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MEETINGS OF THE ROYAL BOTANICAL SOCIETY OF THE NETHERLANDS

MEETING OF THE SECTION FOR PLANT TAXONOMY AND PHYTOGEOGRAPHY ON 15 NOVEMBER 1985

J. C. ARENDS *(Vakgroep Plant Taxonomy, Landbouwhogeschool, Postbox 8010, 6700 ED Wageningen)*

Cytotaxonomy of the Apocynaceae

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Flower differentiation within the Annonaceae

Although the Annonaceae are a large family (c. 120 genera) they are remarkably homogeneous in their morphology. The basic concept of an Annonaceous flower consists of one whorl of 3 sepals, two whorls of 3 petals each, numerous stamens, and many carpels. Genera are delimited by usually small differences in the number, size and/or shape of each of these parts of the flower. Generally the flowers are pollinated by beetles.

An extensive study on the morphology of the flowers, on genus-level, revealed several morphological trends, as well as the importance of the aestivation for the morphology of the flower. The two main aestivation-types in the Annonaceae are the imbricate and the valvate aestivation. Especially when the petals are valvate, there is much variation in the morphology of the flower.

"Imbricate" flowers usually have more of less rounded petals and both petal whorls are (sub)equal.

In "valvate" flowers the following modifications can be observed:
- apex of the petals acutish
- the petals being concave
- the petals being long and narrow
- strong differences in size between outer and inner petal whorl
- loss of one petal whorl
- connivence of the inner petals
- wing-like structures on the petals

Throughout the family two trends can be recognized in the flower-morphology:
1. Transition from "open" to "closed" flowers.
2. The function of the sepals can be taken over by the outer petal whorl, and even by the inner petal whorl.

Also within the stamens, the carpels, and the shape of the receptaculum, much variation can be observed.

L. J. G. VAN DER MAESEN *(Vakgroep Plant Taxonomy, Landbouwhogeschool, Postbox 8010, 6700 ED Wageningen)*

Taxonomy of Cajanus (Leguminosae) and the use of wild species in breeding of pigeonpea.

Cajanus was long considered a monotypic genus, its only wild African species was often overlooked as validly described. *Cajanus cajan* (L.) Millsp. is the pigeonpea, an important protein-rich grain legume, found in 50 tropical countries around the globe but native in India. An important old secondary centre of diversity lies in E. Africa. Pigeonpea is well adapted to semi-arid areas. Since 1956 interspecific hybridization produced viable hybrids with species of *Atylosia* W. & A., the nearest allied genus in Cajaninae, the merger of the genera was time and again advocated. The very last character to distinguish the genera, the seed strophiole present in *Atylosia* and not in *Cajanus*, ceased to be discriminative, because some genotypes in pigeonpea also possess a seed strophiole, and unripe seeds always have one. The character is based on two complementary genes, and is recessive, the merger of the genera was finally effectuated in 1985 (VAN DER MAESEN, 1985). Also the Indo-chinese genus *Endomallus* (1 sp.) was put into synonymy (= *Cajanus goensis* Dalz.).

Cajanus now consists of 32 species. India harbours 17 species, Australia has 13 endemics including 3 newly described ones, one Australian species also occurs in Papua New Guinea, and *C. scarabaeoides* (L.) Thouars has a wide distribution from the Pacific to India, the shores of Africa, Madagascar and even Jamaica.

While most species are equally susceptible to podborers and podflies *C. scarabaeoides* showed antibiosis to *Heliothis armigera*. Resistance to sterility mosaic is shown by *C. albicans* (W. & A.) van der Maesen, *C. lineatus* (W. & A) van der Maesen, *C. sericeus* (Benth. ex Bak.) van der Maesen (species crossing with pigeonpea) and *C. crassus* (Prain ex King) van der Maesen (does not hybridize). *Fusarium* (wilt) resistance is harder to find. Blight (*Phytophtora*) resistance is found in *C. platycarpum* (Benth.) van der Maesen and *C. sericeus* (REMANANDAN, 1981). Segregating populations of interspecific crosses have produced male sterile lines and genotypes with "wrapped" flower which are cleistopetalous throughout flowering.

Most wild species have seeds richer in protein than pigeonpea, perhaps because they are smaller.

Wild sources of genes are difficult to use, but have a place when conventional sources fail.

REMANANDAN, P. (1981): The Wild Gene Pool of Cajanus at ICRISAT, Present and Future. *Proc. Int. Workshop Pigeonpeas* 1980 vol. 2: 29–38

VAN DER MAESEN, L. J. G. (1985): Cajanus DC. and Atylosia W. & A. (Leguminosae). A revision of all taxa closely related to the pigeonpea, with notes on other related genera within the subtribe Cajaninae. *Agric. Univ. Wageningen Papers* (5–4) (in press).

G. E. SCHATZ *(Herbarium, Department of Botany, Birge Hall, University of Wisconsin-Madison, Madison WI 53706, U.S.A.)*

Floral odours and patterns of visitation by dynastine scarab pollinators in a Costa Rican tropical wet forest.

