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Methods and Apparatus for Liquid Media and Semi-automated Micropropagation

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Meristematic or bud clusters of *Syngonium*, *Spathiphyllum*, potato and banana were multiplied on interfacial membrane rafts and in plastic film air lift bioreactors. The clusters were separated by a mechanical cutting device and subcultured either to multiplication or to a growth medium on an interfacial membrane raft. For *Syngonium*, the clusters were multiplied in bioreactors equipped with a filter device to control contamination during proliferation. For *Syngonium*, *Spathiphyllum* and banana, plantlets were regenerated either on agar or on membrane rafts. For potato, microtubers were produced in tuber induction medium. Details on the rates of multiplication and growth will be presented for the various crops.

Phase Change in *Acacia mangium* Willd: A Model System

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The process of phase change from juvenile to adult involves changes in morphology, growth patterns and organogenic ability, and finally culminates in flowering. For many woody plants much effort has been put in reversing phase change by various horticultural techniques, especially by *in vitro* culture. However, these attempts to improve vegetative propagation were invariably hampered by the superficial basic knowledge about this aspect of plant development, and often resulted in what is called '(partly) rejuvenated' offspring. This research was set up to study woody plants during phase change with special attention for the shoot apical meristems, because of their important role in the control of plant development.

Acacia mangium was selected as a model plant, because of its distinct change in leaf morphology: bipinnate in the juvenile phase and phyllodia in the adult phase. This first sign of phase change takes place already 10–12 weeks after sowing and proved to be accompanied by changes in primary stem shape, branching pattern and type of epidermal hairs. Phase change could be reversed by isolation of nodal explants *in vitro*, in this case not by time-consuming repeated subcultures, but by applying cytokinin in the initial culture. At low cytokinin concentrations axillary buds of adult origin developed into adult shoots, whereas higher concentrations of cytokinin induced juvenile development. Decreasing the sugar concentration stimulated phase change reversion induced by cytokinin. In this way, *Acacia mangium* offers a good opportunity to study and compare the processes of *in vitro* culture-induced phase change reversion with phase change during normal development.

Direct Regeneration from Leaf Explants of Five Glasshouse-grown Cut Rose Cultivars

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Our study reports on the direct regeneration of adventitious buds taken from five glasshouse-grown cut rose cultivars. After surface-sterilization young leaves were separated from shoot tips and divided into six types of explants. Explants were inoculated on half-strength Murashige-Skoog medium, supplemented with vitamins, thidiazuron, naphthaleneacetic acid and silver nitrate. Adventitious buds emerged 14–17 days after inoculation. Highest percentage of regeneration occurred at the base of leaflets (70–100%) or at the base of leaf sheaths (40–100%). Silver nitrate significantly promoted the percentage of regenerating explants (91 vs. 62%) and reduced the time to adventitious bud emergence (14·3 vs. 17·1 days).

Mannitol Metabolism in Salt Stressed *Agaricus bisporus*

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Cultivation, trade and processing of edible mushrooms are important economic activities throughout Europe. A better understanding of the molecular and biochemical processes regulating the morphogenetic quality and production of mushrooms is desirable for strain improvement. A transformation protocol is available for *Agaricus bisporus* which allows further study of genes involved in physiological processes such as mannitol metabolism.

Mannitol, a six-carbon sugar alcohol, is the main storage carbohydrate in the edible mushroom *A. bisporus*. Mannitol can contribute up to 20% of the mycelium dry weight and up to 40% of the fruit body dry weight and may play a critical role in regulating growth, development, quality, and osmotolerance of *A. bisporus*. A better understanding of the function and regulation of mannitol metabolism in mushrooms may lead to improved production and (post-harvest) quality. Several strains of *A. bisporus* were grown under normal and salt stressed conditions. Fruit bodies were produced on casing soil that was saturated with either 0 (control) or 150 mM NaCl. Irrigation of casing soil during mycelial development was also conducted with either 0 (control) or 150 mM NaCl. Fruit bodies were harvested at seven developmental stages ranging from buttons to fully mature fruit bodies and were separated into pileus, stipe and gill tissue. The mannitol synthesizing enzyme, NADP-dependent mannitol dehydrogenase (NADP-MtDH), showed highest activity (expressed on a fresh weight basis) early in development, followed by decreased activity during further development. The specific activity of NADP-MtDH decreased only slightly during development. Salt stress resulted in an increased activity of NADP-MtDH throughout all stages of development. Pileus, stipe and gill tissues did not differ greatly in NADP-MTDH activity. Further implications of salt stress on mannitol biosynthesis will be discussed.

Agrobacterium-mediated Transformation of the Apple Cultivars Gala, Golden Delicious and Elstar at CPRO-DLO

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Leaf segments of the commercial apple cultivars Gala, Golden Delicious and Elstar were co-cultivated with a supervirulent *Agrobacterium* strain, containing the *nptII* and *gus* genes. Putative transgenic callus clumps were excised from the leaf explants and GUS-positive shoots were induced on calli which proliferated on kanamycin-containing medium. Southern blot analysis confirmed the integration of both genes with one or two copies per genome being present. The transformation frequencies, based on the number of GUS-positive shoots per leaf explant, were 0·7–8% for Gala, 0·2–6% for Golden Delicious and 0·4–0·8% for Elstar. Some of the plants were successfully transferred to the greenhouse.

Hormonal Regulation of Apical Dominance in Rose *in vitro*

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Apical dominance is the inhibition of outgrowth of axillary buds by an actively growing apex. To study the effects of different plant hormones on apical dominance *in vitro*, single-nodes (stem segments with an axillary bud excised from microcuttings) of the cut rose 'Madelon' were cultured in Petri dishes on media with different types and concentrations of hormones.

Positional effects were evident. Single-nodes from positions close to the apex, sprouted faster and to a higher percentage than single-nodes from positions further down the stem. Outgrowth was inhibited by darkness and/or adding auxin to the medium. Indole-3-butyric acid (IBA) was the most effective auxin: 1 μM strongly inhibited sprouting. After isolation of single-nodes, the inhibition was only maintained if IBA was present between 24–96 hours after cutting the single nodes. No inhibitory effect was observed when IBA was added later than 96 hours after excision.

Simultaneous addition of a cytokinin reduced the inhibitory effect of auxin. The phenylurea-analogue 4-PU acted faster than the synthetic cytokinin 6-benzylaminopurine (BAP) but not stronger: the final sprouting percentage was the same.

BAP-stimulated outgrowth was inhibited by abscisic acid (ABA), and IBA-induced inhibition was reversed by simultaneous addition of fluridone, an inhibitor of ABA-synthesis. This suggests involvement of ABA in apical dominance. However, gibberellic acid nullified the effect of ABA and had no effect on IBA-imposed inhibition, suggesting different working mechanisms for IBA and ABA, respectively.

In histochemical observations, a strong increase in carboxylesterase activity in the axillary bud was observed 48 hours after addition of 8·8 μM BAP to single-nodes. In single-nodes cultured on hormone-free medium this increase started approximately 96 hours after excision. On IBA-containing medium this increase did not occur. Initiation of carboxylesterase activity is often associated with the onset of vascular differentiation (Rana, M.A. *et al.* (1983): *Cell Biochem. Function* **1**: 109–111), which in turn is associated with polar auxin movement (Sachs, T. (1986): *Plant Growth Substances 1985*, Springer-Verlag, Berlin). This suggests that cytokinin can stimulate the export of auxin from the bud.

Regulation of Senescence in *Alstroemeria* Leaves

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Leaves of the cut flower *Alstroemeria pelegrina* L. exhibit rapid senescence in the dark (i.e. loss of photosynthetic activity, decrease in chlorophyll and protein content). Senescence can be studied both in leaves of cut flowering stems and in a model system consisting of detached leaf tips. The characteristics of the senescence process in both systems were identical (Jordi, W. *et al.* (1993): *Physiol. Plant.* **87**: 426–432).

