and long days of 14 h or more, to investigate the influence of photoperiod on plant development. The results showed that long photoperiods could delay or even prevent flowering. Moreover, fruit set was delayed or absent in some of the accessions with day-neutral or delayed flowering. As staining techniques indicated no influence of photoperiod on pollen fertility, the ovaries of plants from short and long photoperiods were embedded in a cold-curing resin and sliced with a microtome for anatomical studies. Embryos were present in ovaries from both photoperiods, indicating normal fertilization. The embryos developed similarly up to 18 days after flowering; by then the average size of an embryo was 0.029 × 0.018 mm. This was the stage at which growth stopped under long photoperiods. In a trial in which plants were transferred from long photoperiods to inductive conditions of 12 h or less, some of the ovaries produced under long photoperiods developed into mature seeds under the short photoperiods. Apparently, photoregulation of development is an important trait in bambara groundnut, providing the plant with a flexible mechanism to adapt to circumstances that create seasonal fluctuations in the length of the growing period.

Ultrastructure of the Apical Apparatus of Asci in *Ombrophila violacea*, *Neobulgaria pura* and *Bulgaria inquinans* (Leotiaceae, Leotiales, Ascomycotina)

G.J.M. Vetkley, Rijksherbarium, P.O. Box 9514, 2300 RA Leiden, The Netherlands

Ascomycetes have, inside the ascus wall, highly differentiated apical structures that play a role in ascospore dehiscence. Features pertaining to the ultrastructure of this ascus wall are now generally regarded to contribute to a better understanding of the classification of ascomycete Fungi.

Comparative T.E.M. studies of this apical apparatus and its dehiscence mechanism have provided valuable diagnostic data for the group of Pezizales (van Brummelen 1978: *Persoonia* **10**: 113–128). Such data are also needed to get a better insight in the large and diverse order Leotiales.

Within the scope of comparative studies of Leotiales, the apical structures of three species of the tribe Ombrophiloideae are presented. The apical apparatus in Leotiaceae seems to consist of a central cylinder surrounded by an amyloid annular structure. On the ultrastructural level certain polysaccharide components of this annulus can be detected by PA-TCH-SP technique. This technique proves to be also useful in the examination of layers that compose the ascus wall.

Ombrophila violacea and *Neobulgaria pura* show similarities in morphology and maturation pattern of the apical apparatus, suggesting a close relation between these species. Both species differ considerably in these respects and in development of the ascospore wall from *Bulgaria inquinans*.

The variation in ultrastructure of the apical apparatus at the genus level within the Leotiales is still unsufficiently known. Therefore research on selected species of several families within this order is continued.

The Use of Immunochemical Techniques in Photoaffinity Labelling of Ligand-Acceptor Complexes

M.H.M. Cornelussen. Department of Plant Physiology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

The photoaffinity labellings technique can be used to identify proteins with a high affinity for specific ligands that lack any enzymatic activity. The ligand used must contain a photochemically active moiety. Upon incubation of a protein solution with the

appropriate ligand, irradiation couples the ligand covalently to its binding site on the acceptor protein. If the ligand is suitably labelled, the protein can be identified by keeping track of the label.

If the acceptor protein is of low abundance or high background labelling occurs, an adapted EIA and/or coupled enzyme technique can be useful. To this end both direct and indirect EIA-methods can be modified.

In the direct method the ligand is coupled to an enzyme. Native protein blots are incubated with the conjugate. Because free conjugate causes an aspecific signal, the blots are rinsed and blocked before irradiation. Crosslinked complexes are identified by EIA through a coupled enzyme reaction.

In the indirect method EIA-plates are coated with a conjugate of the ligand to an inert protein. The normal competition in antibody binding between free and coated ligand is reversed: antibody and acceptor protein in an extract compete for the coated ligand. After incubation the plate is decanted, rinsed, irradiated and assayed.

The methods are being applied to identify ABA-binding proteins in extracts of seeds of *Arabidopsis thaliana* and tomato.

