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MEETING OF THE SECTION FOR VEGETATION RESEARCH ON 12 JANUARY 1997 IN UTRECHT

Grasslands, Origin and History

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The present vegetation of Central and North Western Europe is the result of a lengthy interaction between natural processes and human influence. The predecessors of our present grassland communities are considered to be the open steppe tundra vegetation that existed during the pleistocene glacial periods. It appears that several grassland species immigrated during the Late Glacial and the early Holocene, when the landscape was still relatively open and the climate favourable for a number of Mediterranean and Pontic species to spread throughout Europe (Pott, R. 1995, *Phytocoenologia* **29**: 7–32).

The expanse of the dense primeval woodland of the early and middle Holocene has been increasingly lost since the onset of the Neolithic period, when man introduced a great number of typical anthropozoogenic grassland species (Koerber-Grohne, U. 1990, *Bibl. Bot.* **39**: 1–105).

It is possible, however, that many of the grassland species did survive the period of dense deciduous woodland in Boreal and early Atlantic times. They may have existed in regions where the woodland remained rather open, such as coastal and river flood plains, steep slopes and subalpine grasslands in mountainous areas, or they may have grown in areas that were kept relatively open by the grazing of large herbivores. Analyses of many archaeobotanical records suggest that most of our present grassland species, especially those belonging to the Arrhenatherion and Cynosurion, originally occurred in ruderal communities (e.g. arable and fallow fields), and in forest mantle communities. The majority of the recorded Poaceae species in Neolithic times is typical of ruderal vegetation communities.

Remarkably, the earliest record of the presently common and often dominant meadow and pasture species *Alopecurus pratensis* and *Arrhenatherum elatius* dates from medieval times. These species along with *Cynosurus cristatus* and *Phleum pratense*, have been imported from regions outside Central and North Western Europe.

Interactions in the Bryophyte Layer of Dutch Chalk Grasslands

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The bryophyte layer in the chalk grasslands in South Limburg (NL) is dense with many species growing intermingled. Fine-scale dynamics are high: shoots are frequently being replaced by each other. These replacements seem to be random and competition for light does not seem to play a role. However, light is expected to be a limiting factor for shoot growth in this bryophyte layer since more than 90% of the irradiant light is extinct in the upper few cm of the vegetation. Therefore, the role of competition for light in this layer was tested in a field experiment. Shoot densities in homogeneous stands of three common species, *Calliergonella cuspidata*, *Ctenidium molluscum* and *Rhytidiadelphus squarrosus* were reduced by 50%. Size distributions of the shoots in these stands were analysed and compared to controls. Inequality of the shoot size distribution was not higher at the end of the growing season than at the start. This was explained by the fact that asymmetrical competition for light did not play a role in structuring these bryophyte stands: large shoots did not have a disproportional advantage over small shoots as has often been found for phanerogams. Light limitation took place, but this only resulted in death at the basal end of the all shoots. Probably, the larger shoots could not take advantage of the high light availability at canopy level because growth at their top was restricted by the high evaporation rates they experience when they emerge from the canopy (these species do not have a cuticula). Inequality was highest in thinned stands, mainly due to new recruitment. Many of the thinned stands returned to (nearly) their original natural density within 2 years.

Restoration of Acidified or Eutrophied Nardo-Galion Communities: Possibilities and Constraints

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Plant diversity of the Dutch landscape is mostly restricted to (semi-)natural ecosystems in nature reserves. Many characteristic plant species, however, have been declining in the last decades, because of environmental stresses, such as air pollution, eutrophication or lowering of the water table. Semi-natural ecosystems of high nature conservation importance on the sandy Pleistocene areas have been especially affected. Ecological restoration has to play a vital role in reducing these negative effects and to reinstate the damaged plant communities.

Species-rich dry matgrass swards (Nardo-Galion saxatile) and heaths are mainly threatened by soil acidification, and restoration measures should focus primarily on counteracting these effects. Therefore, field liming experiments have been set up in several deteriorated Nardo-Galion saxatilis and dry heath communities. The plots have also been sod cut to (i) remove the accumulated nutrients, and (ii) initiate germination and establishment possibilities. Topsoil chemistry, establishment of endangered plant species and vegetation development were quantified from 1990 to 1996. It is concluded that liming after sod cutting is an adequate method to restore weakly buffered, oligotrophic soil conditions in acidified Nardo-Galion grasslands. The recovery of the characteristic vegetation composition is, however, seriously limited when the characteristic species disappeared some years before the experimental restoration measures started. This is probably caused by the non-persistent seed bank of almost all endangered Nardo-Galion species and the restricted seed dispersal seed bank of almost all endangered Nardo-Galion species and the restricted seed dispersal capacities of most plants. Experimental re-introduction of the plant species involved should be considered under these conditions, to restore these typical communities.

It was shown that species-rich wet heath and Nardo-Galion grasslands were restricted to oligotrophic, but slightly or weakly buffered soil conditions, originating from seepage with buffered (local) groundwater in the rooting zone or loamy soil conditions. Acidification, caused by atmospheric deposition and desiccation, is thus a serious threat for the maintenance of these species-rich situations, together with enhanced N eutrophication. We found that suitable hydrological measures, in combination with sod cutting, can rehabilitate acidified and eutrophied wet soils by allowing seepage of buffered groundwater. Within 5–6 years, species-rich plant communities recovered in several sites. It is likely that this relatively quick response is related to the presence endangered species in the soil seed bank under these more-or-less wet, eutrophic conditions and seed sources in nearby sites (<500 m). A serious constraint for the restoration of these species-rich wet communities is when the seepage water is also acidified; additional research is needed to solve this problem. It is finally concluded that for the long-term preservation of the restored ecosystems, the inputs of atmospheric pollutants should decrease below the set critical loads in near future.