Senescence was strongly delayed by exogenous gibberellins (GAs), cytokinins (CKs) and red light acting through phytochrome. A comparison of various chemically pure GAs and CKs demonstrated large differences in the ability to delay senescence. Immunoassays demonstrated that GA-induced retardation of leaf yellowing does not involve an increase in the endogenous CK concentrations in the leaves as an intermediate step (Jordi, W. *et al.* (1994): *J. Plant Growth Regul.* **14**: 121–127). Based on studies with GA-synthesis inhibitors, it has been suggested in literature that red light stimulates GA-synthesis. However, our results demonstrate that the endogenous GA-levels quantified by GCMS do not change after red light illumination, indicating that the action of red light is *not* mediated by enhanced GA-levels in leaves.

Tissue Culture under Controlled Atmosphere Conditions

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A large body of literature is currently available, describing, in great detail, the culture medium ingredients necessary for successful micropropagation of a variety of plant species. However, the protocols developed in one laboratory may sometimes fail to give satisfactory results when applied in other laboratories. This may, in part, be due to the lack of definition of the composition of the gaseous atmosphere inside the vessel. Build-up or depletion of carbon dioxide, accumulation of ethylene and high relative humidities in the vessel may affect growth characteristics of plants. The composition of the vessel atmosphere is determined mainly by the physical properties of the culture container (aeration). In addition, the metabolic activity of the plant material and the features of the macro-climate (temperature, light, air velocity) may, in interaction with each other, create a given micro-climate. The interaction of the continuously changing vessel atmosphere with growth and development of plants is hard to study using conventional tissue culture containers. We developed a flow-through system to treat cultures with different air mixtures. This allows us to investigate the effects of different gaseous components on growth and development of *in vitro* cultured plants. In addition the effects of other parameters (e.g. the light level) can be investigated without affecting the composition vessel atmosphere. Results on the effects of different carbon dioxide, ethylene, relative humidity and light levels on physiological and morphological characteristics in different ornamental plant species have been discussed.

Localization of r-RNA during Microspore Embryogenesis in *Brassica napus*

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Nuclear changes are studied during plant embryogenesis and may be used to monitor this developmental process. As a model system, microspores of *Brassica napus* are used which change their developmental pathway from gametogenesis to embryogenesis if cultured under embryogenic conditions (heat shock, at least 8 hours, 32°C).

Both by light and by electron microscopy, we try to relate the patterns of synthesis and transport of both r-RNA and m-RNA to different cell cycle or embryogenic stages. Also, the localization of nuclear proteins which are involved in m-RNA splicing is examined, using antibodies against snRNPs.

Some first results have been presented which were mainly obtained by *in situ* hybridization of various embryogenic stages with a ribosomal 18s RNA probe.

Acknowledgements: the 18s rDNA fragment of *Pisum sativum* was kindly provided by Dr G. McFadden (Melbourne University) and was originally cloned by Dr W.F. Thompson (North Carolina State University).

Postharvest Morphogenesis of *Agaricus bisporus:* Influence of Plant Hormones

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Morphogenesis of common mushroom (*Agaricus bisporus*) sporophores does not cease after harvest. Harvested sporophores, although inhibited in further development, continue to grow at the expense of endogenous storage carbohydrates (mannitol). The opening of the cap is the most important morphological quality parameter and initiates a cascade of senescence events. In the past, evidence has been obtained for the existence of a hormone-based regulation of morphogenesis in developing sporophores. Also, the presence of plant-hormone analogues of auxins and cytokinins has been demonstrated in agaricales. The specific function of these substances in fungi is yet unknown.

Experiments were performed applying auxins and cytokinins and combinations of these hormones to harvested sporophores of *A. bisporus*. Hormone-treated sporophores showed retardation of cap-opening compared to water-treated controls. Furthermore, the extent of cap opening was reduced in hormone-treated sporophores.

Molecular Biology of Common Mushroom *Agaricus bisporus*

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Some major problems strongly affect mushroom production and quality that cannot yet be properly solved by conventional breeding due to the aberrant life-cycle of *Agaricus bisporus*, in which each basidium contains two spores (bisporic) each with two parental nuclei.

ATO-DLO was the first research institute to develop a transformation system for the common mushroom, now resulting in a maximum number of about 100 transformants per integral transformation experiment. Donor DNA is introduced utilizing electroporation of mycelial protoplasts. A dominant selection system is employed based on hygromycin B antibiotic resistance (*hpt*-gene). Southern blot analysis unequivocally shows the presence of donor DNA sequences integrated into the genomic DNA of both a homokaryotic non-commercial strain and a commercial heterokaryotic strain.

By sequential propagation of inocula from the edges of colonies, transformants of the non-commercial strain showed mitotic stability for at least 15 generations. The system has also yielded transgenic fruit bodies, still containing the donor DNA.

Using strong, homologous *A. bisporus AbGPD2*-promoter and terminator sequences around the *hpt*-gene instead of the original heterologous sequences has resulted in a slightly increased transformation efficiency. In addition, highly efficient homologous integration has been accomplished (50–60%) using a genomic *A. bisporus* fragment containing two tandem exo-β1,3-glucanase genes. The techniques to be further developed involve co-transformation, antisense RNA, gene targeting and gene disruption procedures.

Current activities also aim at further improving the transformation system and finding scientific and commercial applications, aiming at the study of browning phenomena induced by *Pseudomonas tolaasii* infection, by mechanical injury and by senescence, and of mannitol metabolism and morphogenetic aspects (details are presented in the next abstract).

Molecular Approaches against Mushroom Browning

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Browning is an important aspect of the quality loss of common mushroom (*Agaricus bisporus*), and is caused by mechanical injury, *Pseudomonas tolaasii* infection and by senescence. Brown discolouration has been associated with the transition of latent to active tyrosinase, possibly mediated by serine proteinase. Tyrosinase catalyses the oxidation of phenolic substrates mainly L-tyrosine, p-aminophenol (pAP) and γ-glutamylhydroxy benzene (GHB) to melanins.

Mushroom tyrosinase has been isolated before and was characterized as a monomeric protein of 43 kDa under denaturing conditions and of 47 kDa under native conditions.

Work on tyrosinase activity, phenolic substrate content or protease activity was carried out to clarify which of these parameters has the highest correlation with the colour and discolouration of mushrooms. Preliminary statistical analyses show that tyrosinase activity significantly contributes to the browning reaction.

Two partial tyrosinase cDNA sequences (600 bp) from *A. bisporus* encompassing the CuA and CuB binding domains of the enzyme have been isolated by PCR. The fragments (*Abtyr1* and *Abtyr2*) have been included in transformation constructs to attempt gene disruption or antisense inhibition. Transformation is based on hygromycin B resistance (*E.coli hpt* gene) driven by the *A. bisporus gpd2*-promoter.

More than 100 putative co-transformants have been produced and are being analysed at the level of the homokaryotic transgenic mycelium, heterokaryotic mycelium (after mating) and fruit bodies. Results have been presented on the initial analyses of the transformants.

Highly Efficient Homologous Integration via Tandem Exo-β-1,3-glucanase Genes in Common Mushroom, *Agaricus bisporus*

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Recently, a transformation system was developed for common mushroom, *Agaricus bisporus*. Plasmids carrying the hygromycin resistance gene controlled by regulatory sequences from either *Aspergillus nidulans* (pAN7-1) or *A. bisporus* were introduced into *Agaricus* protoplasts by electroporation. Approximately 1–100 transformants arose from $0{\cdot}2–1.10^8$ protoplasts using 10 μg linearized DNA. Linearization of plasmid DNA increased the number of transformants 2–5-fold. A random 3 kb genomic fragment from *A. bisporus* was cloned into plasmid pAN7-1, downstream of the *A. nidulans* terminator. The resulting plasmid pHAG3-1 was linearized within this homologous region (called AbGH3) before transformation. Transformation frequency was not enhanced by this plasmid, although it was found that homologous integration occurred in about 50% of the transformants. Homologous integration was found with pHAG3-1 linearized at three different positions within the AbGH3 sequence generating either blunt, 5′- or 3′-overhanging ends. Tandem integrations were also observed at the homologous position with restoration of the restriction sites used for linearization.

