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MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE ON 16 MARCH 1989

LECTURES:

Effects of Plant Growth Regulators on Epigenetic Instability of *Kalanchoe blossfeldiana* Poelln. Propagated *in vitro*

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Epigenetic variation interferes with vegetative propagation *in vitro*, especially when the callus phase has to be passed. Ornamental *Kalanchoe* species show fasciation and aberrant phyllotaxis. The role of plant growth regulators (PGRs) in this form of epigenetic instability was analysed.

In the vegetative propagation from leaf discs of *Kalanchoe blossfeldiana* Poelln. both auxin and cytokinin are required. The measure of instability increases at increasing concentrations of PGRs, especially cytokinin. Lowest instability occurs at low concentrations of such mild regulators as indoleacetic acid (IAA) and zeatin. Cytokinin may induce vitrification which is reduced by low concentrations of auxin.

Shortening of the exposure time to the PGRs considerably decreases epigenetic instability, auxin being required in the first week only. To study the effects of PGRs during different developmental stages of the regeneration process, a system using liquid medium was developed. On this medium sprouts regenerate more rapidly without visible callus. It was found that the explants show a variable sensitivity for cytokinin during regeneration and that epigenetic variation can be reduced by careful timing and manipulating the PGRs required for growth and organogenesis.

Anther Culture of *Lolium perenne* and *Lolium multiflorum*

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Rye-grasses are the most important grass species for west-European agriculture. Homozygous lines of these species can be produced by anther culture. However, a general procedure for *Lolium* anther culture is still lacking due to low anther response and poor plant regeneration.

In an investigation aimed at increasing anther response and plant regeneration efficiency, the effects of anther cold pretreatment, the nitrogen and carbon source in the induction medium and the incubation of the anthers at elevated CO_2 concentrations, were examined.

The culture media were based on the Linsmaier and Skoog salts (LS) together with the Fujii organic supplements. Albino, as well as green, plantlets were obtained from *L. perenne* and *L. multiflorum* anthers. Isozyme and flow cytometric analyses showed the microspore origin of these plantlets.

Nitrogen source. The use of LS induction medium with a low ammonium concentration (2 mM instead of 20 mM) but supplemented with 5 mM glutamine led to an increase in anther response (percentage anthers with calli or embryos), regeneration frequency (percentage calli or embryos forming shoots) and culture efficiency (percentage anthers forming shoots).

To investigate the effect of KNO_3 for *Lolium* anther culture, additions of different KNO_3 concentrations to a LS induction medium containing $(NH_4)_2SO_4$ (1 mM) and glutamine (5 mM) were tested. For *L. perenne*, addition of KNO_3 decreased the anther response and culture efficiency. For *L. multiflorum*, anther response and culture efficiency decreased when the KNO_3 concentration exceeded 20 mM.

Carbon source. Use of LS induction medium with sugars such as maltose, trehalose and maltotriose dramatically increased the anther response as compared with sucrose for both *L. perenne* and *L. multiflorum*.

Cold pretreatment. A 4°C pretreatment of the spikes before plating the anthers increased the anther response for both *L. perenne* and *L. multiflorum*; the optimal duration of pretreatment is 2 and 3 weeks respectively.

Tissue Culture of *Cyclamen persicum* Mill

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Several methods for clonal propagation of *Cyclamen* have been published, using either tuber (Geier, T. *et al. Gartenbauwissenschaft* 1979, **5:** 226–237) or etiolated petioles (Ando, T. & Murasaki, K. *Chiba University Techn. Bull. Fac. Hortic.* 1983, **32:** 1–5) as explant

source. However, these methods are not applicable to all commercially important varieties.

We cultured 8 × 8 mm leaf explants at 20°C on basal MS-medium with modified concentrations of NH_4NO_3 (10·3 mM), KNO_3 (18·8 mM), $Ca(NO_3)_2.4H_2O$ (3·0 mM), $MgSO_4.7H_2O$ (1·5 mM), KH_2PO_4 (1·25 mM) and NaH_2PO_4 (1·1 mM). Best induction of adventitious shoots was obtained at 0·25–0·50 times the concentration of macro-elements mentioned above. A 4-week period of complete darkness immediately after culture initiation increased the percentage of shoot-forming explants from 25 to almost 70. Growth regulators benzyladenine (BA) and naphthylacetic acid (NAA) (concentrations used: 2·2, 4·4, 8·8 μM and 0, 0·27, 0·54, 5·4 μM, respectively) not only influenced the percentage of explants forming shoots, but also showed a strong effect on number and morphology of the shoots formed. At low BA/NAA concentrations high numbers of shoot primordia (> 100 explant^{-1}) were induced, but their growth was arrested. At high BA/NAA concentrations fewer leaf explants regenerated shoots. Moreover, the number of shoots was low (< 10 explant^{-1}) and many shoots were malformed. Optimal temperature for growth of shoots was 15°C, whereas at high temperatures (25°C) leaves were small and pink instead of green.

Rooting of shoots was done by omitting BA from the medium. The optimal concentration of NAA was 2·7 μM. After 6 weeks on rooting medium 70% of the shoots on average had formed roots. Rooted plantlets were transferred to soil and observed during subsequent growth. Plantlets showing very aberrant morphology *in vitro*, maintained that appearance *ex vitro* and did not grow. The plantlets with a normal morphology (50% of the total number), initially stopped growing for 2 months during which time a tuber was formed. Then they resumed growth and flowered normally 10 months after transfer to soil.

Chromosome Elimination in Somatic Hybrids of a Transformed Potato Marker Line and *Nicotiana plumbaginifolia*

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Gene localization can be achieved using interspecific somatic hybrids in which only one or a few target chromosomes are stably maintained after continuous elimination in prolonged cultures (e.g. due to selection pressure, genomic incompatibility). To produce such hybrids, genetic marker lines with low ploidy level, carrying a selectable marker on one chromosome, are indispensable tools.