Effects of Grass-encroachment on Soil and Vegetation of Coastal Dry Dune Grasslands and Management Measures for Regeneration

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In The Netherlands, the economically important functions of the dunes are coastal defence, drinking water catchment and recreation. In spite of this the coastal dunes are of great importance for nature conservation, since they harbour almost 70% of the species of the Dutch flora. The dune area is very dynamic and is characterized by a large variability in biotic and abiotic properties. This research is focused on the species-rich dry dune grasslands, which belong to the xerosere. These grasslands have to a large extent changed into a vegetation dominated by tall grass species. These changes in vegetation structure are associated with a loss of species diversity and thus grass-encroachment leads to a decrease of nature conservation values. The causes are not yet well understood: in the dry dune system more factors – atmosphere N-deposition, P-limitation, rabbit grazing, former management – are involved and it is not easy to separate them. This research focuses on the effects of grass-encroachment and the main questions to be answered are: (i) what are the changes in species-composition and vegetation structure resulting from grass-encroachment; (ii) does grass-encroachment result in changes in light and nutrient availability and, if so, (iii) do these changes give insight into the process of grass-encroachment.

It was found that grass-dominated plots have a low species diversity and number of species, especially of mosses and lichens. This is associated with a high biomass and low availability of light at soil surface. In addition, nutrient availability seems to be important. N-availability by mineralization and its turnover rates are higher in grass-dominated plots. Root biomass and the amounts of both ect- and endorganic matter are higher in grass-dominated plots, which may account for the higher nutrient uptake in the vegetation. It is concluded that a grass-dominated system can maintain and consolidate itself due to

positive feedbacks. A grass-dominated system has changed considerably and it is likely that a return to a state of open dune grassland may only be achieved by removing the biomass or creating nutrient-poor conditions. Suitable management measures are mowing, grazing, sod cutting and the stimulation of aeolian activity, which are further discussed within the context of the Nature Policy Plan.

Dynamics of Salt Marsh Grasslands

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Salt marshes feature nature conservation interest by their large-scale, natural processes of inundation and sedimentation, and the occurrence of characteristic salt-tolerant plant and animal species. Salt marshes show changes superimposed on natural dynamics: (i) the area might decrease as a result of mean sea-level rise, (ii) eutrophication, (iii) decrease of their exploitation by grazing, (iv) development of new salt marshes in summer polders. Predicted mean sea-level rise until 2030 is 6 mm/year. The rate of sedimentation at mainland salt marshes ranges between 10 and 20 mm/year; at island salt marshes between 5 and 7 mm/year. We may conclude that only island salt marshes may become threatened by mean sea-level rise exceeding 6 mm/year.

The ecosystem of a developing salt marsh may be best illustrated at the Wadden Sea island of Schiermonnikoog. This island gradually spreads eastward and features a gradient of salt marshes ranging from a few years up to 200 years of age. The gradient can be seen as a chronosequence that represents the succession at lower-, mid- and higher salt marshes. The plant production of the ecosystem is nitrogen-limited. Along the productivity gradient initially an increase of above-ground green biomass can be seen coinciding with the number of small herbivores, such as geese, hare and rabbit. Further on the productivity gradient the number of small herbivores decreases, however, coinciding with an increase of litter and height of the canopy. The optimum of the number of small herbivores found along the chronosequence, could also be derived from recordings during the last 20 years. The area where most geese were found has shifted several kilometres eastward in that period. Apparently, the small herbivores are forced to move to young successional stages, and they cannot control the succession. However, they may retard succession. Resident hares may facilitate use by winter staging geese by removal of the later successional plant species that are not eaten by geese.

The marked increase in the tall grass species *Elymus athericus* is thought to be favoured by eutrophication. The sediment contains an increasing nitrogen pool along the chronosequence. The rate of nitrogen mineralization strongly increases in exclosures along the productivity gradient, whereas it s low at all sites under grazing by small herbivores. This suggests that the amount of available nitrogen depends more on litter accumulation than on sedimentation. The input of nitrogen by atmospheric deposition is only high at the young successional stages with a low rate of mineralization. The data suggest that large amounts of available nitrogen are due to accumulation of litter during natural succession or from the cessation of cattle grazing.

Cattle grazing on older salt marshes facilitates use by smaller herbivores, as can be shown from experiments in The Netherlands and Germany. Cattle grazing affects the number of plant communities and plant species. Both are lower at abandoned salt marshes as long-term studies show. The effects of cessation of grazing and renewal of grazing after abandoning are reversible.

An unintended burst of a dike suggests that summer polders might be transformed to salt marsh systems. Little is known about the rate of sedimentation, establishment of plant species, and the effects of cattle grazing on the fauna. Seeds of salt marsh plants have a short living seed bank. Hence, new salt marshes have to rely on dispersal by inundating water or by grazing birds moving from existing salt marshes.