The AbGH3 fragment was found to contain two open reading frames in tandem, which showed 60% similarity to exo-β-1,3-glucanases from *Saccharomyces cerevisiae* and *Candida albicans*. The function of these enzymes is unknown, but they are thought to be involved in cell wall metabolism. The upstream gene (*AbEXG1*) encodes a polypeptide of 419 amino acids, whereas only the start of the second gene (*AbEXG2*) is present on the genomic fragment, encoding the N-terminal 159 amino acids. Both polypeptides contain a predicted signal peptide region. The genes are interrupted by numerous short introns at conserved positions. Expression at the mRNA level is low in vegetative mycelium and relatively high in fruit bodies. Exoglucanase mRNA was increased in vegetative mycelium of a transformant with tandemly integrated pHAG3-1 plasmids at the homologous position. Fruit bodies of this transformant are currently grown to study the effect of exoglucanase overexpression.

In Situ Histone H4 Mrna Detection in Shoot Apical Meristems of Juvenile and Adult *Acacia mangium* Willd

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In *Acacia mangium* a clear shift in leaf morphology is associated with the phase change process. Phase changes are thought to be initiated in the shoot apical meristems. Therefore, we hypothesized that patterns of meristematic activity in shoot apical meristems of juvenile plants may differ from those of adult plants. We decided to follow meristematic activity by detection of histone H4 gene activity, an approach also used to detect shoot apex changes in tomato (Brandstädter *et al.* (1994): *Planta* **192**: 69–74). *In situ* hybridizations on sections of paraformaldehyde-fixed and paraffin-embedded shoot tips of 3-week-old seedlings and 5-year-old adult plants were made, followed by detection of DIG-labelled H4 sense and antisense probes by anti-DIG-AP (Alkaline Phosphatase). Our first results show that histone H4 mRNA could be detected in shoot tips of both juvenile and adult plants and colocalized with the expected sites of cell division. In comparison with juvenile, more signal was detected in adult meristems, possibly correlated with their larger size. Obvious differences in H4-gene expression were present in the developing leaves of shoot tips in the juvenile and adult phase. However, in the shoot apical meristems a clear phase change correlated shift in H4-gene expression was not observed.

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Toxicity of Ethanol during Proliferation and Adventitious Root Formation in Apple Shoots *in vitro*

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Ethanol is a convenient solvent for plant growth substances. Usually, the final concentration in tissue culture media is very low. The effect of ethanol has only occasionally been studied. It has been reported that a concentration as low as 0·003% is inhibitory in somatic embryogenesis of *Daucus*. We have examined the toxicity of ethanol in tissue culture of the apple rootstock 'Jork 9'. During proliferation through axillary branching, 0·2% (v/v) ethanol slightly stimulated proliferation whereas significant inhibition occurred at concentrations of 0·4% or higher. In adventitious root formation, significant inhibition occurred at concentrations of 0·1% or higher. The effect of ethanol was stage-dependent: during the induction period (i.e. from 24 to 72 h after the start of the rooting treatment), inhibition was less. During autoclaving, ethanol evaporated to *c.* 50%.

Salicylic Acid Affects Rooting of Apple by Enhancing Auxin Oxidation

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Monophenols are believed to be cofactors of auxin-oxidase. We have examined whether the monophenol salicylic acid (SA) acts accordingly in rooting. We used a test system consisting of 1-mm stem slices cut from microcuttings of the apple rootstock 'Jork 9' (Van Der Krieken *et al.* (1993): *Plant Cell Rep.* **12**: 203–206; De Klerk, G.J. *et al.* (1995): *J. Exp. Bot.* **46**: 964–972). The auxins added in the experiments were indoleacetic acid (IAA) or naphthaleneacetic acid (NAA). In plant tissues, IAA is oxidized and conjugated, whereas NAA is only conjugated. Thus, if SA acts by stimulating auxin-oxidation, it should reverse the effect of IAA more than the effect of NAA. It should be noted that SA may also enhance the oxidation of IAA synthesized by the plant tissue itself. In addition, in literature other effects of SA have been reported.

SA inhibited IAA-induced rooting more than NAA-induced rooting. SA reversed the inhibition of outgrowth of root meristems by IAA, but hardly affected that by NAA. The dose–response curve of IAA added for only 5 days to induce rooting was shifted to the right by SA. Finally, during the first 5 days, SA had most effect during the auxin-sensitive phase in rooting (cf. De Klerk, G.J. *et al.* (1995): *J. Exp. Bot.* **46**: 964–972). Together, these data indicate that SA enhanced the oxidation of IAA.

Induction of Direct and Indirect Somatic Embryogenesis in *Dactylis glomerata* Leaf Explants: Cell Cycle Kinetic Studies

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Developing leaves of gramineous species possess spatial and temporal gradients of embryogenic competence. *In vitro* culture of leaf explants from a highly embryogenic genotype of *Dactylis glomerata* L. (orchardgrass) provides a reliable system for producing somatic embryos directly (without an intervening callus phase) and indirectly. Indirect embryogenesis occurs from the basal leaf parts and direct embryogenesis from middle ones. The duration of exposure to auxin is the second crucial factor.

We studied auxin-induced changes in cell cycle kinetics during the earliest developmental stages: acquisition of embryogenic competence and induction of both types of somatic embryogenesis. Reduction of mitotic activity and accumulation of cells in G_1-phase characterized *in vivo* development of orchardgrass leaves. The basal leaf segments contained mainly meristematic cells which were in different cell cycle compartments. A significant increase in mitotic index was established: from 8–10% in the initial explants to 16–18% after 1 week of culture. Although all cells in the more differentiated middle leaf segments were arrested in G_1/G_0-phase, about 25% of them re-entered the mitotic cycle 10 days after culture initiation. Most probably, a part of these cells were committed to direct embryo formation. Data on changes in the relative distribution of leaf cells into interphase compartments and their mitotic activity during the induction of somatic embryogenesis clearly demonstrate a relationship between cell cycle kinetics and the gradient of the explant embryogenic response in *D. glomerata*.

Antioxidant Enzyme Activity during the Induction of Somatic Embryogenesis in *Dactylis glomerata* L.

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The formation of active oxygen species, such as singlet oxygen ($^1O_2$), superoxide (O_2^-) and hydrogen peroxide (H_2O_2) is a normal event in cell metabolism. A regulated balance between oxygen

radical production and destruction is required if metabolic efficiency and function are to be maintained in both optimal and stress conditions. We determined the changes in activity of peroxidase, including IAA-oxidase (E.C. 1.11.1.7) and catalase (E.C. 1.11.1.6) during the induction of somatic embryogenesis in leaf explants of a highly embryogenic genotype of *Dactylis glomerata* L. (orchardgrass).

The more mature leaf segments (committed to direct somatic embryo formation) exhibited a 2-fold higher catalase activity per mg protein than the younger ones (that produce *in vitro* callus and then embryos). In contrast, auxin-oxidase had a higher activity in the younger leaf parts. The results indicate a strong correlation between antioxidant enzyme activity and the established spatial and temporal gradient of the embryogenic competence of the explants. Some reduction in catalase and slight changes in relative IAA-oxidase activity were detected 5 and 10 days after culture initiation—the shortest periods for induction of indirect and direct somatic embryogenesis, respectively. The significant increase of total peroxidase during the same periods was due rather to *de novo* synthesis of the enzyme than to activation and was also related with auxin-induced stimulation of cell proliferation ability. The data suggest that H_2O_2 formed in both developing orchardgrass leaves and explants during the earlier stages of cell dedifferentiation and induction of somatic embryogenesis are scavenged in different ways.

Increase of Embryogenic Callus Formation in Cucumber by Initial Culture on High Concentration of 2,4-Dichlorophenoxyacetic Acid

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Somatic embryogenesis from cell suspensions is a cheap and efficient method for plant propagation. However, the induction of embryogenic callus often fails. Because embryogenesis, just as other regeneration processes, consists of several phases each with its specific hormonal requirements, we decided to examine the effect of various types and concentrations of auxins applied for various periods of time on embryogenic callus formation.

Leaf segments of cucumber (*Cucumis sativus* L.) cvs. Profito and Cordito were cultured either continuously on standard medium containing 4·5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4·4 μM benzylaminopurine, or first cultured for various periods at different levels of 2,4-D, picloram or naphthalene-acetic acid (NAA), and then transferred to standard medium. The percentages of original explants forming embryogenic callus were scored 30 weeks after the start of tissue culture.