At the La Selva Biological Station of the Organization for Tropical Studies, located in the Caribbean lowlands of Costa Rica, approximately 60 plant species in the families Annonaceae, Araceae, Arecaceae, and Cyclanthaceae are pollinated by 16 species of dynastine scarab beetles in the genera *Cyclocephala* (14 species), *Erioscelis* (1 species), and *Mimeoma* (1 species). Varying degrees of specificity versus overlap, as well as variable pollination effectiveness, are exhibited by the scarab visitors. The organization of this complex assemblage of functionally similar plant species and their closely related pollinators can be attributed to a number of structuring parameters operating in concert, including: 1) floral odours; 2) phenology, both of flowering time and adult beetle emergence time; and 3) vertical stratification. Insofar as inflorescences serve as perhaps the only aggregation sites for mating, floral odours function not only as pollinator attractants, but also as sexual "pheromones" for the scarab species. The marking of over 8000 beetles has yielded data on population sizes, sex ratios, adult longevity, and movement patterns (and therefore gene flow) which vary among species. Analysis of pollen loads and fruit production has revealed that, although *Erioscelis colombica* is the most abundant scarab visitor, it is the least effective pollinator, and should therefore be

considered a "parasite" on the mutualism that has evolved with *Cyclocephala* species. Differential pollinator effectiveness among a coterie of flower visitors is critical to the question of mutualism specialization, and constitutes a necessary precondition of coevolution to occur. The absolute diversity of these two groups of organisms in the Neotropics may well reflect their long history of mutualistic association, adaptations for which, have in turn resulted in patterns of parallel speciation.

E. WARDENAAR (*Vakgroep Bijzondere Plantkunde, Kruislaan 318, 1098 SM Amsterdam*)

Inheritance of allozymes in sexual dandelions, *Taraxacum* section *Taraxacum* from France

In the biosystematic researchproject of *Taraxacum* a start has been made in applying isoenzyme electrophoretic techniques to the section *Taraxacum*. Our previous study of isozyme variation in some species of sections Obliqua and Erythrosperma was restricted to asexual (polyploid) individuals (VAN OOSTRUM et al. 1985) and now we hope to elucidate the genetic patterns of some of the enzymes in the french (diploid) sexuals. Such data are the more interesting as the morphology of the dandelions still remains problematical. Apart from the genetical analysis per se, the present research has two ultimate goals: first, to establish the relationship between the disjunct (western versus central European) distribution areas of the diploid *Taraxacum* specimens, in order to clarify their origin, phylogeny and taxonomic position and, second, to establish the relationship between the sexual and the asexual components in the populations in order to investigate the presence of di-polyploid hybridogenous speciation cycles in such stands.

Electrophoretic techniques as described by MENKEN (1982) were optimized for *Taraxacum* and carried out in diploid parental plants and their (F_1 and F_2) offspring families as produced in the studies by JENNISKENS et al. (1985). Upto now the monomeric enzymes leucine amine peptidase (2 loci both polymorphic), phosphoglucomutase (4 loci, 3 polymorphic) and shikimate dehydrogenase (1 locus) and the dimeric enzymes 6-phosphogluconate dehydrogenase (2 loci, 1 polymorphic) and alcohol dehydrogenase (2 loci, 1 polymorphic) show simple Mendelian inheritance. Several other loci appeared to be monomorphic in the crosses studied. Null alleles (no functional allozyme produced) are observed at one leucine aminopeptidase and one alcohol dehydrogenase locus. Once having unravelled the Mendelian genetics of some of the enzymes we will start the broader genetic analysis of the (di-polyploid) mixed populations.

In order to increase the nummer of loci that can be used in our biosystematic study of *Taraxacum* we are now changing to polyacryl amide gels and the preliminary results are promising.

JENNISKENS, M. J. P. J., J. C. M. DEN NIJS & A.A. STERK (1985): Crossability and hybridization of taxa of Taraxacum section Taraxacum from central and Western Europe. *Proc. Kon. Acad. Wetensch., Series C*, **88**: 297–338.

MENKEN, S. B. J. (1982): Biochemical genetics and systematics of small ermine moths (Lepidoptera, Yponomeutidae). *Z.zool. Syst. Evolut.-forsch.* **20**: 131–143.

OOSTRUM, H. VAN, A. A. STERK, & H. J. W. WIJSMAN (1985): Genetic variation in agamospermous microspecies of Taraxacum sect. Erythrosperma and sect. Obliqua. *Heredity* **55**: 223–228.

MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE AND THE BELGIAN PLANT TISSUE CULTURE GROUP ON 14 MARCH, 1986.

LECTURES

R. L. M. PIERIK (*Vakgroep Tuinbouwplantenteelt, Postbus 30, 6700 AA Wageningen*)

Vegetative propagation of horticultural crops in vitro

General. Over the last 5 years micropropagation of horticultural crops in the Netherlands has grown enormously. A recent enquiry showed that totally 36 million plants were cloned in 1985. From 1981–1985 the number of companies using micropropagation in Holland has increased from 29 to 42.

