Acta Bot. Neerl. **44**(3), September 1995, p. 279–305

Meetings of the Royal Botanical Society of The Netherlands

COMBINED MEETING OF THE KNBV-SECTION FOR PLANT PHYSIOLOGY AND THE SLW-SECTION FOR EXPERIMENTAL PLANT SCIENCE ON 28 OCTOBER 1994

Changes in Levels of Organic Acids and Other Anions During Tuberization in *Solanum tuberosum*

L. Krizkova, D. Vreugdenhil and S.M. de Bruijn. Department of Plant Physiology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

Tuber formation in potatoes was studied in an *in vitro* model system, using single-node cuttings from short-day grown plants (Hendriks *et al.* 1991, *Plant Mol. Biol.* **17**: 385–394). The levels of anions, including chloride, nitrate, phosphate, malate, oxalate and citrate, were determined using high pH anion chromatography, with ion-suppressed conductivity detection. Determinations were done using very small tissue samples (<5 mg fresh weight).

Visible swelling of the buds started at day 5, and all buds formed tubers within 48 hours.

The levels of most anions (PO_4, NO_3, SO_4, oxalate) decreased during the first day of *in vitro* culture. The levels of malate and citrate showed markedly different patterns: the level of malate in the axillary buds increased until tuber formation became visible, and then decreased, whereas citrate exhibited an opposite pattern. As a result, the malate–citrate ratio showed a sharp peak, coinciding with the onset of tuberization. It is suggested that tuber formation is associated with a change in the regulation of the Krebs cycle.

Gene Expression and Carbohydrate Metabolism During Stolon to Tuber Transition in Potatoes (*Solanum tuberosum* L.)

N. Appeldoorn[1,2], S.M. de Bruijn[1], E. Koot-Gronsveld[1], R. Visser[2] and D. Vreugdenhil[1]. [1]Department of Plant Physiology, Wageningen Agricultural University, Aboretumlaan 4, 6703 BD Wageningen, The Netherlands; and [2]Department of Plant Breeding, Wageningen Agricultural University, Lawickse Allee 166, 6709 BD, Wageningen, The Netherlands

Single-node cuttings from short-day *Solanum tuberosum* cv 'Bintje' plants were grown *in vitro* on a highly inductive medium (Hendriks *et al.* 1991, *Plant Mol. Biol.* **17**: 385–394). Visible tuberization on the axillary buds was synchronized and occurred after 5–6 days of culture. 100% of the cuttings formed tubers. The buds or tubers were harvested every day for determinations of enzyme activities and levels of sugars.

The levels of fructose and glucose increased during the first days of culture, and decreased after swelling of the buds. The two enzymes that can hydrolyse sucrose showed a markedly different pattern: soluble acid invertase activity was initially high and decreased upon tuber formation, whereas sucrose synthase activity was hardly detectable during the first 5 days in culture, and then rose to a high level.

Fructokinase activity also increased concomitantly with tuberization. Glucokinase showed an opposite pattern. UPDglucose pyrophosphorylase (UGPase) activity increased steadily until day 4 and then remained constant. The other enzymes tested (phosphoglucomutase, phosphohexoseisomerase, alkaline pyrophosphatase) exhibited less dramatic changes.

It is concluded that major changes in carbohydrate metabolism occur, coinciding with the onset of tuber formation. Imported sucrose is no longer hydrolysed by soluble acid invertase, but by sucrose synthase.

Possible Role of Lipid Membrane Degradation in Tulip Micropropagation from Bulb Scale Explants

M.W.P.C. van Rossum and L.H.W. van der Plas. Plant Physiology, Wageningen Agricultural University, Arboretumlaan 4, 6703 Wageningen, The Netherlands

The propagation of tulip is strongly hampered by the long period (25–30 years) before a new variety can be introduced in the market. For many other crops the application of tissue culture techniques gives good results. In tulip, however, major problems are encountered. Especially when bulb scale explants are used, the explants suffer from extreme browning which eventually results in the death of the explant. Although bulb scales are available throughout the year, successful regeneration only appeared possible during limited periods. The physiological background of browning is still unknown, but a relationship with sensitivity to oxidative stress and loss of membrane integrity has been suggested.

In this line of investigation we are studying the composition of the membranes in the explants, the

degree of saturation of the phospholipids and the presence of protecting enzymes or substances (anti-oxidants). The effect of the presence of compounds in the culture medium which affect the (oxidative) degradation of the membranes is also under study, as well as the effect of those additives on the regeneration.

First results indicate that the fatty acid content of the phospholipids shows a strong decrease in the amount of linoleic acid (C 18:2) from about 70 down to 45% on a molar basis during the tissue culture period and a concomitant rise in the amount of linolenic acid (C 18:3). Also, an initial rise in the total amount of phospholipids (% of dry weight), followed by a gradual decrease was often observed. This might indicate an initial start of cell division, followed by membrane degradation and death of the explant. The degradation of membranes is also shown by the production of MDA in the explants.

Treatments are applied which might affect the degradation of membranes or the antioxidant status of the explant. Experiments with antioxidants show a positive relation with the subsequent activation of cell division leading to callus or shoot formation. This is further evidence for the key role of the membranes in this problem. At this moment the activity of the endogenous protective enzymes are being monitored and compared between positive and negative treatments.

Vitrification of Shoot Regenerants Depends on the Position of the Explant in the Donor Plant

L.J.W. Gilissen, M.J. van Staveren, J.C. Hakkert and J. Creemers-Molenaar. CPRO-DLO, Dept of Cell Biology, PO Box 16, 6700 AA Wageningen, The Netherlands

Vitrification is an incalculable problem occurring during *in vitro* culture in many plant species. Research on vitrification is mainly directed towards abatement and prevention of the problem. Fundamental knowledge on the phenomenon is scarce.

In tobacco, vitrified regenerants on thin-cell-layer (TCL) internodal explants frequently appeared. Vitrification is expressed in the leaves, which developed as thick, pale-green and lanceolate structures, while normal regenerants had thin, dark-green and oval-to-round leaves. The frequency of occurrence of vitrified regenerants on the TCL explants from the second to the fifth upper internodes from young plants (with a mean height of 11 cm) and in older plants (with a mean height of 23 cm) was measured and related to the internodal length. Nearly all (95–100%) regenerants on the explants from the young plants were vitrified. However, from the older plants, hardly any vitrified regenerants occurred on explants from the fourth and the fifth internodes (with lengths of 15 mm or more). Highly variable frequencies (ranging from 0 to 85%) of regenerant vitrification were found on the TCL explants excised from the second and third internodes (with lengths below 15 mm).

The results indicate that the occurrence of vitrified regenerants on the explant is determined by its position in, and the developmental stage of the donor plant. Because of the high degree of predictability of occurrence of vitrification, the tobacco TCL regeneration system will be useful in the investigation of cellular parameters related to vitrification, especially those related to the (intra- and inter-)cellular organization in the primary meristem and the formation of the leaf primordia of the regenerants.

Plant Regeneration from Suspension Culture-derived Protoplasts of Leek (*Allium ameloprasum* L.)

J. Buiteveld and J. Creemers-Molenaar. Department of Cell Biology, DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO), PO 26, 6700 AA Wageningen, The Netherlands

To apply biotechnological methods like somatic hybridization for the improvement of leek, an efficient regeneration system for protoplasts is essential. A method was described for the initiation of a friable, embryogenic callus type with a high regeneration potential. To initiate this type of friable callus the age of the explant played an important role. The friable callus was used for the establishment of embryogenic suspension cultures from which protoplasts were isolated. Protoplasts from these cultures were highly cytoplasmic and capable of sustained cell divisions. Different culture conditions were tested, but it appeared that the plating density, gelling agent and the quality of the suspension culture were the most important factors to obtain microcalli. After transfer of these microcalli to regeneration medium, well-rooted plants could be regenerated at a high frequency.

The applicability of these results were discussed in relation to the transfer of cytoplasmic male sterility via protoplast fusion.

Competence of Cells for Regeneration and Transformation in Thin Cell Layer Explants of Tobacco

J. Creemers-Molenaar, J.C. Hakkert, M.J. van Staveren and L.J.W. Gilissen. Department of Cell Biology, DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO), PO Box 16, 6700 AA Wageningen, The Netherlands

To study the localization and frequency of cells that are both competent for transformation and regeneration, thin cell layer explants (TCLs) from stem internodes of young tobacco plants were used. The optimal response for the induction of shoots, roots or callus from TCLs was determined by varying the concentrations of benzyladenine (BA) and naphthalenacetic acid (NAA) in the culture medium. The origin of shoots was determined by histological examination of TCLs after different periods of culture on shoot induction medium. Subsequently, explants were bombarded using the particle gun after culture of TCLs for defined periods on media with different hormonal compositions. GUS was used as a marker for transformation.

The origin of lateral shoots was multicellular; one subepidermal and at least one or more epidermal cells were involved. The origin of polar shoots was unicellular; these arose from one epidermal, subepidermal or cortex cell. Depending on the type of promotor, up to 142 GUS-expressing cells per TCL (size TCL 0·2 × 0·8 mm) were obtained after particle bombardment. The frequency and the location of GUS-expressing cells was markedly affected by the hormonal composition of the culture medium prior to and following bombardment. The results were discussed in relation to the competence of cells for transient and stable transformation.

The Effect of Temperature on the Level of Erucic Acid in the Oil of Oilseed Rape (*Brassica napus* L.)

J.A. Wilmer[1], J.P.F.G. Helsper[1] and L.H.W. v.d. Plas[2]. [1]Plant Physiology, AB-DLO, PO Box 14, 6700 AA Wageningen, The Netherlands; and [2] Department of Plant Physiology, WAU, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

The effect of temperature on fatty acid composition of plant lipids has mainly been described in terms of desaturation ratio, an effect confined to the C18 fatty acids. In 1965 an effect was reported of growth temperature on the levels of erucic (C22:1) and oleic acids in seed oil of *B. napus* cv. Nugget.

In this study, plants were grown at 15°C or 25°C or transferred from one temperature to the other during seed development. In the cultivar Reston the level of erucic acid in seed oil was about 40% of total fatty acids for plants grown at 15°C and about 20% for oil obtained at 25°C. Changes in erucic acid content are completely compensated by oleic acid. The cultivar Gulle showed hardly any response in its oil composition, levels of C22:1 varied between 45% and 40%.

In vitro experiments show hardly any effect of temperature on the level of C22:1 in the oil of microspore-derived embryos of cvs Reston and Gulle. At temperatures of 15–25°C, levels of about 20% C22:1 were found for both Reston and Gulle. We are presently studying the possible role of abscisic acid as a mediator in the temperature response of embryonic tissue.

Formation of Blackspot in Potato

L.H. Stevens, J.P.F.G. Helsper and A.J. Haverkort. DLO-Research Institute for Agrobiology and Soil Fertility (AB-DLO) PO Box 14, 6700 Wageningen, The Netherlands

Upon mechanical impact, potatoes may develop a dark discoloration below the intact skin which is known as blackspot, a negative quality trait in tubers for human consumption and for the processing industry. The mechanism of blackspot formation is studied with emphasis on the chemical identity of the compounds involved, on the enzymology of their biosynthesis and on the physiological aspects, such as accessibility of oxygen and cell decompartmentalization. Preliminary studies showed that the dark pigments, developing after mechanical disturbance can be fractionated by centrifugation in water-soluble and water-insoluble components. The water-soluble components precipitate in 5% TCA solutions, which indicates linkage to a proteinaceous matrix. Subsequent studies will involve analysis of the constituents and further physicochemical characterization of the water-soluble pigments.

Tissue Sensitivity of 'Madelon' Roses to Cytokinins

J.A. Dieleman and D. Kuiper. Research Institute for Agrobiology and Soil Fertility (AB-DLO), PO Box 14, 6700 AA Wageningen, The Netherlands

In rose combination plants, bud break of the scion is influenced by the genotype of the rootstock, probably caused by differences in its cytokinin production. Branching of scions on one rootstock varies consequent on differences in sensitivity of the scion tissue for cytokinins. The tissue sensitivity of 'Madelon' roses for BA, BAR, Z, ZR, iPA, iPAR, DHZ and DHZR was tested and reported. Axillary rose buds from the second, third and fourth five-leaflet leaf counted from the apex were sterilized and placed on MS medium with glucose (45 g l^{-1}) and 0·32, 3·2 and 32 μM filter sterilized cytokinin. After 28 days, fresh and dry weight and number of shoots were recorded. In general, at a low concentration of cytokinin, the axillary bud developed into a primary shoot, increasing the concentration of cytokinin caused an increase in dry weight of the plant and formation of axillary shoots on the primary shoot (secondary shoots). A further increase of the concentration of cytokinin caused a decrease in the total dry

weight; the share of the secondary shoots in the total plant dry weight increased up to approximately 50%. All cytokinins appeared to be able to induce this maximal response, although each cytokinin required a characteristic amount to do so. The different cytokinins are either recognized by one, not specific receptor with different binding capacities for the cytokinins, or are converted to one form that is recognized by a specific receptor. Further research has to be done to elucidate this.

Carbohydrate Uptake by Rose Petals during their Development

D. Kuiper. DLO-Research Institute for Agrobiology and Soil Fertility, Box 14, 6700 AA Wageningen, The Netherlands

Flower bud opening by expanding petals requires a substantial supply of carbohydrates from other plant parts. The uptake of sucrose and D-glucose is partly mediated by a H^+/carbohydrate cotransporter. This facilitated transport is especially prominent and dependent on gibberellins (GAs) at young developmental stages. The addition of several GAs showed that their number of hydroxyl-groups determined largely the sensitivity to GAs of the system. In the presence of orthovanadate, FCCP or β-erythrosine carbohydrates were exclusively taken up by the low affinity system (diffusion) and the stimulating effect of GA was lost. Lowered pH-values were measured after GA was added to the solution. Orthovanadate and β-erythrosine abolished the GA-effects. It was concluded that GA enhances the activity of the proton pumps and activity and steepens the H^+ gradient.