When cultured continuously on standard medium, less than 10% of the explants formed embryogenic callus. Initial culture on media with picloram or NAA, or with 2,4-D at a low concentration (1·4 μM) did not result in formation of embryogenic callus. Embryogenic callus formation increased to 40% if during the initial phase of the culture (1·5 week), the 2,4-D concentration was raised to 14 or 45 μM, but decreased after a longer period of culture. Similar results were obtained in both cultivars.

Propagation of Tulip *in vitro*

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For propagation of tulip *in vitro* a protocol is available that is used by tissue culture companies. According to this protocol, thin slices (1 mm) of young flowerstems are used. Shoots that regenerate on these slices are used for further propagation. In these shoots, bulb formation is induced by a cold treatment. Growth of the bulblets takes place at high temperatures. This protocol is far from optimal. Our research has focussed on the initiation phase.

On the thin slices, shoots and leafy structures regenerate. This is undesirable since leafy structures do not have a meristem and thus cannot be used for axillary propagation. The percentage of shoots can be as low as 5–10%. Moreover, the leafy structures cannot be distinguished from shoots. With addition of paclobutrazol (PP333) or methyl-jasmonate (MeJa) the percentage of meristems was increased. Further research is needed since bulblets were formed instead of shoots and it might be that these bulblets require special treatment for subculturing. Silverthiosulphate (STS, an inhibitor of ethylene action) also increased the percentage of shoots, but less than PP333 or MeJa.

For propagation of shoots, they were either cut longitudinally or in thin slices. So far, no definite conclusion about the best method can be given.

Bulblets grew well after planting in soil. When bulblets were grown under artificial light, their weight doubled in the first growing season. In the second growth cycle, weight increased 2·5 to 4·5 times. It was possible to have two growing seasons in 14 months.

Improved Protocol for the Propagation of *Narcissus in vitro*

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Narcissus is propagated *in vitro* using 'twin-scales' (two longitudinal segments cut from adjacent scales, connected by the basal plate) as initial explant. Shoot clusters or bulblets regenerating on these twin-scales were used for further propagation. When shoot clusters were subdivided, the propagation rate decreased after some cycles, possibly because the basal plate, from which the shoots originate, lost the capacity to regenerate. The propagation rate was improved by an intermediate bulb formation phase. For this, shoots were cultured separately on bulbing medium. From the bulblets, 'mini twin-scales' were cut: depending on the size, two to four twin-scales per bulblet. On every twin-scale two to three new shoots regenerated. The procedure of mini twin-scales can also be used for bulblets regenerated on the original explant. The propagation rate of longitudinally cut bulblets was very low. After propagation, bulblets were planted in soil. A very important factor for good performance after planting was the presence of roots. Without roots, a cold treatment was necessary for sprouting. However, the leaves remained short (0·5–1 cm) and died after a few weeks. When roots were present at planting, bulblets sprouted and grew very well, even without a cold treatment.

In vitro Propagation of Rose Rootstock 'Sturcinq'

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Rose rootstock *Rosa canina* 'Inermis' clone 'Sturcinq' gives a very high production of good quality flowers of the scion cultivar grafted upon it. Propagation of 'Sturcinq' by cutting or grafting is difficult due to a low rooting percentage. In many woody plants, rooting of cuttings from tissue-cultured stock-plants is greatly improved compared to cuttings from conventional stockplants. Therefore a tissue culture protocol for rose rootstock 'Sturcinq' has been developed.

For initiation, surface-sterilized axillary buds were placed on full strength MS-medium solidified with 7 g/l agar, containing 45 g/l sucrose, 0·1 mg/l 6-benzylaminopurine (BAP) and 96 mg/l FeEDDHA (Van der Salm *et al.* (1994): *Plant Cell Tiss. Org. Cult.* **37**: 73–77). Glucose was slightly negative for initiation. There was no difference in yellowing of the plantlets growing on medium with NaFeEDTA or with an equimolar amount of FeEDDHA.

Outgrowth of axillary buds of the shoots was barely increased by adding higher concentrations of BAP. Propagation was strongly stimulated by adding liquid MS-medium with 45 g/l sucrose on top of the solid medium (with 1 mg/l BAP). The highest fresh and dry weights were obtained if 4 ml liquid medium per tube were added after 2 weeks of culture. The highest propagation rate was obtained when 3 ml were added after 2 weeks.

Shoots were rooted on 1/2 MS, 20 g/l sucrose, 48 mg/l FeEDDHA, 7 g/l agar and 0–3 mg/l indole-3-butyric acid (IBA). Even without addition of auxin all shoots rooted. With 0·1 mg/l IBA significantly more roots per plantlet were formed. Shoots could be rooted directly in rockwool plugs. The highest rooting percentage (97%) was obtained after rinsing the plugs with 0·1 mg/l IBA. The highest percentage of acclimatized plants (91%) was reached at 0·05 mg/l IBA.

Effect of Cytokinins on Axillary Bud Growth of 'Madelon' Roses

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An *in vitro* single node system was used to study the response of axillary buds of 'Madelon' roses to the cytokinin free bases BA, Z, iP, (RS)DHZ, R(+)DHZ, S(−)DHZ and their ribosides. Addition of cytokinins was necessary for the bud to grow out to a fully developed shoot. Bud break, expressed as the number of days until the first compound leaf was visible, was not affected by the presence or the type of cytokinin.

Cytokinin ribosides were equally active as their cognate free bases. When the cytokinin concentration in the medium was raised from 0 to 32 μM, the dry weight of the total plant increased. Depending on the type of cytokinin added, the optimum was reached at 3·2 or 32 μM. The number and dry weight of secondary and tertiary shoots increased with exogenous cytokinin concentrations. The cytokinin activity was in the decreasing order

$$Z = BA \gg iP \gg S(-)DHZ > (RS)DHZ > R(+)DHZ.$$

Anthraquinone Production in *Agrobacterium rhizogenes* Transformed Roots and Cell Suspension Cultures of *Rubia* and *Morinda*

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Roots of *Rubia tinctorum* and *Morinda citrifolia* are used to study the role of cellular differentiation in anthraquinone biosynthesis. Root cultures of *Rubia* and *Morinda* transformed with *Agrobacterium rhizogenes* have been established and they provide a structured *in vitro* system that will be used to relate the biochemical events to processes in the intact plant. Anthraquinone accumulation was quantified and the distribution in the root tissue was studied. The localization of anthraquinones at the (sub)cellular level was determined with confocal laser scanning microscopy. Cell suspension cultures of *Rubia* and *Morinda* which are inducible by changes in the composition of the medium were studied for comparison. These cell cultures are also well suited to investigate the effects of rapid anthraquinone accumulation on cellular metabolism.

Future studies will focus on immunolocalization of the important key enzymes of the shikimate pathway and of anthraquinone biosynthesis, in an attempt to specify the cell types in which this biochemical process occurs.

Effects of Glyphosate on Cell Suspensions of *Morinda citrifolia*

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One important question in secondary metabolite research is how plants divide available resources over primary and secondary metabolic routes. Many biosynthetic routes have several branching points where precursor molecules are channelled either into secondary routes or remain in primary pathways. Secondary metabolites known as anthraquinones are produced by a route which branches off the shikimate pathway at the point of chorismate. Chorismate remaining in the shikimate pathway is converted into the aromatic amino acids tryptophan, tyrosine and phenylalanine. This means that chorismate, being a common precursor for both secondary and primary metabolites, marks an important regulatory point in the flow of precursors through the shikimate pathway.

In order to understand more about the metabolic regulation of this system, the activities of several important enzymes are being investigated in both anthraquinone-producing and non-producing cells. The first enzyme of interest is isochorismate synthase, which catalyzes the conversion of chorismate into isochorismate, the first committed step in the production of anthraquinones. Chorismate mutase converts chorismate into prephenate (a precursor of the amino acids tyrosine and phenylalanine) and phenylalanine ammonia lyase is involved in the conversion of phenylalanine into trans-cinnamic acid, from which many secondary compounds are formed via the phenylpropanoid pathway.

