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MEETING OF THE SECTION FOR VEGETATION RESEARCH ON 26 NOVEMBER 1992

The International Importance of Plant Communities in The Netherlands

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The international importance of Dutch vegetation is analysed (1) by compiling the available information and (2) by presenting some new quantitative methods. The association, according to the Braun–Blanquet approach, is the level of the items to be considered.

The criteria by which the international importance can be determined are discussed and a number of examples of Dutch associations of international importance are given. Two criteria seem obvious, but they are operational in a few cases only: (1) presence of endemic species and (2) surface occupied by the unit in a given country in relation to the European surface. Overall criteria used by the authors are: (1) European area and relative rarity inside it, (2) central vs marginal position, (3) presence of species on 'red data' lists, and (4) other data such as rate of endangerment, replaceability, rate of persistence. The plant communities of greatest importance concentrate in sea dunes, salt marshes, broads and fens, and poor sandy soils with heathland and oligotrophic pools. The authors have evaluated the associations of the Dutch, German and Danish Wadden Sea including all of the Frisian Islands. Within this area about 35 associations are considered to be of international importance, viz. 5 in the xerosere, 10 in the hydrosere, 13 in the hygrosere (wet dune slacks), and 7 in the halosere.

Two quantitative methods, which are likely to lead to more reliable conclusions, are discussed: (1) drawing up distribution maps of associations within The Netherlands, and (2) calculating frequency diagrams of characteristics of species composing the plant communities. A map of the Cirsio-Molinietum is shown as an example of the first approach. Frequency diagrams are named ecological and chorological spectra, and can be analysed for any syntaxon (vegetation unit). In this study two chorological characteristics are considered, resulting in (1) spectra of syntaxa concerning the Dutch situation within the total area, and (2) spectra of syntaxa concerning the relative frequency of European phytogeographic elements.

The Significance of Vegetation Statistics to Environmental Policy

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The primary aim of vegetation statistics in connection with environmental policy is to describe the current state of the vegetation, as accurately as possible, on a national and regional scale. Another major aim is to understand the causes of the observed changes in vegetation and determine the effectiveness of actions taken. A distinction should be made between data from atlases and data from monitoring programmes. An atlas reflects the distribution of species in a certain area within a certain period. A monitoring programme is based on the frequent count of species within sample-plots. This makes an atlas more suitable for an area-directed policy, while monitoring is more appropriate for rapidly observing problems and evaluating the measures taken.

At the moment the possibilities for compiling an atlas of plant communities are being studied, in collaboration with the Institute of Forestry and Nature Research. This atlas will be compiled from different sources. The main source will be the vegetation records collected in The Netherlands in the last 50 years. The project 'Plant communities in The Netherlands' will produce a new vegetation classification for The Netherlands which will make many records easily accessible. The vegetation surveys are another source although they are less useful because of their incompatibility. Apart from these sources, data are available about the probability of appearance of certain plant communities such as data about characteristic plant species, abiotic circumstances and types of landscape.

Nowadays, emphasis is put on monitoring programmes. These monitoring programmes are important because they link up well with all kinds of regular government reports. In collaboration with Floron, an organization of flora-volunteers, much effort is being put into developing a flora monitoring programme. In this monitoring programme the quantity of species will be observed on line transects in certain landscape types. Because of the large number of monitoring points needed, at first the choice will be limited to certain landscape types. The possibility of integrating other data sources with 'Floron-data' will therefore be

studied. These other sources being the time series of vegetation records at universities and research institutes, vegetation data of nature conservation authorities and data collected in provincial monitoring programmes.

Perspectives of Dune Slack Regeneration

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Calciphilous pioneer vegetation types on the dune slacks of the Dutch Wadden Sea islands (*Samolo–Littorelletum, Junco baltici–Schoenetum nigricantis*) are endangered by the lowering of groundwater tables, increasing deposition and intensified dune fixation. To safeguard the many 'red list' species present in such dune slacks, the following measures are often carried out: sod-cutting; stimulation of new blow-outs; and restoration of natural hydrological systems. Such measures are only successful if (1) the pH in the rooting zone remains above 6, (2) accumulation of organic matter and consequently the availability of nutrients remains low, (3) inundations occur.