Elevation of Cytokinin Levels in the Lateral Suppression Mutant of Tomato by Introduction of the *ipt* Gene

S.P.C. Groot, R. Bouwer and J.J.M. Dons. Department of Developmental Biology, Centre for Plant Breeding and Reproduction Research (CPRO-DLO), PO Box 16, 6700 AA Wageningen, The Netherlands

The lateral suppressor (*ls*) mutant of tomato is characterized by lack of axillary meristem formation and aberrant flower development. We studied the role of cytokinins in the mutant phenotype, by introduction of the isopentenyl-transferase (*ipt*) gene from *Agrobacterium tumefaciens*. Transformants varied in phenotype, those with a mild 'cytokinin-like' phenotype exhibited reduced internode length and reduced root development. Transformants with a severe phenotype showed even shorter internodes, loss of apical dominance, reduction of leaf size, production of callus at the basis of the shoots and inhibition of root development. The severity of the phenotype correlated well with the level of *ipt* gene expression, as measured by northern analysis. Transformants with a severe phenotype exhibited increased levels of zeatin riboside, but zeatin levels were not elevated. The increase in endogenous zeatin riboside levels in the *ls* mutant did not restore axillary meristem formation, but sometimes bulbous structures were formed in the initially 'empty' leaf axils. Several adventitious meristems and shoots developed from these structures. Also, the aberrations in flower development were not restored by expression of the *ipt* gene. It is concluded that increase in cytokinin level in the *ls* mutant shoots does not restore its phenotype.

The Influence of Surfactants on the Uptake of Glyphosate by Protoplasts from Couch Grass

H. de Ruiter and E. Meinen. Research Institute for Agrobiology and Soil Fertility (AB-DLO), PO Box 14, 6700 AA Wageningen, The Netherlands

Glyphosate (N-(phosphonomethyl)glycine) is a phloem mobile compound which means that foliarly applied glyphosate enters the symplast by moving through the cell membrane. The octanol/water partition coefficient of glyphosate (log $K_{o/w} = -4{\cdot}3$ at pH 4·5) indicates that the cell membrane is relatively impermeable to this hydrophilic compound if glyphosate enters the symplast by simple diffusion. We investigated whether surfactants may enhance the foliar uptake of glyphosate by increasing the permeability of the cell membrane to glyphosate thereby facilitating the transport of glyphosate out of the treated area of the leaf.

Protoplasts isolated from couch grass (*Elytrigia repens* (L.) Nevski) were used to investigate the influence of the surfactants Ethomeen T/25 (polyoxyethylene (15) tallow amine) and Atplus 201 (polyoxyethylene (20) hexitan laurate ester) on the uptake of glyphosate. Without surfactant and after an incubation period of 20 min there is hardly any uptake of glyphosate. Addition of Ethomeen T/25 induced diffusion equilibrium (internal concentration of glyphosate=external concentration) at a surfactant concentration of 0·01% (w/v). Addition of Atplus 201 up to a concentration of 1% (w/v) did not induce diffusion equilibrium. Uptake experiments with whole plants demonstrated that glyphosate was easily absorbed by the leaves of couch grass. The absorption 24 h after treatment was 70% of the applied amount. Ethomeen T/25 and Atplus 201 did not enhance the uptake of glyphosate into the leaves.

The permeability of the cell membrane of couch grass protoplasts was increased by Ethomeen T/25.

However, this did not result in facilitated uptake when glyphosate was applied to the whole plant. Therefore, the outcome of this study does not confirm the idea that the cell membrane is a substantial barrier to the foliar uptake of glyphosate.

Molecular Analysis of Bacterially Induced Systemic Resistance in *Arabidopsis*

C.M.J. Pieterse, J.A. van Pelt, S.C.M. van Wees and L.C. van Loon. Department of Plant Ecology and Evolutionary Biology, Section Phytopathology, Utrecht University, PO Box 800.84, 3508 TB Utrecht, The Netherlands

Induced systemic resistance is the phenomenon that a plant reacts earlier and/or to a greater extent to challenge inoculation, once it has been appropriately stimulated. Besides necrosis-inducing pathogens and selected chemicals, non-pathogenic, root-colonizing *Pseudomonas* bacteria can induce resistance without provoking any symptoms. To study the mechanistic basis underlying systemic resistance as induced by non-pathogenic *Pseudomonas* bacteria, we developed a model system using *Arabidopsis thaliana* as host plant, *Fusarium oxysporum* as pathogen and *Pseudomonas fluorescens* strain WCS417r as inducing agent. Treatment of the lower parts of the roots of 2-week-old seedlings with WCS417r, 3 days before inoculation of the upper parts of the roots with *Fusarium oxysporum*, resulted in a significant reduction of disease severity. Systemic resistance as induced by necrosis-inducing pathogens and abiotic agents (SA, INA) is commonly associated with increased PR-gene expression. However, studies on PR-gene expression revealed that bacterially induced systemic resistance is not accompanied by an increase in PR mRNA accumulation. The model system is currently being employed to study the changes in gene expression upon induction of systemic resistance and to search for molecular markers which are correlated with bacterially induced systemic resistance.

Localization of ^{14}C-labelled 2,4D in Cultured Zygotic Embryos of Embryogenic and Non-embryogenic Maize Inbred Lines

F.B.F. Bronsema, W. van Oostveen and A.A.M. van Lammeren. Department of Plant Cytology and Morphology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

Immature zygotic embryos were cultured on modified Chu medium with ^{14}C-labelled 2,4D to induce formation of somatic embryos and embryogenic callus. After 16 h of culture embryos were fixed, embedded in Technovit and processed for autoradiography. Median sections of the embryogenic inbred line A188 showed 2,4D in the coleoptile, in the supensor, and in the basal part of the scutellum and in its top part opposite the shoot meristem. In the non-embryogenic line A632 more 2,4D was found: in all parts of the scutellum except for the procambium strands, in the coleoptile and in a part of the shoot. Controls were negative.

Subculture of embryos on medium with unlabelled 2,4D for another 24 h or 72 h revealed that A188 embryos showed most labelling in the scutellum: predominantly in the basal part and opposite the shoot. This distribution is comparable to that of the highly mitotic areas from which embryogenic callus originated (Fransz *et al.* 1990, *Acta Bot. Neerl.* **39:** 65–73). In A632 labelling was still observed in the scutellum but its intensity had diminished in areas of growth. The high accumulation of 2,4D in A632 might cause the formation of rhizogenic callus at the scutellum surface and the repression of regeneration.

Differential Expression of mRNA Partially Homologous to the Yeast Ubiquitin-activating Enzyme Gene During Pollen Development of *Brassica napus* L. and *Arabidopsis thaliana* L.

G. Hause[1], K. Angelis[2], T. Havlicky[1], H. Körber[2], B. Hause[1], A.A.M. van Lammeren[1] and P.M. Pechan[2]. [1]Department of Plant Cytology and Morphology, Wageningen Agricultural University, Arboretumlaan 4, NL 6703 BD Wageningen, The Netherlands; and [2]Max Planck Institute für Biochemie, Am Klopferspitz 18a, D 82152 Martinsried, Germany

A cDNA clone; exhibiting 28% protein homology to the yeast UBA1 gene coding for ubiquitin activating enzyme (E1), was isolated from a *Brassica napus* microspore cDNA library. Expression of corresponding mRNA was investigated by *in situ* hybridization during pollen development of *B. napus* and *Arabidopsis thaliana*. In both species comparable patterns of gene expression were detected. There was neither expression in microspores nor in vegetative cells of pollen. However, cytoplasm of generative cells exhibited strong hybridization signal, as did the sperm cell cytoplasm after division.

Since E1 is an enzyme important in the ubiquitin-dependent pathway of proteolysis, it is possible that the isolated clone might be involved in the degradation of various proteins in the generative and sperm cells and could thus contribute to the differentiation of these cells.

Expression of Cytoskeletal and Heat Shock Proteins in Embryogenic Microspore Cultures of *Brassica napus* L. Visualized by Immunocytochemistry

A.A.M. Van Lammeren, T. Havlicky, P. Binarova, K. Straatman, B. Hause and G. Hause.
Department of Plant Cytology and Morphology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

Microspores and pollen of rape, cultured at 32°C for 8 h, change their developmental pathway to embryogenesis. Induced microspores exhibited a turned division plane at metaphase or microspore nuclei migrated towards the cell centre before mitosis. Both phenomena resulted in symmetrical division. In embryogenic pollen vegetative cells divided. Changes in cytoskeleton, subcellular distribution of heat shock proteins (HSP) and DNA synthesis were analysed immunocytochemically.

Induced microspores and pollen exhibited changed microtubular arrangements which play a role positioning nuclei and in the induction of symmetrical divisions. In pollen, vegetative cells divided only when generative cells were attached to the pollen wall. The temporary disruption of the cytoskeletal may be responsible for that arrest of the generative cell (Hause *et al.* 1993, *Cell Biol. Int.* **17**: 153–168).

The distribution of HSP68 and HSP70 at non-embryogenic culture conditions was comparable to that found *in vivo*. HSP68, restricted to mitochondria, appeared not to change qualitatively by elevated temperatures. HSP70, however, clearly showed temperature-induced subcellular changes in distribution. Its presence in the vegetative nucleus is probably associated with the reentry of that nucleus into the cell cycle, also shown by newly induced DNA synthesis (Binarova *et al.* 1993, *TAG* **87**: 9–16).

Pollen Tube Guidance Towards the Ovules in *Brassica napus* L.

F. Mittempergher[1], C.J. Keijzer[2] and J.L. van Went[2]. [1]Department of Plant Biology, University of Perugia, Borgo XX Guigno 74, Perugia, Italy; and [2]Department of Plant Cytology and Morphology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

In *Brassica napus* L. var *oleifera* Del. we found low seed set among the ovules within each ovary. As a part of a project on this decreased fertility, we studied factors influencing the attraction of the pollen tubes by the ovules.

In mature flowers, minute amounts of exudate were observed on the placenta, the funicle and in front of the micropyle. This exudate was found to be present exclusively on the border between the cells, and could only be observed when using high SEM magnifications, in combination with specific preparation methods. The exudate, like any other liquids inside the ovary, is removed when using conventional microscopical fixation and dehydration procedures. Therefore, we used both Cryo-SEM and freeze-substitution techniques to study the presence and locations of exudate in the ovary. The goal of this project is to find a correlation between the presence of exudate and the receptivity of an ovule for a pollen tube, which may explain the irregular fertilization and seed set we found. Furthermore, we studied the relationship between floral age and the first appearance and amount of exudate in the ovary. The origin of the exudate will be analysed to understand whether its production occurs in different sites.

Pollen Tube Refusal by Rapeseed Ovules

F. Mittempergher[1], C.J. Keijzer[2], F. Ferranti[1], B. Romano[1] and J.L. van Went[2]. [1]Department of Plant Biology, Perugia Agricultural University, Borgo XX Guigno 74, 06100 Perugia, Italy; and [2]Department of Plant Cytology and Morphology, Wageningen Agricultural University, Arboretumlaan 4, NL-6703 BD Wageningen, The Netherlands

The fertilization process in *Brassica napus* L. var *oleifera* Del. has been studied to identify the phenomena which lead to a lower number of seeds than the potential of the species. Pechan 1988, *Ann. Bot.* **61**: 201–207 and Ferranti *et al.* 1993, *Gior. Bot. Ital.* **127**: 527 observed that from 31 ovules present on average at anthesis in each ovary from flowers over the terminal raceme, only about 22 will develop into seeds.

4–6 days after pollination three types of ovule development could be recognized: seeds with a normal development (71%), early aborted seeds (13%) and ovules that stopped their development at the anthesis state (16%). Further observations indicated that in the latter ovules the fertilization process did not take place, since pollen tubes were not found in the micropyles. Our present research is focused on the unravelling of the mechanism leading to failure of fertilization.

Analysis of hand-pollinated pistils at anthesis showed that even the most basal ovule has been reached by pollen tubes only 26 h after pollination. On the surface of the outer integument, pollen tubes were found growing along the funiculus, and their growth was directed towards the micropylar region. However, in case of unfertilized ovules, the pollen

tubes changed their direction before reaching the micropyle and turned around.

Since the quantity of pollen grains on the stigma surface of the pollen tubes inside the ovary seemed not to be a limiting factor, we examined the ovule to observe any signs that could explain the refusal of pollen tubes. By using critical point dried ovules for SEM observation followed by re-embedding of selected ovules in Technovit 7100, we had a good method to check the megagametophyte condition at the light microscopy level, both for fertilized and unfertilized ovules. Repeated pollination will give an answer to the question of whether the seed set problem is either caused by maturation gradient along the placenta or whether it is due to an abnormal development and functioning of the megagametophyte.

Tracing Fertilization in Crop Plant

C.J. Keijzer. Department of Plant Cytology and Morphology, Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

The earliest step to find out whether pollination has been successful is tracing the fertilization in the narrow sense. This is difficult since it deals with tiny nuclei inside two particular embryo sac cells that are generally well-hidden by the nucellus and the integuments of the ovule. A number of light-microscopical techniques to show the fertilization were demonstrated as were some further implications for plant breeding.

Freshly fertilized ovules of different crop plants were isolated and histochemically treated using different stains and tissue-clearing techniques, thus avoiding the need to section them. Subsequently they were observed using either light, fluorescence or Nomarski microscopy.

Depending upon the chosen technique, it is possible to observe either the pollen tube inside the micropyle and inside one of the two synergids or to distinguish the sperm cells inside one or two of the embryo sac cells. The choice of the technique in its turn depends upon the plant species, since it has to do with the stainability of the pollen tube, the possibility of staining the sperm cells preceding pollination or fertilization, the thickness of the integuments and the cytoplasmic constitution of the embryo sac cells.

Cryo Scaning Electron Microscopy in Plant Breeding Research

C.J. Keijzer and A.C. van Aelst. Department of Plant Cytology and Morphology, Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

The combination of freeze (=cryo) fixation and scanning electron microscopy offers excellent possibilities to produce high quality three-dimensional images of both fully hydrated plant structures and free water. For many plant breeders knowledge of de- and rehydration of drought-resistant structures (e.g. pollen, seeds, pills, somatic embryos) is important. Apart from showing the very natural situation, cryo fixation takes place within a second and, accordingly, the timing and proceeding of hydration processes can be very precisely followed. Some examples from the practical plant breeding situation and allied disciplines were presented.

The Mechanics of the Maize Stamen at Anthesis

C.J. Keijzer, F.A. Hoekstra, M.C. Reinders and H.B. Leferink-ten Klooster. Department of Plant Cytology and Morphology, Department of Plant Physiology, Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

Flowering of Poaceae is a crucial process for human nutrition, since the world production of wheat, rice and maize depends upon this process. Amazingly, almost nothing is known about the structural and physiological processes that accompany the mechanical movements in these flowers at anthesis. As a part of our broad-based study on this topic, we presented an analysis of the water movements and their induction in the staminal filament at anthesis.