In potato no such marker lines are available. Attempts were therefore made via *Agrobacterium*-mediated transformation to introduce genetic markers in diploid potato. The diploid genotype *Solanum tuberosum* HH260 (2n = 2x = 24), which is stable in ploidy level during *in-vitro* culture and genetic transformation, was infected with *Agrobacterium tumefaciens* strain LBA 1060KG (containing the *A. rhizogenes* plasmid pRi1855 with TL- and TR-DNA, and the plasmid construct pBI121 carrying the genes for kanamycine resistance and β-glucuronidase activity). Using this procedure several transformed potato lines carrying various introduced markers have been produced. Among these lines, one (line 413) contained the complete set of markers (hormone autotrophy, hairy root phenotype, production of agropine and mannopine, kanamycine resistance, β-glucuronidase activity). This line was used for protoplast fusion with *Nicotiana plumbaginifolia* (wild type, 2n = 2x = 20). After selection on increasing concentrations of kanamycin (10→30→100 mg/l) a high number (> 300) of hybrid calli was obtained. Karyotype analysis revealed that in a number of hybrids elimination of potato chromosomes (up to 70%) had occurred, whereas in some hybrids chromosomes of *N. plumbaginifolia* have been eliminated (up to 100%). Further characterization of the chromosome elimination process in sublines of the hybrids is currently in progress.

Dormancy Induction in Lily Bulblets Cultured *in vitro*

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Lily bulblets cultured *in vitro* are used in our laboratory as a model to study the induction of dormancy *in vitro*. Under standard conditions (culture of scale explants on MS medium with 3% sucrose at 20°C), the bulblets are formed after 3 weeks but become dormant after only 9–10 weeks of culture. Dormancy is broken by a cold treatment of 6 weeks at 2°C.

ABA is supposedly involved in the induction of dormancy in seeds. In our experiments though ABA applied during the culture had no effect. From the many factors examined, only sucrose and temperature affected dormancy. High sucrose concentration (> 1%) and high temperature (25°C) speeded-up development and resulted in deeper dormancy. In contrast, at low temperature (15°C), the bulblets never became dormant whereas at low sucrose concentration (< 1%) dormancy still developed, albeit later. The length of the cold treatment necessary to break the dormancy increased both with the length of the culture and the temperature applied during the culture. We have also examined whether the change of the physiological status of primordia during the induction and breaking of dormancy coincided with a change in isoenzyme patterns. No such change was observed.

In-vitro Storage of Potato Plant Material

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Storage of sterile, healthy plant material may be important for two purposes: 1. to be used as a source for the rapid multiplication 'in vitro'; and 2. to be stored for a long period in a gene bank.

The aim of the experiment was to store plant material of four potato cultivars for a period of several years with maintenance of the growth vigour.

Plantlets were grown at 17°C in darkness on a medium containing 8% sugar, 0·5 mg/l benzylaminopurine (BAP), 4 g/l Murashige and Skoog (MS) nutrients and 0·9% agar (pH 6). After tuberization from each cultivar equal numbers of plantlets were transferred to 4 and 8°C, under 8 h daylength (light intensity 3 W/m^2). After 1 year of storage from each treatment 10 tubes were taken. To determine the reproduction value of this plant material tubers and cuttings from the stems were transferred to tubes with medium containing 3% sugar, 4 g/l MS and 0·9% agar (pH 6). The tubes were placed at 18°C under 16 h daylength (24 W/m^2). This procedure was repeated every 3 (after 2 years of storage every 6) months.

During storage at 8°C three cultivars produced several generations of tubers. New stems were also formed at this temperature.

At 4°C no tuber formation occurred with all cultivars. After 18 months of storage the plantlets and tubers of cv. Prominent were dead, whereas after 30 months with cvs Jaerla and Bintje only 30 and 40% respectively, of the plantlets and tubers were dead.

Cultivar Kennebec showed relatively few differences between both storage temperatures, with a tendency to less senescence at 8°C than at 4°C after 30 months of storage.

Because of the formation of new tubers at 8°C the possible duration of storage will probably be extended by several years in three of the four cultivars.

POSTERS:

Distribution of ^{14}C-Labelled 2,4 D in Cultured Immature Embryos of Maize

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Callus formation and somatic embryogenesis in *Zea mays* L. are stimulated by 2,4-dichlorophenoxy-acetic acid (2,4 D). These processes occur in distinct regions of excised immature embryos cultured *in vitro*. We studied whether the distribution of 2,4 D corresponded with the distribution of cellular differentiation in the embryo.

Immature embryos were cultured in the dark on N6 medium supplemented with 1 mg/l 2,4 D for 1 or 4 days and then cultured on N6 medium with 1 mg/l [^{14}C]2,4 D for 16 h. Semi-thin sections of Technovit-embedded embryos were attached to slides and coated with Ilford L4 emulsion for autoradiography. After an exposure time of 3 weeks films were developed and silver grain densities, visualizing the location of ^{14}C, were determined on photographs.

It was found that living parts of embryos took up [^{14}C]2,4 D. The distribution of ^{14}C in young and older embryos showed similarities in parts of the scutellum where no structural changes were observed. Differences in the distribution between young and older embryos were observed in differentiating parts of the embryo, e.g. the scutellum base and the root–shoot axis. Regularly, many silver grains were found at cell borders and in the cytoplasm, but few in vacuoles and nuclei indicating a distinct subcellular distribution of 2,4 D. Degenerated tissues did not show label.

It is concluded that tissue-specific accumulation of [^{14}C]2,4 D occurs. The uptake and distribution of 2,4 D probably takes place by selective transport towards, or selective accumulation into various regions of the embryo.

Transfer of Genetic Information Between Unrelated Plant Species by means of Asymmetric Somatic Hybridization

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By means of somatic hybridization genetic material of unrelated plant species can be combined. After the fusion event the development of a fusion product depends on the cooperation of both genomes and plastomes. Somatic hybrids of unrelated species often give rise to abnormal plants, do not differentiate or sometimes fusion products do not even divide.

When only a small part of the genome of an unrelated donor species is combined with the total genome of a receptor species, chances to obtain asymmetric somatic hybrid plants are higher. In several asymmetric somatic hybrids extra chromosomes or chromosome fragments have been detected. It is also possible that chromosome fragments are incorporated in the genome of the receptor species.

Tomato is a useful species for investigating the possibilities of asymmetric hybridization because genetically it is very well characterized, allowing

detailed analysis of fusion products. For these experiments we used as a receptor tomato genotypes with a good regeneration capacity that contained a cell-selectable antibiotic resistance. As donor parents, a range of plant species transformed with the T-DNAs, with either kanamycin resistance (NPTII) and β-glucuronidase (GUS) or hygromycin resistance (AphIV) is used.