The number of plant species propagated in the Netherlands in numbers more than 100.000 per year is only 16. This indicates that there is, even in a highly developed horticultural country as the Netherlands, still a wide gap between our knowledge from publications and its application in the horticultural industry. The most important problems met when cloning plants *in vitro* are: internal infections, regeneration of adventitious organs and embryos, rejuvenation of woody plants, genetic instability, uncovering of chimaera, vitrification, adaptation problems after the transfer from test tube to soil, production of toxic compounds, too high cost prices and no automation in the laboratories.

Micropropagation of Syringa vulgaris. Shoot tips of three rootstocks and two cultivars of *Syringa vulgaris* were isolated *in vitro* and further propagated by single node culturing. Culture took place at 21 °C, 16 hrs daylength, 4–5 W/m^2 and on a modified MS-medium. Stem elongation was induced by applying cytokinin (2iP or zeatin) at a concentration of 0.6–1.0 mg/l. Induction of axillary branching by high cytokinin levels resulted in leaf curling and sometimes vitrification. Rejuvenated shoots could easily be rooted, even without auxin in the medium. Transfer from test tube to soil was successful.

Propagation of ferns in vitro by sowing. When ferns are sown in soil very often competition with micro-organisms (molds and bacteria) and higher organisms (mosses and algae) occurs. To prevent contamination various fern species were sown *in vitro* to develop a procedure to raise ferns from spores under sterile conditions. After surface sterilization spores were sown on modified MS-media. Almost all species germinated and formed prothallia. In a number of ferns no sporophyte development took place *in vitro;* in another group sporophyte development only occurred after inundation with sterile water; a third group of ferns formed prothallia on which apogamous sporophyte development took place. It is also possible to transplant gametophytes from test tube to soil, where after fertilization sporophyte formation can occur.

M. JACOBS (*Laboratorium voor Plantengenetica VUB, Paardenstraat 65, B 1640 St. Genesius-Rode, België*)

Biochemical mutants isolated in in vitro cultures and their use for genetic manipulation

The use of induced genetic variability under the form of mutants with the goal of dissecting and manipulating plant systems has been generally underexploited in higher plants.

Examples of mutants affecting biochemical pathways or developmental processes have been reviewed in 1973 by NELSON and BURR. They showed that, despite the restrictions imposed by the life cycle characteristics and the genome complexity of a higher plant a wide range of biochemical mutations were already available. However, one of the merits of the *in vitro* culture methodology has been to create a new attraction of higher plants as an experimental system and to offer the prospect of applying to crop plants sophisticated techniques developed in prokaryote genetics; in particular we have in mind the impact on selection of biochemical mutants. As a matter of fact, progress has also been achieved by applying mutagenesis-selection schemes on whole plants, as illustrated by the obtention of nitrate reductase deficient mutants, thiamine-less mutants in *Arabidopsis,* defective mutants in the photorespiratory pathway or maize mutants lacking alcohol dehydrogenase. The development of novel techniques for modifying the genome of plants highlights the need for good biochemical markers characterized by a simple genetic lesion and a readily identifiable molecular basis. These will be most valuable in selecting for transformed cells at an early stage after gene transfer and in extending such methodology to cell-mediated transfer of a limited amount of genetic material by protoplast fusion between a donor cell inactivated by irradiation and a receptor mutant cell. They also represent valuable tools to study gene expression and to isolate key genes governing essential metabolic functions or important in crop production.

In this communication, we describe examples of biochemical markers obtained mainly by the use of in vitro cultures and discuss their potential value in the development of genetic manipulation procedures in higher plants.

H. A. VERHOEVEN (*Onderzoeks-instituut ITAL, Postbus 48, 6700 AA Wageningen*)

Physical methods for the transfer of cellular components to recipient protoplasts

Conventional breeding procedures are by and by being supplemented with novel techniques: e.g. the use of in vitro propagation, selection at the cellular level and genetic manipulation. In this lecture, the benefits and drawbacks of genetic manipulation will be discussed, with emphasis on the application of physical methods for the transfer of cellular components to recipient protoplasts.

The major techniques involved in genetic manipulation are based on cell-mediated or direct and vector-mediated transfer of genetic traits.

A. *Cell mediated gene transfer*

Protoplast fusion has been achieved by means of chemical (PEG, Ca^{2+}/high pH) and electrical treatments. In this way, two genomes (nuclear as well as cytoplasmic) are joined, the natural sexual barriers between more or less related species being circumvented. The heterofusion products, however, seldom result in true hybrids. Chromosome loss, segregation and/or recombination of organelles and the formation of chimeras are well known to occur during the first cell divisions after fusion.

If limited transfer of nuclear genes is required, irradiation of the donor protoplasts is often used. A major drawback of this procedure is the random character of the so induced genome fractionation, which demands for an extensive selection procedure. This technique suffers from the concomitant transfer of cytoplasmic genes. In principle, transfer of isolated nuclei is the only way out, presently. For the transfer of only cytoplasmic genes, fusion with cytoplasts has been carried out successfully.