At anthesis, sucrose is converted into glucose and fructose in the epidermal and subepidermal cells of the filaments. The resulting increasing osmotic pressure attracts water from both the anther and the receptacle to the filament, allowing the latter to extend. Together with evaporation, the water retraction from the anther causes its dehiscence.

Apart from its scientific importance, this analysis aims at solving the problem of an (undesired) disturbed flowering in certain maize inbred lines.

Isolation and Culture of Embryo Sacs of *Petunia*

M.H. Thijssen and J.L. Van Went. Department of Plant Cytology and Morphology, Graduate School Experimental Plant Sciences, Wageningen Agricultural University, Arboretumlaan 4, NL-6703 BD Wageningen, The Netherlands

In vitro culture of isolated embryo sacs creates new possibilities for analysis and manipulation of gametophyte development, leading to insight in nature and regulation of gametophytic differentiation. An enzymatic maceration method, using driselase, was developed to release mature living embryo sacs from

ovules of *Petunia hybrida* L. (Van Went, J.L., Kwee H.-S. 1990, *Sex. Plant Prod.* **3**: 257–262).

Megaspore mother cells, megaspores and coenocytic embryo sacs can be isolated by maceration of the ovules with a different enzyme mixture, containing Cellulase Y-C and Macerozyme. Isolated embryo sacs were cultured in microculture chambers, and in small Petri dishes; different culture media were used. Directly after isolation, the mature embryo sac and its composing cells show a morphology, organisation, and structure similar to those *in situ*. After a few hours in culture, the embryo sac cells become spherical in shape, but they retain their position and polarity. The central cell possesses a large vacuole and numerous starch grains. Throughout the vacuole mobile strands of cytoplasm are formed in which a rapid migration of organelles is visible, indicating cell viability. After 2 days of culture in a sucrose depleted medium, starch grains have completely disappeared, which shows the metabolic activity of the central cell *in vitro*. The embryo sac remained viable in culture for up to 1 week after isolation.

The Origin of an Exotestal Phytomelan Layer in the Seed Coat of *Gasteria verrucosa*

P.E.Wittich[1] and P. Graven[2]. [1]Department of Plant Cytology and Morphology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen; and [2]Hugo de Vries Laboratorium, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands

During seed development of *Gasteria verrucosa* the mechanical layer is formed from the exterior epidermis of the outer integument. It evolves into a thick layer of black-brown cell wall material which is said to be phytomelan.

The development of this wall was studied by light and electron microscopy. Between 14 and 16 days after pollination (DAP) the tangential walls increase in thickness. Between 15 and 17 DAP a tertiary cell wall of 20 microns thick is formed at the exterior side of the epidermis between the plasma membrane and the existing cell wall. This tertiary cell wall is periodic acid-Schiff (PAS) and calcofluor white (CFW) positive. Between 17 and 29 DAP the cytoplasm degenerates and the tertiary wall shrinks to 10 microns. In the tertiary wall two layers become discernible. The exterior layer shows globules surrounded by fine electron dense material, leading to a brown-black, PAS and CFW negative layer (i.e. the phytomelan layer). The interior layer changes at the ultrastructural level too, but remains PAS and CFW positive.

Finally, the exotestal layer consists of a frame work of thickened secondary cell walls filled with blocks of tertiary cell wall (phytomelan). The secondary wall permits water penetration while the phytomelan is impermeable and solid.

Temporarily Red Light-insensitive Mutants of Tomato are Phytochrome B1 Receptor Mutants

L.H.J. Kerckhoffs[1,3,4], A. van Tuinen[2], A. Nagatani[3], M. Koornneef[2], L.H. Pratt[4], M.-M. Cordonnier-Pratt[4] and R.E. Kendrick[1,3]. [1]Departments of Plant Physiology and [2]Genetics, Wageningen Agricultural University, Arboretumlaan 4, NL-6703 BD, Wageningen, The Netherlands; [3]Laboratory for Photoperception and Signal Transduction, FRP-RIKEN, Hirosawa 2-1, Wako-shi, Saitama 351-01, Japan; and [4]Department of Botany, University of Georgia, Athens, GA 30602, USA

Recessive tomato (*Lycopersicon esculentum*) mutants called temporarily red light-insensitive (*tri*), which have a slightly longer hypocotyl under white light than wild type (WT) have been studied. While continuous low fluence red light (R) is relatively ineffective in hypocotyl inhibition, both blue and far-red (FR) light are just as effective as in WT. Western blot analysis showed that the *tri*^{1} mutant is deficient in a relatively light-stable phytochrome (116 kDa), recognized by a tobacco phytochrome-B antibody in WT; *tri*^{3} has a reduced 116-kDa band; *tri*^{2} and *tri*^{4} have bands running at different positions, 105 and 95 kDa, respectively. These patterns were retained in light-grown plants. Northern blot analysis for *PHYB1* mRNA showed a *c.* 2-kb larger transcript for *tri*^{2}; a much reduced transcript of WT size for *tri*^{4}; *tri*^{1} and *tri*^{3} were equivalent to WT. Other members of the tomato phytochrome gene family (*PHYA, PHYB2, PHYX, PHYZ*) were normal in these mutants. This indicates that the *tri* locus specifically affects *PHYB1* gene expression. Unlike other phytochrome-B mutants (e.g. cucumber *lh, Arabidopsis hy-3, Brassica ein*), de-etiolated seedlings of the *tri* mutants exhibit normal responses to end-of-day FR and day-time supplementary FR.

Analysis of Secretory Proteins from the Potato Cyst Nematode *Globodera rostochiensis* Using Monoclonal Antibodies

A. Goverse[2], G. Smant[1], J. Stokkermans[2], J. de Boer[1], H. Overmars[1], J. Zilverentant[2], H. Pomp[2], M. van Gent-Pelzer[2], J. Bakker[1] and A. Schots[2]. [1]Department of Nematology, Binnenhaven 10, 6709 PD Wageningen, The Netherlands; and [2]Laboratory for Monoclonal Antibodies, Binnenhaven 12, 6709 PD Wageningen, The Netherlands

Monoclonal antibodies raised to the subventral glands of second stage juveniles (J2) of *Globodera rostochiensis* (MGRs) were used to analyse secretory proteins. Induced stylet secretions containing secretory proteins from preparasitic J2 were solubilized and used for dot-blot assays. This resulted in positive reactions of 10 MGRs including MGR48, which also labelled specifically isolated stylet secretion particulates in immunofluorescence assays. Immunogold labelling of the subventral gland granules proved that the antigens of the used MGRs are located in the granules. Immunofluorescence of induced preparasitic J2 resulted in decreased labelling of the MGR antigens showing individual granules in the subventral gland extension and ampulla. The results point to the secretion of subventral gland antigens *in vitro*. Immunogold labelling has to confirm the secretion of subventral gland antigens *in planta*. Western blot analysis of J2 homogenates using MGRs resulted for MGR48 in binding to a 32 kD, 39 kD and 45 kD protein band, whereas two MGRs bound to only one, 39 kD, protein band, five MGRs to a 32 kD and 39 kD band and three MGRs to a 39 kD and 45 kD protein band.

Recently, we constructed a large ($2{\cdot}6 \times 10^6$ primary recombinants) cDNA library of preparasitic J2. Immunological screening of this library together with induced stylet secretions, is a promising approach for further analysis and characterization of secretory proteins which are probably involved in the induction and maintenance of syncytia in plant roots.

Functional Analysis of the *in planta* Induced Gene *ipi*O of *Phytophthora infestans*

P. van West[1], C.M.J. Pieterse[1,3], N.N. van der Wel[1], H.S. Judelson[2] and F. Govers[1]. [1]Department of Phytopathology, WAU, Binnenhaven 9, 6709 PD, The Netherlands; and [2]CEPRAP, UC Davis, CA 95616, USA. [3]Present address: Department of Plant Ecology and Evolutionary Biology, University of Utrecht, The Netherlands

An *in planta* induced gene, *ipi*O, of the potato late blight pathogen *Phytophthora infestans* (Mont.) de Bary, was isolated from a genomic library by differential hybridization. *P. infestans* has two *ipi*O genes, *ipi*O1 and *ipi*O2, which are very similar and closely linked. The encoded proteins, IPI-O1 and IPI-O2, have no homology with known protein sequences (Pieterse *et al.* 1994, *Gene* **138**: 67–77). The *ipi*O genes are expressed at high levels in the early stages of the pathogenic interaction of *P. infestans* with its host plants potato and tomato suggesting that the IPI-O proteins have a function in pathogenicity. In *in vitro* grown mycelium *ipi*O gene expression is induced by nutrient deprivation (Pieterse *et al.* 1994, *MGG* **244**: 269–277). In order to assay the role of IPI-O in pathogenicity, we transformed *P. infestans* with constructs carrying a strong oomycete promoter fused to the *ipi*O coding sequence in anti-sense orientation. These transformants were tested for their ability to cause disease on potato leaves. To determine where and when during the interaction the *ipi*O genes are expressed, we transformed *P. infestans* with constructs containing the *ipi*O1 promoter fused to the GUS-reporter gene. Characterization of the transformants was presented and the possible role of IPI-O during pathogenesis of *P. infestans* on its hosts discussed.

Non-destructive *In Vivo* Transgene-expression Studies Using the Luciferase Reporter System and a 2D-luminometer

D. Haarsma, C. Voogd, D. Willemen, O. Vorst and S. van der Krol. Department of Plant Physiology, Agricultural University, Arboretumlaan 4, 6703 BD, Wageningen, The Netherlands

The 2D-luminometer, in combination with the firefly luciferase reporter gene (LUC), offers a unique system to monitor and localize *in vivo* transgene expression. The luminometer set-up consists of an extreme light-sensitive ccd video camera, imaging software and additional peripheral equipment. To visualize the presence of the luciferase enzyme, transgenic plants are sprayed with the substrate luciferin. The LUC enzyme catalyses the reaction:

$$\text{luciferin} + O_2 + \text{ATP} \rightarrow \text{Oxyluciferin} + CO_2 + \text{AMP} + \text{PP} + \text{light}.$$

Since this way of measuring gene activity is non-destructive, gene expression can be followed throughout plant development, and in the same plant grown under different physiological conditions. An additional advantage of the LUC gene is that the enzyme it encodes is not stable in the presence of the substrate. In order to monitor transgene promoter activity, first conditions need to be established under which substrate and ATP are not limiting for the luciferase activity. Under these conditions, in the continuous presence of luciferin, changes in the bioluminescence are a direct measure of changes in luciferase steady state levels. Several experiments were presented that illustrate how the 2D-luminometer and the luciferase reporter system can be used in plant gene expression and plant transformation studies.

Carbohydrate Metabolism of Embryogenic Cell Cultures of *Daucus carota* Measured by Means of *In Vivo* Nuclear Magnetic Resonance

J. Krook[1], A. van 't Slot[1,3], D. Vreugdenhil[1], L.H.W. van der Plas[1] and C. Dijkema[2]. [1]Department of Plant Physiology, Agricultural University, Arboretumlaan 4, 6703 BD Wageningen; and [2]Department of Molecular Physics, Agricultural University, Dreyenlaan 3, 6703 HA Wageningen, The Netherlands

Embyrogenic cell cultures of carrot (*Daucus carota*) contains two different cell types: proembryogenic masses (PEMs) and non-embryogenic, highly vacuolated cells. To study the differences in carbohydrate metabolism between embryogenic and non-embryogenic cells, these two cell types were separated; subsequently the conversion of $^{13}C_1$-labelled glucose added to the nutrient medium was followed by means of NMR.

(i) $^{13}C_1$-labelled glucose was rapidly taken up from the medium: after 6 h no glucose is present in the medium.

(ii) Inside the cell $^{13}C_1$-labelled glucose was converted into $^{13}C_1$-labelled fructose, probably by hexose isomerase and both labelled hexoses were found in newly synthesized sucrose. Sucrose accumulation was higher in PEMs than in the vacuolated cells. There are also indications that more starch was formed in the PEMs from $^{13}C_1$-labelled glucose.

(iii) Next to $^{13}C_1$-labelled glucose and fructose, these hexoses were also present in the $^{13}C_6$-labelled form (as free hexoses as well as in sucrose). This C_1–C_6-exchange will occur at the level of triose phosphates in the glycolysis; in combination with gluconeogenetic activity after this exchange has taken place, this will result in both hexoses labelled at the C_1- and at the C_6- position. In this gluconeogenetic pathway, pyrophosphate-dependent fructose-6-phosphate phosphotransferase (PFP) probably plays a key-role.

(iv) The labelling of sugars in the cells was transient, both in PEMs and in vacuolated cells. After 8–10 h all the NMR-visible label had disappeared, probably by a combination of sugar degradation (yielding $^{13}CO_2$) and synthesis of (NMR-invisible) macromolecules.

Freezing Tolerance of Scots Pine Needles: The Effect of Ammonia Fumigation

H. Clement and P.R. van Hasselt. Department of Plant Biology, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands

The effect of ammonia fumigation on the freezing tolerance of current year and 1-year-old Scots pine needles (*Pinus sylvestris* L.) was examined. Needles were collected from 20-year-old pine trees from a heavily ammonia polluted forest and a forest with low ammonia concentrations. In addition, needles from 5-year-old trees, which were fumigated in open top chambers for 8 months with ammonia concentrations of 0, 53 or 106 nl l^{-1} NH_3, were studied. Needles were collected at (2)-monthly intervals from October until May. The freezing tolerance of needles was determined in a laboratory freezing test and frost injury was measured with chlorophyll fluorescence.

Needles from either field-grown or in open-top-chamber-grown trees hardened against frost during autumn and early winter and de-hardened in late winter and early spring. Current-year needles were more frost-tolerant than 1-year-old needles. Ammonia fumigation in the open-top chambers did not affect the freezing tolerance significantly. However, freezing tolerance of 1-year-old needles from the polluted forest decreased markedly compared to the control site.

From these results it is concluded that short-term exposure to NH_3 does not affect freezing tolerance directly. The observed decrease in frost tolerance of needles from the polluted forest site may possibly be explained by long-term disturbances of the nutrient status of the trees.