Salt-marsh management according to the wilderness concept takes place in large areas along the mainland in Germany. The cessation of all human interference, including drainage and grazing, seems a goal in itself. A point of discussion may be that no ecological reference exists for natural mainland salt marshes. These have been agriculturally exploited since 2500 years, as soon as they emerged after slowing down of the mean sea-level rise after the last glacial period. The only salt marshes never exploited can be found at the eastern points of Wadden Sea islands, such as Terschelling and Schiermonnikoog. They only exist for a maximum of 60 years. It should be realized that ceasing to maintain the groynes in the pioneer zone will eventually result in cliff formation and erosion of the marsh. Salt-marsh management in Denmark and The Netherlands tends to follow the biodiversity concept, formulates target plant communities and chooses the necessary management practice. Hence, the latter serves as a tool, not as a goal in itself.

Most important for nature conservation, management and development is the exchange between (often long-term) fundamental research into the mechanisms of recorded changes, and its integration into the field of application in order to lift the latter

beyond the level of trial and error. The formulation of well defined targets is important as it enables an evaluation of the effects of management practices. If predictions come true we may be satisfied, as we apparently have understood the ecosystem concerned. However, we can learn much more from predictions that do not come true, as they generate new research.

Grasslands, Habitat (Re)creation and Development

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In The Netherlands, wild grasslands have disappeared for the greater part and have been transformed into cultural grassland and field. Nowadays, measures are being taken to reverse this process and to increase the natural area.

The quality of a community is based on the environment. In the interaction between environment and vegetation, the former dominates the latter; the influence of the environment on the vegetation is greater than the reverse. This is also true for grassland. A varied grassland rich in species requires a varied environment.

As an effect of agriculture, the original environment has been degraded by levelling down the relief, by drainage and by fertilization. In such uniform environments nature development leads to uniform communities poor in species. To obtain better results, it is necessary to restore the original environment.

In some cases nature can repair the environment itself. This is true of landscapes where geological processes are still active, such as coastal areas and river margins. New habitats have been formed by sedimentation and erosion by water and/or wind. Former fields in the dunes can disappear under sand drifts. Cultural grassland in polders along the sea can be changes into salt marshes by cutting the dike. This will be done in some polders along the Frisian coast. Along rivers, banks and river dunes can arise.

It is best for nature development to stimulate such natural processes and to reduce or replace the level of human influence. However, active geological processes are lacking in most landscapes in The Netherlands. Therefore, the environment can only be restored by man, especially its relief, hydrology and soil quality.

Grasslands develop spontaneously under a grazing or mowing regime, particularly the semi-natural grasslands. These management measures are necessary for their subsistence; otherwise vegetation succession will develop, mostly into woodland. Natural or sub-natural grasslands in The Netherlands are more or less confined to the coastal area: the outer dunes and salt marshes. They can survive without grazing, but the best development and greatest diversity will be obtained by low-density grazing (originally by wild herbivores).

Guidelines for habitat (re)creation and community and landscape development are given in a handbook about nature development (Londo, G. 1997, *Natuurontwikkeling*, Backhuys, Leiden).

MEETING OF THE SECTION FOR VEGETATION RESEARCH ON 6 FEBRUARY 1998 IN UTRECHT

On the Importance of Metapopulation Theory for Plant Population Studies

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A metapopulation is a 'population of populations' (Levins, R. 1969. *Bulletin of the Entomological Society of America* **15**: 237–240) in which local populations wink in and out of existence, and the metapopulation consists of a shifting mosaic of local populations linked through dispersal with only a fraction of the available habitat patches occupied at one time. The key processes are local extinction and colonization. There exists a viability threshold for the landscape characteristics, below which extinction rate exceeds colonization rate. This is the case if the patches are too small, the habitat quality is too low, or the patches are too isolated. Metapopulation theory has been applied widely to explain the dynamics of fragmented and endangered animal populations (Hanski I. & D. Simberloff 1997. *Metapopulation Biology: Ecology, Genetics and Evolution.* Academic Press). More recently, the theory has been applied to plants (see for a review Husband, B.C. & S.C.H. Barrett 1996. *Journal of Ecology* **34**: 461–469). The main problems are that (i) the time scale of metapopulation dynamics (colonization, extinction) tends to be long compared with the duration of an average empirical study, making it difficult to measure extinction and colonization rates, especially for long-lived plants; (ii) seed dormancy makes it difficult to distinguish between real colonizations (through seed dispersal) and re-establishment from the seed bank; (iii) dispersal is restricted for many plants, especially those threatened by fragmentation, and difficult to measure; (iv) local adaptation, usually not incorporated in animal metapopulation models, may be crucial for plant populations. We show examples of studies in which

extinction risk was found to be related to population size and isolation (Ouborg, N.J. 1993. *Oikos* **66**: 298–308) and colonization probability to distance to nearest seed source (Grashof-Bokdam, C. 1997 *IBN Scientific Contributions 5*). We recommend a hierarchical approach to plant metapopulation dynamics, and population processes on the landscape level. An understanding of the dynamics of fragmented plant populations is badly needed in order to design strategies for conserving biodiversity in a changing and stressed environment.

Do Corridors Benefit Pollen Flow Between Small Populations?