B. *Direct and vector mediated gene transfer*

Once a breeding aim has been formulated, it is desirable to reduce the material to be transferred as far as possible. Ideally, genes can be identified, cloned and transferred by plant DNA vectors e.g. derived from *Agrobacterium tumefaciens*, or via direct DNA transfer. For gene(s) involved in a certain character, located on a specific chromosome or in certain organelles, the isolation and transfer of cell components is an attractive method. For both the transfer of naked DNA and isolated organelles a number of techniques may be adopted. In addition to the *A. tumefaciens* transformation, the use of PEG has made it possible to transfer DNA into protoplasts, whith transformation frequencies of up to 0.01%. With a similar method, the uptake of organelles has been demonstrated: the integrity of the organelles, however, seems to be impaired by the PEG treatment. Another method, electroporation, employs strong electric fields to induce pores in the cellular membrane, to get DNA or RNA into the cell. In this way, the uptake and subsequent integration of naked DNA with a frequency of up to 10% was proven. This procedure can also be used to check the nature and behaviour of different DNA constructs due to so-called transient expression in approximately 90% of the protoplasts.

Micro-injection is widely used in mammalian systems for several purposes, such as the production of transgenic animals. The vital microinjection of protoplasts, expression of viral messenger RNA and stable integration of injected DNA has been described. Recently, the technique has been applied for chromosome transfer successfully. Its major benefits are the ability to preselect the cells to be injected, and the possibility to identify succesful injections, thus enabling to monitor the processes immediately following micro-injection. One of the interesting aspects is the possibility to inject into plant cells and organs, offering prospects for monocot transformation. Cells survive this treatment, and in this way it is possible to circumvent the problems which are connected with the isolation and subsequent regeneration from protoplasts, such as genetic instability.

Last but not least, naked DNA can be entrapped in liposomes which can be fused either chemically or electrically with recipient protoplasts.

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The influence of initial sucrose and nitrate concentrations on the growth of Cinchona ledgeriana cell suspension cultures and the production of alkaloids and anthraquinones therein

In a previous study (HARKES et al. 1985) it was demonstrated that the growth of and the production of alkaloids and anthraquinones in *Cinchona ledgeriana* tissue cultures is strongly influenced by the medium composition. It is known from the literature that sucrose and nitrate concentration can have a profound effect on the production of secondary metabolites in plant cell cultures. Some authors have reported on the influence of the ratio between these two medium components (C/N ratio) on growth and secondary metabolite formation (e.g. WICHERS et al. 1985).

Seven media were prepared in such a way that four different C/N ratios were obtained. One medium was the standard B_5 medium. With the normal nitrate level maintained three other media were prepared with 0.5, 4.0 and 8.0% sucrose and with the sucrose level fixed at 2.0%, three media were prepared with 0.625 g/l, 1.25 g/l and 10.00 g/l nitrate.

Optimum growth was obtained using a B_5 medium with 4% sucrose and the normal level of nitrate (2.50 g/l). Biomass doubling times were ca. 100 hours for all media except for the medium containing 10.00 g/l nitrate, on which medium the doubling time was much higher.

Looking at the productivity of secondary metabolites, expressed as µg/g dry weight, the medium with 10.00 g/l nitrate gave the highest contents of secondary metabolites, both the quinoline alkaloids and the anthraquinones. The high levels of potassium and nitrate ions probably represent a stress factor for the cells. When looking at the yield of quinoline alkaloids (mg per liter suspension culture) it emerges that the medium with 4% sucrose and the normal level of nitrate gave the best results. For the yield of anthraquinones 8% sucrose is within this experimental optimal. From these experiments it is clear that the medium components investigated play an important role both in growth and secondary metabolite formation in *Cinchona ledgeriana* cell suspension cultures. It is also clear that sucrose of the two is the predominant factor influencing growth and production.

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WICHERS, H. J., R. WIJNSMA, J. F. VISSER, TH. M. MALINGRÉ & H. J. HUIZING (1985): Production of L-DOPA by cell suspension cultures of Mucuna pruriens. II. Effect of environmental parameters on the production of L-DOPA. *Plant Cell Tissue Organ Culture* 4: 75–82

PH. BOXUS (*Station des Cultures Fruitières et Maraîchères, Centre de Recherche Agronomiques, B-5800 Gembloux, België*)

Main problems related to mass propagation of horticultural plants

The main news of te last Symposium "In vitro problems related to mass propagation of horticultural plants, 16th–20th September 1985" were reported and commented.

A great place was made to woody species. Different new schemes to propagate fruit trees, small fruits, forest trees, tropical trees and some shrubs were described. Two reports presented a solution to propagate *in vitro* some *Citrus* rootstocks.

The big problem of mature tree rejuvenation was discussed. Meristem tip culture and *in vitro* micrografting were proposed to juvenilize different ornamental and forest trees. It was also effective for mature *Sequoiadendron giganteum*. Pretreatments by cytokinin pulses or sprays were very useful to raise bud break and subsequent proliferation of axillary shoots.

Juvenility induces high proliferation rate after some subcultures. M-A.. measurements may be a good marker of subcultures number.

Experiments with woody plants require always a larger number of replications during more than

one subculture. Two important disorders can disturb the experiments with woody plants: vitrification and shoot tip necrosis.