Evaluation of Chilling Tolerance of Tomato Genotypes with Chlorophyll Fluorescence Measurements

J.H. Venema and P.R. van Hasselt. Department of Plant Biology, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands

A number of different tomato genotypes were analysed for their chilling susceptibility after growth under near-optimal (24/20°C) and suboptimal (15/13°C) temperatures. To determine the role of the growth temperature on chilling tolerance of the photosynthetic apparatus, the maximal rise of chlorophyll fluorescence induction (F_R) was assessed during incubation at 0°C. We also measured the temperature dependence of the maximal fluorescence peak of chlorophyll fluorescence induction (F_p) and characterized the low and high temperature (LTB, HTB) breakpoints of F_p. Other temperature-dependent fluorescence parameters which are supposed to vary between species of different chilling susceptibilities, as photochemical quenching (q_p) and the quantum yield of PSII electron transport (φ_{PSII}), were also determined.

In addition to dark chilling, the sensitivity of the chloroplast to photoinhibition at a temperature of 1–3°C and an illuminance of 300 μmol m^{-2} s^{-1} was studied.

The results of these experiments were primarily discussed with emphasis on the chilling susceptibility of the wild genotypes in comparison to the domestic tomato. Secondly, the role of the growth temperature was reviewed.

Measurements of *In Vivo* Ubiquinone Reduction Levels in *Petunia hybrida* Cell Suspensions

A.M. Wagner and M.J. Wagner. Fys. & Bioch. Plants, VU, de Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

A method is described for the determination of *in vivo* ubiquinone reduction levels in non-green tissues, by extraction and subsequent detection of Q10 and Q-10H_2 with HPLC. In *Petunia hybrida* cell suspensions Q reduction levels remained at a stable level of about 60%, despite the changing conditions during the batch culture (from excess sugar to starvation) and the concomitant variations in respiration. Also, in the presence of uncoupler, which causes a huge increase in respiration via both the cytochrome pathway and the alternative pathway, Q reduction levels stayed at the 60% level. The increased engagement of the alternative pathway at low reduction levels can be explained by a change in kinetics of the oxidase caused by accumulating organic acids (e.g. pyruvate). It is postulated that the Q reduction level is maintained at a stable level in order to prevent free radical formation and subsequent membrane damage. Only in non-physiological conditions, such as under anaerobiosis or in the presence of azide, did reduction levels increase up to 80%.

An Oat Root Plasma Membrane Bound Protein Kinase with PKC-like Properties Characterized with a Novel Non-radioactive PKC Assay

P.C.J. van der Hoeven, H.A.A.J. Korthout and A.H.de Boer. Department of Plant Physiology and Biochemistry, Institute of Molecular Biological Sciences, Vrije Universiteit Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

An oat root plasma membrane bound protein kinase was characterized with a novel non-radioactive method using a protein-kinase-C-specific fluorescent peptide substate. The specific activity was 20–40 nmol Pi min^{-1} mg^{-1} plasma membrane protein. The kinase was sensitive to the very specific PKC inhibitor calphostin C (IC_{50} 0·5 μM). The kinase is calcium dependent and is stimulated twofold at 1·3 mM Ca^{2+} by polyunsaturated fatty acids, but hardly by phosphatidylserine. The presence of polyunsaturated fatty acids uplifted the calcium dependency. Preliminary results indicate that calphostin C inhibits the fusicoccin induced proton extrusion by the plasma membrane bound H^+-ATPase (IC_{50} 1 μM) in oat coleoptiles; therefore, it is possible that this kinase plays a role in fusicoccin signal transduction.

Isolation and Characterization of the Fusicoccin Binding Protein from Oat Roots

H.A.A.J. Korthout, P.C.J. van der Hoeven and A.H. de Boer. Department of Plant Physiology and Biochemistry, Vrije Universiteit, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands

Fusicoccin (FC), a phytotoxin produced by the fungus *Fusicoccum amygdali* Del., elicits a number of physiological responses after binding to the fusicoccin binding protein (FCBP) located in plasma membranes of higher plants. In order to understand the nature of the FCBP and to elucidate its biochemical characteristics, it is crucial to isolate the FCBP in pure and active form. Here we describe two different and independent techniques to purify the protein from oat roots: (i) purification using avidin/biotin affinity chromatography; and (ii) purification by means of a number of conventional protein purification methods. An important step in both purification protocols was charging plasma membrane vesicles with biotinylated fusicoccin. Precharging resulted in stabilization of the binding pocket of the FCBP during the purification.

Purification of the FCBP by avidin/biotin affinity chromatography resulted in the elution of two polypeptides from the affinity-column, with an apparent MW of 30 kD and 31 kD on SDS-PAGE. Purification of the FCBP using this protocol yields a 150-fold increase of specific binding activity compared to crude solubilized plasma membrane proteins.

Purification of the FCBP using the second purification protocol (involving anion-exchange and hydrophobic interaction chromatography) also revealed two major polypeptides of 30 kD and 31 kD. The 31 kD polypeptide was isolated from SDS-PAGE, eluted from gel, digested with trypsin and digests were used for sequence analysis. A search on the EMBL database revealed that the 31 kD polypeptide was homologous to the family of so called 14-3-3 proteins. Polyclonal antibodies raised again the BMH1-gene translation product, a 14-3-3 protein in yeast, showed that both the 31 kD and the 30 kD polypeptides, isolated by both purification protocols, were recognized on immunoblots. Two-dimensional SDS-PAGE revealed that the isoelectric point of the 31 kD subunit was 5·0. Gelfiltration

showed that both FC-binding activity and the two immunostained bands were eluted in fractions representing proteins with molecular masses of 70 ± 10 kD, indicating that the native FCBP does exist as a dimer in native form. The isoelectric point and the dimeric native form of the FCBP are two properties shared with certain members of the 14-3-3 protein family.

Ion Channels in the Plasma Membrane of Cortical and Xylem Parenchyma Cells of Maize Mesocotyl

P. Giesberg and A.H. de Boer. Department of Plant Physiology and Biochemistry, Vrije Universiteit Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

The mesocotyl of maize can accumulate Na^+ from the ascending transpiration stream, thereby acting as a sink to protect the shoot from excess Na^+. Two processes are involved: uptake of sodium from the xylem vessel and (temporary) storage. The uptake from the xylem vessels is thought to be mediated by the xylem parenchyma cells (xpc): the cells adjacent to the xylem. Sodium is then relocated within the mesocotyl, where it is stored. Johanson *et al.* postulated that sodium is finally stored in the cortex of the mesocotyl. With this in mind we tried to identify the ion transport mechanisms in the plasma membrane of both xpc's and cortical cells using the patch-clamp technique.

The cortex was separated from the stele and incubated in a cell wall digesting solution. Protoplasts thus obtained were used for patch-clamp studies. All 19 protoplasts possessed a time-dependent inward current and no outward current. The inward current started to activate at potentials negative from -100 mV. Tail current analysis revealed a reversal potential close to the Nernst potential of potassium. When extracellular potassium was replaced for sodium the inward current was completely abolished.

Due to their position, embedded by several other cell types, and their strong lignification the isolation of protoplasts from xpc is more cumbersome. We have chosen to remove all non-xpcs with a mixture containing cellulase and pectolyase, leaving the lignified cell walls of xpc intact. Protoplasts from xpcs were then released by a mechanical step. An outward current, activating at potentials higher than 20 mV was seen in approximately 65% of these protoplasts. The reversal potential of this current was around 0 mV and was unaffected by changes in potassium or chloride concentration. Approximately 50% of the protoplasts showed an inward current, which activated at potentials more negative of -100 mV and reversed close to the potassium Nernst potential. Preliminary experiments where extracellular potassium was changed by sodium indicate that unlike cortical cells, a time-dependent inward current carried by sodium exists.

Delayed Fluorescence in Site-specific Mutants (YM210) of the Photosynthetic Reaction Centre from *Rhodobacter sphaeroides*

R.W. Visschers[1], S. Vulto[1], L.P. Beekman[2], R. van Grondelle[2], M. Jones[3], C.N. Hunter[3], K. Krab[1] and R. Kraayenhof[1]. [1]Department of Molecular and Cellular Biology, and [2]Department of Biophysics, BioCentrum Amsterdam, Vrije Universiteit, de Boelelaan 1087, 1081 HV Amsterdam, The Netherlands; and [3]Department of Molecular Biology, Krebs Institute, University of Sheffield, UK

Time-resolved fluorescence in the nanosecond range was measured for several site-directed mutants of the photosynthetic reaction centre (RC) from *Rb. sphaeroides* in which the Tyrosine M210 residue was changed for a number of other residues, including phenylalanine, leucine, histidine, serine and tryptophan. In these mutants the initial charge separation rate is slower than in the wild type. Chemical titration shows that the reduction potential of the P/P^+ couple varies in the mutants, in good agreement with Nagarajan *et al.* (1993: *Biochemistry* **32:** 12323). Moreover, when LH1 is present the reduction potentials are 20–30 mV lower than for the RC-only samples. The rate and relative amplitudes of the recombination fluorescence that is emitted upon reduction of Q_a by dithionite was measured for all mutants. This allows a direct comparison of the change in free energy and the reduction potentials, yielding a reorganization energy of 150–300 cm^{-1} in good agreement with measurements for similar mutants of *Rb. capsulatus* (Jia *et al.* 1993, *J. Phys. Chem.* **97:** 13180).

Rapid turnover of Phosphatidylinositol(3)phosphate in Plant Cells

T. Munnik[1], J. Brederoo[1], R.F. Irvine[2] and A. Musgrave[1]. [1]Institute for Molecular Cell Biology, BioCentrum, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands; and [2]Department of Biochemistry, AFRC Institute, Babraham, Cambridge CB2 4AT, UK

In animal cells, much attention is focused on polyphosphoinositides due to their involvement as second messenger precursors in calcium mobilization and the activation of protein kinase C ($InsP3/Ca^{2+}$/DAG signalling). This group of minor phospholipids

includes PtdIns(4)P and PtdIns(4,5)P2 that are synthesized by sequential phosphorylation of PtdIns. Recently, the group has been extended by lipids phosphorylated at the D-3 position of the inositol ring (viz. PtdIns(3)P, PtdIns(3,4)P2 and PtdIns(3,4,5)P2), which are thought to represent a novel signal transduction pathway. Their levels rapidly increase upon stimulation, but even then do not exceed more than a few per cent of the total phosphoinositide pool. Their route of synthesis is still unclear. We are using the green alga (*Chlamydomonas*) to show that the basic elements for InsP3/Ca^{2+}/DAG signalling are present in plants and that these signals are generated during sexual fusion. New analysis of the polyphosphoinositide pool revealed the presence of PtdIns(3,4)P2, representing 15% and 1% of the PtdInsP and PtdInsP2 pools, respectively. The turnover of PtdIns(3)P in unstimulated cells is shown to be equivalent to that of PtdIns(4,5)P2, and much faster than that of structural phospholipids. Since the PtdIns(3,4) pool is too small to account for the PtdIns(3)P turnover, we suggest that it is directly synthesized from PtdIns.

Although these data were obtained from HPLC analysis, our results using TLC to enrich and to separate the isomers were also presented.

Calcium-stimulated Kinase Activity in *Chlamydomonas eugametos* Gametes

M. Siderius, A. Porto-leBlanche, J. van Himbergen and A. Musgrave. Institute for Molecular Cell Biology, BioCentrum, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands

The green alga *Chlamydomonas eugametos* is an excellent model system for studying signal transduction in plants. During sexual reproduction. cell–cell recognition via the flagella gives rise to several responses that lead to fusion of the gametes. Signal transduction during mating is mediated by several second messengers, such as Ca^{2+}, cAMP and IP3/Ca^{2+}/DAG. In the further signal transduction protein, phosphorylation is thought to be of great importance.

To study protein phosphorylation during sexual signalling in *Chlamydomonas*, we started to characterize kinase activity in the flagella of gametes. As Ca^{2+} is thought to be an early messenger, calcium-stimulated kinase activity was studied *in vitro* and *in situ*. A 67 kDa calcium-stimulated kinase was present in extracts of flagella.

Activity was stimulated by calcium alone, without an extra stimulation by calmodulin. It therefore resembled the calcium-dependent protein kinase (CDPK) activity characterized in higher plants. An antibody directed against the soybean CDPK (Dr Harmon, University of Florida, Gainesville, USA) cross-reacted with the *Chlamydomonas* protein. The 67 kDa kinase appeared to be flagellum-specific and membrane-associated, although other cross-reacting bands in the cell body suggested a small family of immunologically related proteins.

Using the antibody to screen an expression library, we were able to clone several CDPK-like cDNAs. Each clone generated a characteristic 700 bp fragment in PCR experiments with primers to consensus sequences in the kinase and calmodulin domains of the known CDPKs. One of the cDNA clones of 2600 bp (C13) was studied further. A fragment from this clone recognizes a 3 kB mRNA. Southern analysis using the same fragment indicates that more than one gene is present in *Chlamydomonas eugametos* and that similar genes are also present in the distantly related species *Chlamydomonas reinhardtii*. The sequence of the C13 clone is homologous to the known CDPKs, although the 5′ end contains some extra sequence which might be important in regulating activity or localization of the protein.

Plant Tyrosine Phosphatase Signalling: Oxidative Stress and Cell-cycle Regulated Expression of the *Chlamydomonas eugametos* VH-PTP13 Gene

M.A. Haring, M. Siderius and A. Musgrave. Institute for Molecular Cell Biology, BioCentrum, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands

We present the first evidence for phosphotyrosine signalling pathways in plants by characterizing a protein tyrosine phosphatase gene from the unicellular green alga *Chlamydomonas eugametos*. The cDNA, referred to as VH-PTP13, contains an open reading frame specifying a protein with a Mr of 30·3 kDa, that has significant homology with a distinct group of dual-specificity phosphatases. The highest homology is found with CL-100, a human stress-response gene that regulates MAP kinase activity. The purified VH-PTP protein expressed in *E. coli* had phosphatase activity. Non-dividing *C. eugametos* gametes did not express the VH-PTP13 gene, whereas synchronously dividing vegetative cells only expressed VH-PTP13 in the early G1-phase of the cycle, implying a function there. When vegetative cells were subjected to oxidative stress (treatment with hydrogen peroxide), expression of the VH-PTP13 gene was strongly induced, analogous to the human CL100 gene. The presence of this tyrosine phosphatase gene implies that the mitogenic MAP-kinase signalling pathway recently elucidated for other eukaryotes is conserved in algae and plants. Preliminary data indicate that at least two

homologous PTP genes are present in *C. eugametos* UTEX 9. We are currently introducing the *C. eugametos* genomic DNA fragment into *C. reinhardtii* cells to investigate its expression, and to establish whether the introduction of an extra PTP gene alters the vegetative growth or other cellular responses.