When investigating enzyme regulation an interesting feature of the shikimate pathway is the possibility to block the formation of chorismate via the herbicide glyphosate (N-(phosphonomethyl)-glycine). Adding glyphosate to the culture medium results in cells no longer producing aromatic amino acids and therefore reduces growth. However, it may be possible to reverse the growth inhibitory effect of glyphosate by providing the cells with aromatic amino acids in the growth medium. In that case it would be possible to study metabolic regulation of shikimate pathway enzymes in normal growing cells while part of the pathway is blocked.

Effect of Elicitation on Isochorismate Synthase in Anthraquinone-producing Cell Cultures of *Rubia tinctorum*

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Cell cultures of *Rubia tinctorum* produce substantial amounts of secondary metabolites in contrast to most cell cultures. These secondary metabolites are called anthraquinones. In the Rubiaceae, anthraquinones are synthesized via the shikimate-o-succinylbenzoic acid pathway. One important step in this pathway is the conversion of chorismate into isochorismate, a reaction catalyzed by the enzyme isochorismate synthase. This reaction is the branchpoint of anthraquinone biosynthesis and the primary shikimate pathway and is therefore a potential site for regulation of flow into secondary metabolism. We investigate whether this enzymatic conversion is indeed a rate-limiting step in the biosynthesis of anthraquinones.

Elicitation with a fungal extract resulted in a substantial increase in anthraquinone production, which is preceded by a large rise in isochorismate synthase activity. Application of inhibitors of translation or transcription annihilates the effect of elicitation on isochorismate activity and anthraquinone production. These results indicate that elicitation requires *de novo* RNA synthesis.

Partial purification revealed the presence of at least two isoenzymes. Native PAGE showed a molecular weight of about 95 kD for both enzymes. The

enzymes are characterized with respect to kinetic properties.

Genetically Stable Cell Lines of Cucumber for the Large-scale Production of Diploid Somatic Embryos

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We studied the initiation of embryogenic cell lines from excised radicles of cucumber (*Cucumis sativus* L.) cultured in liquid medium. The culture regime, explant density and type and concentration of hormones were adjusted so that pro-embryogenic masses (PEMs) were formed within about 8 weeks. The established cucumber cell lines were maintained for several years without loss of embryogenic and genetic stability. The ploidy level of somatic embryos from different cucumber cell lines was either diploid or tetraploid and depended on the ploidy level of the cell line. Cucumber cell lines that produced only diploid embryos were obtained by selecting completely diploid explant material and growth in the dark during the initiation phase. Mixoploid explants could lead to tetraploid or mixoploid cell lines. Isolation and additional selecting and subculturing of single PEMs resulted in either completely diploid or completely tetraploid cell lines, indicating that all cells of individual PEMs are either diploid or tetraploid. The embryogenic cucumber cell lines differing only in ploidy level were indistinguishable in growth rate and embryogenic potential and were genetically stable over several years.

MEETING OF THE SECTION FOR PLANT SYSTEMATICS AND GEOGRAPHY ON 15 MARCH 1996

On the Genus *Trichosanthes* (Cucurbitaceae) in Java

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Cucurbitaceae is a middle-sized family of pantropical and temperate areas. All species are susceptible to frost. Most are trailing. There are *c.* 120 genera with 850 species, indicating that most genera contain few species. Cucurbitaceae are at present being studied in Leiden for Flora Malesiana. In that area 27 genera occur (including those known only in cultivation), with 60–70 species. One of the larger SE Asian genera is *Trichosanthes* L., with some 50 species, ranging from India and China (and Japan) through Malesia into the Pacific and Northern Australia. *Trichosanthes* is readily recognizable by the longly fringed petals. Malesian *Trichosanthes*, some 20–25 species, will be revised in Bogor, in cooperation with Leiden.

Some characters of Cucurbitaceae, also present in *Trichosanthes*, are dioecism, night-flowering, the presence of a pro-bract, the typical lateral insertion of the tendril and the typical construction of the androecium. Pollen and seed morphology will probably be of great importance taxonomically, but sufficient research is still lacking.

Study of the taxonomy of the species of *Trichosanthes* in Java will result in accepting nine species, compared with eight species in Backer (*Flora of Java* **1**, 1963) and 10 species in Blume (*Bijdrage Fl. Ned. Ind.*, 1826). However, Backer's revision contains important differences: *T. trifoliata* (L.) Merr. should be renamed as a new species because of misinterpretation of the type; *T. anguina* L. should be regarded as a cultivar of *T. cucumerina* L., and the species accepted in Backer (1963) under the name *T. bracteata* (Lam.) Voigt (a continental SE Asian species), appears to represent three different quite distinct species, especially evident when examining living specimens in the field: *T. tricuspidata* Lour., *T. pubigera* Blume and *T. quinquangulata* (A. Gray).

Morphological Variation of Recent Invaders in Northern Central America: the case of *Malmea* (Annonaceae)

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Cluster analysis was used to reveal patterns of morphological variation in a species complex of *Malmea* (Annonaceae), distributed in eastern Mexico, Guatemala, Belize and Honduras. Initially *M. depressa*, *M. gaumeri*, *M. leiophylla* and *M. guatemalensis* belonged to this species complex.

In total 53 characters were used for the cluster analysis, among which were 50 leaf, flower and fruit morphological characters. Of these, 24 characters significantly determined clustering of 238 herbarium specimens into 12 clusters. No cluster is exclusively specified by any character or combination of characters, nor can any geographical pattern be detected, except for the clustering of specimens from Los Tuxtlas Biological Station, Veracruz, Mexico. A new

subspecies from this area will be described. *M. gaumeri* and *M. leiophylla* are brought into synonymy with *M. depressa*. Distribution patterns of Mexican and Central American taxa of *Malmea* are largely concordant with those of South American taxa which spread into Central America after the Pliocene closure of Isthmus of Panama. Absence on the West Indies and little differentiation on species level in Central America are the main clues.

A phytogeographical novelty is presented, however, as the distribution of the new subspecies within the Sierra de los Tuxtlas shows a hitherto unknown pattern. In contrast to other endemic taxa of this Sierra, it is not spread throughout the whole mountainous range, but only found on the wet northern slopes.

Amorphophallus: Variation in (Almost) Everything!

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The genus *Amorphophallus* (Araceae), presently under revision, is estimated to contain *c.* 170 species. They are found from West Africa eastwards to Polynesia and all are tropical species, mostly in secondary forests. Within the framework of the aroid family, *Amorphophallus* displays a surprising variation in several character suites, e.g. spathe morphology, appendix morphology, pistil morphology, underground parts and pollen. Another remarkable feature is the tremendous heat development in the male part of the spadix and the appendix. The array of volatiles, emitted during the female flowering phase, is equally variable and ranges from agreeable (e.g. aniseed-like, fresh carrots-like, fruity, etc.) to highly upsetting (rotting meat, decaying vegetables, LPG), the latter often brought about by organic oligosulphides. Pollination data are scanty but, more than once, carrion beetles have been found in the spathes as well as tiny beetles of other families. The sheer size of, especially, one of the genus' members (*A. titanium*) has always attracted much attention. Its tuber may weigh up to 75 kg and the leaf may reach as high as 5 m, with a lamina diameter of 7 m. By contrast *A. pusillum* does not exceed 7 cm in height. The systematic position of *Amorphophallus* in the aroid family is still debated.

The Caesalpinioideae Project in Wageningen

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The Leguminosae-Caesalpinioideae are studied in Wageningen, as this subfamily (or family Caesalpiniaceae) (1) lacks a worldwide revision; (2) because its species are most diverse in tropical Africa, the focal area of the taxonomic research in Wageningen; (3) its species are as important as Dipterocarpaceae in Asia; and (4) because several tribe and genus circumscriptions are unclear (Cowan, 1981, Cowan & Polhill, 1981, in *Adv. Leg. Syst.* **1**: 57–64, 117–142).

Tribal delimitation is an aspect under review, and monographic treatments of certain genera (*Aphanocalyx*, *Eurypetalum*, *Monopetalanthus*) are being carried out (Breteler, Wieringa, in preparation).

Cowan (1981) and Cowan & Polhill (1981) recognize five tribes in Caesalpinioidae: Caesalpinieae, Cassieae Bronn, Cercideae Bronn, Detarieae DC and Amherstieae Benth. The latter two were emended by Breteler (1995, in *Adv. Leg. Syst.* **7**: 53–61) by the placement of the *Amherstia* group (three genera) in Detarieae, and the main part of the former Amherstieae as a monophyletic group became Macrolobieae Breteler.