Asymmetric protoplast fusions with the tomato as a receptor have been made with *Nicotiana plumbaginifolia, N. tabacum* SR1 and *Solanum lycopersicoides*. Antibiotic-resistant calli grow and show shoot formation on selective media. In addition fusions have been made between cytoplasmic albino cell suspension protoplasts of tomato as a receptor and 0, 50 or 500 Gy γ-irradiated leaf mesophyll protoplasts of a *S. tuberosum* NPTII + GUS transformant as a donor. Green calli were selected as putative fusion products. Greening is assumed to be due to chloroplast transfer from the *S. tuberosum* donor. Cytological observations on fusion products show retarded nuclear fusions after irradiation, which may result in the formation of more cybrids. Further analysis of the fusion products using species-specific repeat probes is currently underway.

Analysis of the Cytoplasm of Somatic Hybrids of *Lycopersicom peruvianum* (+) *Lycopersicom esculentum*

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The aim of this study was to examine the effect of various doses of irradiation on the organelle trait in fusion products obtained after symmetric and asymmetric fusion of *Lycopersicon peruvianum* with *Lycopersicon esculentum* (Wijbrandi *et al. Plant Cell, Tissue Organ Cult.* 1988, **12:** 193–196). In the asymmetric fusion the *L. peruvianum* protoplasts were irradiated with various doses of γ-ray (50, 300 and 1000 Gy). For the analysis of chloroplast and mitochondrial DNA present in these fusion products the chloroplast probe PCY64 (De Haas *et al. Mol. Gen. Genet.* 1986, **202:** 48–54) and the mitochondrial probe, the EcoRI-SalI fragment (Young, E.G. & Hanson, M.R. *Cell* 1987, **50:** 41–49), were used. Autoradiographs of Southern blots containing total DNA prepared from the parents and the fusion products hybridized with PCY64 show that only one of the chloroplast types is present in the fusion products and that they segregate randomly. In the case of the mitochondria the analysis shows that the mitochondrial DNA of several fusion products is different from those of the parents and from their mixture. From these results it can be concluded that high irradiation dose rates do not influence the segregation pattern of the chloroplasts nor the rearrangement events in the mitochondria. Furthermore, high irradiation dose rates (up to 1000 Gy) do not inactivate the chloroplasts.

Differentiation of Hairy Roots from Leaf Protoplasts of Ri-Transformed *Nicotiana plumbaginifolia*

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Light and electron microscopical studies on root formation from callus or from *in-vitro* cultured stem tissue are very scarce. Here, a system is described which enables investigation of the initiation and early development of hairy roots from protoplast-derived microcalli of a Ri-transformed *N. plumbaginifolia* line.

Leaf discs of *N. plumbaginifolia* (wild type) were infected with *Agrobacterium tumefaciens*, strain LBA 1020, containing pRi 1855 with TL- and TR-DNA. Various transformed root clones showed spontaneous plant regeneration on hormone-free MS medium (Murashige T. & Skoog, F. *Physiol. Plant.* 1962, **15:** 473–497) containing 30 g/l sucrose. Leaf protoplasts were isolated from one of the regenerated plant clones (line Np99) using cellulase (1%) and macerozyme (0·2%) during overnight incubation. Leaf protoplasts of the wild type line of *N. plumbaginifolia* were used as control.

The protoplasts were cultured at a density of 5×10^4 per ml in 1/2 V-KM medium (Bokelmann, G. S. & Roest, S. *Z. Planzenphysiol.* 1983, **109:** 259–265) to which 0·3 mg/l naphthylacetic acid and 0·1 mg/l zeatin were added for callus formation and root development.

Within 3–4 weeks, development of a single hairy root occurred with a high frequency from protoplast-derived microcalli of the line Np99. Microcalli from protoplasts of the wild type very rarely responded with root formation. Light and electron microscopy indicated that root differentiation most probably started from one single cell. Root initial cells could be observed in cross-sections of microcalli from the 10th day after protoplast culture. These cells contained large numbers of small starch granules, but also numerous electron-dense granules. These osmiophilic structures were characteristic for initial cells and resulting morphogenic tissue since they were not found in non-morphogenic callus cells and in the microcalli from the wild-type protoplasts. The initial cell developed into a meristematic globular structure

by organized cell divisions. After approximately 14 days the globular structure showed polarity by forming one root apex. This apex gave rise to further root development, resulting in a visible root of 2–5 mm in length at 3–4 weeks after protoplast culture.

The present system is attractive to study cytological, histochemical and (molecular) biochemical determinants of root differentiation from undifferentiated cells or tissues.

Basic Peroxidase Activity and Rooting in Microcuttings of *Malus*

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We have examined in microcuttings of *Malus* cv. Elstar (1) the occurrence of enzymatic markers for rootability and (2) the timing of inhibition of rhizogenesis in poor rooting shoots.

1. We found a positive correlation ($P < 0{\cdot}001$) between the basic peroxidase activity (basPox) at the time of transfer to rooting medium and the number of roots at 21 days after the transfer. However, this correlation only occurred if the non-rooted shoots had been omitted from the calculations.

2. The course of basPox is thought to be closely correlated with the successive stages in rhizogenesis (Gaspar, Th. In: Jeffcoat, B. (ed.) *Aspects and Prospects of Plant Growth Regulators* 1981, 39–49. British Plant Growth Regulator Group, Wantage). We also found an increase in basPox during the first stages of the rooting process, but not a subsequent decrease. This increase also occurred in non-rooted shoots on rooting medium and in shoots on propagation medium. In both types of non-rooted shoots callus was formed. Microscopical observations revealed that poor rooting was not caused by inhibition of the outgrowth of root primordia, but by reduction of the initiation of root primordia.

We suggest that the increase in basPox is related to cell divisions that may result in root primordia and/or callus on rooting medium or in callus on propagation medium and that in poorly rooting shoots redifferentiation into root primordia is inhibited.

The Influence of the Substrate during Acclimatization of *Quercus robur in vivo* after Induction of Rooting *in vitro*

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Woody species produced *in vitro* require a period of acclimatization to adapt functioning of roots and leaves to natural surroundings: a lower humidity, a lower temperature, a higher light intensity, etc. Experiments were carried out using seedling-derived shoots with an *in-vitro* age of 3 years. In the literature rooting is said to be stimulated by an increased carbon supply. Two concentrations of sucrose were therefore tested: 2% (control) and 3%. Rooting was induced on woody plant medium (WPM) with addition of activated charcoal (8 g/l), sucrose (20 g/l) and various concentrations of IBA. The effect of IBA on growth and development *in vivo* was tested in peat–perlite (1:1). Two modified rooting media containing activated charcoal (8 g/l) were tested simultaneously: WPM sucrose (30 g/l) lacking indolebutyric acid (IBA), and WPM sucrose (20 g/l) with IBA (1·0 mg/l). In an earlier experiment an efficiency of over 95% was obtained on these media. The *in-vivo* development of rooted plantlets from that experiment was studied during 5 months on various substrates; the plants spent the first 2 weeks in the mist tunnel (relative humidity 95%).