Though several communications treated the vitrification phenomenon, it was impossible to give a complete definition of this trouble. A few reasons of vitrification are accepted by all the participants: high cytokinin level, low agar concentration, hypolignification. The role of ethylene was doubtful. Some remedies were suggested: use of high fructose concentration, or adding of hydrolysed agar in the liquid medium.

The double phase culture medium was very interesting to keep a very high rate of proliferation without vitrification induction.

The control of contaminants was also discussed: use of antibiotics, detection of "endogenous bacteria".

Rooting was improved by brief inductive treatments, pulses or sprays of high auxin solutions during short time. The use of textile fiber plugs was also proposed.

The water losses and the photosynthetic activity of the *in vitro* plantlets during the acclimatization period were discussed. And an optimum system to improve quality of *in vitro* plantlets with a minimum of manipulation was suggested.

A. J. KOOL, M. Q. J. M. VAN GRINSVEN, M. A. HARING, J. M. DE HAAS, F. VAN DER MARK and M. M. C. TAN (*Vakgroep Moleculaire Genetica VU, De Boelelaan 1087, 1081 HV Amsterdam*)

Identification and genetic manipulation of cytoplasmic properties in plants

Posters

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Substrate utilization during carrot somatic embryogenesis measured by natural abundance ^{13}C-NMR

Introduction: In plant media predominantly sucrose is employed as the main carbon source. Since the composition and levels of substrates are of great importance for the outcome of successful in vitro growth and regeneration experiments, the utilization of sucrose in embryogenic cell suspension cultures of *Daucus carota* cv. Flakkese was followed non-destructively employing natural abundance ^{13}C-NMR.

Summary of results:

1. During in vitro culture of carrot cells the uptake and metabolism of sugars is regulated in a complicated fashion. The activities of both internal and external invertase, as well as isomerase, sucrose synthase and differential uptake mechanisms for glucose and fructose are apparent.
2. Regenerated embryo's and plantlets are characterized by the absence of preferential uptake of glucose and relatively high internal levels of free sucrose.

In addition, regenerated plantlets contain high levels of components characteristic for cell wall formation.

3. Natural abundance ^{13}C-NMR is a fast and reliable method for studying metabolism in single cells, cell clusters, embryo's and plantlets.

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Comparison of the plant habit of pot roses propagated in vitro and by cuttings

In the Dutch pot rose industry plants of dwarf cultivars of various descendance are produced all-year-round. Propagated by softwood cuttings, they have a cultivation cycle of 8–12 weeks. Current cultivars usually are too tall, too upright, branch too little, and lack clustering. Using 36 cultivars it was investigated whether plant habit might be favourably modified by *in vitro* propagation. For

the start-up and proliferation phases, modified MS-media were used, containing 0.1–2.0 mgl^{-1} BAP and 0.1–0.5 mgl^{-1} IBA, depending on the cultivar; the rooting medium contained 0.5–1.0 mgl^{-1} IBA without cytokinin. Single-node softwood cuttings were planted in TRIO 17, without the use of auxins. Both types of plants were grown in pots and observed until anthesis. *Ex-vitro* plants flowered earlier, had shorter shoots with fewer and shorter internodes, and more and longer laterals than plants from cuttings. Both types of plants did not differ for number of: bottom-breaks, flower buds or petals. *Ex vitro* plants seems promising but are too expensive at the moment. Therefore, breeding genotypes of dwarf roses with lower apical dominance, improved dwarfness and better clustering habit is imperative.

H. M. PETERS, A. F. CROES, R. H. W. POUWELS and G. W. M. BARENDSE (*Botanisch Laboratorium KUN, Toernooiveld, 6525 ED Nijmegen*)
Accumulation of thiophenes in calli of Tagetes erecta

In roots of Marigolds (*Tagetes* spp.) several thiophenes, heterocyclic sulfur compounds, are accumulated which display a nematicidal activity both *in vivo* and *in vitro*. The NOVAPLANT biotechnology group investigates the possibility to produce thiophenes by cell and tissue culture techniques at an industrial scale. Young undifferentiated calli of *T. erecta* hardly accumulate any thiophene. Roots begin to appear spontaneously after three weeks in culture. The onset of differentiation is accompanied by a sudden rise in thiophene content. Three thiophenes are accumulated in large amounts, especially under weak light: bithienylbutene (BBT) and two derivatives *viz.* an alcohol (BBTOH) and an acetoxy compound (BBTOAc).

L. P. PIJNACKER and M. A. FERWERDA (*Vakgroep Genetica, Biologisch Centrum RUG, Kerklaan 30, 9751 NN Haren*)
Polyploidization in potato callus

Cells of leaf explants of a monohaploid potato (*Solanum tuberosum;* $2n = x = 12$), genotype 7322, were stimulated to mitosis on a solidified MS-medium supplemented with NAA and BAP as hormones and 5-bromodeoxycytidine as DNA precursor during a period of 0 to 7 days. The chromosomes were stained by the fluorescent plus Giemsa technique, and showed differential staining of the sister chromatids and sister chromatid exchanges after two rounds, i.e. S-phases, of BrdC incorporation.