With respect to dune valley systems, three main types can be distinguished. The primary dune slacks are frequently inundated with brackish water, their calciphilous vegetation types may last for 10–20 years, and regeneration of such slacks is not difficult. Secondary dune slacks in blow-outs, with well-developed calciphilous pioneer vegetation are very rare at the moment. Those present may harbour calciphilous 'red list' species for 10–25 (max. 40) years. These dune slack systems are difficult to regenerate since the areas where wind blowing is permitted are often much too small. Most blow-outs, therefore, do not reach the groundwater level. The third type of dune slack is situated in seepage areas, nourished by calcium-rich groundwater. These areas can maintain oligotrophic and near neutral conditions for a very long time: 40–80 years, possibly even longer. Regeneration of such systems is extremely difficult and only possible in places with a regular supply of calcium-rich groundwater as, for instance, in some inner dune fringes.

Perspectives for *Littorelletea*-vegetations in The Netherlands

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In The Netherlands the number of waters inhabited by soft-water vegetations belonging to the *Littorelletea* has considerably decreased during this century. Besides reclamation, an important part of the degeneration can be attributed to acidification and concomitant nitrogen enrichment or eutrophication (Arts, G.H.P. (1990): *Deterioration of Atlantic soft-water systems and their flora, a historical account*. Thesis, Nijmegen). Restoration of acidified or eutrophicated waters requires the stopping of the input of nutrients, a removal of the organic top layer, and a supply of buffering substances via water inlet or liming. In addition, the removal of surrounding trees to stimulate wind action and also hydrological measures may be part of a restoration project. These measures have been successfully applied in a number of Dutch waters. However, in many waters the result is not a long-lasting re-establishment of *Littorelletea*-vegetations, because of the input of air-borne nitrogen and the fall of the groundwater level. The atmospheric deposition of nitrogen compounds causes acidification as well as nitrogen enrichment of the environment of *Littorelletea*-communities. This favours *Sphagnum cuspidatum, S. denticulatum* and *Juncus bulbosus*, which overgrow and smother the soft-water plants. Therefore, in order to maintain *Littorelletea*-communities under the present circumstances, a removal of the organic material has to be carried out repeatedly, and buffering substances have to be supplied frequently. As a result of the fall in the groundwater table in many regions, the fluctuations in the water level are high. These conditions are not favourable to isoetid communities and result in other vegetation types of drier biotopes. Thus, if the emissions of nitrogen and acidifying substances don't decrease, intensive and expensive nature management will continue to be necessary to guarantee the survival of *Littorelletea*-vegetations. Moreover, without improvement of the groundwater situation, restoration in a number of waters is impossible. The present aims of policy with respect to the reduction of emissions of nitrogen and acidifying substances are insufficient and need to be reconsidered.

Ecology of Remote Areas as a Reference for Dutch Nature

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The recently launched Dutch policy for nature has raised an increased interest in ecology. A reference for what valuable nature could look like is requested for the setting of specific goals. The policy states that human steering and management should be avoided, unless 'hard' social constraints do not allow any results in excess of some trivial level. Reference studies thus qualify as key documents for the justification of a substantial political move.

Reference studies have for a long time indicated factors and constraints determining the trivial level, and

management alternatives for better results. Hypotheses about the rules and quality of 'virgin' nature now drive ecologists abroad in search of corroborative evidence. However, the ecological community is not prepared to provide any substantial product control in this applied field!

Nevertheless, foreign references for nature conservation can provide:

(1) an international scale for the estimation of conservation value: identification of factors and constraints that set the trivial level;
(2) a model for the transformation of present farmlands into 'new' nature;
(3) an actual 'model' for factors and processes that can no longer be studied in The Netherlands.