The distinctive traits between the Detarieae and Macrolobieae are: Detarieae bracteoles small (sometimes absent) or large and conspicuous (*Amherstia*, *Hymenostegia*) but without protective function and always imbricate; Macrolobieae bracteoles always present, always valvate, with a clear protective function, i.e. remaining till anthesis without any time delay between expansion of the bracteoles and the opening of the flower. In some Macrolobieae genera this has resulted in the calyx being reduced or almost disappeared: for instance in *Aphanocalyx* and *Cryptosepalum*, where the genus names also express this property.

The geographic distribution of Detarieae and Macrolobieae is peculiar. Australia lacks any representative. A majority of its genera are endemic to a continent.

Detarieae (60 genera): America 19 genera (14 endemic); Africa 37 genera (29 endemic); Asia 15 genera (nine endemic). Only two genera, *Crudia* and *Cynometra*, are circumtropical.

Macrolobieae (29 genera): America two genera (one endemic); Africa 27 genera (25 endemic); Asia one endemic genus.

Problems exist where genera of different continents are similar, but have not been critically submitted to revision on a worldwide basis. Examples are the Detarieae pairs *Eurypetalum*/*Eperua*; *Schotia*/*Phyllocarpus*, the Macrolobieae *Berlinia*/*Dicymbe* from Africa and America, respectively, and the Detarieae pair *Colophospermum*/*Hardwickia* from Africa resp. Asia. These may turn out to be congeneric.

For such revisions macromorphology has to be aided by other disciplines. In flower ontology, Dr Shirley Tucker from Berkeley has agreed to collaborate, in seed chemistry Dr Geoffrey Kite from

Kew is contributing his findings, Dr Keith Ferguson from Kew studies the pollen morphology, molecular data are supplied by Dr Anne Bruneau in Montreal, and Dr Peter Gasson (Kew) investigates the wood anatomy. These experts collaborate informally, and for this purpose the Wageningen Herbarium Vadense has provided many samples of flowers, seeds and wood samples obtained during field missions in Africa.

Paraphyletic Genera: their theoretical basis

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The Tree of Life can be subdivided according to various hierarchical models. Two of these, the monophyletic model and the Linnaean model, are highly relevant for systematics. They are, however, incongruent and no general model can be constructed that satisfies all conditions of both these models. Therefore, monophyletic classification is impossible when applying present day nomenclature which follows the Linnaean model. Some attempts have been made to construct such an intermediate model, notably by Wiley (Wiley, E.O. (1981): *Phylogenetics*, John Wiley, New York) who called it the 'annotated Linnaean classification', but all these were doomed to fail because of theoretical imperfections.

Strict monophyletic classification requires a new set of nomenclatural principles and rules and a first impulse to such a new system has already been published (de Queiroz, K. & Gauthier, J. (1992): *Ann. Rev. Ecol. Syst.* **23**: 449–480). One could also opt for the acceptance of the Linnaean model which would lead to the recognition of both mono- and paraphyletic taxa. Evolutionary processes, however, do not only lead to a dichotomously branched phylogenetic tree. Through hybridization followed by speciation and by the merging of formerly distinct species, reticulate phylogenetic relationships are formed. It is impossible to divide a reticulate phylogenetic tree solely into monophyletic entities. Therefore, evolution itself does not allow the application of the monophyletic hierarchical model. The Linnaean model, however, can cope with reticulate patterns and the acceptance of paraphyletic higher taxa has become inevitable.

Distribution Data of Higher Plants in The Netherlands: at what scale?

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This presentation focuses on the use of biogeographic data in policies. Since the end of the 1960s the Dutch government has been interested in quantitative comparative valuations and prognoses of increase or decrease of the value of reserves. I discuss the nature of the data, and whether a quantitative approach only is sufficient.

The first aim towards quantification of biogeographic information was presented by Adriani & van der Maarel in 1968 (Voorne in de branding), by comparing the species number of several areas. The Red List of higher plants, published in 1990, was based on a combination of rarity of the species and its percentage of decline at hour-square level. The question is: when is a species rare, and which problems do we have to deal with when analysing distribution data?

Seven forms of rarity are described, depending on geographical area, specificity of habitat and local population size. The latter is not considered for the draft of the Red List. With five examples, the difficulties with interpreting distribution maps are illustrated. First, the density of inventarization is not constant over several periods. Also, the level of knowledge of the observers has increased. Thirdly, when comparing the number of squares before and after 1950 one has to bear in mind that the sizes of the squares before and after 1950 are different. Finally, the presentation of the data is of influence. Shown at hour-square level a species may seem common, and the presence not changed during time, but on a square kilometer level the distribution may show to be much more fragmented, and possible changes in presence become visible. For several species even extra information on abundancy is needed to trace real changes earlier.

It is concluded that quantitative data (presence or abundancy) at a square kilometer level, form a good basis for the decision process in policies and conservation. However, qualitative knowledge is always needed to distinguish artefacts from real changes.

Biodiversity of Non-Timber Forest Products in North-West Guyana

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Non-Timber Forest Products (NTFP), defined in this study as all plant products extracted from the forest except commercial timber, are of great importance for forest-dwelling people because they offer a source of food, shelter, household equipment, medicine or cash income.

Within the Tropenbos–Guyana Programme, a new research has started on the use of NTFP by Carib, Arawak and Warrao Indians in the North-West District of Guyana.

The first 2 months of fieldwork were spent in the remote Carib settlement of Kariako on the Barama River. An almost complete inventory was made of the plants used by the Kariako Caribs. The first results of this study point out that there are about 250 plant species used for a wide variety of purposes (firewood not included).

Among the most important NTFP in this area are two species of *Ischnosiphon*, Marantaceae (used for plaiting household equipment such as cassava sifters, fans and cassava squeezers), as well as the aereal roots of three hemi-epiphytic Cyclanthaceae (used as binding material and in basketry). The small understorey palm *Geonoma baculifera* is the most common source of roof thatch. A wide range of plants are used in medicinal decoctions. Most medicinal infusions are made from herbs of secondary vegetations or from the barks of primary forest trees and lianas. Wild fruits form an important part in the diet of, especially, young children. Melastomataceae, Palmae (*Astrocaryum*), and *Inga* (Mimosoideae) were the most important wild fruit sources.

During the second field work period, 1-hectare plots will be laid out to assess the diversity and abundance of NTFP in different forest types in the region: swampy Mora forest, well drained Licania forest and secondary forest.

MEETING OF THE SECTION FOR VEGETATION RESEARCH ON 18 MARCH 1996

On the Vegetation of Eastern North Greenland

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North Greenland stretches from about 78°N–83°N and lies within the Greenland National Parc. Climate is very cold and dry (mean annual T< − 11°C; 2–3 months with mean T>0°C; precipitation often <150 mm/yr; strong winds). Topography is mountainous with alpine terrain or undisturbed plateaus up to 1000 m intersected by fjords; 30% of the area is covered by ice. Bedrock is precambrian to quaternary, sand- and limestones dominate. Permafrost, solifluction, frost boils, sorted rings and polygons are common. Soil development is poor; rego- and lithosols dominate. It belongs to the high arctic and Canada–Greenland flora-province (Yurtsev, B.A. (1994): *J. Veg. Sci.* **5**: 765–776). Vascular plant flora (172 species) is fairly well known (Bay, C. (1984): *Meddr. Grönl. Biosci.* **36**). During July 1995 we studied the semi-desert vegetation of the inland of Kronprins Christian Land and Mylius Erichsen Land (about 80–81°N) according to the Braun-Blanquet approach.