Using IBA to induce rooting *in-vitro* stimulated growth *in vivo*; the optimum was 0·25 mg/l. However, the sucrose concentration had a larger impact on growth *in vivo* than IBA. During the acclimatization period the plantlets in potting compost developed faster than those in peat. In contrast, acclimatization in potting compost–perlite strongly depended on which type of induction had been used to induce rooting *in vitro*. A decrease in absolute extension growth was caused by the combination of dormancy and the death of some of the fastest growing plants.

It is concluded that the fastest growth *in vivo* does not depend on the best type of induction of rooting *in vitro* but on the substrate during acclimatization.

Callus Induction, Plant Regeneration and Protoplast Culture in Onion (*Allium cepa* L.)

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In-vitro procedures are being developed in order to facilitate the breeding of commercially valuable *Allium* species.

Callus induction and plant regeneration. Callus was induced using embryos isolated from mature seeds. For callus induction and maintenance Murashige and Skoog's (MS) medium (Murashige, T. & Skoog, F. *Physiol. Plant.* 1962, **15**: 473–497) was used, supplemented with (per litre) 1 mg 2,4-dichlorophenoxyacetic acid, 20 g sucrose and 10 g agar (pH 5·8; 25°C; darkness). Plants were regenerated from morphogenic callus of cvs Balstora, Jumbo, Marcia, Noordhollandse bloedrode, Oporto and Robot.

Plant regeneration medium was MS-medium supplemented with 1 mg/l kinetin (25°C; light). Morphogenic callus was rough and/or nodular. Plant regeneration occurred via organogenesis. Somatic embryos were not observed on the callus surface. Regenerated plants retained the diploid chromosome number (2n = 16). Green shoots could be regenerated from callus of up to 13 months old (cv. Jumbo).

Protoplast isolation and culture. Protoplasts were enzymatically isolated (cf. Tan *et al.*, *Plant Cell Reports* 1987, **6:** 172–175) from leaves of young seedlings and also from callus. Protoplasts were cultured in RY2-medium (Yamada *et al.*, *Plant Cell Reports* 1986, **5:** 85–88) modified VKM-medium (Bokelmann, G. S. & Roest, S. *J. Plant Physiol.* 1983, **109:** 259–265) and MS-medium. Callus protoplasts (cv. Jumbo), cultured in agarose-solidified RY2-medium (500–700 mOsm), regenerated a cell wall and showed budding. Sustained cellular divisions have not yet been observed.

Cryopreservation of Lily Meristems

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Cryopreservation of plant material may become an important tool for long-term conservation of germplasm. Meristematic tissues are the most appropriate material because of their genetic stability. On the other hand cryopreservation of organized tissues like meristems is more difficult to achieve than cryopreservation of cell cultures or callus.

We used meristems induced on lily scale explants as a model system and pretreated them in two ways: 1. freeze-hardening. The scale fragments and/or excised meristems were grown at low temperature and/or on high sugar medium for 1–14 days; and 2. cryoprotection. The meristems were incubated in solutions with various concentrations of DMSO, glycerol and sucrose during 2 h at 20°C or 24 h at 4°C. Both pretreatments were applied in several combinations.

The meristems were then frozen slowly at a cooling rate of 0·6°C/min to −40°C and subsequently plunged into liquid nitrogen; alternatively, they were plunged directly into liquid nitrogen and then kept at −196°C for at least 1 day. After thawing in a water bath at 37°C they were transferred to propagation medium and initially held in the dark at 20°C. Resumption of growth occurred after 1–3 months. Surviving meristems were transferred to fresh medium and grown in the light.

Only four pretreatments, all including a long preculture of the meristems at 4°C, were successful. Preculture on MS 1/1 with 10% sucrose followed by treatment with 5% DMSO + 15% glycerol, 5% DMSO + 10% sucrose, and 5% DMSO + 10% sucrose + 5% glycerol resulted in a survival percentage of 2, 5 and 2 respectively. Preculture on MS 1/1 with 5% DMSO + 10% sucrose, followed by plunging into liquid nitrogen resulted in 8% survival.

Reduction of Contamination in Twin-Scale Explants of *Narcissus*

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Cultures of twin-scale explants of *Narcissus* 'Golden Harvest' were contaminated with fungi, especially *Fusarium*. By intensification of the disinfection procedure of halved bulbs in 1% (w/v) sodium hypochlorite we tried to reduce this contamination. In a first attempt the hypochlorite treatment was extended from 30 min to 60, 90 or 120 min. This affected neither contamination nor regeneration in the explants. In a second attempt halved bulbs were treated with 1% hypochlorite for 30 min and rinsed with sterile water. Subsequently, large-sized twin scales were cut, disinfected again in 1% hypochlorite for 10 min and rinsed with sterile water, before normal-sized twin scales were cut. This double disinfection procedure also failed to reduce contamination. We therefore concluded that the contamination was inside the tissue.

The next approach was to investigate the effect of a warm-water treatment (WWT) of whole bulbs before the disinfection procedure with hypochlorite was carried out. For this purpose field grown bulbs were harvested and immediately stored at 30°C. After 1, 3 or 5 months of storage a WWT was performed. We tested the effects of immersion in water of 54°C for 0, 1, 2 or 3 h and immersion for 1 h in water of 50, 54, 58 or 62°C. After the treatments the bulbs were dried on filter paper in a clean room for 1 day, halved, treated with 1% hypochlorite for 30 min and rinsed with sterile water before the explants were cut. After 12 weeks of culture at 15°C the contamination and regeneration in the explants was scored. Twenty-eight to sixty per cent of the twin scales were contaminated when no WWT was carried out. All of the non-contaminated twin scales regenerated bulblets. A treatment of 1 h at 50°C reduced the contamination to 18–55%. Almost no contamination (0–7%) was observed after a treatment of 1, 2 or 3 h at 54°C and after 1 h at 58 or 62°C. In the latter two treatments no regeneration in the twin scales was observed. A reduction of regeneration (72–100%) in the twin scales was found after a treatment of 2 or 3 h at 54°C. In all the experiments with a WWT of 1 h at 54°C the contamination was almost zero (0–5%), while no reduction in regeneration (96–100%) was observed. We tested the 1 h 54°C WWT on five other cultivars of *Narcissus* and

found the same results. We concluded that a treatment of 1 h at 54°C effectively reduced the contamination without affecting the regeneration in twin-scale explants.