Through the staining patterns of the chromosomes three cell cycles could be followed. Induction of cell divisions was evident at day 3 of culture and from then onwards. A cell cycle as well as the dedifferentiation processes lasted minimally 1 day. The metaphases showed 12, 24 or 48 mono- or diplochromosomes. The diplochromosomes arose through endoreduplication of G2-phase cells. The polyploid metaphases originated from resident polyploid cells (leaves were mixoploid) and from the endoreduplicated cells. The polyploid cells had a selective advantage over haploid cells in being triggered to mitosis. The percentage of polyploid mitoses increased from 10 to 70 in 7 days.

In the same explant the number of sister chromatid exchanges per basic set of chromosomes ranged from 0–8.

N. PRAS (*Vakgroep Farmacognosie RUG, Ant. Deusinglaan 2, 9713 AW Groningen*)
Transformation spectrum of alginate entrapped cells of Mucuna pruriens

In vitro grown cells of *Mucuna pruriens* entrapped in calcium alginate are able to transform L-tyrosine into L-DOPA.
On the enzymatic level this biotransformation is conducted by a phenoloxidase. In general para-substituted phenols can be transformed into their corresponding catechols, which are biological active substances or fine chemicals. Several substrates were tested on their conversion by a cell homogenate of *Mucuna pruriens*.

The initial rates of synthesis (V_0) and transformation percentages (t_p) after one hour have been determined for the expected biotransformations.

Concerning the substrate specificity it was concluded that with para-hydroxyphenylpropionic

acid, para-hydroxyphenylethanol, tyramine and para-hydroxymandelic acid as substrates, high V_0's and t_p's can be measured.

In the present study the same range of substrates was tested on entrapped cells of *Mucuna pruriens*.

On basis of the aforementioned parameters the transformation spectrum on the enzymatic level (i.e. of the cell homogenate) was compared with the transformation spectrum of entrapped cells.

Purification by preparative chromatography and subsequent identification by combined LC/MS and D/CI-techniques were successfully carried out for each product.

Final conclusion is that entrapped plant cells of *Mucuna pruriens* are suitable for the biotechnological production of a whole range of catechols.

J.H.N. SCHEL and H. KIEFT *(Vakgroep Plantecytologie en -morfologie, Arboretumlaan 4, 6703 BD Wageningen)*

A method for plant tissue culture with a continuous flow of fresh nutrient medium

The use of solidified media in plant tissue culture often has the disadvantage that the medium composition is not exactly known caused by the presence of agar. Moreover, accumulation of toxic metabolites might occur. This is also the case in batch cultures with liquid media. Using continuous flow chemostat cultures in general is elaborate and expensive because of the large volumes of media which are needed.

Here, a method is described which uses commercially available disposable filter units as culturing chambers. The filter units are sterile Nalgene filters, type S, Sybron Corp., Rochester, N.Y. The upper lid of the filter is sprayed with 70% ethanol and, after that, perforated with a sterile needle, attached by silicone tubing to a vessel containing liquid medium. The medium is dispended by a multi-channeled peristaltic pump, connected with a timer, giving e.g. about 6 droplets in 1 hour (i.e. about 10 ml daily). The lid is sealed with Parafilm; the whole combination might be placed in an incubator.

The system is thought to be particularly suited in the study of plant embryogenesis where gradients of nutrient supply have to be simulated and toxic metabolites have to be removed continuously. We have used it in the study of embryo and endosperm development during ovary culture of maize (*Zea mays* L., strain A-188) in which the effect of calcium depletion also was examined. Some first results are presented.

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Structural changes during in vitro culture of ovaries from Zea mays L.

The development of maize ovaries after various periods of in vitro culture was studied by light and electron microscopy. A culture method was used which allows the continuous supply of fresh fluid medium and which prevents the accumulation of toxic metabolites.

Embryo development *in vitro* in general showed the same ultrastructure as is known for the *in vivo* situation. On the contrary, the formation of the endosperm was strongly distorted which often resulted in the absence of cellularization of the inner endosperm. In some cases, only the endosperm developed without any indication of embryo formation.

After calcium depletion the embryo developed normal but again endosperm formation was aberrant. No cells were formed in the central part of the endosperm; near the placental region degeneration took place, resulting in vacuoles with dark inclusions, clots of RER membranes and cellular breakdown.

The events, occurring after *in vitro* culture, strongly resemble those taking place after intergeneric crosses or crosses between diploid and tetraploid strains. It is concluded that, most probably, in all cases defective endosperm development is the main factor for the failure of grain formation. The absence of a central conducting tissue might be the reason for this.