Closed vegetation is local and confined to wet sites; polar desert vegetation dominates. Open *Pleuropogon sabinei* vegetation (1) occurs locally in shallow streams, ponds and lakes, which are bordered by wet dense, often moss-rich *Carex stans* vegetation (2) and *Eriophorum triste* mire-vegetation (3) on sites with lateral water supply. Both types are preferably grazed by musk-oxen. *Salix arctica-Carex stans* vegetation (4) occurs on silty flood plains. *Luzula arctica* vegetation (5) prefers late snowbeds. *Carex misandra-Dryas integrifolia* vegetation (6) grows on temporary moist, active, silty soil. *Dryas integrifolia-Cassiope tetragona* heaths (7) are confined to early snowbeds. Open *Saxifraga cespitosa-Papaver radicatum* vegetation (8) occurs between coarse debris at higher altitude, lichen-rich *Carici-Dryadetum integrifoliae* (9) occurs in dry, wind-exposed sites. *Papaver radicatum* vegetation (10) occurs in manured stony sites. *Poa abbreviata-Melandrium affine* vegetation (11) is confined to sheltered, warm, pockets and gullies on manured, loamy soil. Vegetation types 1–5 and 11 have a high-arctic distribution. Thanks are due to the Deutsche Forschungsgemeinschaft for support.

Vegetation Development in Relation to Land Use and Management of the Coastal Village Landscape Near Zandvoort

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Like many other villages along the Dutch coast, Zandvoort has a long history of using the surrounding dune area. Doing studied the landscape ecology of the Dutch coast and defined the coastal village landscape (*Landscape ecology of the Dutch coast*; EUCC/-Stichting Duinbehoud, 1991). The semi-natural dune landscape, which is restricted to the surroundings of the old fishing villages, differs from other parts of the coastal dunes, particularly as a result of intensive use by man. The dunes were used for grazing cattle, growing potatoes and gathering brushwood, which resulted in a very specific vegetation. Common species are *Silene otites*, *S. nutans*, *Anthyllis vulneraria*, *Orobanche picridis* and

Ballota nigra subsp. foetida. In two of these areas, the Zeeduinen and the Zuidduinen, the vegetation development was studied in order to evaluate land use and management.

The Zuidduinen and the adjacent Zeeduinen are situated south of Zandvoort. The typical land use in the Zuidduinen has almost disappeared. Nowadays it is used as an amenity area for dog-walking and other kinds of recreation. In 1994 the Zuidduinen were added to the Amsterdam Waterwork Dunes. The Zeeduinen belong to the Amsterdam Waterwork Dunes and have been fenced since approximately 1920. It is now a nature reserve and protected water catchment area. In 1988 cattle grazing was introduced in the Zeeduinen in order to counteract grass and scrub encroachment and to restore the coastal village landscape (Ehrenburg *et al.* (1995): *De Levende Natuur* **96**: 202–211).

In 1935 and 1990 a floristic inventory was conducted, which could be used to reconstruct ecotope development. Interpretation of aerial photographs from 1958 and 1990 enabled a study of the vegetation structure development, especially concerning processes such as blow-out and scrub development. In 1988 and 1992 the vegetation structure was mapped in the field before and after 4 years of cattle grazing.

Typical ecotopes of the coastal village landscape, such as fields and tracks, dry ruderals and open and dense herb-rich dune grasslands seem to have become more complete since 1935 in the Zuidduinen. Vegetation structure development since 1958 shows that the vegetation has changed little in the Zuidduinen in 1990, while the Zeeduinen have suffered scrub encroachment. Detailed field mapping of the vegetation structure in the Zeeduinen makes clear that as a result of cattle grazing since 1988 long and dense grasslands have changed into short herb-rich grasslands, while scrub encroachment has stopped. The open character with a mosaic of blow outs, short grasslands and scrub has been restored.

In the Zeeduinen a change in the grazing intensity (summer grazing) is proposed in order to enable a better dispersion of the plant species, which are characteristic of the coastal village landscape. In the Zuidduinen the present use is maintained and the area remains an amenity area for the local people. Recreation seems to fit well as a management tool to safeguard the characteristic vegetation of the coastal village landscape.

Population Dynamics and Genetic Variation in *Circaea lutetiana*, a Pseudo-annual Clonal Plant

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Parent shoots of most clonal plants are able to support their vegetative 'offspring' (ramets) with resources. Early mortality risks of such ramets may therefore be very low. In pseudo-annuals, which are clonal plants surviving the winter only as seeds and hibernacles produced by the rhizome apices, parent shoots do not support their ramets. Preliminary studies on the pseudo-annual *C. lutetiana* indicate that (i) production and mortality of hibernacles in a field population is more or less in balance, (ii) mortality risk of hibernacles can be 90%, and (iii) no mortality of vegetative offspring occurs at the ramet level. Furthermore, seedling establishment is rare or absent in *C. lutetiana* populations. Using a simulation model in which the production and mortality of hibernacles is in balance, hibernacle mortality is random, and in which no seedling establishment takes place, a rapid decrease in the number of genets and dominance of some genets can be found with increasing population age. To test this hypothesis, genet sizes were studied in an established *C. lutetiana* population. Using RAPD profiles of 43 sampled plants, 36 genets could be identified. This result is in contrast to the outcome of the simulation model. The observed high number of genets may be the result of (i) recurrent input of new genotypes and/or (ii) low extinction risk of present genets. These two hypotheses can be tested with a revised version of the simulation model. When 0·1% of all produced seeds are established, genetic variation is maintained, although this process cannot prevent the presence of large sized genets. When one produced hibernacle per ramet has a much higher chance to survive than all other hibernacles of that very ramet, a loss of genets is almost prevented.

The Influence of a Delayed Removal of Cuttings on the Nutrient Balance in Roadside Vegetation

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As part of a research project on the synecology of roadside vegetation, the effect of an extended timelag between cutting and hay removal on the amount of macronutrients withdrawn from different plant communities was studied. In road verges, contrary to agricultural or nature conservation practices, cuttings are often left in the field for a considerable time before removal takes place.

A litterbag experiment was conducted at eight sites. On five occasions during a period of 6 weeks, five random litterbags were taken to the laboratory. Dry weight and the amounts of N, P and K were determined for each litterbag. The remaining

fractions of the original amounts of N, P and K appeared to be well explainable by a decreasing exponential relation with a non-zero asympote. The average remaining fraction after 4 weeks amounted to roughly 67% for nitrogen, 50% for phosphorus and 25% for potassium, the latter further decreasing to an average asymptotic value of 10%. The differences between the elements were already significant after 1 or 2 weeks.

Regression models showed that the differences between sites could be explained best by the chemical composition of the vegetation at the time of cutting. The remaining fractions of the original dry weight and N-amount were explained best by the C/N ratio at time of cutting, whereas the remaining fractions of P and K were explained best by the P-concentration of the vegetation at time of cutting.

With this knowledge, we were able to model the withdrawn amount of nutrients for 11 roadside communities for which data were available from the main research project. The amounts were modelled for different timelags, ranging from 0 to 6 weeks or more. It appeared that, for many communities, the yearly withdrawal of N is larger than the annual atmospheric deposition only when the cuttings are removed within the first 2 or 3 weeks. For some communities the atmospheric N deposition can never be withdrawn, not even by a direct removal of the cuttings. On the other hand, the withdrawal of K exceeds the atmospheric deposition in all communities when the cuttings are removed within the first week. However, the heavy losses for this element result in the withdrawal of K falling below the annual deposition in nearly all communities when the cuttings are left for longer periods.

Changes in the Vegetation of Meijendel Influenced by Grazing

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Meijendel is a calcareous coastal dune system near the city of The Hague, which is used as a water catchment area. A combination of a decreased intensity of grazing by rabbits together with a strongly increased load of nitrogen caused by the pollution of the precipitation has led to a dune vegetation with a less open character and an increasing dominance of *Calamagrostis epigejos* and *Carex arenaria*. To stop this process, grazing by cattle was introduced in some parts of Meijendel in 1990. After 5 years of grazing a first evaluation report was planned. This research is part of the evaluation and contains the development of the vegetation as influenced by grazing. It consists of three parts:

- Vegetation classification; the present vegetation types in three transects (both grazed and ungrazed) are described.
- Permanent plots; in the grazed area there are 19 permanent plots, from which development has been observed since 1960.
- Structure comparison; with the help of false-colour aerial photo-series, a comparison of the vegetation structure in the three transects has been made between 1990 and 1995.