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Populations genetic theory predicts that, as a consequence of genetic drift and inbreeding, small populations will have decreased levels of genetic variation. This genetic erosion may ultimately increase the chance of extinction of populations and of species. However, the deleterious effects of genetic erosion can be counteracted by gene flow. In plants, pollen flow in particular contributes to preservation of genetic variation. The movements of flower-visiting insects, made to their own benefit, will have an impact on pollen flow and seed set of plants. We studied how pollen flow between patches was affected by the presence of a flower corridor. Our target plant species was *Scabiosa columbaria* (Dipsacaceae) and as corridor species we used either *S. columbaria*, *Origanum vulgaris* or *Aster* spp. Main pollinators were large syrphid flies and occasionally bumblebees. These insects visited the flowers of the corridors which resulted in a loss of *S. columbaria* pollen but, on the other hand, they were guided to the next patch. The distance flown by syrphids between visited flowers in the corridor was larger when *Aster* was used, compared to *S. columbaria*. We expected that this would result in a lower loss of pollen and consequently more pollen flow between the patches. However, the presence of *Aster* corridors resulted in a net negative effect on pollen exchange, whereas a corridor of *S. columbaria* had a positive effect on pollen flow. This positive effect of a *S. columbaria* corridor was even observed when receptive female flower heads were used, which we expected to maximize pollen losses.

Rare Plants in Peril: The Problems of Small, Isolated Populations

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We summarize data from our studies on a number of rare plant species, belonging to different plant families and life histories (viz., *Gentiana pneumonanthe*, *Gentianella uliginosa*, *G. germanica*, *Arnica montana*, *Phyteuma spicatum* subsp. *nigrum*, *Orchis morio*, *O. militaris* and *O. purpurea*). Small populations are in peril because of their small size, which makes them prone to local extinction by stochastic variation of various kinds. We report on such extinctions by long inundations, management accidents, being run over by a tractor and robbery for collections. When population size decreases, genetic variation is reduced in most of the species studied. In some cases, we could link this genetic erosion to reduced plant vitality through inbreeding. Most of the species are dependent on insects for successful pollination and seed set. Small populations of these species have reduced seed production, probably wing to a lack of visitation by pollinators. The annual *Gentianella uliginosa* is a notable exception. This species is probably a habitual selfer, and – as a consequence – has no detectable genetic variation in The Netherlands.

We conclude that especially long-lived, insect-pollinated plant species are experiencing problems with habitat fragmentation and the associated small population size. Annuals do not have similar problems, but are more likely to suffer from ecological deterioration, because they lack the genetic variation to cope with or respond to changes. Biennials seem to take an intermediate position. In any case, small populations deserve more attention from reserve managers, because they are more sensitive than their larger counterparts. Habitat restoration – although positive in the long run – increases the extinction risk of small populations as a consequence of 'management accidents' and unpredictable fluctuations in ecological conditions.

Enhancing Wildflowers in the Linear Structures of the Arable Landscape: Perspectives

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In the arable landscape, the linear structures with permanent vegetation (e.g. hedgerows, ditch banks) are generally devoid of wildflower species. Although 77% of the species encountered in herbaceous field boundaries in central and eastern regions of The

Netherlands were dicotyledoneous (wildflower) species, these species contributed only 14% to the total boundary biomass production. To determine whether it is possible to maintain viable populations of wildflower species in well managed field boundaries along normally cultvated arable fields, a mixture of these species was introduced in 1993 in a newly created vegetation strip. The 4-metre wide strip was created on the outer metres of the arable field bordering the pre-existing boundary. Three years after establishment, tall species such as *Tanacetum vulgare* were found dominating a *c.* 1 metre wide zone near the arable field. Prostrate species (e.g. *Hieracium pilosella*) had gone extinct in all but the zone 3–4 metres from the arable field (Kleijn *et al.* 1997, *Acta Bot. Neerl.* **46**: 175–192). Establishment from seed of tall, early successional grassland species such as *Tanacetum vulgare* was severely reduced in the second and third year of the old-field succession. In contrast, establishment of grassland species such as *Campanula rotundifolia* and *Galium mollugo* was highest in the closed vegetation of the 3-year-old vegetation strip. In 1997, plumed Asteraceae species (e.g. *Crepsis capillaris*, *Leontodon autumnalis*) had spread furthest laterally through the vegetation strip while heavy seeded species (e.g. *Galium mollugo*, *Leucanthemum vulgare*) had covered the least distance from their point of introduction. Finally, there seem to be good perspectives for enhancing wildflower species in field boundaries once boundary management has improved (no herbicide use, removal of the cuttings each year, see Kleijn, D. 1997. *Species richness and weed abundance in the vegetation of arable field boundaries.* PhD thesis, Wageningen Agricultural University). However, lack of seed sources nearby may delay colonization of boundaries once habitat conditions have improved.

Survival in Dynamic Landscapes: A Matter of Space and Time

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The survival of wild plants in agricultural landscapes depends mainly on non-productive landscape elements like ditch banks and hedgerows. The quality of these landscape elements is heterogeneous in space. Moreover, due to changing management practices or agricultural activities on adjacent fields, this quality can change in time. Thus, the habitat for many plant species is discontinuous in space as well as in time. Plant populations will be divided into several local populations.

Due to demographic stochasticity and environmental factors, local populations can go extinct. Survival on a larger scale is possible, when empty patches are recolonized. The colonization process depends for plants on seeds from the seed rain or the soil seed bank (Bakker, J.P. *et al.* 1996, *Acta Bot. Neerl.* **45**: 461–490).

In large-scale landscapes, distances between suitable patches will on average be large. Successful colonization of empty sites is then most probable for species adapted to long-distance dispersal (e.g. *Epilobium hirsutum*). In highly dynamic situations, periods of unsuitability frequently occur. In this situation species that can stay and wait in the soil with dormant but viable seeds (e.g. *Malva sylvestris*) have higher survival probabilities than species that do not. In such a situation it is also advantageous to have adaptations for long-distance dispersal.