Use of $^{11}CO_2$ in Long Distance Transport Studies in Pea Plants

M.A. van Oene and S. Jahnke*. Department of Plant Ecology and Evolutionary Biology, Transport Physiology, University of Utrecht, P.O. Box 800.84 3508 TB Utrecht, The Netherlands and *Botanisches Institut, Fachbereich 9, Universität GSH Essen, Universitätsstrasse 5, D-4300 Essen 1, Germany

To study photoassimilate transport from source leaves to other plant parts under different sink strength conditions, $^{11}CO_2$ (half life time 20·4 min) was used. Sink strength was manipulated by means of the empty seed coat technique. (1984, *Physiol. Plant.* **61**, 172–182). Because of the short half lifetime and the strong radiation of ^{11}C, permitting a non-destructive method of measurement, it was possible to use the same plant several times. A ^{11}C-pulse of 1 min could be measured with time intervals of 10 s during 2·5 h. Hereby, 12 detectors were placed along the fruit, peduncle, stem, petiole and source leaf.

Recovery of the sink strength in the seeds resulted in an elevation of ^{11}C-transport in the direction of the seeds at the expense of the ^{11}C-transport towards the roots. Export of ^{11}C-photo-assimilate out of the source leaf did not change after recovery of sink strength in the seeds. Results are strongly dependent on the pretreatment of the plants. After sink manipulation, changes in ^{11}C-export from the source leaf could be shown, when the phloem of the main stem was blocked by means of a stem girdling one day before the experiment. ^{11}C is a very powerful tool in assimilate transport studies in plants, with other possibilities than ^{14}C.

The Effect of Sink Strength Reduction on Phloem Loading in Source Leaves of *Pisum sativum* L.

M.A. van Oene and P. Wolswinkel. Department of Plant Ecology and Evolutionary Biology, Transport Physiology, University of Utrecht, P.O. Box 800, 84 3508 TB Utrecht, The Netherlands

Sink strength may effect the rate of phloem loading of assimilates in source regions. The sink strength of developing fruits of *Pisum sativum* L. cv. Marzia was manipulated by the empty seed coat technique. The turgor of the unloading sites in the seed coat was influenced by the osmolality of the substitute medium. Seeds were operated three hours before labelling the source leaves with $^{14}CO_2$. Autoradiography was used to visualize the distribution of labelled assimilates between mesophyll and veins. When the sink strength was low, accumulation of labelled assimilates in the veins was reduced. Pulse-chase experiments with $^{14}CO_2$ showed a reduction of vein loading when the turgor at the end of the phloem pathway in operated seeds was enhanced by a substitute medium with a low osmolality. This reduction was visible in autoradiographs 10–15 min after applying the $^{14}CO_2$. Measurements of ^{14}C in different plant parts after destruction of tissues, showed also a higher transport velocity of assimilates when the sink strength was kept high. Under our experimental conditions sink strength appears to modulate the rate of vein loading and transport from source leaves to sink regions.

The Use of Autoradiography and Densitometry as a Tool for Quantification of ^{14}C-Valine Transport Through the Source Leaf

A. Hartman, M. A. van Oene and P. Wolswinkel. Department of Botanical Ecology and Evolutionary Biology, Transport Physiology, University of Utrecht, P.O. Box 800,84 3508 TB Utrecht, The Netherlands

In a study on sink-source interactions, ^{14}C-valine transport through the source leaf was studied in explants (one node, one leaf, three fruits) of *Phaseolus vulgaris* L. Sink strength was manipulated by means of fruit removal. The braided tip of the source leaf was placed in a solution containing ^{14}C-valine. Transport of the ^{14}C (through the phloem) was visualized by means of autoradiography. Densitometry of the auto-radiographs was used to quantify the differences in ^{14}C-content in different parts of the source leaf. Special attention was paid to differences in patterns of

blackening on the place of larger veins in relation to the distance from the abraded area of the leaf.

In explants with high sink strength (fruits present), there was a higher rate of ^{14}C-transport into proximal parts of the source leaf than in explants with low sink strength (fruits removed). The reduction of vein loading and transport of ^{14}C-valine through the source leaf, after sink strength reduction, presents evidence supporting the view that phloem loading in the source leaf is regulated by sink strength.