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Evidence for a role of auxin transport in the polar or non-polar development of flower buds on explants from tobacco floral stalks

Explants from floral stalks of *Nicotiana tabacum* L. are cultured *in vitro*. At the optimal cytokinin concentration (10^{-6} mol.l^{-1} benzylaminopurin) the concentration of the auxin 1-naphthylacetic acid determines a) whether flower buds are formed and b) how they are distributed over the surface. At low concentrations, more polar buds are formed. Their number is maximal at 4.5×10^{-7} mol.l^{-1} NAA. At higher concentrations bud formation takes place over the whole surface (maximum at 2.2×10^{-6} mol.l^{-1} NAA), and the polar buds disappear. The shape of the concentration *vs* response curve is the same for the two groups of buds. With the auxin transport inhibitors 1-N-naphthylphthalamic acid and 2,3,5-triiodobenzoic acid in the medium, the buds develop evenly spread over the whole explant at all NAA-concentrations used. The maximum for both groups of buds is now reached at 10^{-6} mol.l^{-1}. This is a strong indication that auxin transport through parenchymous cells is responsible for the pattern of bud development. This transport takes place both at low and at high NAA concentrations. This is surprising since the explant is open to the medium over its whole length, and taking up a large amount of hormone.

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Embryoculture as an aid in overcoming hybridization barriers between sections of Lilium

Broadening the genetic base of commercial lily will be of great importance for introduction of desired characteristics in the crop, e.g. virus- and *Fusarium* resistance from *Lilium henryi*, low temperature and low light tolerance from *L. candidum*, year round forceability and growth vigour from *L. longiflorum*.

To overcome pre-fertilization barriers cut-style method and mentor pollen were used. Embryoculture was practised to avoid postfertilization barriers (endosperm degeneration and embryo abortion). The low fertility of certain F_1-hybrids was restored by chromosome doubling using colchicine.

Crosses between species out of four different sections were carried out. *L. longiflorum* (section Leucolirion) has been crossed successfully with *L. candidum* (section Lilium), the asiatic hybrid "Mont Blanc" (section Sinomartagon) as well as *L. rubellum* (section Archelirion). In crosses of oriental hybrids with *L. auratum* × *L. henryi* and "Shikayama" × *L. henryi* only a triploïd progeny was obtained. This was caused by the production of 2n-gametes in the case of the interspecific hybrid. A crossing polygon was compiled of all data including those from literature. Within and between four sections of the genus *Lilium* all crosses are marked, making a distinction between crosses realised with and without embryoculture. It can be concluded that crosses between different sections succeeded only when embryoculture is applied. In crosses within the same section, this technique leads to a considerable improvement in the results.

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Isolation and transfer of metaphase chromosomes of N. plumbaginifolia

Partial genome transfer by means of isolated chromosomes as an intermediate between somatic hybridization which joins two complete genomes, and the monofactorial transformation which introduces a very limited number of genes, is atractive for a number of reasons. We have shown that metaphase chromosomes, isolated from synchronized *H. gracilis* cultures, could be identified and sorted by flow cytometry (DE LAAT & BLAAS, *Theor. Appl. Genet.* 67: 463–467, 1984). Because of the lack of regeneration capability and the absence of suitable markers in *H. gracilis*, we switched to *N. plumbaginifolia* as a model system for further research on transfer and expression.

Current research emphasizes the mass isolation and transfer of chromosomes isolated from synchronized Kan^r suspension cells of *N. plumbaginifolia*. These chromosomes are being transferred to wild type or to auxotrophic (NR$^-$) *N. plumbaginifolia* protoplasts.

Protoplasts derived from Doba suspension cells, (BARFIELD et al. *Plant Cell Rep.* **4**: 104–107, 1985) (Obtained from SHIELDS, Sittingbourne) were transformed by cocultivation with *Agrobacterium tumefaciens* (LBA 4404/pAGS 129) at a frequency of 1 to 5%, according to VAN DEN ELZEN et al. *Plant Mol. Biol.* **5**: 149–155, 1985. A fast growing suspension culture, selected on 200 g/ml kanamycin, was subcultured with 3 day intervals. For synchrony, cells were exposed to hydroxyurea (HU) (4mM, 24h) 1 day after subculturing and colchicine (0,05%, added 8h after washing). Routinely, metaphase indices of approximately 25% were reached at 14h after the HU wash.

The procedure for chromosome isolation (DE LAAT & BLAAS, *Theor. Appl. Genet.* **67**: 463–467, 1984) was improved by preincubation of the synchronized cells in the enzyme medium on ice for 15 minutes, followed by cell wall digestion at 15°C for 2h. After protoplast lysis, relatively pure preparations were obtained by sieving with Nucleopore filters (20, 10, 5µm, respectively). Flow cytometric analysis and sorting yielded two chromosome peaks, probably representing ana- and metaphase chromosomes.

The mass isolation and sorting of chromosomes is largely hindered by chromosome stickyness. Therefore, we are currently investigating whether alternative spindle toxins are superior in this respect.

After having gained extensive experience with the vital injection of protoplasts and protoplast-derived cells with dyes and viral RNA's using very small capillaries (less than 0.5 µm), the use of larger needles was investigated. For passage of chromosomes, tip diameters of 2–5 µm are necessary. Such capillaries, which are shaped as an epidermal needle, were prepared by bevelling and were used for vital injection of latex beads and chromosomes.