In order to control a sound use of references, ecologists should:

(1) strive for a thorough understanding of the special Dutch situation, and build easily accessible and reliable data archives;
(2) develop an applied branch of conservation ecology; derive requirements and criteria (not just motivation!) from the field of application;
(3) in all studies abroad, whenever possible, let the local experts do the job; check for biogeographic, climatic, and other differences and include these in the 'models';

and do all the obvious things that are so easily forgotten when the market place is crowded and the public is innocent and desperately seeking help.

MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE ON 24 SEPTEMBER 1992

Plant Protoplasts as a Research Tool: Fundamental and Practical Aspects

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The Netherlands has a distinguished history of research on plant protoplasts including early osmotic response studies on mechanically isolated protoplasts, and in more recent years a whole range of studies using protoplasts isolated using enzymatic procedures for cell-wall degradation. The availability of these single plant cells has greatly stimulated various aspects of plant cell and tissue culture, particularly with the objective of plant genetic manipulations.

The exposure of plasma membrane at the surface of protoplasts has enabled studies to be undertaken of the role of the plasma membrane in plant cell endocytosis and exocytosis and in cell wall synthesis. It has also enabled studies to be undertaken of the interaction between the plasma membranes of protoplasts leading to fusion and the production of both homokaryons and heterokaryons. From these fundamental physiological and cell biological investigations a very broad range of plant genetic manipulations has become possible, utilizing protoplast fusion for somatic hybridization and direct uptake of DNA and viruses. The production of plants from cultured protoplasts is a basic requirement if these cell and molecular studies on protoplasts are to be applied to plant improvement.

A unique feature of isolated protoplasts is that they regenerate a cell wall thereby stabilizing themselves osmotically and triggering a developmental pathway leading under suitable conditions to sustained cell division and ultimately plant regeneration. A major challenge for protoplast researchers is to have a better understanding of the factors controlling cell division and of somatic embryogenesis leading to plant regeneration. Whilst major advances have been made in recent years in plant regeneration from cereal protoplasts and other crop plants, and those from a wide range of tree species, the procedures are still largely empirical.

The interest of plant breeders in plant protoplasts has been a major stimulus to fundamental studies on protoplast fusion, including more recently attempts to transfer single chromosomes by such fusion procedures. A range of somatic hybrid plants have become available and also a range of transgenic plants derived from protoplasts interacted with suitable plasmids. Because plant protoplasts have provided cell and tissue culturalists with a convenient single cell system such protoplasts have provided an experimental system linking fundamental and applied research.

Microprotoplast Fusion as a Tool for Partial Genome Transfer

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Somatic hybridization is a promising approach for the transfer of economically important agronomic and horticultural traits across sexual borders or taxonomic distance and for the genetic analysis of traits. This method is also valuable for transfer of traits controlled by polygenes and those unidentified genes, which are not yet amenable for the transfer through DNA transformation techniques. However, as protoplasts with whole genomes are fused, the resulting somatic hybrids are genetically complex with several unwanted chromosomes and genes. Irradiation treatment of donor

protoplasts prior to fusion has been employed previously by several authors to eliminate the undesired chromosomes, but without much success. In this regard, microprotoplast fusion can be a unique approach to partial genome transfer (i.e. one or a few chromosomes carrying desirable genes). Recently, we have optimized the microprotoplast system as well as the protocols of microprotoplast fusion and selection of fusion products. Essentially, the development of the microprotoplast system involves mass induction of micronuclei by spindle toxins, such as amiprophos-methyl in donor cell suspension lines carrying selectable markers (e.g. kanamycin resistance), isolation of microprotoplasts using high-speed centrifugation (100 000 g) on iso-osmotic Percoll gradients, and purification and enrichment of smaller microprotoplasts with one or a few chromosomes by sequential filtration through nylon sieves of decreasing pore size. Using polyethylene glycol-based protocol, the enriched fraction of smaller microprotoplasts from a donor cell-line of potato (K^r, GVS, OP, HR, HA) were fused with leaf mesophyll protoplasts of the shoot cultures of recipient partner *Lycopersicon peruvianum*. When compared to symmetric, protoplast-to-protoplast fusions, microprotoplast-to-protoplast fusions gave a higher frequency of plant regeneration in a shorter time. The regenerated plants derived from microprotoplast fusions expressed kanamycin resistance character from the donor parent potato and resembled very much the recipient parent *L. peruvianum*, whereas those derived from symmetric fusions were grossly aberrant in phenotype. The data on dot-blot analysis using species-specific DNA probes and GVS assay of plants regenerated from microprotoplast fusions revealed partial genome (limited gene) transfer from potato to tomato (*L. peruvianum*). Thus, the results suggest that partial donor genome transfer through microprotoplast fusion is better tolerated by the recipient genome than the transfer of whole genome through symmetric fusion, as adjudged from plant regeneration efficiency and the quality of regenerated plants. The results obtained on plant regeneration with other microprotoplast fusion combinations, i.e. potato (donor) (+) tobacco, and *Nicotiana plumbaginifolia* (donor) (+) *L. peruvianum* and the applications of microprotoplast systems were discussed.