Species having high dispersal capacity and a persistent seed bank are common in agricultural areas. They are often competitive, so that other species are out-competed. On the contrary, species that lack adaptations for dispersal in space and time, often represent more species-rich vegetations. They need a landscape that is continuous in space and time: a dense network of stable ditch banks for example. If we want to increase the diversity (or nature values) of agricultural landscapes we should take this into account.

Habitat Fragmentation: The Application of Multi Criteria Analysis (MCA) for the Assessment of Effects on Plant Populations

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The problem of fragmentation has been approached from the viewpoint of information analysis. Up to now, most attention in fragmentation research has been focused on fauna. The same is true for proposed measures to mitigate fragmentation. However, over 30% of higher plants of national and international importance are decreasing in population size. Fragmentation is considered to be one of the most important threats. To fill the botanical gap, this study will focus on potential effects of habitat fragmentation on plant species as a result of change in land use. A MCA is used to compare the potential effects of sand excavation at two locations in the 'Maas and Waal' area. Because of its high nature value and high species richness, the plant community

Arrhenatherion is chosen as an indicator for habitat fragmentation. The Arrhenatherion consists of low-fertilized grasslands and the herbaceous layer includes a relatively high percentage of Fabaceae.

With a GIS we analysed the spatial effects of fragmentation on the habitat of Arrhenatherion. Aspects of fragmentation are resistance of the landscape and distance from the habitat. Increased resistance of the landscape for dispersal of seeds and an increased distance from habitat are both aspects that lead to increased fragmentation of the habitat.

It is shown how scientific data on fragmentation can be used in decision making. It has been concluded that an information system does not lead to better decisions, but being based on a rational model it can change the process of decision making. The study shows the role of information systems in decision making and assesses the importance of including the aspect of habitat fragmentation of plant species in the decision process.

Feasibility of Target Communities in the 'Drentse A Brook Valley System'

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For the 'Drentse A brook valley system', target communities and target plant species have been assessed by the Province of Drenthe. A model is developed that predicts the number of target plant species for four semi-natural target communities; namely, species-rich meadow, heathland, arable field and species-rich woodland. In the model, data on the present and past occurrence of target plant species in the study area are used. The probability of germination from the seed bank and the probability of seed dispersal are estimated for each of the four target communities. The model predicts a large variance in number and percentage of species between the four target communities in the future situation. For species-rich meadow, half of the target plant species can be expected. For heathland, 38% of the target plant species can be expected. However, for species-rich woodland and arable field not more than 16% and 8% respectively of the target species are predicted to occur. For these target communities, a large deficit is found between the number of predicted plant species and a completely developed target community.

MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE, WAGENINGEN, ON 20 MARCH 1998

Discovering the Rooting Potential of Adult Lilac *in Vitro*

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To the frustrating experience of many propagators, cuttings of adult woody plants often prove highly recalcitrant in rooting. In several cases, the rooting ability of adult material has been gradually restored by serial subcultures *in vitro*; so called (partial) rejuvenation. Usually, the rooting of adult material is attempted following the protocol optimized for juvenile material, although the adult material may well have different requirements. Therefore, adult lilac (*Syringa vulgaris* 'Mme Florent Stepman') was serially propagated *in vitro*, followed by rooting tests on a series of rooting media with different concentrations of MS-macrosalts and sucrose. A control group of frequently subcultured lilac rooted well on many of these media. Adult lilac only formed roots when isolated *in vitro* on one particular rooting medium. On the same medium, rooting performance after the initial culture was already fully comparable to that of the control group. On other rooting media, the isolated adult material showed a low level of rooting after the initial culture, which gradually increased to the level of the control group within the next 5–6 subcultures. Hence, the adult material of lilac retains its rooting potential. It is therefore only the sensitivity for the rooting conditions that decreases with the subculturing, and which may be interpreted in terms of a phase change reversion.

Fruit-specific Activity of a Receptacle-specific *Petunia* Promoter in Transgenic Strawberries

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Whereas in most plant species the fruits are of ovarian origin, in strawberry (*Fragaria* × *ananassa*) the fruit originates from the receptacle, a non-ovarian tissue. It is, therefore, regarded as a false fruit. For this reason, fruit-specific promoters that are isolated from other plants species, e.g. tomato, might not be suitable to direct transgene expression in the strawberry (false) fruit. In order to obtain expression specifically in those parts of strawberries that are eaten and so well-appreciated, we tested the activity of the *Petunia* MADS-box floral binding protein 7 (*FBP7*) promoter

fused to the β-glucuronidase (GUS) reporter gene. This chimeric gene construct was introduced into two cultivars of strawberry, i.e. Polka and Gariguette, by *Agrobacterium*-mediated transformation. As a control, transgenic plants were produced containing the same reporter gene combined with the constitutive cauliflower mosaic virus (CaMV) 35S promoter. The GUS expression patterns were compared. GUS activity was found in all tissues analysed when the CaMV 35S promoter was used. On the other hand, in transgenic strawberry plants containing the *FBP7-gus*-gene expression was restricted to some specific parts of the flowers and to fruits at all developmental stages. This is the first report presenting a reliable promoter for fruit-specific expression in strawberries.

The Role of 2,4-D during the Induction of Embryogenic and Non-embryogenic Callus in *Zea mays* (L.)