Utility of Available Selection Markers for Transformation of *Lilium* Bulb-Scale Tissue

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In the past transformation experiments have been performed on bulb-scale tissue of *Lilium* using *Agrobacterium tumefaciens* as vector and kanamycine as selection marker. Plants were obtained on medium containing 100 mg l^{-1} kanamycine, but no biochemical evidence for their transformation could be obtained. In this investigation we examined the utility of available selection markers for transformation of bulb-scale tissue of *Lilium*. Bulb scales of 'Star Gazer' produced *in vitro* were cultured in Petri dishes on medium with or without 100 mg l^{-1} kanamycine, neomycine, metha-trexaat or hygromycine B. The scales were cultured in the dark at 20°C and transferred every 14 days to fresh medium (Murashige and Skoog salt mixture, 100 mg l^{-1} myo-inositol, 0·4 mg l^{-1} thiamine, 30 g l^{-1} sucrose, 0·1 mg l^{-1} 1-naphthylacetic acid, 6 g l^{-1} agar, pH 6·0). After 12 weeks the regeneration of bulblets and their weight was scored.

We found that 70·5% of the bulb scales cultured on kanamycine-containing medium regenerated bulblets. For neomycine this value was 64·5%, for metha-trexaat 6·7% and for hygromycine B 0·0%. Bulb scales cultured on medium without selection markers regenerated (97·5%) and produced bulblets with a mean weight of 43·7 mg. The mean weights of bulblets produced on medium containing neomycine, kanamycine or metha-trexaat were 35·3, 23·5 and 14·0 mg, respectively. From these results we conclude that hygromycine B is the most suitable selection marker to use for transformation of lily bulb-scale tissue.

MEETING OF THE SECTION FOR PLANT MORPHOLOGY, ANATOMY AND CYTOLOGY ON 26 MAY 1989

Ovule and Seed of the African Saprophyte *Voyria primuloides* (Gentianaceae)

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The development and structure of the seeds in the neotropical species of *Voyria* have been described by Bouman & Devente, (In: Maas & Ruyters. Voyria and Voyriella (saprophytic Gentianaceae). *Flora Neotropica* Monograph 1986, **41**: 9–25). Within the genus, trends in reduction from unitegmic, anatropous ovules towards ategmic, orthotropous ovules could be demonstrated. Based on the micromorphology, four different groups are recognized.

Voyria primuloides Baker is the only paleotropical species and grows in the rain forest of tropical West Africa. Its ovule and seed belong to the most reduced ones within the angiosperms. The ovule is ategmic and atropous. During seed development the ovule and embryo sac do not increase further in size. The embryo is 3- to 4-celled only and surrounded by about eight endosperm cells. The seed coat is undifferentiated and one cell thick. Mean seed size is $75 \times 235 \times 10^{-6}$ m. The mature seeds are liberated by damage or decay of the fruit wall. The seeds are probably dispersed by rain-wash and epizoochory in mud on legs, pelt or feathers of animals. On the basis of the embryological characters *Voyria primuloides* is most closely related to the neotropical *Voyria rosea* group.

Characterization of Isolated Spinach Sperm Cells

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Gametes are haploid cells. They can fuse with other cells and transmit genes from one generation to the next. These characteristics could all be of importance in biotechnology and molecular biology. In order to use sperm cells for biotechnology, and in general, to know more about their capacities and functions in the fertilization process, sperm cells are isolated from pollen grains and examined in different ways.

Sperm cells inside the pollen grain are spindle shaped, and the two sperm cells of one pollen grain are connected to each other, forming a pair. In contrast, isolated sperm cells (Theunis & Van Went, *Sex. Plant Reprod.* 1989, **2**: 97–102) become spherical and separate from each other. Their diameter varies from 4 to 8 μm, depending on the osmolarity of the isolation medium. Ultrastructurally, the isolated sperm cells show the same organelles as the sperm cells inside the pollen grain, except for microtubules. After staining with DiOC6(3) (3,3' dihexyloxacarbocyanine iodide) and examination by fluorescence microscopy, individual mitochondria can be seen inside the cells. Isolated spinach sperm cells have an average number of $12{\cdot}4 \pm 3{\cdot}2$ mitochondria. Dimorphism of the sperm

cells with respect to their number of mitochondria per sperm was not found. The isolated sperm cells could be kept alive (fluorescein-diacetate positive) for 10 h at room temperature, 20 h at 4°C, and 30 h at 4°C in a medium containing 1% vitamin C.

Changes in Calmodulin Distribution During Somatic Embryogenesis in *Daucus carota* L.

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Carrot somatic embryogenesis is used extensively as a model system for early plant development. Somatic embryos of carrot develop from small clusters of cells designated pro-embryogenic masses (PEMs). Morphological, physiological, and biochemical polarization are considered to be important factors in embryogenesis. Ca^{2+} participates in triggering and maintaining polarized growth. Calmodulin is a Ca^{2+}-binding protein which is important during plant growth and development.

The distribution of Ca^{2+} and calmodulin was studied during the process of somatic embryogenesis in carrot. Calcium distribution was visualized by chlorotetracycline (CTC) fluorescence, which is a marker of membrane-bound calcium. Calmodulin distribution was studied by fluphenazine fluorescence, which is a marker of the activated Ca^{2+}-calmodulin complex and by immunocytochemistry, which gives an image of the total calmodulin level.

The CTC signal in embryo primordia and embryos was very intense in comparison with the signal in PEMs. This means that embryonic cells possess a higher Ca^{2+} concentration or contain more membranes than the cells of the PEMs. Fluphenazine fluorescence was present in certain regions of PEMs and absent in other regions. In the globular, heart-shaped, and torpedo-shaped stage the fluorescence was restricted to the basal part of the embryo. Only in old torpedo-shaped embryos was the shoot apex also fluorescent. The immunocytological observations showed that the total amount of calmodulin was evenly distributed in PEMs and the early phases of somatic embryogenesis. In late torpedo-shaped embryos, calmodulin appeared to be present in plastids in the outer layers of the embryo.