Acta Bot. Neerl. 40(4), December 1991, p. 377–380

Meetings of the Royal Botanical Society of The Netherlands

MEETING OF THE SECTION FOR VEGETATION RESEARCH ON 22 JANUARY 1991

Changes in the Flora of Macrofungi in The Netherlands

E.J.M. Arnolds and E. Jansen.* Biological Station, Kampsweg 27, 9418 PD Wijster and *Research Institute for Nature Management, Kempenbergerweg 67, 6816 RM Arnhem, The Netherlands

Macrofungi are an artificial group of fungi, characterized by macroscopic sporocarps. In The Netherlands ±3400 species have been observed, mainly basidiomycetes (2750 species), for the rest ascomycetes (650 species). They are essential in nutrient cycles of ecosystems, in particular in forests and (semi)natural communities, as decomposers of e.g. litter and wood (mainly of cellulose and lignin compounds) and as ectomycorrhizal symbionts of woody plants.

Analysis of long-term changes in the mycoflora are hampered by methodological problems such as dependence on short-living sporocarps, which may not reflect occurrence of mycelia and which show strong periodicity and annual fluctuations, which are entangled with natural succession of vegetation and environment. In addition, many taxonomic and nomenclatural problems exist, relatively few floristic data are available which are unevenly distributed over different periods, data before 1960 being scarce. Nevertheless three methods have proved to be successful: (i) A critical analysis of excursion reports, (ii) mapping of selected species and (iii) repeated mycocoenological research.

All methods reveal similar trends: a strong decrease in abundance and species diversity of ectomycorrhizal fungi; a strong increase of wood-inhabiting fungi and relatively few changes in the saprotrophic soil inhabiting species. For instance, the average number of ectomycorrhizal species observed during an excursion decreased from 45 (46% of all macrofungi) in the decade 1950–1959 to 21 (26%) in 1980–1989; that of wood-inhabiting fungi increased from 20 (20%) to 29 (37%) over the same period. Three plots (each 1000 m^2) in oak forests on windblown sand dunes in Drenthe were investigated with mycocoenological methods during three periods: 1972–1973, 1976–1979 and 1988–1990. During this time span the average number per plot of species of ectomycorrhizal fungi decreased from 41 to 14; whereas the number of wood-inhabiting species increased from 16 to 29 and the number of soil saprophytes fluctuated between 13 and 20. Numbers of sporocarps changed from 4800 to 660 for ectomycorrhizal fungi, from 250 to 2100 for wood-inhabiting species and varied between 500 and 1300 for soil saprophytes. Many species have become rare or even extinct during this century, in particular ectomycorrhizal fungi. A preliminary Red List of threatened and extinct macrofungi in The Netherlands comprises 944 species names.

The changes are partly ascribed to natural processes, such as forest succession, in particular the increase of wood-inhabiting saprophytes. The decrease of ectomycorrhizal species is mainly attributed to nitrogen input in forest ecosystems from air pollution and to a lesser degree, to acidification. Changes in the saprophytic communities of grass- and heathlands are mainly caused by loss of habitats and the negative effects of nitrogen enrichment, acidification and drainage in the remaining areas.

Ectomycorrhizal Succession during Forest Stand Development

T.W. Kuyper. Biologisch Station, Kampsweg 27, 9418 PD Wijster, The Netherlands

Not much attention has been given to fungal succession in relation to forest succession, and to the way in which are pollution influences fungal succession. Data from several tree species in plantation forests from The Netherlands show a monotonous decrease in ectomycorrhizal fungal diversity after 5–10 years, leading to a (almost) complete disappearance of fruitbodies of ectomycorrhizal fungi in stands that are only 50 years old. This decrease is stronger in sites in which nitrogen input via air pollution is higher. Data from other European countries and from North America indicate a different successional pattern under unpolluted conditions, namely an increase in ectomycorrhizal species diversity up to canopy and root closure, and a slow decline afterwards. This decrease after canopy closure is faster under more nitrogen-rich conditions. Succession of saprotrophic macromycetes has not yet conclusively been demonstrated. Patterns of individual ectomycorrhizal species show that during tree ageing, new species appear close to the tree and other species shift to the peripheral root zones. Such observations gave rise to the recognition of two groups, namely early-stage (ES) and late-stage (LS) ectomycorrhizal fungi. Species of both categories, which can be roughly

equated with r-strategists and K-strategists, differ in both ecological and physiological respects.