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The growth regulator 2,4-D is commonly used to induce callus formation. Induction, maintenance, differentiation and embryogenic capacity of callus obtained from immature embryos by *in vitro* culture were compared in the inbred maize lines A188 and A632. Callus of A188 was embryogenic and maintained embryogenic capacity for at least 1 year, whereas A632 only formed non-embryogenic callus that produced roots.

Uptake and metabolism of 2,4-D were compared for A188 and A632 by culture of embryos on medium with ^{14}C-2,4-D. Until 5 days after the start of culture, lower levels of 2,4-D per g fresh weight were observed in A188. Hereafter, concentrations per g fresh weight were comparable in both inbreds. The ^{14}C label that had accumulated was analysed biochemically. After 24 h of culture, in A188 up to 70% was free, 20% conjugated and 10% metabolized 2,4-D. For A632, these values were 37, 22, 41%, respectively.

The distribution of ^{14}C-2,4-D was visualized on sections of embryos by autoradiography. In A188, ^{14}C was observed in distinct regions of the scutellum, coleoptile and suspensor. A632 contained more label after 16 h of culture, and in a different distribution. Subculture for 24 and 72 h revealed that the pattern of 2,4-D label did not codistribute with regions of proliferation, indicating that 2,4-D is not the only trigger for proliferation.

Regulation of Bulblet Growth and Stem Formation in Lily Regenerated *in Vitro*

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Lily bulblets (Oriental hybrid 'Star Gazer') were regenerated *in vitro* from scale explants, cold-treated, weighed and transferred to soil. After one growing season, the weight of the bulblets was again measured. We found that the weight of the bulblets at planting determined growth after planting: large bulblets gained more weight than small bulblets. We also observed that bulblets that sprouted with a stem did grow more than bulblets that sprouted with only leaves. Thus, it is desirable to produce in tissue culture large bulblets that are capable of stem formation. We examined how such bulblets could be produced.

In addition to the length of the tissue culture period, three factors determined the size of the bulblets produced *in vitro*. First, bulblets regenerated in the dark were larger than those regenerated in the light. Secondly, high sucrose concentration stimulated bulblet growth. Thirdly, explant size determined bulblet size: from a large explant large bulblets regenerated. Experiments with ^{14}C-sucrose showed that bulblets regenerated from large explants used more reserves from the explant than bulblets from small explants.

To obtain bulblets that sprouted with a stem, it was essential to give during the final weeks of the tissue culture period a proper temperature treatment (15°C). The capability to form a stem was correlated with bulblet and explant size. Large bulblets formed a stem more frequently than small bulblets. Furthermore, irrespective their weight, bulblets regenerated from a large explant showed an increased capacity for stem formation.

Are there Limits to Commercial Micropropagation?

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Micropropagation companies produce starting material for growers. In The Netherlands, more than 100 million microplantlets are used by growers annually. For the companies, the most important external factors are the globalization of the market, the growing demand for tissue-cultured plants by growers and the available know-how present with the growers. The factors that determine the success of micropropagation companies also include entrepreneurship, know-how in the field of tissue culture

and logistics (scaling-up multiplication factor, management). Labour costs make up *c.* 50% of the total costs. Therefore, investment in human resources is of utmost importance. The possibilities for commercial micropropagation are still very large.

Genetic Transformation of Bulbous Crops

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Genetic transformation of bulbous crops has only been achieved in a limited number of cases. Protocols have been reported for tulip, gladiolus and lily but, until now, inheritance of the transgenes introduced into these crops has not been reported. The majority of bulbous crops can be propagated efficiently by *in vitro* techniques. In this way, excellent starting material can be obtained for various transformation protocols. Different culture techniques have to be applied to match the requirements for genetic transformation to produce non-chimeric transgenic plants.

The key problem we try to solve is how to obtain uniformly transformed tissues from which plantlets can be regenerated by either embryogenesis or organogenesis. Our approach is focused on the induction of cell division and the dedifferentiation processes in organized tissues from *in-vitro* grown plantlets. This is accomplished by plant hormones. After induction of the cell dedifferentiation process in explants, transient marker DNA expression (GUS, introduced by particle gun bombardment) is monitored to select the right moment for transformation and selection of the transformed tissue. During the selection process, transformed tissues are kept on callus induction medium until callus with a uniform pattern of GUS expression is obtained. Then, the callus is transferred to regeneration medium for production of new shoots or bulblets.

In this way, we established a transformation procedure for lily. The transgenic plants exhibited the original cultivar features and their transgenes were inherited by the seedlings produced. For iris and hyacinth, the same approach is used to achieve genetic transformation. The iris protocol now includes full dedifferentiation into callus, regeneration of shoots on this callus and finally, maturation of shoots into bulblets.

Identifying Phase Changes in *Acacia mangium*

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In general, ontogenetic development in plants is expressed as a series of transitions in morphological, anatomical and/or biochemical characteristics. The main question is whether these transitions are controlled by individual programmes or by a single master programme. Ontogenetic development in *Acacia mangium* is marked by a phase change from juvenile bipinnate leaves to adult phyllodes following a consistent pattern. The present study reveals that seedlings also show an increase in the size of the shoot apical meristem and the apex, a transition in the shape of the pith in transverse sections from pentagonal to sharply triangular, and a transition in the shape of the epidermal hairs from monocellular to multicellular. All these transitions seem to take place in the same window of development. However, microscopic examination of the transitions in leaf shape, pith shape and shape of the epidermal hairs in individual seedlings revealed that neither their positions nor their ranges of (inter)nodes in the primary axis are strictly correlated. Hence, we conclude that the three phase changes are to some degree independently controlled. The phase change in leaf shape can be reversed by isolation of phyllodinous shoots *in vitro*, and is correlated with a reversion to unicellular epidermal hairs.