The Actin Cytoskeleton in Sub-Protoplasts from Pollen Tubes of *Nicotiana tabacum*

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Actin filaments in regenerating and growing pollen tube sub-protoplasts of *Nicotiana tabacum* were studied using fluorescent probes. The filaments in freshly produced sub-protoplasts were severely disordered and often fragmented. During sub-protoplast regeneration the F-actin became re-organized into a cytoplasmic network with a random orientation, and into a cortical network of highly ordered filaments.

The cortical actin network was highly dynamic. At first circumferential arrays of filaments were formed, evenly distributed over the cell ortex. Progressively, the cortical filaments were either concentrated into bundles or converged into two opposite cortical foci. Actin filament organization reflected a polarity which determined the direction of outgrowth. The presence of a generative cell and/or vegetative nucleus had no influence on the distribution of the actin filaments. We therefore conclude that the cytoplasm of the pollen tube sub-protoplasts has the intrinsic capacity to reorganize the actin cytoskeleton into a series of highly ordered cortical arrays.

Electron Microscopical Investigation of *Vicia hirsuta* Root Nodule Cells Infected with *Rhizobium leguminosarum* Biovar *Viciae* Strain 248 Mutants, with Altered Lipopolysaccharides

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Lipopolysaccharides (LPS) present in the outer membrane of the bacterium *Rhizobium* seem to play a role in the establishment of an effective symbiosis, i.e. the formation of root nodules which can fix atmospheric nitrogen in a leguminous host plant.

In order to study the role of LPS in nodulation, LPS mutants of strain 248 were selected (de Maagd *et al. J. Bacteriol.* 1989, **171:** 1143–1150). The mutants do not react with a monoclonal antibody (mAb3) which recognizes an epitope on the 'O-antigenic' part of LPS (de Maagd *et al. J. Bacteriol.* 1989, **171:** 5901–5907). Based on their LPS profiles after SDS–polyacrylamide gel electrophoresis the mutants were divided in different classes: (I) various mutants without the slower moving LPS species, which contains the 'O-antigenic part', (II) a mutant with a small amount, and (III) a mutant with a normal amount of slow moving LPS.

Class I and II mutants produced spherically shaped ineffective nodules in *Vicia hirsuta*. The nodules lacked the zones of cells in different stages of infection as found in the case of infection with the wild-type bacterium; they just had a small group of infected cells in various stages in the middle of the nodule. Nodules infected with the class III mutant were similar to those infected with the wild-type bacterium.

Ultrastructural studies showed that at 4 weeks after inoculation, the nodules infected with a class I mutant

(Ia) had normal infection threads (IFs) but no bacteria had left the IFs. Another class I mutant (Ib) had produced very wide IFs. However, most intracellular IFs degenerated before the bacteria had left IFs. Nodules infected with a third class I mutant (Ic) had IFs of normal size and some mature bacteroids were observed. Nodules infected with class II mutants were similar to those infected with mutant Ic, except that in some cells bacteroids were degenerated already. Class III mutants produced normal root nodules.

These results also point to a relationship between the presence of the 'O-antigenic' part of LPS and the establishment of an effective symbiosis. When nodules are infected with mutants with LPS lacking the 'O-antigenic' part, IFs are delayed in reaching the nodules and few bacteria leave the IFs.

Cellular Polarization and Spatial Control of Cell Division in the Unicellular Green Flagellate *Brachiomonas submarina* Bohlin

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In an attempt to determine the possible role of the flagellar apparatus (FA) in polarization of the division process, multiple fission ('Vielfachteilung') in sporangia of the unicellular flagellate *Brachiomonas submarina* has been investigated using serial section analysis. Since, during the second and third sporangial division sequence, cytokinesis is initiated before mitosis, we were able to localize a premitotic organizer of the division site, the (paired) four-stranded flagellar root. Prior to semiconservative replication of the FA, these two roots lie below the premitotic plasma membrane invagination, but once a spindle and two daughter FA's have been formed, their proximal ends are re-oriented such that they are more or less co-aligned with the spindle axis. This re-orientation appears to be mediated by lateral association of the two strongly curved roots in the plane of division via microtubules of opposite polarities. The data strongly suggest that these roots perform multiple functions, including: (1) segregation of the daughter FA's, (2) (indirectly) positioning of the spindle, (3) establishment and maintenance of a plasma membrane invagination prior to and throughout mitosis, and (4) these roots are actively involved in the formation of the phycoplast (Segaar *et al. Nova Hedwigia* 1989, **49**: 1–23) that polarizes the cytoplasm for directed growth of the cleavage furrow once daughter nuclei have been widely separated. It is concluded that the parental four-stranded roots are essential cytomorphogenetic tools for the spatial control of multiple fission in walled flagellates, which may also be present in related flagellated organisms such as the (colonial) Volvocales.

MEETING OF THE SECTION FOR VEGETATION RESEARCH ON 12 APRIL 1989

Population Dynamics of *Gentiana pneumonanthe* L. (March Gentian) After Sod Removal: a Model Study Based on Long-Term Field Observations

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In The Netherlands *Gentiana pneumonanthe* L. is a rare species in wet heathlands and grass heaths. Since 1950 it has disappeared from 25% of the sites in which it once occurred. It has been suggested that this decline is related to increasing competitive ability of companion species and by humus accumulation as there is no more sod removal by farmers today.

In spring 1979 six experimental populations of *G. pneumonanthe* were established from seed after sod removal in a nutrient-poor *Erica* heathland and in a somewhat more nutrient-rich *Molinea*-dominated grass heath. Until now relevant demographic information has been collected each season with 6-week intervals such as time to first reproduction, seed production, germination success, survival of seedlings, juveniles and adults and decline of seed viability in the soil. This information is incorporated into a stage-classified matrix projection model. Those transition probabilities in the model that showed significant trends over time, as a result of increasing density dependence, have been replaced by explicit time functions. This was the case for seed production, germination success and establishment.

Projected time trajectories for both populations fell within the range observed in the field. In the long run the population from the *Erica* heathland will probably go extinct. In this habitat adults take too long to become reproductive, notwithstanding good opportunities for germination and establishment in the relatively open vegetation. In the plots in the *Molinea*-dominated grass heath the reverse is true. Adults start flowering fairly quickly, whereas opportunities for seeds and seedlings are rapidly diminishing. Nevertheless, this population will probably survive. To a large

extent, however, survival or extinction depends on local small-scale variability within the habitat. Such variability affects long-term population growth more severely than variability between years as shown by stoichastic simulation of the model.