Molecular Analysis of Pollen Coat Proteins of *Brassica oleracea* L. in Relation to Anther Development and Pollination

R.K. Ruiter, D.M.C. van Laarhoven, J.H.B. van de Bovenkamp, G.J. van Eldik, M.M.A. van Herpen, J.A.M. Schrauwen and G.J. Wullems. Department of Experimental Botany, Research Group Molecular Plant Physiology, University of Nijmegen, Toernooiveld, NL-6525 ED Nijmegen, The Netherlands

The interaction between the pollen grain and the stigma determines the outcome of a pollination event. This means that molecules at the outer faces of both cell types are thought to play a key role in the process of pollination. In the exine (the outer layer of the pollen grain) of mature *Brassica* pollen, a pollen coat is formed by tapetal remnants (=tryphine). It mainly consists of lipids, proteins and carbohydrates. The importance of the pollen coat during pollination is emphasized by the description of a conditional male sterile *Arabidopsis thaliana* mutant, missing the tapetum-derived pollen coat in the exine of mature pollen grains.

We are focusing on the molecular analysis of the proteins present in the pollen coat of *Brassica oleracea*. Therefore, proteins have been extracted from the pollen coat according to the method of Doughty *et al.* (1993 *PNAS* **90**: 467–471). This extract consisted of proteins up to 45 kDa, as shown by SDS-PAGE, and was used to raise a polyclonal antiserum. After western blot analysis, the antiserum showed specificity for proteins present in the pollen coat and whole anthers.

The polyclonal antiserum was used to screen an anther cDNA library of *B. oleracea*. This library was made of mRNA isolated from whole anthers during the post-tetrad development of the pollen grains. This screening resulted in the isolation of six different cDNA clones, called bopc (*Brassica oleracea* pollen coat) clones. The deduced amino acid sequences of bopc15 and bopc34 have homology with proteins differentially regulated during dehydration-stress. Bopc39 has homology with tonoplast intrinsic protein (TIP) encoding genes. TIPs are attached to protein vacuoles, and are thought to play a role in the transport of molecules across the tonoplast. The regulation of these tip genes is also dehydration dependent. Bopc17 shows homology with genes encoding thioredoxins, enzyme-regulating proteins. A fifth clone, bopc25, is highly homologous to profilin encoding genes. Profilins are actin-sequestering proteins, probably also involved in signal transduction systems. Bopc4 encodes a glycine-rich protein, harbouring some characteristics of oleosins.

Bopc4 has been studied into more detail. It encodes a 363 amino acids long protein rich in glycine, with some typical domains as a hydrophobic domain and an amphipathic domain, both characteristic for oleosins. The oleosin-like hydrophobic domain is also found within the deduced amino acid sequences of five other anther-specific genes of the Brassicaceae. These data suggest that the hydrophobic domains of the oleosin-like proteins have the same function as those of oleosins.

Oleosins are the major oil-body associated proteins. The hydrophobic domain is used to anchor the protein into the oil-body. Oleosins are suggested to play a role in establishing the stability of oil-bodies during seed desiccation. A similar role can be assigned to the bopc4 gene product, attached to oil-bodies in the pollen grain or pollen coat during pollen dehydration and rehydration.

In the near future promoter-GUS fusions will be used to determine the site of gene expression within the anther. Furthermore, antisense constructs may show the importance of this protein in anther development and pollination, especially in relation to the stability of oil-bodies in the pollen coat. BOPC4 protein synthesized *E. coli* will be used to raise an antiserum which enables us to study the site of protein synthesis and protein accumulation within the anther.

Pistil Genes with a Putative Role in Pollen Tube–Pistil Interaction in Potato

G.J. van Eldik, R.K. Ruiter, M.M.A. van Herpen, J.A.M. Schrauwen and G.J. Wullems. Department of Experimental Botany, Research Group Molecular Plant Physiology, University of Nijmegen, Toernooiveld 1, NL-6525 ED Nijmegen, The Netherlands

The interaction between pollen and pistil is of great importance for fertilization in plants. So far, little research has been done on the molecular basis of a successful pollen tube–pistil interaction. To isolate genes with a modulated expression after pollination, both expressed in the pollen tube or the pistil, a cDNA library of cross-pollinated pistils of *Solanum tuberosum* was constructed.

Normal differential screening resulted in the isolation of several pistil-specific genes. One of these

genes, STS14, is a single copy gene; its expression levels in pollinated and unpollinated pistils are equal. Homologous transcripts have been found in pistils of *Petunia hybrida, Nicotiana tabacum* and *Brassica oleraceae*. Sequence analysis of the genomic clone (STSg14) revealed that the C-terminal part of the protein has 43% amino acid identity with pathogenesis-related proteins (PR-1) from tobacco. *In situ* hybridization experiments resulted in the localization of STS14 in the stigma and stylar cortex. No STS14 transcripts were detected in the transmitting tissue.

A cold-plaque screening (Hodge *et al.* 1992, *Plant Journal* **2**: 257–260) resulted in the isolation of several genes expressed in the pistil. Two of these genes are involved in the flavonoid biosynthetic pathway. The first gene encodes isoflavone reductase (IFR), an enzyme that catalyses the reduction of 2'hyroxyformononetin into vestitone in alfalfa. This gene showed an increased expression in pistils after pollination. The second gene encodes flavonol synthase (FLS), a key enzyme in the flavonol biosynthesis. This enzyme hydrolyses dihydroflavonols into flavonols. Flavonols are essential for pollen germination. The expression of other genes in the flavonoid biosynthetic pathway is analysed for a modulated expression after pollination.

Specific Antibodies for Localization of Thiophenes in *Tagetes*

C.C.F.M. Schrijnwerkers, A.F. Croes and G.J. Wullems. NOVAPLANT Cell Biotechnology Group, Department of Experimental Botany, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

In the study on the regulation of secondary metabolism it is very important to have information on the localization of secondary metabolites at the level of cells and tissues. Therefore, a procedure has been developed to produce specific antibodies against thiophenes in *Tagetes*. Naturally occurring thiophenes in *Tagetes* species (marigolds) are characterized by one, two or three heterocyclic thiophene rings, and are known for their biocidal effects. In plants, thiophenes are abundant in roots and hypocotyl. For that reason transformed root cultures were made using *Agrobacterium rhizogenes* to study thiophenes in tissue culture.

Polyclonal antibodies were raised against α-T, a particular thiophene consisting of three rings and no side chain. For conjugation to a carrier protein a special spacer to α-T was constructed. An α-T-BSA conjugate was used for the immunization of rabbits. The serum was screened for specificity with an indirect competitive enzyme-linked immunosorbent assay (ELISA), using α-T-casein conjugate.

The most abundant thiophene in roots in 5-but-3-en-l-ynyl-(2,2')bithienyl (BBT). The antibodies react very specifically with BBT and other thiophenes. Thiophenes in extracts of transformed root cultures of *Tagetes patula* were also reactive. There was no cross-reactivity of pyrrol, monothiophene, and root extracts of *Rubia*, tomato and bean, which is indicative of high specificity of the antibodies.

Future studies will be focused on the identification of the cell types that contain thiophenes using immunohistochemistry. For localization, unembedded hypocotyl tissue will be sectioned, immunolabelled with the anti-thiophene antibodies, and examined under the light microscope.

Isochorismate Synthase in Anthraquinone-producing Cell Suspension Cultures of *Rubia tinctorum*

L.J.P. van Tegelen[1], M.H.H. van der Bruggen[1], R.J.M. Bongaerts[2], A.F. Croes[1], R. Verpoorte[2] and G.J. Wullems[1]. [1]NOVAPLANT Cell Biotechnology Group, Department of Experimental Botany, University of Nijmegen; and [2]Leiden/Amsterdam Centre for Drug Research, Division of Pharmacognosy, University of Leiden, Leiden, The Netherlands

Cell suspension cultures of *Rubia tinctorum* produce substantial amounts of anthraquinones. This is in contrast with the situation in most cell suspension cultures, in which yields of secondary metabolites are generally low compared to those in the whole plant. Therefore, *Rubia* cell suspension cultures provide an interesting model system for studying regulation of secondary metabolite production.

Anthraquinones in *Rubia tinctorum* are synthesized via the shikimate-o-succinyl-benzoic acid pathway. The immediate precursor of o-succinylbenzoic acid is isochorismate, which is synthesized from chorismate through the action of isochorismate synthase (ICS). Chorismate is also a precursor in the biosynthesis of aromatic amino acids. The isochorismate synthase reaction therefore is the branchpoint at which anthraquinone biosynthesis diverts from the primary shikimate pathway. There are indications that this branchpoint reaction is a limiting step in the biosynthesis of anthraquinones. Therefore, we decided to study the regulation of isochorismate synthase at the molecular level. In order to isolate the gene for ICS from *Rubia* we are purifying the enzyme from cell suspension cultures. Partial sequencing of a purified protein will enable us to make DNA probes which can be used to screen a *Rubia* cDNA library.

One of the problems we are dealing with is the low abundancy of the protein. The use of a production medium or elicitation with a fungal extract are successful ways to increase ICS activity.

Analysis of Self-incompatibility in Diploid Potato by Sense and Antisense Transformation

E. Abad i Cantero, R. Eijlander, M.S. Ramanna and E. Jacobsen. Graduate School Experimental Plant Sciences, Wageningen Agricultural University, Department of Plant Breeding, PO Box 386, 6700 AJ Wageningen, The Netherlands

Diploid potato (*Solanum tuberosum*, $2n=2x=24$) exhibits one locus gametophytic self-incompatibility (SI). Little is known about the pollen side in the SI reaction. Some S-genotype linked glycoproteins with RNase activity are regarded to be responsible for the stylar side in the SI reaction. At the MPI, Köln, Germany, DNA clones were obtained from some of these S-RNases. Some have been used in studying the SI reaction by promoter analysis of the S2-promoter and by trying to get sense and anti-sense S-constructs expressed in transgenic potato. The promoter analysis of some minimal S2-promoter versions showed a partial loss of the style specific expression in heterologous constructs. Sense constructs did not introduce new incompatibility factors. Unstable co-suppression is probably found, however. In one case the anti sense approach caused a total breakdown of the S2-incompatibility reaction when transformed with S2-as. The biological reaction pattern could be confirmed by Iso Electric Focusing of stylar extracts, showing a strongly reduced S2-glycoprotein production. This confirms for potato the results of Hyun-Sook *et al.* and Murfett *et al.* 1994, *Nature* **367**: 560–563; 563–566), obtained in *Petunia* and *Nicotiana*.

Chromosome Behaviour at Meiosis of Somatic Potato (+) Tomato Hybrids and Derivatives Using Genomic *In Situ* Hybridization

F. Garriga Caldere[1], H. de Jong[2], M.S. Ramanna[1] and E. Jacobsen[1]. [1]Graduate School of Experimental Plant Sciences, Wageningen Agricultural University, Department of Plant Breeding, PO Box 386, 6700 AJ Wageningen; and [2]Department of Genetics, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands

Potato (+) tomato fusion hybrids were backcrossed to the tetraploid potato ($2n=4x=48$) and BC and BC2 progenies were produced. Using the technique of genomic *in situ* hybridization (GISH), the chromosomes of potato and tomato were stained differentially. The number of chromsomes of each of the parental genomes were accurately estimated in both BC1 and BC2 progenies. In a pentaploid BC1 in which four of potato and one genome of tomato were expected to be present, only nine of the expected twelve tomato chromosomes were present. Besides GISH, the individual chromosomes of tomato were identified through RFLP analysis, which confirmed the cytological observations. The causes of these anomalies were described and explained.

Comparison of Genomes of a Tuberous and a Non-tuberous *Solanum* Species Through GISH of Somatic Hybrids and their Backcrosses

K. Horsman and E. Jacobsen. Graduate School of Experimental Plant Sciences, Wageningen Agricultural University, Department of Plant Breeding, PO Box 386, 6700 AJ Wageningen, The Netherlands

Through somatic hybridization, fusion hybrids between the black nightshade (*Solanum nigrum*, $2n=6x=72$) and diploid potato ($2n=2x=24$) were produced. The resulting octaploids were backcrossed to *S nigrum* as well as to the tetraploid *S. tuberosum* in order to obtain eleven BC1 plants of the former and one plant of the latter combinations. In both cases embryo rescue was required. Both fusion hybrids and BC1 plants were analysed through genomic *in situ* hybridization (GISH). The genomes of both of the parents could be distinguished from each other through differential fluorescent staining. The genome compositions in both cases were according to expectations.

Photosynthetic Redox-regulation of the pH-polar-reaction of *Potamogeton lucens*: The Response of Photosynthetic Electron Transport to Free-CO_2-limiting Conditions

L.C. van Ginkel. ECOTRANS, Department of Plant Biology, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands

Photosynthesis and growth of submerged aquatic macrophytes is often limited by the availability of free CO_2. Several species show strategies like CO_2-concentrating mechanisms to overcome this limitation. *Potamogeton lucens* can make use of bicarbonate as a carbon source for photosythesis.

A so-called pH-polar-reaction proton transport is responsible for converting HCO_3^- into CO_2 by acidification of the apoplast at the abaxial side of the leaf. This process of bicarbonate utilization is

regulated by the ratio of light and CO_2 available for photosynthesis. Upon changes in the light–CO_2 balance, the ratio NAD(P)H/NAD(P) in the chloroplast and the cytosol changes. The idea of redox-regulation of the pH-polar-reaction is that this change in redox-state is responsible for the regulation of the plasma membrane H^+-ATPase.

Chlorophyll-*a* fluorescence quenching *in vivo* can describe the redox-state of the chloroplast. By measuring simultaneously the chlorophyll-*a* fluorescence and the pH in the abaxial apoplast it has been shown that the pH-polar-reaction is strongly related to a highly oxidized state of photosystem II.

Photosythetic Nitrogen Use Efficiency of *Galinsoga ciliata* and *Origanum vulgare*, Plant Species Differing in their Maximum Relative Growth Rate

M. Houssa and T. Pons. Department of Plant Ecology and Evolutionary Biology, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands

Fast-growing herbaceous plant species have a higher photosynthetic nitrogen use efficiency (PNUE) than slow-growing species (Lambers, H & Poorter N. 1992, *Advances in Ecological Research* **23**, 187–261). This difference in PNUE has been investigated in a comparison of two plant species differing in their inherent relative growth rate. The species chosen were *Galinsoga ciliata*, a fast-growing annual weed and *Origanum vulgare*, a perennial with an inherently lower relative growth rate. The present results show the nitrogen partitioning among the various components of the photosynthetic apparatus and the photosynthetic performance of the plants. The plants were grown outdoors in pots at different levels of nutrient supply.