Mechanisms that can explain this transition from ES to LS-ectomycorrhizal fungi during succession include both changes in tree physiology and in soil characteristics. Early stage fungi have a lower carbohydrate demand (or are a less effective carbon sink) and probably show a higher sensitivity to fresh litter extracts. It is therefore not clear whether this transition should be interpreted as soil-mediated or tree-mediated. In order to separate both effects, a large-scale experiment with removal of the litter layer and adding these organic horizons to other plots is presently being executed in various stands of *Pinus sylvestris* of different age in Drenthe, The Netherlands. Although it is not clear whether ectomycorrhizal succession under unpolluted conditions can be a driving force for forest succession, the hampered ectomycorrhizal species composition under conditions of nitrogen excess will probably have adverse effects on stand development and longevity.

Changes in the Bryophyte Layer in Rich Fens in The Netherlands

A.M. Kooijman. Department of Plant Ecology and Evolutionary Biology, University of Utrecht, Lange Nieuwstraat 106, 3512 PN Utrecht, The Netherlands

As an indicator of the present state of (base-rich and nutrient-poor) rich fens in the The Netherlands, the former and present distribution of the bryophyte species *Scorpidium scorpioides* (Hedw.) Limpr., *S. cossoni* (Schimp.) Hedenas and *S. revolvens* (Sw. ex Anonymo) Rubers is studied. Until recently, rich fens were widely distributed. The three *Scorpidium* species have decreased considerably since, and have been replaced by *Sphagnum* species, such as *S. subnitens* Russ. & Warnst. or *S. squarrosum* Crome, or by *Calliergonella cuspidata* (Hedw.) Loeske. Changes in the bryophyte layer are attributed to natural succession and eutrophication.

A study of the mechanisms behind these replacements suggests that (in)tolerance of abiotic environmental factors is important only in the replacement of *Scorpidium* by *Sphagnum subnitens*, where the latter is strongly inhibited by base-rich ground water. Interspecific interactions (competition) seem to play a role in the replacement of *Scorpidium* by *Calliergonella* and *Sphagnum squarrosum* in a more eutrophic environment. A transplantation experiment with the same four species revealed that growth of *Sphagnum squarrosum* is retarded in mesotrophic environment, but stimulated in nutrient-rich ground water. A study of acidification capacity suggests that, while *Sphagnum subnitens* hardly releases any acid, acidification capacity in *Sphagnum squarrosum* is considerable. This points to essential differences between succession lines in mesotrophic and those in more eutrophic environment, notably with regard to species composition, stability and velocity of succession. In a mesotrophic environment, succession from *Scorpidium* to *Sphagnum subnitens* is slow and species may co-exist in a relatively stable, fine-patterned mire for a long time. In a more eutrophic environment, succession from *Calliergonella* to *Sphagnum squarrosum* is fast. Once established in an early phase of succession, *Sphagnum squarrosum* will expand, run over and dominate the fen in a short period of time.

Diatoms as Indicators for the Management of Moorland Pools

H. Van Dam and M.J.S. Bellemakers.* Research Institute for Nature Management, Department of Hydrobiology, P.O. Box 46, 3956 ZR Leersum, and *Catholic University of Nijmegen, Department of Aquatic Ecology and Biogeology, Toernooiveld, 6525 ED Nijmegen, The Netherlands

Acidity changes in three isolated moorland pools were reconstructed from diatoms in sediment cores. Pollen, other micro- and macrofossils and ^{210}Pb were used for dating (Dickman *et al.* 1987, *Arch. Hydrobiol.* **109**: 377–408). The pools showed considerably larger pH-changes than acidifying lakes in the Northern temperate zone do. The pH increased from 4 to 5 in the first half of the 19th century to 6 around 1900, due to eutrophication by washing of sheep, rearing of ducks and other agricultural activities. The pH decreased to recent values between 4 and 5, when eutrophication stopped and acid deposition increased.

Leuven *et al.* (1986, *Experientia* **42**: 495–503) demonstrated that the reproduction of the moor frog (*Rana arvalis*) was inhibited by the acidification of moorland pools. Therefore liming experiments were conducted in eight acid shallow moorland pools in the heathland area 'Tongerense Heide' from February 1988 until November 1989. The effects on water chemistry, diatoms, macrophyte vegetation and the fungal infection percentage of the moor frog eggs were studied.