Fusion of Irradiated Protoplasts of *Solanum brevidens* and *S. tuberosum*. Dot-blot, RFLP and flow cytometric analyses

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Somatic hybridization offers the possibility to transfer desirable traits across sexual barriers. However, the fusion of protoplasts with whole genomes from two different plant species often results in genetically complex hybrids with many unwanted characters. In this regard, asymmetric somatic hybridization using irradiated donor protoplasts is one approach for partial genome transfer. In our study, several asymmetric somatic hybrid calli were obtained after fusion of gamma-irradiated protoplasts from kanamycin resistant *Solanum brevidens* and protoplasts from untreated *S. tuberosum*. No shoots could be regenerated from these calli. The relative nuclear DNA content of the hybrid calli was measured by flow cytometry (FCM). Ideally, asymmetric hybrids should contain slightly more DNA per nucleus than the recipient fusion partner. However, practically all calli tested contained more DNA per nucleus than the sum of both parents. A dot-blot analysis using species-specific probes was carried out to determine the fraction of the hybrid genome derived from each parent. From the combined data from FCM and dot-blot analyses, the estimated ploidy levels of *S. tuberosum* and *S. brevidens* genomes present in the hybrids were calculated. For most hybrids the ploidy level of the *S. tuberosum* genome had increased considerably, while occasionally also polyploidization of the donor genome occurred. In a number of calli, partial elimination of *S. brevidens* DNA could be ascertained, ranging from 18 to 62% of the diploid genome. Chromosome-specific restriction fragment length polymorphism (RFLP) probes were used to investigate the presence or absence of *S. brevidens*-specific markers in the hybrids. In one hybrid, which contained only a restricted amount of *S. brevidens* DNA, nine out of eleven *S. brevidens*-specific markers were lost, whereas in another hybrid which contained a high amount of *S. brevidens* DNA, at least one copy of all nine *S. brevidens*-specific markers tested was present. It can be concluded that asymmetric fusion with the partner combination used in this study, including gamma-irradiation of the donor and selection for a nuclear trait (kanamycin resistance) results in limited elimination of donor DNA, together with polyploidization of the recipient genome.

Does the Organelle Genome Play a Role in Chromosome Elimination in Somatic Hybrids of Unrelated Species?

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The chloroplast and mitochondrial DNA of 34 *Lycopersicon* + *Nicotiana* fusion products and of 21 *Solanum tuberosum* + *Nicotiana plumbaginifolia* fusion products has been analysed. Total DNA was isolated

from the somatic hybrid calli and the fusion parents and Southern blots were hybridized with two chloroplast DNA specific probes and four mitochondrial DNA specific probes.

All somatic hybrids contain only one type of chloroplast DNA. Eighteen of the *Lycopersicon*+*Nicotiana* hybrids possess *Lycopersicon* chloroplasts and sixteen hybrids possess *Nicotiana* chloroplasts. Seven of the *Solanum*+*Nicotiana* fusion products contain potato chloroplast DNA and fourteen have *Nicotiana* chloroplast DNA.