Do Plants Cry For Help?

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Herbivory is a major determinant of plant fitness. To defend themselves plants may use a direct mode of defence, such as the production of toxic substances and digestibility reducers (Rhoades & Cates, *Rec. Adv. Phytochem.* 1976, **10:** 168–213), or they may use an indirect mode of defence such as production of substances that increase the effectiveness of natural enemies of herbivores (Price *et al.*, *Ann. Rev. Ecol. Sys.* 1980, **11:** 41–65). These modes of defence can be classified as inducible and constitutive, depending on whether the defensive action occurs before or after herbivore attack.

Most defensive actions of plants against herbivores studied to date are examples of constitutive and direct defence (Rhoades, *Am. Nat.* 1985, **125:** 205–238), but evidence for inducible direct defence is increasing. In addition, evidence for inducible indirect defence has recently been presented in a system consisting of predatory mites, spider mites and their host plants (Dicke, PhD Thesis, *Wageningen Agricultural University*, 1988).

Spider mites are a serious threat to their host plant because in the absence of predators they over-exploit the plant as a food source. When predatory mites discover the spider mite colony in an early phase of population growth this is to the benefit of the plant because the predators are capable of eliminating the spider mite population before the plant becomes over-exploited. Recent investigations showed that upon attack by spider mites plants release volatile chemicals, some mono-terpenes and a phenolic compound, which are not produced after artificial damage to the plant. These chemicals appear to contain very helpful information to predatory mites in search of prey, i.e. not only to find prey but also to select suitable prey species. This plant response to herbivore attack may have the advantage of getting protection from appropriate natural enemies at the earliest possible time (though after at least some damage has been done). Clearly, inducibility of the alarm system is a major feature of the plant's defence strategy. At an evolutionary time scale there may well have been an 'arms race' between plants leading to changes in the quantity and quality of the chemical information produced and even to polymorphisms in investment patterns (Sabelis & De Jong, *Oikos* 1988, **53:** 247–252).

VAM and Acid Rain

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It has been demonstrated that disturbance of the balance in the ectomycorrhizal symbiosis by air pollution affects forest decline. Not only forests are affected by air pollution. Low vegetation, both semi-natural and agricultural crops, also suffer from air pollution. For semi-natural vegetation the input of atmospheric nitrogen is the most important factor, especially for vegetation on nutrient-poor sandy soils, such as heather vegetation. The disappearance of some herbaceous plants from heather vegetation is evident. Analogous to trees, herbaceous plants have mycorrhiza, namely vesicular arbuscular mycorrhiza (VAM). The disappearance of herbaceous plants from heather vegetation could possibly be the result of a disturbance of this mycorrhizal symbiosis. Therefore the influence of ammonium sulphate and pH on VAM was investigated in greenhouse and field experiments. Plants used were *Arnica montana* L., *Viola canina* L., *Antennaria dioica* (L.) Gaertner, *Hieracium pilosella* L. and *Deschampsia flexuosa* (L.) Trin. Plants were rained upon artificially with ammonium sulphate solutions in several experiments. Neither a negative nor a positive effect on VAM infection could be detected. However, increased ammonium availability did increase nitrogen content of the plants and enhance growth.

Plants drained weekly with nutrient solution of different pH values showed a reduced growth with the most acid treatments, but VAM infection was not affected.

Plants without VAM showed reduced growth compared to plants with VAM in all greenhouse experiments.

It is concluded that pH stress did affect plant growth in these experiments, but did not affect VAM. Ammonium sulphate, a major acid rain component in The Netherlands, enhanced growth of the plants and VAM infection was not affected.

Variability in Morphology and Life History of *Plantago major* in Relation to Small-Scale Environmental Heterogeneity

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At a primary beach plain, embanked in 1966, a mosaic environment was distinguished with spatial variability

in nutrient availability and water content of the soil as well as in vegetation structure (Lotz & Spoormakers, *Oecol. Plant* 1988, **9:** 11–18). Contemporary selection on *Plantago major* L. was studied in three sub-sites. In one sub-site, patches (*c.* 1m^2) with a high availability of nutrients and a dense cover of grasses, plants with a relatively high number of large leaves (both long and broad) had a higher total seed production than other plants. In the other sub-sites, an area with a low availability of nutrients and a low cover of higher plants and an area with shrubs of *Hippophaë rhamnoides* L., leaf width, and not leaf length, was positively correlated with relative fitness.

The effect of nutrient supply and waterlogging on morphology and life history was studied on lines from the three sub-sites in a greenhouse. For most of the traits studied high levels of phenotypic plasticity were observed, almost entirely covering the observed phenotypic variability of *P. major* at the beach plain. However, in all treatments, lines from the shrubs had a higher leaf–area ratio, but lower growth rates, as well as a delayed flowering when compared to lines from more open sub-sites. In addition, in a reciprocal transplant experiment it was demonstrated that lines from the shrubs had higher above-ground dry weight and, e.g. broader leaves in the shady environment of the shrubs than other lines.

From the experiments no indications were obtained that lines from any sub-site were specially adapted to specific levels of nutrient supply or water content of the soil. With respect to these environmental factors *P. major* might occur and reproduce in all sub-sites by performing phenotypic plasticity, e.g. in plant form. It is suggested that spatial variability in vegetation structure (i.e. light intensity at the soil surface), however, caused a population sub-division in allocation patterns, leaf form and life history of *P. major* at the beach plain during primary succession over a period of 20 years.

The Mechanism and Significance of Competition for Nutrients

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In many natural ecosystems plant growth is limited by the availability of nutrients (Chapin, *Ann. Rev. Ecol. Sys.* 1980, **11:** 233–260). For the acquisition of this growth-limiting soil-resource plants invest heavily in their roots (Caldwell & Richards, *On the Economy of Plant Form and Function* 1986, 251–273).

The efficiency at which plants acquire nutrients is likely to have important implications for growth in competition. This is especially relevant as competition experiments investigating the relative importance of below and above-ground competition showed that the balance between species is usually more affected by root competition than shoot competition (Wilson, *J. Appl. Ecol.* 1988, **25:** 279–296).