At a high total nitrogen content of the leaf, the maximum PNUE (calculated from photosynthesis at light saturation) did not differ between species. With decreasing nitrogen content, only the maximum PNUE of *Galinsoga* increased significantly, due to the higher amount of N invested in the photosynthetic capacity. The daily PNUE (calculated from photosynthesis on an average day during the growth period) was highest at low-nitrogen supply, but no significant difference between the species was apparent.

Technetium in Tomato Plants: Uptake and Transport

G.C. Krijger, E.M. Brokx, D.A. van Elswijk and H.Th. Wolterbeek. Interfaculty Reactor Institute, Delft University of Technology, Mekelweg 15, 2629 JB Delft, The Netherlands

The fission of uranium or other actinides in nuclear reactors leads to the production of a number of technetium isotopes, of which ^{99}Tc (half-life $2{\cdot}1 \times 10^5$ years) forms a persistent presence in nuclear wastes. In an aerobic environment the predominant Tc species, pertechnetate (TcO_4^-), is water soluble and highly mobile. A rapid uptake of technetium by and transport within many plant species has been observed.

Several nutrient ions, like $H_2PO_4^-$, SO_4^{2-} and MoO_4^{2-} metabolic inhibitors are shown to be effective in reducing TcO_4^- uptake by whole roots. This suggests analogue (carrier-mediated) uptake mechanisms for pertechnetate and mentioned ions (Cataldo, D.A., Wildung, R.E. and Garland, T.R. 1983, *Plant Physiol.* **74**, 849–852.) However, the reduction is only marginal and observed in the presence of the complicating cell interior and at relatively high Tc-concentrations (>10 nmol l^{-1}). To exclude this possibly interfering effect, in the present study we make use of plasma membrane vesicles and lower, more realistic Tc-concentrations (>fmol l^{-1}). The plasma membrane vesicles are obtained by partitioning of a microsomal fraction in an aqueous polymer two-phase system (Larsson, C., Widell, S. and Kjellbom, P. 1987, *Meth. Enzymol.* **148**, 558–568.)

As yet, little is known about the causes of rapid distribution of technetium to and accumulation in the leaves (mainly). Some experiments suggest that TcO_4^- is biologically available as sulphate-analogue, namely protein labelling experiments indicated the formation of Tc-cysteine (Cataldo, D.A., Garland, T.R., Wildung, R.E. and Fellows, R.J. 1989, *Health Phys.* **57**, 281–287). This compound may act as a Tc-deposit, because of its inability to form disulphide bridges, thus leading to nonfunctional proteins.

Investigations of the Tc-species within plants may lead to a deeper understanding of technetium behaviour in plants. To this end, the selective extraction of TcO_4^- by a chloroform solution of tetraphenylarsonium-chloride was used to determine the pertechnetate fraction of technetium present in the xylem, the phloem and the nutrient solution of our model plant tomato. The determination takes only a few minutes and requires no pretreatment. The selectivity of this method for TcO_4^- was tested by attempting to extract several Tc-complexes out of a nutrient solution, namely Tc-cyclam, Tc-chloride, Tc-tartrate and Tc-citrate. None of them appeared to be co-extractable with TcO_4^-. Some other relevant Tc-complexes will be tested as well, like Tc-DTPA, Tc-oxide, Tc-cysteine, Tc-methionine and Tc-proteins. Size-exclusion chromatography and ion-interaction chromatography is used for removal of the excess of ligand and oxidized reducing agent. The

results obtained so far indicate that TcO_4^1 is the main Tc-species in the xylem, the phloem and the used nutrient solution of tomato plants.

Growth and Development of Seven Woody *Ficus* Species

E. Veneklaas and F. den Ouden. Department of Plant Ecology and Evolutionary Ecology, Utrecht University, PO Box 80084, 3508 TB Utrecht, The Netherlands

Seven *Ficus* species of tropical origin, six of which were in use as indoor ornamental plants, were studied during a 5-month period in a glasshouse. Growth was assessed by destructive harvesting and by leaf demographical observations. There are both ontogenetic and species differences in growth and biomass allocation. The species differ in leaf size, rate of new leaf formation, internode length and branching patterns. Possible relationships with growth are discussed. Relative growth rates are well-correlated with leaf area ratios (leaf area/plant dry weight). Species with greater leaf area ratios invest less dry matter per unit leaf area. Acclimation to decreased light intensity was studied in a shading treatment. The project aims at identifying mechanisms underlying high potential growth rate and good acclimation to lowered light intensity, characteristics that are considered important for potential indoor plants.

MEETING OF THE SECTION FOR FERTILIZATION RESEARCH IN PLANTS ON 30 SEPTEMBER 1994

Cell Biological Aspects of Embryo Sac Development in *Petunia*

M.H. Thijssen and J.L. Van Went. Department of Plant Cytology and Morphology, Graduate School of Experimental Plant Sciences, Wageningen Agricultural University, Arboretumlaan 4, NL-6703 BD Wageningen, The Netherlands

The embryo sac develops from one single cell, the megaspore, deeply embedded in the sporophytic tissues of the ovule. After three mitotic divisions, resulting in an eight-nucleate, coenocytic embryo sac, cellularization takes place. The seven cells formed differentiate to perform their specific functions during fertilization.

In spite of a vast number of microscopical studies of embryo sac development, the regulation of this process and the mechanisms behind it are still poorly understood. Isolation and *in vitro* culture of embryo sacs opens prospects for the analysis and manipulation of embryo sac development and can afford insight into factors and mechanisms involved in regulation.

Mature living embryo sacs of *Petunia hybrida* L. can be isolated by enzymatic maceration of the ovules, using the enzyme Driselase. By using the enzymes Cellulase Y-C and Macerozyme, viable embryo sacs in the one-nucleate, two-nucleate and four-nucleate stage can be isolated. The morphology of the embryo sacs directly after isolation is comparable to the morphology of the embryo sacs *in situ*.

Mature isolated embryo sacs were cultured in small droplets of medium in micro-culture chambers which are suitable for microscopical studies or in 3·5-cm plastic dishes in which the embryo sacs are easy accessible for manipulation.

Embryo sacs could be kept alive in culture for up to 1 week after isolation. The changes occurring during the culture period which were most prominent in the central cell were the formation of cytoplasmic strands throughout the vacuole, the fusion of the two polar nuclei, and the disappearance of starch grains.

Pistil Genes with a Putative Role in the Pollination Process

Gerben J. van Eldik, René, K. Ruiter, Marinus M.A. van Herpen, Jan A.M. Schrauwen and George J. Wullems. Department of Experimental Botany, University of Nijmegen, Toernooiveld 1, NL-6525 ED Nijmegen, The Netherlands

Successful pollination is necessary to achieve complete fertilization followed by seed set. Despite the importance of pollination in the fertilization process, little research has been done on the molecular basis of successful pollination. To elucidate the changes in gene expression in the pistil during successful pollination, a cDNA library was constructed of *Solanum tuberosum* pistils 24 h after cross-pollination and screened using the cold-plaque screening method (Hodge *et al.* 1991, *Plant Journal* **2**: 257–260). Several genes were isolated which are expressed at a medium or low level in pistils. Two of these genes encode enzymes, isoflavone reductase and flavonol

synthase, involved in the flavonoid biosynthetic pathway.

Isoflavone reductase (IFR) catalyses the reaction of 2'hydroxyformononetin into vestitone in alfalfa. In potato *ifr* was expressed in all tissues. However, the expression of *ifr* in pistils was increased upon pollination with a maximum pollination effect 24 h after pollination. No increased expression of *ifr* was shown in other flower parts upon pollination, even after 48 h.

Flavonol synthase (FLS) hydrolyses dihydroflavonols (dihydrokaempferol, dihydroquercetin and dihydromyricetin) into flavonols. Flavonols are required for pollen tube growth and can be delivered either from the tapetum (anther) into the pollen coat or from the pistil into the pollen tube. In petals, flavonols are important co-pigments in flower colour. In potato, *fls* was expressed in petals, pistils and anthers, but not in pollen. During the development of potato flower buds *fls* is firstly expressed in 5–6 mm flower buds with a maximum at 7–9 mm buds. Using northern blot analysis to investigate the expression in different flower parts during development the *fls* gene is already expressed at a low level in pistils, anther and petals of 3–4 mm flower buds.

We have isolated and described different genes expressed in the pollinated pistil, of which some have a possible role during the pollination process. Further characterization of the *ifr* and *fls* gene expression in relation with pollination can elucidate the role for these enzymes in pollination processes.

Callose Deposition During Megasporogenesis in the Tropical Grasses *Brachiaria* and *Paspalum*

M. Wagenvoort[1], Th.S.M. de Bock[1] and T.N. Naumova[2]. [1]Centre for Plant Breeding and Reproduction Research, CPRO-DLO, PO Box 16, 6700 AA Wageningen, The Netherlands; and [2]Komarov Botanical Institute of the Academy of Sciences, Laboratory of Embryology, 2 Prof. Popov Street, 197022, St Petersburg, Russia

Apomixis in tropical grasses was described about 30 years ago and is known as apospory. In *Brachiaria* and *Paspalum* apospory results in an embryo sac with diploid nuclei because it originates from a diploid nucellar cell. In displosporous apomictic species there is no callose deposition around the megaspore mother cell (MMC) and at later stages of megasporogenesis. The aim of this study was to investigate callose deposition in aposporous species. In a sexual biotype of one accession of *Brachiaria decumbens* callose was deposited around the MMC and at the dyad, triad and tetrad statge of megasporogenesis in 89·1% of the ovules studied. In two aposporous biotypes of *B. decumbens* about the same percentage of ovules with callose deposition, 85·8 and 89·1%, was found. Also, no difference was found in callose deposition between sexual and aposporous biotypes of the species *Paspalum notatum* and *P. simplex*.

It is concluded that in the apomictic species investigated there is no difference in callose deposition between sexual and aposporous biotypes.

MEETING OF THE SECTION FOR VEGETATION RESEARCH ON 7 FEBRUARY 1995

History and Development of Floodplain Forest in The Netherlands

R.J.A.M. Wolf. Institute for Forestry and Nature Research (IBN-DLO), PO Box 23, 6700 AA Wageningen, The Netherlands

The study of the history and development of Dutch floodplain forests is part of the project 'Classification of Dutch Forest Ecosystems'. In this project, the Dutch forests are described as a series of related ecosystems. The classification is based on present-day ecosystems; the approach aims at integrating vegetation types, site types and forest history and management.

The majority of the higher parts of the river area was already deforestated in the Roman period. After a period of woodland regeneration, in the Carolingian period (8th century AD) woodlands on natural levees, stream ridges and river dunes were cleared again and disappeared forever. After completion of the embankment of the large rivers at the beginning of the 14th century, woodland in the embanked floodplain mainly consisted of willow coppice.This willow coppice was situated either on tidal silt flats in the freshwater tidal area ('gorzen') or on sand bars and sand banks in the river clay area ('rijswaarden').

Today the majority of the woodlands in the Dutch large river floodplains consists of old abandoned willow coppice in the (former) freshwater tidal area. Further upstream virtually all old willow coppice woodlands have been converted into pasture. Small-scale willow woodlands which sprang up spontaneously on river banks or on sites from which clay had

been extracted for brick production, have taken over (Wolf 1995, *History and Management of Floodplain Forest in The Netherlands*, IBN, Wageningen).

An example of a floodplain forest ecosystem is the Colenbrandersbos. Sedimentation changed the site from river bed (17th century) into a river beach. On the higher parts of the river beach, willow planting and coppice management changed the potential natural vegetation (PNV; *Salicetum albae*) into willow coppice. As a result of further sedimentation, the site developed into a low natural levee (c. AD 1900); willow coppice substituted the PNV of this site (*Fraxino–Ulmetum*). At present, the Colenbrandersbos is situated on a high natural levee (PNV: *Violo odoratae–Ulmetum*). Development of the PNV has been disturbed by poplar planting.

The example shows that the integrated approach of the classification project leads to a good understanding of relationships between different forest ecosystems and forest development. However, intense human interference in the embanked river landscapes and absence of old, more-or-less natural floodplain forests complicate this.

The Effects of Extensive Livestock Farming on the Colombian Páramo Ecosystem

Robert G.M. Hofstede. Hugo de Vries-Laboratory, Department of Palynology and Paleo/Actuo-ecology. University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands

Páramos are cold and wet alpine grasslands, which are found in the Northern Andes. They are ecologically important owing to their high degree of endemism and their regulative role in the hydrology of the Northern Part of the South American continent. Moreover, páramos are economically important because they function as a water resource for the large interandean population and because they are used by cattle- and potato-farmers. Because of the limited infrastructure and the harsh circumstances, the present agricultural practices are extensive but the number of farmers is growing. Recently, there is increasing concern that the ecological and hydrological functions of the páramo will be endangered.

The natural páramo grassland has a high above-ground biomass (>3 km m^{-2}), but a relatively low below-ground biomass (1–2 kg m^{-2}). Tall growing tussock grasses (*Calamagrostis* spp.) and stem rosettes (*Espeletia* spp.) constitute a tall vegetative structure which consists of more than 75% of dead material. The natural páramo grassland soil is humid and organic and has low extractable nutrient concentrations. Phosphorus plays a key role in the volcanic soils studied. Its fixation by the soil absorbtion complex is high and it is the primary limiting element for plant growth.

Grazing and burning results in grasslands with far less total above-ground biomass (1 kg m^{-2}) but the mass of living leaves and roots remains almost constant. This decrease in dead leaf mass of the grazed tussocks corresponds to a lower productivity. The decomposition rate of litter is low, but increases significantly in grazed conditions and after fire. In the first year after a monitored fire, there was a decrease in total above-ground vegetation mass due to enhanced decomposition of the remaining dead material and the lack of quick regeneration of green material. Grazing and burning has significant effects on soil physical characteristics, but nutrient concentrations remain low among differently managed sites. The extra amount of mineralized nutrients deposited during a fire or liberated by increased decomposition after a fire or after grazing is immediately immobilized and has no effect on nutrient concentrations nor on productivity. Overall palatability of the vegetation was higher at grazed and burned sites but only because of the disappearance of dead material: a higher share of nutrients is allocated to living above-ground material. However, a higher share of nutrients still is allocated below ground (roots and soil): this is considered undesirable in the present limited circumstances where nutrients are immobilized to a large extent in the soil.