After initial treatment with 0·2 kg limestone per m^3 water in March 1988 the pH increased from *circa* 4·0 to *circa* 5·0 in those pools which desiccated in summer and to *circa* 6·0 in the permanent pools. Alkalinity increased from $\leqslant 0$ to 0·02–0·2 meq l^{-1} in intermittent and from $\leqslant 0$ to 0·3–0·5 meq l^{-1} in permanent pools. As desiccation of the pools caused re-acidification, the intermittent pools were relimed after refilling in March 1989. No significant changes were found in concentrations of phosphate and nitrogen compounds.

After liming the percentage of infected moor frog eggs decreased from *circa* 95 in the untreated to *circa* five in the treated pools. No changes were found in the species composition of the vegetation of the pools, which was dominated by *Eriophorum angustifolium*,

Carex rostrata and *Sphagnum cuspidatum*. The vitality of *Sphagnum* decreased in the limed permanent pools.

Eunotia paludosa, which is characteristic of oligotrophic, very acid pools and bogs with fluctuating water table, was the dominant diatom in the untreated pools. Particularly in the limed permanent pools, this species was replaced by eurytopic, eutraphentic and saprophilous species, e.g. *Achnanthes minutissima*, *Fragilaria capucina* and *Gomphonema parvulum*. In the limed intermittent pools only small amounts of these species were present.

In contrast to the chemical measurements, the diatoms indicate a release of nutrients by accelerated decomposition of organic material at higher pH-levels. This eutrophication is low in intermittent pools. Thus liming for improving breeding conditions of the moor frog should be focused on this type of pool. Continued chemical and biological monitoring is necessary to registrate and assess the long-term effects.

Our case studies demonstrate that diatoms are well preserved in the sediments of moorland pools. Therefore, they can be used for retrospective monitoring of the effects of acidification and eutrophication. Also they registrate environmental changes rapidly, because their life-cycle is very short. So, diatoms are very suitable indicators for both long- and short-term changes in management practice of moorland pools.

The Decrease of Bryophytes and Lichens in Dutch Heathland since 1975

J.T. de Smidt and P. van Ree.* Department of Environmental Studies, University of Utrecht, The Netherlands and *Service of Environment and Water, Province of Gelderland.

Since the end of the 1970s, Dutch heathland rapidly lost its rich lichen and bryophyte flora, due to high atmospheric N-deposition and the subsequent change from dwarf shrub dominance to grass dominance.

This loss of cryptogams could be documented for the Veluwe, thanks to 800 relevés from the period 1965–1975. They are compared with 800 additional relevés made in 1988 on the same 10 heathland areas.

Between the two observation periods were lost or nearly lost: 12 of the 18 lichen species, 10 of the 13 hepatics. Also several bryophytes were lost. The total cover of the remaining moss-layer reduced from 50–60% to 2–10%. The neophyte *Campylopus introflexus* is the only species that strongly increased.

A consequence of this dramatic loss is the disappearance of 6 of the 10 subassociations of dry heathland vegetation.

Long term experiments started in 1981 on the Hoorneboegse heide (Goois Natuurreservaat) show that addition of fertilizer (0, 100, 200, 400, 800 kg NPK/ha/y) causes a rapid and complete extinction of lichens within 4–5 years, followed by most of the hepatics and bryophytes 1 or 2 years later. Only a few species survive in low numbers.

Atmospheric deposition appeared to have the same effect on the control plots from 1983–1984.

In the fertilized plots after 6–8 years a group of six bryophytes, characteristic of eutrophic conditions immigrated. The strong increase of *Deschampsia flexuosa* in response to fertilizer is only partly the cause of the decrease of cryptogams. A toxic effect is apparently the other cause, as the loss of cryptogams starts one or two years before the expansion of the grass. More evidence comes from heathland areas in the North-Veluwe. These have lost their lichens although they have not (yet) been turned into grass.