Sustainable Production of Biodegradable Polyesters in Starch-storing Crop Plants

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Bacterial poly-hydroxy-alkanoates (PHAs) are considered valuable alternatives for fossil oil-derived plastics, because they can be produced from renewable resources and they are biodegradable. However, a technological problem is the production by conventional fermentation which is relatively expensive and has a limited capacity. A few years ago research was initiated on the production of PHAs in plants, which has already resulted in the generation of PHA-producing *Arabidopsis thaliana* and *Brassica napus*. For the current project (EU/FAIR-CT96. 1780), the application of starch-storing crop plants which are considered more suitable for the large-scale accumulation of PHAs is anticipated.

The objectives are (i) isolation and characterization of bacterial genes involved in PHA synthesis; (ii) isolation and application of plant genes that mediate the formation of PHA precursors (fatty acids, acetyl-CoA, acyl-CoAs); (iii) definition of minimum gene

sets required *in planta*; (iv) mutant or genetically modified crop plants suitable as hosts for bacterial PHA-genes; and (v) transgenic, hybrid crop plants producing PHAs. Microtubers from the first generation of transgenic potato plants containing the *P. oleovorans phaC2* gene under the control of the potato granule bound starch synthase (*GBSS*) promoter and plastidial targeting sequences, are currently being evaluated.

NADP-mannitol Dehydrogenase Abundance and Activity during Fruit Body Development of *Agricus bisporus*

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In the edible mushroom *Agricus bisporus*, the six carbon sugar alcohol mannitol can contribute up to 20% of the mycelium dry weight and up to 40% of the fruit body dry weight. Mannitol metabolism has been postulated to play a critical role in growth, development, salt tolerance and post-harvest shelf life of the edible mushroom. Synthesis of mannitol occurs by the reduction of fructose in a reaction catalysed by a homomultimeric NADP-dependent mannitol dehydrogenase (MtDH, C 1.1.1.138). To better understand the function and regulation of mannitol metabolism, we purified MtDH to homogeneity from *A. bisporus* fruit bodies. Native MtDH molecular mass, determined by calibrated gel filtration was approximately 70 kD, whereas MtDH subunit size was 29 kD. Polyclonal antibodies against SDS-gel purified MtDH were raised in rats. Mannitol concentration in cap, stem and gill tissue increased early in fruit body development and remained constant throughout further development. MtDH activity expressed on a g fresh weight basis was highest early in development. Specific activity of MtDH remained relatively constant throughout development. Western analysis using polyclonal antibodies raised against the purified MtDH subunit indicated that the abundance of MtDH protein was relatively constant throughout fruit body development. These results indicate that MtDH activity is directly related to the amount of MtDH protein present in fruit body.

Oxidation of Indoleacetic Acid by Apple Stem Slices during Rooting Treatment

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When apple stem slices were cultured on medium with carboxyl labelled indoleacetic acid (IAA), massive formation of $^{14}CO_2$ occurred. This demonstrates the incidence of massive IAA oxidation. When 4-h pulses with ^{14}C-IAA were given at various times after preparing the slices, the rate of IAA oxidation increased after excision, reached a maximum at 16 h (fourfold the initial value), and then decreased to the initial value at 48 h. Oxidation occurred mainly at the cut surface, probably by peroxidases that were synthesized and secreted into cell walls and medium after wounding. Chemical oxidation in the light (35 µmol/m^2/s provided by cool white fluorescent lamps) was also determined. After 1 day, 12% of the IAA in a Petri dish had been oxidized. This value is much lower than the rate of photo-oxidation of IAA reported in literature.

Root and Callus Formation from Apple Stem Segments

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Indoleacetic acid (IAA) is more efficient in *in vitro* rooting of apple microcuttings than indolebutyric acid (IBA) or naphthaleneacetic acid (NAA). 2,4-Dichlorophenoxyacetic acid (2,4-D) hardly induces any roots (G.J. de Klerk, J. ter Brugge & S. Marinova 1997, Effectiveness of indoleacetic acid, indolebutyric acid and naphthaleneacetic acid during adventitious root formation *in vitro* in Malus 'Jork 9'. *Plant Cell Tiss. Org. Cult.* **49**: 39–44). This finding may be partially explained by differences in the stability amongst the various auxins: IAA is not very stable and therefore present at low concentration after the root primordia have been formed. An additional explanation is that for some auxins (IAA and IBA) root formation is the chief response, whereas auxins like 2,4-D are more effective with respect to other responses, in particular callus formation. Here we examine both responses (root and callus formation) in old or young apple segments with different lengths.

When 1.6 mm stem slices were exposed to IAA, NAA or 2,4-D, these auxins showed very different efficiencies with respect to callus and root formation: IAA resulted in many roots and little callus, 2,4-D in a few roots and abundant callus, while NAA had an intermediate response. 3-mm stem slices (compared to 1.6 mm stem slices) produced many roots and hardly any callus in the presence of NAA. We suppose that this difference is due to the larger wounding response in 1.6 mm segments having a much larger ratio of cut surface versus volume. Old segments (excised

from 10.5-weeks-old instead of 7-weeks-old shoots) exposed to NAA produced a similar number of roots as young segments, but hardly any callus.