It can be concluded that grazing does not result in a change in the vegetation types. The different types are present in both grazed and ungrazed areas. The cover of *Calmagrostis epigejos* and *Carex arenaria*, however, is significantly lower in the grazed area. The total number of plant species, decreasing since 1960, shows an increase after the introduction of cattle in 1990. With regard to the structure comparison it can be concluded that in grazed areas the amount of high grass vegetation has almost totally disappeared and the amounts of open sand, sand with moss and lichens and low grass vegetation have increased. After 5 years of grazing the pattern of the several structure types has become more small-scale.

Compensating for Water Shortage in Wet Grasslands: does sulphate-enriched water cause problems?

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Many wetlands in The Netherlands deal with serious water shortage, because of agricultural activities and water extraction. To compensate for the decrease in groundwater levels, allochthonous water, originating from the rivers Rhine and Meuse, is often used. The concomitant eutrophication was originally completely attributed to the external input of nutrients, especially of phosphates.

It was hypothesized that sulphate-enrichment, due to the use of this river water, is a major cause for the observed eutrophication in wet grasslands. Therefore, the effects of waterlogging with sulphate-enriched water (0, 2 and 4 mmol l^{-1}) on soil processes were tested in mesocosms. Humic soil cores including vegetation, from a mesotropnic wet grassland dominated by *Carex* species, were investigated in a laboratory flow-through system. Soil water chemistry was determined, using permanent moisture samplers, and the above-ground biomass was harvested several times during the experiment. In the soils treated with sulphate-enriched water, enhanced

sulphate reduction generated alkalinity and caused accumulation of free sulphide in soil pore water. Nutrient concentrations increased considerably within a few months, compared with the control treatment. Because of iron sulphide precipitation during sulphate enrichment, less free iron was available for the binding of phosphates, resulting in a rapid increase of the phosphate concentration. The increase in alkalinity probably stimulated the decomposition and mineralization of organic matter, causing a significant increase of macronutrient concentrations. However, the vegetation regrowth was reduced in the sulphate-treated pots, indicating sulphide toxicity. During the subsequent desiccation of the sulphate-treated soils, soil pH dropped substantially due to proton production by iron sulphide oxidation. Moreover, heavy metal (e.g. aluminium) concentrations increased to potentially toxic levels.

The outcome of this experiment indicates major changes in soil chemistry due to sulphate enrichment, which might contribute to the observed deterioration of wetland vegetations affected by water, rich in sulphate. Future research will focus on the sensitivity of different soil types and plant species, in relation to the involved biogeochemical processes. The occurrence of internal eutrophication due to sulphate enriched water has important implications for the management of nature reserves, as a decrease of phosphor and nitrogen levels in the inlet water may be insufficient to prevent eutrophication. It is suggested that the restoration of the original hydrology is the only way to conserve endangered wetlands.

Pollen Exchange in Small Populations of *Salvia pratensis*

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Recently, many plant populations have become fragmented, often due to habitat destruction, resulting in small and isolated populations. These populations not only face an increased probability of extinction by stochastic processes, but, in addition, may also experience a loss of genetic variation and fitness due to genetic drift and inbreeding. Gene flow can alleviate the deleterious genetic effects of small population size and isolation. In many plant species with limited seed dispersal gene flow largely depends on insect-mediated pollen flow.

In *Salvia pratensis* pollen flow depends on the behaviour of bumble bees, foraging for nectar. Since the number of open flowers per plant can have important consequences for their behaviour, we have investigated how pollinator movement and thus potential pollen exchange is affected by plant size (measured as number of open flowers). Therefore, all plants and all bumble bees in a patch were marked individually, and we tracked the foraging paths of these numbered bumble bees and constructed the possible pollen flow between plants.

A positive correlation was found between the number of open flowers per plant and the percentage intra-plant movements. In other words larger plants experience more geitonogamous visitation, that can cause relatively higher selfing rates. On the other hand plants with many flowers attracted more bumble bees and were connected with a larger number of neighbouring plants. This promotes outcrossing and may result in a genetically more diverse progeny. As the balance between outcrossing (connections to other plants and gene flow) and selfing (and the possible occurrence of inbreeding depression: Ouborg & Van Treuren (1994): *Evolution* **48**: 996–1008) depends on the foraging behaviour of bumble bees, changes in their behaviour in relation to population fragmentation can have important consequences for the persistence of plant populations.

A Landscape Ecology Survey of Edgeoya Svalbard

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A land ecological survey was executed of the island of Edgeoya (Svalbard, Spitsbergen) during a Reindeer Environment Expedition (REES) summer 1977. For the methodology see I.S. Zonneveld (1995) *Land Ecology*, SPB Academic Publishing, Amsterdam. 199 pp. Sub-objectives were to describe the vegetation communities and their ecology and to assess the environment of the reindeer. This survey was carried out parallel to studies on the reindeer population. The result was expressed in a four-colour map, scale 1:200 000, cartographically treated and printed in 1995 using the newest techniques. The legend of the map is expressed in 25 land units, each described according to landform (geomorphology), soils and vegetation. The latter was expressed in (complexes of) seven floristically defined plant communities, structural data and (peak) standing crop of the vascular plants and (negligible) lichens. The communities distinguished (A, *Eriophorum—Carex subspathaca*; B, *Tomenthypnum—Saxifraga flagellaris*; C, *Tomenthypnum—Luzula arctica*; D, *Papaver—Cardamine bellidifolia*; E, *Phippsia algida*; F, *Papaver—Oxyria digyna*; G, *Papaver—Saxifraga cernua*) reflect temperature and moisture gradients.

They are composed of 72 plant species of vascular plants, 78 species of Musci, 10 species of Hepaticae and 80 Lichenses. The total production appeared to be enough for *c.* 30 000 animals, a factor 20 times higher than the actual population of reindeer being an average *c.* 1500. The limiting factor, apparently, is the isolation from the food through the snow cover, especially in early spring: often sealed with ice as a cause of intermittently freezing and thawing on the places most favourable for vegetation. For this reason only a limited part, moreover of lower quality, of the food source is accessible.

A Pioneer Community Along the Large Dutch Rivers

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The valleys of the large Dutch rivers are phytogeographically characterized by some hundreds of species, most of them thermoxerophilous ones, with a continental, Eastern European centre of distribution. The key plant community of this 'Fluviatile District' is a closed dry grassland, *Medicagini-Avenetum pubescentis* (W.C. de Leeuw 1936). Its stands are preceded or accompanied by an open pioneer community, establishing itself on bare sand which has been turned over by river floods in winter. As yet, this subruderal vegetation type has been considered a pioneer phase of *Medicagini-Avenetum*. It is more correct, however, to assign it to the subruderal alliance of *Convolvulo-Agropyrion repentis*, order *Agropyretalia* (Th. Müller in Oberdorfer, E. (1983): *Süddeutsche Pflanzengesellschaften*, vol. 3: 278–299, G. Fischer, Stuttgart), which we assign now to the ruderal class *Artemisietea vulgaris*. The *Agropyretalia* have an optimal development in Central and Eastern Europe, our riverside community being a sub-Atlantic outpost. The Dutch pioneer community is characterized by the dominance of *Eryngium campestre* in combination with the character-species *Bromus inermis*, *Rumex thyrsiflorus*, *Saponaria officinalis* and *Euphorbia esula*. In the adjacent German foreland of the Lower Rhine a similar association has been described as *Poo-Eryngietum* (Passarge, H. (1989): *Tuexenia* **9**: 121–150). *Eryngium campestre* and *Medicago falcata* are constant species as well in the Dutch pioneer community as in the sympatric *Medicagini-Avenetum*; the latter association is mainly characterized by *Salvia pratensis*, *Veronica austriaca* ssp. *teucrium* and *Thalictrum minus*.

When we compare the recent floristic assemblage of the *Medicagini-Avenetum* with the classic, but unpublished relevé table by the late G. Sissingh, dating from 1939–1954, it is notable that the presence degrees of *Rumex thyrsiflorus* and *Bromus inermis* have considerably increased, particularly in the most typical subassociation *centaurietosum*. *Medicagini-Avenetum* and the Dutch counterpart of *Poo-Eryngietum* seem to merge during the course of decades. Probably, we are dealing here with a process of ruderalization as a consequence of increasing human disturbance and air pollution.