Management of Dutch Chalk Grasslands and the Species Richness of the Cryptogam Layer

B.F. van Tooren,* B. Odé and R. Bobbink. Department of Plant Ecology and Evolutionary Biology, Lange Nieuwstraat 106, 3512 PN Utrecht and *Natuurmonumenten, Noordereinde 60, 1243 JJ, 's-Graveland, The Netherlands

Although the Dutch chalk grasslands are still rich in bryophyte species, several characteristic species have disappeared or have become very rare nowadays (During, H.J. & J.H. Willems, 1986, *Biol. Cons.* **36**: 143–158). Furthermore, almost all lichens have disappeared completely, especially many species of the genus *Cladonia*.

The main causes for this impoverishment are the disappearance of chalk grasslands, the change of management of the remnant sites, and the increased air pollution.

Can the former species richness be restored? Which management regimes are favourable? If the species richness is increasing again, what is the origin of the diaspores: are they present in the local soil, are they being dispersed from local or even distant populations, and is establishment from air-transported diaspores possible? Inventories of the present chalk grasslands as well as management experiments and diaspore bank investigations were carried out to obtain answers on these questions.

As expected, in mown or grazed sites the species richness is much higher than in burnt or abandoned sites. If a mowing or grazing regime is reintroduced on formerly abandoned sites the species richness increases within 2–4 years. On sites which are traditionally mown once a year in late autumn, species richness increases when mowing is performed 2–4 times a year, or is replaced by grazing.

In the experiments invading pleurocarpous species are nearly absent, probably due to their lack of vegetative propagules and the relative rarity of sporulation.

The invading acrocarps are colonists and annual shuttle species (sensu During, H.J., 1979, *Lindbergia* **5**: 2–18). The colonists are all common species with low-weight spores and high densities of diaspores in the local soil. The annual shuttle species are mainly rare species, e.g. *Ephemerum recurvifolium*, with relatively heavy spores. However, these species already occurred on the sites, e.g. on ant-hills. Lichens and also many rare bryophytes, especially those occurring on very open and bare spots, remain nearly absent.

It is concluded that the former species richness can be partly restored, especially via spreading of local remnant populations and via the soil diaspore bank. Grazing is favoured as the best form of management.

Recent Changes in the Epiphytic Lichen Flora of The Netherlands

H.F. van Dobben. Research Institute for Nature Management, P.O. Box 46, 3956 ZR Leersum, The Netherlands

Decline of epiphytic lichens is ascribed to exposure to atmospheric SO_2. The recovery during the past decade is correlated with decreasing SO_2 concentrations and possibly also with increasing NH_3 concentrations. According to the acidification hypothesis (de Bakker, A.J. 1989, *Acta Bot. Neerl.* **38**: 337–342) the effects of SO_2 and NH_3 on epiphytes come about through changes in bark pH: SO_2 deposition acidifies and NH_3 deposition alkalizes. As a consequence, SO_2 and NH_3 are largely antagonistic and changes cannot be attributed to any single one of these pollutants. Epiphytic lichen data collected in 1988 by the National Air Pollution Monitoring Survey in various parts of the country (1100 releveés, 100 species) were used to test the acidification hypothesis.

In simple linear regression, highly significant correlations were found between species number and atmospheric SO_2 concentration, both on a releveé basis ($r = -0{\cdot}58$, $n = 1102$) and on a 5×5 km grid square basis ($r = -0{\cdot}72$, $n = 266$). Correlations with atmospheric NH_3 concentrations (inferred from emissions using the TREND model; Asman, W.A.H. & van Jaarsveld, H.A. 1990, RIVM Report 228471007) were not significant, but in a multiple regression model significant positive effects of NH_3 concentration on species number were found, both per releveé and per grid square. However, the multiple regression model shows that the negative effect of exposure to SO_2 far exceeds the positive effect of NH_3.

According to the acidification hypothesis, single species cannot be positively correlated with both SO_2 and NH_3 concentration. Therefore redundancy analysis (performed by the program CANOCO, ter Braak, C.J.F. 1988, GLW Report LWA-88-02) was used to detect correlations between species abundance and atmospheric SO_2 and NH_3 concentrations, and the significance of these correlations was tested for the 49 species with >20 occurrences. All these species appeared to be significantly ($P < 0{\cdot}05$) negatively correlated with SO_2 concentration, with only three exceptions. As to NH_3, species were found with significant positive, significant negative or no significant correlations. Of the species that are positively correlated with SO_2, one is negatively correlated with NH_3 and one has no correlation with NH_3. All these findings support the acidification hypothesis.