The management and conservation of the páramo ecosystem is difficult if the various functions of the páramo are to be respected. If plant diversity and water storage are to be protected, but the same production of beef and milk is to be achieved, grazing should be concentrated to some areas only. On these areas, which are preferably flat and have a good ground coverage, the production might be intensified by an input of organic and inorganic fertilizers (phosphorus). Other areas can remain ungrazed and left for recovery of the natural, tall vegetation structure with its high degree of endemism.

Rewetting of Desiccated Peat Soils: Changing Nutrient Limitation?

Iris van Duren. University of Groningen, Laboratory of Plant Ecology, PO Box 14, 9750 AA Haren, The Netherlands

Drainage and fertilization have led to an increase in soil fertility which resulted in the disappearance of many species-rich wet grasslands. Therefore, management practices in nature reserves are often aimed at reducing the nutrient availability, and rewetting of desiccated peat soils could be one of those measures. The type and extent of the nutrient limitation was studied in relation to rewetting.

Intact sods from both a well-developed and a desiccated *Calthion palustris* and a *Caricion nigrae* stand were grown on nutrient solutions. Biomass production, resulting from omitting N, P or K from the solution, was used as a measure for nutrient limitation. The sods taken in the desiccated fields were treated under both drained and rewetted circumstances, while the sods from the well-developed stands were grown only under wet conditions.

In all stands co-limitation of N and K was observed. Potassium limitation appeared stronger in the desiccated *Calthion palustris* stand. This could be due to leaching of this mobile nutrient in this community. In both the well-developed and desiccated *Caricion nigrae* stand N-limitation appeared stronger. Phosphate was hardly limiting plant growth in any treatment. This was confirmed by a fertilization experiment in the field.

It was concluded that rewetting does not seem to reduce biomass production or give a relevant change in nutrient limitation in both vegetation types. Long-term experiments could give more insight in the perspectives of restoring peat soil by rewetting. Probably other aspects, such as stress factors, have to be taken into account.

Secondary Succession on the Low Terrace of the Caqueta River (Amazonia Colombia): An Architectural Analysis

H.F.M. Vester. Dutch Canopy Foundation; Tropenbos Programme, Colombia; Hugo de Vries Laboratory, Kruislaan 318, 1098 SM, Amsterdam, The Netherlands

Research on the dynamics of long-living, species-rich systems like the tropical rain forest is hampered by the lack of time for long-term observation. Diffeent development stages of the same system are often studied in a chronosequence. Large samples or many replicates are often investigated to obtain a reliable picture of the development. In this research, the development of a secondary forest, regeneration after the use of the land in slash-and-burn agriculture is analysed from five transect studies. Instead of replications in a similar age group, tree architecture is used to understand development processes during succession, and to relate the observations from the studied transects.

The development of the secondary forest up to 35 years can be divided into three development phases, each characterized by the maximum crown extention of one or more species.

Phase 1 is reached at *c.* 4 years when *Vismia glaziovii* and *Miconia minutiflora* reach their maximum crown extension.

Phase 2 is reached at *c.* 8 years when *Vismia japurensis* and *Vismia macrophylla* reach their maximum crown extension.

Phase 3 is reched at *c.* 10 years when *Miconia poeppigii* reaches its maximum crown extention. This phase continued to determine forest development up to an age of 35 years.

Comparison with a transect made in old forest reveals that a secondary forest of 35 years is still very different from primary forest, although species richness and basal area may have recuperated considerably.

3000 Years Before Ooievaar: Vegetation Patterns and Dynamics in the Dutch Fluvial Area Near Leerdam

Pim de Klerk. Laboratory of Palaeobotany and Palynology, Utrecht University, Heidelberglaan 2, NL-3584 CS Utrecht, The Netherlands

Palynological investigation of cores in the Dutch fluvial clay district may give insight into pre-human vegetation patterns and dynamics, important as reference for nature development projects. In order to trace vegetation patterns, time-synchronous cores have to be studied. Since pollen deposition values depend on the distance to the pollen sources (i.e. vegetation units) the cores must be located at different distances from various geomorphological landscape elements, containing different vegetation units. In the surroundings of Leerdam (Central Netherlands) two cores, located in the residual channel and the flood-basin of the Schaik system (a former Rhine distributary), have been studied palynologically. Calibrated ^{14}C dates show that the time period studied is between approximately 3500 and 2000 years BP. The cores partly cover the same time period.

During river activity the vegetation in the flood-basin resembled a *Thelypterido–Phragmitetum*. An alder/willow carr developed after river activity ended. No clear succession is visible in the pollen diagrams, suggesting continuous paludification, due to rising groundwater levels. Grazing of wetland mammals in the flood-basin is indicated by a sample probably representing an excrement.

During river activity local vegetation succession in the residual channel started with reed marshes along the channel margins, with *Typha angustifolia* and occasional *Sparganium* in the deeper parts. After river activity ceased a *Bidentetea*-vegetation developed on clay on the lower parts of the levees, later succeeded by extension of reed marshes. Vegetation succession ended with an alder/willow carr. There are indications of the occurrence of a fire in the surroundings of the channel, temporary disturbing the vegetation.

Levee vegetation consisted of a deciduous forest, containing *Corylus, Fagus, Acer* and *Fraxinus. Pinus* probably was present as well. Other trees might have been present, though this is not clearly indicated in the pollen diagrams.

Perspectives of Nature Regeneration on Sandy Soils: The Case Study of the Dellebuursterheide

Marco Boeringa, Rudy van Diggelen, Julia Klooker, Renee Bekker and Jan Bakker. Laboratory of Plant Ecology, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands

Intensification of agricultural exploitation during the 20th century resulted in enormous losses of flora and fauna in The Netherlands. Nature reserves were established in an attempt to safeguard the endangered species but they were mostly too small to cope with the negative influences from outside. Moreover, the landscape had become highly fragmented and corridors between these reserves hardly existed. Recently, nature conservation has focused on nature regeneration in sites which were previously used under intensive agricultural exploitation. The treatment generally consists of a removal of the nutrient-rich topsoil up to 50 cm in order to create optimal conditions for low productive, species-rich plant communities.

The Dellebuursterheide is presented as a case where measures were carried out to enable the re-establishment of plant communites of semi-natural conditions which were present prior to agricultural intensification. The nature reserve is presently dominated by communities typical of acid, nutrient-poor conditions belonging to the *Nardo-Callunetea, Ericion tetralicis, Violion tetralicis* and *Junco molinion* assemblages.

Removal of the topsoil of a 25 ha agricultural field in conjunction with heathland was performed in the winter of 1992/1993. Research started in September 1993 and included measurements of the hydrology and soil chemistry and determining the seedbank. Ground water levels indicated that infiltration of rain water took place. The groundwater in the nature development area was slightly acid and contained some $CaSO_4$, while the water in the heathland area was very acid and contained hardly any minerals. The geology was the same in both areas and consisted of a sandy soil overlaying an impermeable boulder clay stratum, so $CaSO_4$ probably indicated former agricultural land use. Organic matter content was rather low (1–5%) after topsoil removal. Soil-pH (pH-H_2O) was 4·6–5·8 in the nature development area as compared to 3·4–4·9 in the heathland.

The optimal hydrological conditions of the above-mentioned plant communities were computed from data sets of reference areas. Results showed a preference of the *Violion caninae* and *Junco molinion* assemblages for slightly enriched $CaCO_3$-water while the *Nardo-Callunetea* and *Ericion tetralicis* assemblages preferred acid water. Optimal ground water level was lowest for *Nardo-Callunetea*, followed by *Violion caninae, Ericion tetralicis* and *Junco molinion*, which had the highest optimal water level.

Predictions of regeneration perspectives were made on the basis of the spatial distribution of the abiotic conditions within the nature development area and showed best perspectives for plants associated with *Nardo-Callunetea* and *Ericion tetralicis*. However, the predicted communities did not reappear in the first and second year after topsoil removal. Analysis of the buried seedbank showed that seeds of the characteristic species were no longer present. Instead, the seed bank was dominated by ruderal species like *Gnaphalium uliginosum, Rorippa sylvestris, Polygonum mite, Juncus bufonius* and *Poa pratensis*. It was concluded that seed dispersal is of utmost importance for re-establishment of the predicted plant communities.

The Vegetation in Agricultural South-Holland Between 1976 and 1993

A.J. den Held. Provincie Zuid-Holland, Dienst Ruimte en Groen, Prinses Beatrixlaan 15, 2595 AK Den Haag, The Netherlands

The province of South-Holland has a particular landscape and vegetation in connection with the flat and broad coastal zone along the North Sea. This zone is characterized by dunes, salt and brackish water and mud-flat areas, extensive polders with a peat or sea clay soil with grasslands, ditches and marshes, and freshwater tidal areas. Extensive ecosystem complexes like this are very rare internationally.

For purposes of provincial rural planning as well as nature conservancy and environment policies, the vegetation of the agricultural landscape in South-Holland has been investigated by means of French-Swiss School relevés. During the first investigation stage (1976–83) about 55 000 relevés have been made in grasslands, banks, ditches, dikes, etc. In 1984–91 another 25 000 relevés were added, including 12 000 in exactly the same locations as relevés from the first stage. (Anon. 1992, *Vegetatie van Zuid-Holland 1976–1991*. Volume 1 & 2. Provincie Zuid-Holland, Den Haag). Since 1993, a monitoring project has started existing of a representative sample of about 2000 relevé locations analysed every other year to allow a more prompt evaluation of nature

conservancy policy. All relevés are stored in a database, and special applications have been developed, e.g. an indicator system for trophic state, a system for the floristic value of vegetation based on provincial, national and international frequency (presence and abundance) and vulnerability of species, and a system for temporal comparisons.

In 1976–83 high floristic values (vegetations rich in species, with many more-or-less rare and vulnerable marsh and water plants) were still found in large parts of the agricultural landscape, especially in the numerous ditches and grassland banks in the polders with a peat soil, and in particular in zones distant from the farms. This in spite of the fact that the strong agricultural development between 1950 and 1970 had already seriously impoverished flora and vegetation of the agricultural landscape. Also, ditch vegetation in the deep reclaimed polders with a *katteklei* (acid marine clay) soil appeared to have an extraordinary vegetation with a mix of oligotrophic and eutrophic water plants. The floristic value of the most valuable ditch and bank vegetation in the agricultural areas equaled that of the vegetation of nature reserves such as peat marshes. In the southwestern island region, elements such as the dikes and salt grasslands and ditches had a large floristic value.

The investigation during 1984–91 showed that there was once more a severe decrease in the floristic value of the vegetation in the agricultural landscape. The average decrease was more than 30%, but for vegetations with high floristic values the decrease mounted up to 70%. Not only rare species have declined but also more common species, even plants such as *Nuphar lutea* and *Bellis perennis*. This deterioration also implied a spatial levelling of botanical diversity in the agricultural landscape. Nevertheless, relatively high floristic values still can be found in large parts of the grassland polders.

The most important cause of the impoverishment of vegetation and flora is the large increase in the amount of nutrients, mainly as a consequence of more intensive farming. Besides, the lowering of the water table plays an important part, resulting not only in stronger drainage but also in mineralization of the peat and a more intensive agricultural use.

Preliminary results of the monitoring indicate a continuing decline in the floristic value of the vegetation (of 25%) between 1987/91 and 1993.

Conservation and restoration of the *Diantho–Armerietum* pastures along the river Dinkel

P.W.F.M. Hommel[1], A.H. Prins[2] and H.P. Wolfert[1]. [1]DLO Winand Staring Centre for Integrated Land, Soil and Water Research, PO Box 125, 6700 AC Waginengen, The Netherlands; and [2]DLO Institute for Forestry and Nature Research, P.O. Box 23, 6700 AA Wageningen, The Netherlands

Over a distance of nearly 20 km, the Dinkel is still a highly sinuous meandering river. Occasionally, very high discharges cause large parts of the valley to be flooded. During the floodings, the land is exposed to erosion and sedimentation. Within the Dinkel valley, a rare type of grassland is found: the *Diantho–Armerietum* ('Dinkel pastures'). The community is characterized by a combination of species such as *Dianthus deltoides, Thymus pulegioides, Pimpinella saxifraga* and *Galium verum*.

The distribution and species composition of the *Diantho–Armerietum* pastures in the Dinkel; valley were studied in relation to water management and soil development. In nine small-sized study areas, vegetation, characteristic plant species, soil characteristics and altitude were mapped in detail. By means of a computer-aided overlay of maps (using ARC/INFO), site preferences of both species and communities were determined (Hommel, P.W.F.M., Dirkx, G.H.P., Prins, A.H., Wolfert, H.P. and Vrielink, J.G., 1994. Rapport 304. DLO-Staring Centrum, Wageningen). It was proved that *Diantho–Armerietum* is best developed on dry, nutrient-poor, sandy and relatively young soils. Occasional flooding is essential to the conservation and restoration of Dinkel pastures. The altitude above the bankful discharge water-level is of great importance. The lower zones are strongly influenced by the nutrient-rich surface water; the higher zones are vulnerable to soil acidification.

Maintaining the current flooding frequency is favourable to the conservation of exiting Dinkel pastures; a higher frequency favours the development of new ones. This is only possible in certain river reaches because the physiography determines the potential for nature restoration. The most promising sites are those in which sedimentation of sand is predominant, resulting in overbank formation, and those which are located directly downstream from erosion-dominated river reaches.

MEETING OF THE SECTION FOR PLANT SYSTEMATICS AND GEOGRAPHY ON 9 DECEMBER 1994

Taxonomy and Phylogeny of *Arctium* (Compositae): 6 or 600 species?

H. Duistermaat. Rijksherbarium/Hortus Botanicus-FLORON Foundation, University of Leiden, PO Box 9514, 2300 RA Leiden, The Netherlands

The genus *Arctium* L. (Compositae) was revised in 1950 by Arènes, who recognized 22 taxa. However, recent floras still do not agree on the delimitation of the species, and the occurrence of hybrids is frequently mentioned. Further systematic studies, including a revision of the species, and a phylogenetic analysis, seemed appropriate.