Current literature on the relationship between root characteristics and competitive ability showed that the size of the root system, expressed in relative weight, is an important parameter determining the competitive ability of plant species (Baan Hofman & Ennik, *Neth. J. Agric. Sci.* 1982, **30:** 275–283). Nutrient uptake models and experimental work has emphasized the importance of root length for the acquisition of nutrients (Silverbush & Barber, *Agron. J.* 1983, **75:** 851–854).

The root length per unit plant weight (RLR, m/g) is the product of the root weight ratio (RWR, root weight/total plant weight (g/g)) and the specific root length (SRL, root length/root weight (m/g)).

In a comparative growth experiment with *Molinia caerulea* and *Erica tetralix*, two species of wet heathlands, of which *Molinia* is competitively superior to *Erica* when the availability of nutrients is raised (Berendse & Aerts, *Acta Oecol. Plant.* 1984, **5:** 3–14), the question was addressed: what is the size of the root system (RLR) of both species, and what is the relative importance of the components of the RLR: the RWR and SRL?

The results show that *M. caerulea* realizes its root length by a high RWR (0·64 g/g) and a low SRL (63 m/g), whereas *E. tetralix* is characterized by a high SRL (280 m/g) and a low RWR (0·28 m/g). This negative correlation between RWR and SRL suggests that both parameters should be included in studies relating the competitive ability of plants with characteristics of their root system.

Effects of Herbivory on the Population Dynamics of the Biennial Plant Species *Senecio jacobaea* and *Cynoglossum officinale*

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Two contrasting ideas on plant–herbivore dynamics have caused a long debate in population ecology. On the one hand, the impact of herbivores is considered marginal: 'cases of obvious depletion of green plants by herbivores are exceptions' (Hairston *et al. Am. Nat.* 1960, **94:** 421–425). On the other hand, herbivores are thought of as 'a prime factor in regulating the abundance of all plants' (Brues, In: *Insect Dietary* 1946). Both extreme views have been subject to criticism, but conclusive information on the subject is lacking.

The present study (1985–1988) on the effects of herbivores on the population dynamics of *S. jacobaea* and *C. officinale* aims to contribute to the discussion.

Natural populations of both plant species were compared with populations that were protected against herbivores. Effects of herbivory on the number of seedlings, rosettes and flowering plants were measured and effects of herbivores on vegetation change were also monitored.

Leaf herbivory was much higher for *S. jacobaea* than for *C. officinale* for all 3 years studied (complete defoliation versus only 5% leaf area damage). Direct effects of herbivory on establishment of seedlings, rosette growth and flowering were found in populations of *S. jacobaea*. In contrast, no effect of herbivores was found in populations of *C. officinale*.

The impact of herbivores on the plant community by an increasing vegetation cover in protected populations was found in both *S. jacobaea* and *C. officinale*.

With respect to the controversy in plant–herbivore dynamics mentioned above we conclude that for *S. jacobaea*, herbivores do have the potential to affect plant populations. For both plant species, the effects of herbivores on the plant community are obvious and must be taken into account in studies on plant–herbivore dynamics.

Populations and Pollination

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Where does pollen go? This simple inquiry offers a rich diversity of possible interactions. Visiting a certain area pollinators encounter a vast array of potential food plants which vary manifestly in their density (and phenology), nutritional value and ease of handling. Faced with this diversity a pollinator must decide where to search, which species to feed from and which plants (and how many flowers per plant) to feed from and in what sequence. Foraging behaviour has consequences for the amount and organization of genetic variation within populations.

In co-operation with Dr O. Jennersten (University of Uppsala, Sweden) some data are presented of a 2-year study on bumble-bee visitation patterns in two co-occurring, simultaneously flowering species. *Melampyrum pratense (Scrophulariaceae)* and *Viscaria vulgaris (Coryophyllaceae)*. Observations were made in June 1986 and 1988 in South-western Sweden, covering the start of both species and peak flowering of *Viscaria*.

The two species share their main visitors: *Bombus hortorum* queens, but *Melampyrum* is also visited by four other bumble-bee species. Both plant species present pollen and nectar. Early flowers of both species had the highest visitation rates; during the season visitation decreased. Bumble-bees switched between both species, especially in 1988 (17% of all interfloral movements, 1·4% in 1986). Summarizing the data of both years, visitation of early flowers of both species was sufficient, but later flowers received too few visits. For *Melampyrum* this was due to high flower density of *Melampyrum*. *Viscaria* was faced with an increasing rate of improper pollen transfer. The presence of other flowering species during peak and late flowering in combination with the changing demand of foraging *B. hortorum* queens resulted in switching and the abandonment of *Viscaria vulgaris* as a food source.

MEETING OF THE SECTION PROTECTION OF THE WILD FLORA ON 24 APRIL 1989

Long Term Demographic Research on Rare Orchid Species in The Netherlands

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A fairly large number of plant species in The Netherlands can be considered endangered. The decline in sites of many species is well documented on the basis of the number of 5 × 5 km squares in which plant species occurred before and after 1950 (Mennema *et al.*, 1980; Mennema *et al.*, 1985). However, for determining the fate of rare and/or threatened long-lived plant species, detailed information on the life cycle and the size and age structure of the population are necessary. These data can be obtained by monitoring a number of individual plants during many years.

The establishment and development of the only population of *Orchis simia* Lamk. in the country, originating from one flowering plant, was monitored from 1972 onwards (Willems 1982). After 17 years of yearly recording, this population can be seen as established. The age structure of the population in 1988 showed that almost every cohort was represented, yet in a few individuals. Individual plants can be very long-lived. The original plant ('motherplant') is at least 20 years old and flowers almost every year. Shoot predation by rabbits and invertebrates (slugs, snails) is a serious threat.

Another orchid species, *Spiranthes spiralis* (L.) Chevall., decreased tremendously in site number in The Netherlands: before 1950 the species was present in at least 35 sites, of which only two remain, namely, Goeree and South Limburg (Mennema *et al.* 1980;

Mennema *et al.* 1985). A number of individual plants on the South Limburg site were monitored yearly from 1981 onwards. In that year there was an alteration of the management regime of the site as a consequence of gaining the status of Nature Reserve. After 8 years of recording, the age structure of the population showed a low recruitment level. Mortality exceeded recruitment, especially during the last 3 years and the population seems to vanish. However, based on the life cycle of the species, it is possible that after a 13–15 year period the alteration of the site management in favour of *S. spiralis* with result in an increased recruitment. Because of the almost yearly production of seeds at this site, there is a chance for this population to survive. Only another 8 years period of monitoring can give a decisive answer.