Most somatic hybrids contain mitochondrial DNA (mtDNA) fragments from both parents and often new fragments. Calli with mtDNA fragments from only one parent also possess the chloroplasts of this species.

The chloroplast DNA type appears to be strongly correlated with the nuclear DNA composition. *Lycopersicon*+*Nicotiana* hybrids containing more than 2C (up to 7C) *Nicotiana* DNA and 3C–9C *Lycopersicon* DNA possess *Nicotiana* chloroplasts, whereas hybrids containing 2C or less *Nicotiana* nuclear DNA and 3C–11C *Lycopersicon* nuclear DNA possess *Lycopersicon* chloroplasts. In the *Solanum*+*Nicotiana* hybrids the chloroplasts originate from the parent which contributes most DNA to the nucleus. Apparently in somatic hybrids between unrelated species the chloroplasts of one parent can only be fully functional when sufficient nuclear DNA (probably genes encoding chloroplast proteins) from the same parent is present.

One question remains: does a rapid unilateral chromosome elimination determine which chloroplast type is maintained, or does a rapid chloroplast segregation determine which chromosomes will be eliminated? Since extensive chromosome elimination and chloroplast segregation have already taken place in the calli 2 months after fusion, an answer to this question is difficult to obtain.

The Cytoskeleton of Protoplasts

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The two major elements of the cytoskeleton in plant cells, i.e. microtubules and actin filaments, co-localize to a high extent in tobacco protoplasts. A quantitative analysis of length, density and bundle formation showed no differences from walled cells. The distribution of coated pits on the plasma membrane showed a relationship with the distribution of the cortical microtubules. Filaments connecting coated pits with microtubules are frequently observed in the electron microscope.

Sub-protoplasts from tobacco showed no co-localization between microtubules and actin filaments. The patterns of microtubules and actin filaments show no resemblance to those in pollen tubes or to protoplasts from cell cultures. However, the organization of the microtubules in the generative cells of the pollen tubes remained intact.

Commercially available antibodies were used to identify and localize microtubule- and actin filament-associated proteins. Anti-tau appeared to co-localize with anti-tubulin. In animal cells tau protein is associated with microtubules and is possibly involved in cross-linking of microtubules. Spectrin-like proteins were present at the plasma membrane, but also on the outer membrane of mitochondria and plastids. In animal cells spectrins are associated with actin and the plasma membrane. The results imply that in plant cells these proteins have a similar function to that in animal cells.

Regeneration and Fusion of Protoplasts of Grasses

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Recent progress in the culture and regeneration of protoplasts from monocotyledonous species has stimulated research to apply protoplast fusion techniques in cereals and grasses. Several agronomically important traits, e.g. cytoplasmic male sterility (CMS), an important trait for the production of hybrid seeds, have been shown to be encoded by organellar DNA. In this study, which has been carried out in co-operation with Barenbrug Research, we investigated the possibility of transferring cytoplasmic male sterility from a sterile to a fertile perennial ryegrass (*Lolium perenne*) breeding line by donor/recipient protoplast fusion.

Gamma-irradiated protoplasts of a cytoplasmically male sterile breeding line of perennial ryegrass (B200) were fused with iodoacetamide-treated protoplasts of a fertile breeding line (Jon 401). After fusion, twenty-five putative cybrid calli were characterized to determine the mitochondrion type and the composition of the nuclear genome. Analysis of PGI isozyme profiles and determination of the ploidy level by flow cytometry indicated that all calli tested essentially contained the nuclear DNA of the fertile line. However, the presence of parts of the nuclear DNA from the sterile line could not be excluded. Southern blotting of total DNA isolated from the parental lines and putative cybrids, combined with hybridizations using the mitochondrial probes *cox*1 and *apt*6, revealed that the mitochondria of the calli originated from the fertile line (5 calli), or from the sterile line (5 calli), or from both parental lines (15 calli). The hybridization patterns of the mtDNA from the cybrid calli showed extensive quantitative and qualitative variation, suggesting that fusion-induced inter- or intramolecular mitochondrial recombination had taken place.