In a scatterdiagram it is shown that two species can be recognized in the *Arctium tomentosum*-complex, based on the length of the anthers and corolla lobes. Similarly, scatterdiagrams for numerous combinations of characters were made for the complex of *Arctium minus, A. pubens*, and *A. chaberti*. These diagrams did not show any correlation between independent characters. Although synonymy can only be proved by negative demonstration, these names are lumped. Six out of the 22 taxa of Arènes are maintained, all at species level. Hybrids are rather rare. A search for suitable outgroups suggested six *Arctium*-like species within the multiform genus *Cousinia*. Together with 14 other species they form the subgenus *Cynaroides*. *Cousinia* subg. *Hypacanthoides* contains 10 species, and *C.* subg. *Cousinia* is the largest genus with 570 species. A cladistic analysis of *Arctium* using Hennig86 was performed, also including the following species: the six *Arctium*-like species, and one species each from the remaining four sections of *C.* subg. *Cynaroides*; one species of *C.* subg. *Hypacanthoides*; nine species, all from different sections of *C.* subg. *Cousinia*. *Onopordum acanthium* was chosen as outgroup. From the strict consensus tree it is apparent that the genus *Arctium* is included within *Cousinia*. Inclusion of *Cousinia* in *Arctium* is not preferred, because the former is very incompletely known. *Arctium* forms a monophyletic group with *Cousinia* subg. *Cynaroides* with trichotomies at two crucial branch points. A final analysis will be necessary to resolve them, possibly making *Arctium* and the *Arctium*-like species a paraphyletic tail to the other four sections of subgenus *Cynaroides*. *Arctium* and the *Arctium*-like species are nevertheless treated as an utilitarian genus with 11 species. Inclusion of the four sections would blur the distinctiveness of the genus.

Bottegoa Chiov. Transferred to the Ptaeroxylaceae

R.W.J.M. van der Ham, P. Baas, M.E. Bakker, B.J. Van Heuven and R.K.W.M. Klaassen. Rijksherbarium/Hortus Botanicus, University of Leiden, PO Box 9514, 2300 RA Leiden, The Netherlands. F.D. Boesewinkel & F. Bouman. Hugo de Vries Laboratory, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands

Bottegoa is a monotypic genus occurring in Ethiopia, Kenya, and Somalia. The only species, *B. insignis*, was described and placed in the Sapindaceae by Chiovenda (1916. Resultati scientifici della missione Stefanini-Paoli nella Somalia italiana 1. Le collezione botaniche) on the basis of a single fruiting specimen. Recently, a flowering collection was mentioned by Vollesen (1989. In: I. Hedberg & S. Edwards, *Flora of Ethiopia* 3: 490–510), and sampled for a survey of Spaindaceae pollen. However, the pollen appeared to be unlike that of the Sapindaceae, showing instead great resemblance to the pollen of the Ptaeroxylaceae and members of the Rutaceae and the Simaroubaceae. Consequently, a multidisciplinary approach was used to elucidate the proper systematic position of *Bottegoa*. Clearly, the genus is very atypical for Sapindaceae. On account of macromorphological, leaf, wood and seed anatomical features it is more satisfactorily placed in the Ptaeroxylaceae. Important features are the presence of solitary oil cells in the leaflets, flowers, fruits and seeds (mesotesta), and extrafloral nectaries on the upper side of the leaflets. The small African-Malagasy family Ptaeroxylaceae (including *Bottegoa, Cedrelopsis*, and *Ptaeroxylon*) is more or less intermediate between the Rutaceae and the Simaroubaceae. The genus *Harrisonia*, another peculiar taxon with affinity to both families, might be its closest relative. Phytochemical (quassinoids, limonoids, ptaeroxylines) and molecular work (rbcL sequences) on the Ptaeroxylaceae might throw more light on the apparently very close relationship between the Simaroubaceae and the Rutaceae.

How Good is the Best Cladogram?

P.H. Hovenkamp. Rijksherbarium/Hortus Botanicus, University of Leiden, PO Box 9514, 2300 RA Leiden, The Netherlands

Datasets with high amounts of homoplasy can be analysed with a standard parsimony protocol, and

will then yield a cladogram which can appear to be well-supported. However, the reliance that can be placed on such cladograms is limited, as such cladograms turn out to be highly sensitive to small changes in the data. I analysed a dataset of 24 taxa × 34 characters and found the best cladogram to have a consistency index (CI) of *c.* 0·30. After 50 small changes in the dataset were analysed in a similar way, only 24 of the resulting cladograms (48%) were identical to or consistent with the original cladogram: 24 cladograms were substantially different, of which 22 (44%) showed differences in parts where the data were left unchanged. Similar effects were found when single taxa were removed from the dataset before reanalysis. Similar effects have been reported for datasets with CIs that are considerably higher, and appear to be absent only when the data are virtually without homoplasy (CI very nearly 1). These results raise the following questions. (i) Data-errors are nearly impossible to avoid in a first analysis. How can we iteratively improve a dataset if causes (data-errors) and effects (changes in a cladogram) are not clearly linked? (ii) Species may be absent from a study-set for several reasons (extinction, incomplete sampling, incorrect generic assignment). How can we be confident that a cladogram can be used as an indication of historical relationships if omission of a single species may distort the relationships of other species?

Botanical Research in Madagascar and the Mascarene Islands

F.I. van Nek. Department of Plant Taxonomy, Agricultural University, PO Box 8010, 6700 ED Wageningen, The Netherlands

While working on the taxonomical revision of the genus *Carissa* (Apocynaceae) and doing fieldwork in Madagascar, Mauritius and Reunion, some interesting facts about botany in that region came to my attention. Although Madagascar (594 180 km^2), with approximately 10 000 higher plant species, was visited relatively early in history (compared with continental Africa) by botanists and plant collectors, it still remains botanically poorly known. In particular, the remaining rain forest areas are threatened by the growing human population, but many (often small and remote) forest areas are still un(der)explored and not in jeopardy. Many areas are legally protected, but there is hardly any government control in these areas.

Many common plant species have been described from Madagascar with the epithet 'madagascariense' while being actually more common in continental Africa. Other species have been described from Mauritius with the epithet '*mauritiana*' while being common in Africa and/or Madagascar and rare, extinct or unknown in Mauritius. Many botanical specimens from the Mascarene Islands or Madagascar appeared not to be labelled properly (or the field data did not survive the long journey) and have been described from the wrong island.

During the last few years botanical field-research has improved under the guidance of The Missouri Botanical Garden. So-called para-taxonomists (local plant collectors) have been employed and Malagasy students are educated at the MSc and PhD level, in both Madagascar and the United States.

The *Flore de Madagascar et des Comores* is rather outdated and needs revision for many families. Some taxa with many (new) species are at present difficult to identify, e.g. the genera *Diospyros* (Ebenaceae, *c.* 100 species) and *Oncostemon* (Myrsinaceae, *c.* 100 species).

Mauritius (1865 km^2) and Reunion (2510 km^2), with about 800 species each, are floristically well-known. Many species however survive in low numbers, and protected areas are often very small. Exotic plant species cause an enormous threat to the native vegetation, especially in the Mascarene Islands.

The *Flore des Mascareignes* is rather complete but, unfortunately, many treatments have not been published yet due to financial problems with the flora project.

Species and Subspecific Entities in the Mainly Tropical Algal Genus *Caulerpa* (Ulvophyceae, Chlorophyta)

W.F. Prud'homme van Reine. Rijksherbarium/Hortus Botanicus, University of Leiden, PO Box 9514, 2300 RA Leiden, The Netherlands

The genus *Caulerpa*, together with its monotypic sister genus *Caulerpella*, forms the family Caulerpaceae in the Ulvophyceae. The two genera differ in reproduction, which is holocarpic in *Caulerpa* but not in *Caulerpella*, where reproductive structures are separated from the rest of the coenocytic thalli by special transverse walls.

Most species of *Caulerpa* have a thick and strong creeping stolon, downward growing rhizoids and upward growing erect parts. These erect parts can be branched or unbranched, and their form is very diverse and often also very variable within the species. Although the most recent monograph of this genus was published long ago (Weber-van Bosse, 1898, *Ann. Jard. Buitenzorg* **4**: 243–401), that classification is still used in the most recent list of all 73 accepted species and 110 subspecific taxa (Calvert, 1974. *Comparative plastid fine structure in the genus*

Caulerpa *Lamouroux, with special reference to phylogenetic relationships*. PhD thesis, University of South Florida). Several authors have shown that occasionally erect parts growing from a common stolon may have morphologies that are characteristic of separate subspecific taxa. This was also observed in plants growing in aquaria. Peterson (1972. *J. Agardh. Micronesica* **8**: 63–86) had already suggested use of the disignation 'ecological phenotype' for some of these growth forms and that suggestion is now often followed in *Caulerpa* studies (Coppejans & Prud'homme van Reine, 1992, *Meded. Zitt. K. Acad. overzeese Wet.* **37**: 667–712). These ecological phenotypes or ecads differ from ecotypes because ecads indicate phenotypic flexibility and morphological plasticity while ecotypes indicate genetic fixity. Advantages and disadvantages of the use of the ecad entity were discussed and suggestions were given on a potential use of *Caulerpa* plants as indicators for a degree of biodiversity in warmer coastal waters.

Biogeography and Phylogeny of *Begonia* in Relation to Glacial Forest Refuges in Africa

M.S.M. Sosef. Department of Plant Taxonomy, Herbarium Vadense, Wageningen Agricultural University, PO Box 8010, Wageningen, The Netherlands

In tropical Africa the area of lowland rain forest has shrunk considerably during the last Glacial period (± 70 000–12 000 years BP) and ultimately disintegrated into a number of comparatively small refuge areas as a result of the drier and cooler climate. It is plausible that the former refuge areas have retained a high degree of biodiversity for forest organisms, hence knowledge about their exact locations is highly interesting in the light of nature conservation. The 40 species of *Begonia* sect. *Loasibegonia* and *Scutobegonia* may be regarded as bioindicators of former rain forest refuges. The falling apart of the rain forest area, which must have resulted in the isolation of populations of individual species, may have led to vicariance events. To find out whether this is correct, a thorough phylogenetic analysis followed by a cladistical historical biogeographic analysis of the *Begonia* species concerned was performed. One hundred and thirty-two characters formed the matrix for the phylogenetic analysis, which was analysed with the computer program Henning86. A straightforward analysis showed a weak and unstable cladogram structure. Via a method of a posteriori weighting and ordering of characters, seven stable monophyletic groups of species could be defined. The position of five species remained uncertain, possibly due to the hybrid origin of several of them. The seven monophyletic groups were used to perform the historical biogeographical analysis using Brooks' Parsimony Analysis. Only very few vicariance events were shown in the results. The relationships between the areas seemed to be more of floristic nature rather than reflecting a common history (Sosef, M.S.M., 1994, *Wagen. Agric. Univ. Papers* **94–1**: 1–306).

Sapindaceae and the Biogeography of East Australia and Adjacent Areas

Hubert Turner. Rijksherbarium/Hortus Botanicus, University of Leiden, PO Box 9514, 2300 RA Leiden, The Netherlands

The biogeographic relations of East Australia and surrounding areas were analysed using seven genera of Sapindaceae. In total, 168 species were included; the region was split into 25 areas of endemism for this study. Because the initial analyses were rather uninformative, the areagrams for the individual genera were inspected in detail. It was found that both the western Pacific areas and the East Australian ones showed two different patterns; these areas were split into two occurrences. Re-analysing the data with Brooks' Parsimony Analysis, coding 'missing taxa' as unknown data, gave 1941 different areagrams (l=461, ci=0.70, ri=0.76).

Despite the large number of trees, the strict consensus areagram showed considerable resolution: (((New Caledonia, Loyalty Islands), (West Pacific areas)), ((East Australia), ((West Pacific areas+New Britain+Central Papuan Mountains), (remaining New Guinean areas+West Malesia+East Australia)))). South New Guinea is remarkable in that it is one of the last areas to split off, as sister area to two secondary occurrences of East Australian areas.

Thus, the biogeographic history of at least the Sapindaceous component of the biota of this region is found to be dominated by a number of major events. (1) An origin in the rain forests of the Australian/Papuan plate, with an initial vicariance when New Caledonia split off *c.* 60–80 Ma (or a dispersal when it was still close to the eastern edge of the Australian plate). (2) Extension of the range over northern New Guinea as parts of the Outer Melanesian Arc (OMA) accreted. (3) Vicariance between East Australia and New Guinea. (4) Dispersal from New Guinea eastward over the OMA, concomitantly with dispersal from New Caledonia into the OMA. (5) Dispersal from New Guinea westward into West Malesia. (6) A recent re-invasion of East Australia from southern New Guinea, probably during a Pleistocene period of sea level lowering. The relative timing of these events is still speculative, in particular the order of events (2)–(5).

Fern Spores in Systematics and Evolution

Gerda A. van Uffelen. Rijksherbarium/Hortus Botanicus, University of Leiden, PO Box 9514, 2300 RA Leiden, The Netherlands

Land plant spores have been found from the Silurian onwards. They have to perform three main functions: protection of the protoplast and genetic material during storage and transport; transport itself; germination in the right conditions.

Many spore characters can be explained in functional terms, and will therefore show homoplasy (parallellisms and convergence). However, some spore characters have remained remarkably constant during the evolutionary process. Examples of such widespread traits are: the tripartite lamellae on which the exine/exospore of most hepatics, vascular cryptogams, and a number of seed plants is deposited; the fact that in all fern spores the different sporoderm layers are deposited centrifugally; characters of the exospore and its formation, which may be remarkably constant in one group.

Stabilizing forces play an important role in evolution: a change usually is not an improvement in a system once it works well. Even in adaptive traits, a genetic base needs to be present for such a trait to develop: the repeated occurrence of a similar adaptation within a group indicates the presence of such a genetic basis common to group members. On the other hand, one and the same set of genetic data may lead to dramatically different spores, e.g. in heterosporous species.

Variability may also result from an environment that does not require many adaptations: the idea of 'patio ludens' in favourable circumstances.

Convergence may be caused by several factors: for one functional demand, only one or a few solutions work well; a trait has to submit to the laws stated by size, surface, and available material; developmental processes are subject to strict laws of, e.g. condensation, which may explain the similarity of many surface patterns found in pollen and spores.

There are many instances of characters that originated several times. Although they occur in more or less distantly related groups, they may be characteristic for quite large taxonomic groups: heterospory (Marsileales, Salviniales, lycophytes, seed plants); an amoeboid tapetum (psilotophytes, equisetophytes, and polypodiophytes, and in some angiosperm families); monolete spores may characterize whole fern families, but they may also occur in one genus with predominantly trilete spores.