Acta Bot. Neerl. 40(4), December 1991, p. 381

Meetings of the Royal Botanical Society of The Netherlands

MEETING OF THE SECTION FOR PLANT SYSTEMATICS AND GEOGRAPHY ON 7 DECEMBER 1990

Glacial Rain Forest Refuges and Speciation in *Begonia* sect. *Loasibegonia*

M.S.M. Sosef. Department of Plant Taxonomy, Agricultural University Wageningen, P.O. Box 8010, 6700 ED Wageningen, The Netherlands

During the last Glacial, climatic conditions that prevailed in tropical Africa were markedly different from those today. Data provided by various disciplines of research indicate that the environment must in general have been much cooler (3°–6° Centigrade) and less humid. Such conditions were probably not suitable for the persistence of lowland tropical rain forest. This type of forest dwindled in consequence and could survive only in a few secluded areas, the refuges.

Major changes in the composition of the vegetation are best demonstrated by the results of palynological research. Invariably data from tropical east Africa show that the forests were replaced by vegetation types dominated by grasses and sedges. Maley (1987: in E.M. van Zinderen-Bakker, *Palaeoecology of Africa* **18**: 307–334) discovered the persistence of a forest vegetation during the last Glacial in western Cameroun at an altitude of 300 m above sea level. Additional postulated refuges are located in: (i) the highlands of Liberia-Guinee; (ii) hilly areas in southern Cameroun and Gabon; and (iii) eastern Zaire.

The occurrence of tropical rain forest refuges has two very interesting consequences for living organisms. Firstly, the location of a former refuge area is likely to be a centre of diversity, which can be revealed by the investigation of distributional data. Secondly, the breaking up of a large distribution area into several isolated parts provides a stimulating force for speciation and this can be studied by means of phylogenetic relations.

Begonia sect. *Loasibegonia* seems to be perfectly tailored to study both effects; the slightly more than 40 species of herbs with a short lifecycle are in majority narrow endemics; they occur in humid and deeply shaded conditions in primary rain forest; their seed dispersal does not seem very efficient as their indehiscent fruits often curve down to the substrate and disintegrate with age; the monophyletic origin of the group seems beyond doubt. The preliminary results are promising as several centres of diversity within the group coincide remarkably well with the postulated refuge areas. A phylogenetic reconstruction followed by a cladistic biogeographical analysis of this group is now in preparation. Additional data are gathered from the anatomy of the ovaries and leaves while De Lange & Bouman (University of Amsterdam) have investigated the micromorphology of the seeds.

Phylogeny and Biogeography of *Spatholobus* Hassk. (Legum.-Pap.)

J. Ridder-Numan. Rijksherbarium/Hortus Botanicus, P.O. Box 9514, 2300 RA Leiden, The Netherlands

A phylogenetic reconstruction of *Spatholobus* and the closely related *Butea* is in progress. *Kunstleria* has been selected as outgroup. This genus resembles *Spatholobus* in many respects, but has some primitive characters. *Butea* and one group of *Spatholobus* species occur on the mainland of S.E. Asia, another group of *Spatholobus* in the eastern Malay Archipelago. *Kunstleria* is found here also, and recently an additional species was described from the S.W. part of India (Kerala), where patches of rainforest still occur. From the same place recent material was collected of *Spatholobus purpureus*, only known from literature and old collections.

The origin of the group under study is probably Africa from where the ancestors migrated to India and then rafted towards S.E. Asia where they were distributed up to China and into the Malay Archipelago. According to recent literature (Briggs, J.C. 1989, *Syst. Zool* **38**: 322–332) India moved northwards more closely to Africa than was thought before and collided with S.E. Asia about 50 Ma ago. The recent discovery of the Indian *Kunstleria* makes the use of the genus as outgroup all the more acceptable.

The construction of a data matrix for the computer assisted phylogenetic and biogeographical analysis is in progress. It will contain macromorphological, pollen and leaf anatomical characters. Results will be used together with data from other unrelated groups in the biogeographical analysis and will be compared with geological data and information on climate and ecology.