The results may be explained by the occurrence of two auxin receptors: one with high affinity for IAA resulting in the formation of root meristemoids from certain cells in stems, and the other with high affinity for 2,4-D resulting in callus formation. The second receptor is present at high levels in plant tissues that are severely wounded and in young tissues. These data are relevant for rooting of microcuttings during which little callus and many roots should be formed.

Elicitors Induce Tissue Competence for Plant Hormones

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Wounding of plant tissue leads to the release of catabolic enzymes from cell compartments. These enzymes catalyse the breakdown of cell structures resulting in the formation of molecules (elicitors) that trigger plant defence reactions.

Physiological studies using a test system of thin discs of apple (Van der Krieken *et al.* 1993, *Plant Cell Rep.* **12:** 203–206) showed that some of these elicitors have a synergistic effect on auxin-induced adventitious root formation and growth. Application of several elicitors and auxin led to a higher rooting response compared to application of auxin alone. This result is consistent with the situation in nature: after wounding, the plant starts producing molecules that are used for defence purposes. The induction of competence of plant cells for growth regulators will subsequently result in the repair of damaged tissue or regeneration of organs. The effect of elicitors on regeneration is not at the level of auxin uptake, auxin metabolism or endogenous auxin synthesis (radioisotope and GCMS analysis). A possible explanation for the mode of action of wound-related molecules on regeneration and growth is that they enhance tissue competence for plant hormones.

Tissue competence on the molecular level can be defined on the level of genes involved in the control of the cell cycle. In particular, the cyclin-dependent protein kinase *cdc2a* plays a key role in the induction of competence (Hemerly *et al.* 1993, *Plant Cell* **5:** 1711–1723). Molecular studies are being carried out to isolate genes involved the induction of the *cdc2* gene and other genes involved in the control of the cell cycle. The method used in these studies is a mRNA fingerprinting method: cDNA-AFLP (Bachem *et al.* 1996 *Plant J.* **9:** 745–753).

Embryogenic Suspension Cultures of Cyclamen with Different 2,4-D Concentrations

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In cyclamen, a protocol for somatic embryogenesis from cell suspensions has been reported (Winkelmann & Schwenkel 1996, Somatische embryogenese bei *Cyclamen persicum* Mill. – Analyse verschiedener Kalluslinien. *Vortr. Pflanzenzuechtg.* **32:** 178–180). In this protocol, excised ovules have been used as starting material. However, the callus is not homogeneous and shows precocious formation of somatic embryos. These embryos have to be removed to allow long-term culture. Transfer of the embryogenic callus to liquid medium results in suspension cultures with the same undesirable properties as the callus cultures.

We examined the effect of increased 2,4-D levels on embryogenic callus formation. An increase of the 2,4-D concentration in the medium from 9 to 22.5 or 45 μM resulted in homogeneous embryogenic callus that showed no precocious embryo formation. Regeneration from this callus was slow on hormone-free medium and showed abundant root growth. Nevertheless, at both 2,4-D concentrations large numbers of plantlets could be grown and the resulting adult plants were normal. From the 22.5 μM callus we initiated a suspension culture which grew well with a doubling time of *c.* 5 days; from the 9 μM callus the suspension culture had a doubling time of 7 days. Regeneration also started with abundant root growth, but recent experiments with a short period on a cytokinin-containing medium before transfer to hormone-free medium solved this problem: embryo development was more synchronous and only little root development occurred.

Kanamycin Uptake by Control (Sensitive) and Transgenic (Resistant) Potato in the Presence of Increasing Amounts of External Ca^{2+}

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Aminoglycoside antibiotics such as kanamycin (Km) are frequently used for the selection of transgenic plant cells. However, in many species Km selection is inefficient. It is reported that the phytotoxic effect of Km is strongly dependent on the Ca^{2+} concentration in the medium (Joersbo & Okkels 1996, *Physiol. Plant.* **97:** 245–250). In the present study, we used two potato varieties (Kondor and Achirana Inta) each with (Km-resistent) and without (controls,

Km-sensitive) the *npt*II gene confering Km resistance. We studied the effect of Ca^{2+} on shoot and root elongation in Km-sensitive and Km-resistent plants in the presence of Km (172 µM, 100 mg l^{-1}). Another goal was to examine the interaction between Km and Ca^{2+} in the sensitive and resistent plants, measuring the endogenous Km content.

External Ca^{2+} in the range of 3, 6, 10 and 15 mM did not affect shoot and root elongation of both the controls and the transgenic clones. At a concentration of 3 mM Ca^{2+}, Km reduced root elongation of the resistent Kondor and Achirana Inta clones to 36% and 47%, respectively. The inhibition by Km was (almost) completely reversed at 15 mM Ca^{2+}. In the controls, Km completely blocked root elongation, while increasing Ca^{2+} in the medium did not reverse this inhibition.

In the controls, we found about a two- to fourfold higher endogenous Km level at 15 mM as compared to 3 mM Ca^{2+}: in Achirana Inta 34 and 125 mmol kg^{-1} and in Kondor 31 and 74 mmol kg^{-1} at 3 and 15 mM Ca^{2+}, respectively. In the resistent Kondor clone, the Km level after 26 days was about 80 mmol kg^{-1} and not influenced by the Ca^{2+} concentration. In the resistent Achirana Inta clone, the Km level after 26 days was about 45 mmol kg^{-1} at 3 mM Ca^{2+} and about 90 mmol kg^{-1} at 6–15 mM Ca^{2+}. These results show that there is no clear correlation between the endogenous Km levels and the extent of inhibition of root elongation.