In addition to microinjection, transfer of chromosomes through entrapment in liposomes and subsequent fusion with protoplasts, anologous to mammalian systems (MUKHERJEE et al. *Proc. Natl. Acad. Sci. USA* **75**: 1361–1365, 1978) is of interest as a large scale method. Developing a modification of the method of MAKINS (Protoplasts 1983, Birkhauser Verlag, Basel pp. 197–207) we prepared large unilamellar liposomes up to 20 µm with neutral, or negatively or positively charged membranes. In this way, high encapsulation efficiencies of at least 40% were scored employing the dye Ponceau S. Entrapment of DNA was very efficient, whereas proteins (BSA and hemoglobin) could not be included, due to denaturation at the chloroform/water interface. From a sample of nuclei of *N. plumbaginifolia*, 1% were entrapped in liposomes, as shown by flow cytometry.

Liposomes were fused with *N. plumbaginifolia* protoplasts by electrofusion (100 Vpp/cm, 20 kHz alignment; 1000 V/cm, 100 µm pulse). Fusion frequencies were low (0.1%), due to differences in membrane composition between protoplasts and liposomes, which prevented an effective alignment in the RF-field.

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Biotransformation of corynantheal by cell suspension cultures of Cinchona ledgeriana. Part 1. Non-alkaloid producing cultures

The biosynthesis of the quinoline alkaloids, that are typical of the genus *Cinchona*, starts just like the general biosynthetic pathway leading to the monoterpenoid indole alkaloids with the condensation of secologanin and tryptamine to strictosidine. After the formation of strictosidine, this key compound is converted to the quinoline alkaloids. A number of steps between strictosidine and the quinoline alkaloids still remains to be investigated. It is generally thought that corynantheal is the first step in the typical biosynthetic route leading to the quinoline alkaloids. BATTERSBY & PARRY (1971) were able to demonstrate the incorporation of radioactively labelled corynantheal in the quinoline alkaloids using intact *Cinchona* plants. However as the percentage of incorporation was rather low (ca. 0.2%), there is still doubt whether or not corynantheal is a true intermediate in this biosynthetic pathway.

The results of feeding experiments in which corynantheal was added to cell suspension cultures of *Cinchona ledgeriana* Moens, that do not produce any quinoline alkaloids under the culture conditions employed, and that did not produce quinoline alkaloids even after the feeding of L-tryptophan, tryptamine or secologanin are presented.

Corynantheal was rapidly taken up by the cells and was extensively metabolized. The main metabolite could be identified as corynantheol. A number of blue fluorescing compounds was also formed after the feeding of corynantheal, but the identification of these compounds has not been completed yet.

No production of quinoline alkaloids could be demonstrated in this study. The most important conclusion from these experiments is that either corynantheal is no intermediate, or that the biosynthetic pathway is (also) blocked at the level of the corynantheal converting enzyme.

BATTERSBY, A.R. & R.J. PARRY (1971): Biosynthesis of the Cinchona alkaloids, late stages in the pathway. *Chem. Comm.*: 30–31.

P. BERTIN et al. *(Université Catholique, Louvain, België)*
Multiplication du Thuja plicata et du Sequoiadendron giganteum par bourgeonnement axillair en culture in vitro

C. M. COLIJN-HOOYMANS[1], M. C. C. TAN[2] and W. H. LINDHOUT[1] *([1]Instituut voor de Veredeling van Tuinbouwgewassen, Wageningen; [2]Vakgroep Genetica, Vrije Universiteit, Amsterdam)*
Tomato: regeneration and somaclonal variation

J. J. M. DONS, J. B. M. CUSTERS, J. H. W. BERGERVOET and R. BOUWER *(Instituut voor de Veredeling van Tuinbouwgewassen, Wageningen)*
Development of in vitro culture procedures for the genetic manipulation of cucumber (*Cucumis sativus L.*)

I. FAMELAER *(Instituut voor Molekulaire Biologie, Vrije Universiteit, Brussel, België)*
Cellular engineering by means of "gamma fusion" in Nicotiana plumbaginifolia

P. F. FRANSZ and J. H. N. SCHEL *(Vakgroep Plantecytologie en -morfologie, Landbouwhogeschool, Wageningen)*
The in vitro response of two genotypically different maize strains. A comparative cytological study

Ch. H. HÄNISCH TEN CATE *(Onderzoeks-instituut ITAL, Wageningen)*
Effects of in vitro differentiation on genetic stability in potato

L. VAN HEE *(Rijksstation voor Plantenveredeling, Merelbeke, België)*
Clonal propagation of Scorzonera hispanica in vitro

J. HUITEMA *(Instituut voor de Veredeling van Tuinbouwgewassen, Wageningen)*
Selection and in vitro characterization of low-temperature tolerant mutants of Chrysanthemum morifolium Ramat

A. C. HULST, J. TRAMPER and K. VAN 'T RIET *(Afdeling Proceskunde, Landbouwhogeschool, Wageningen)*
Biotechnological production of secundary metabolites with immobilized plant cells

D. H. KETEL, H. BRETELER and B. DE GROOT *(Onderzoeks-instituut ITAL, Wageningen)*
Thiophene biocides from cell cultures of Tagetes patula (marigolds)

A. M. M. DE LAAT and J. BLAAS *(Onderzoeks-instituut ITAL, Wageningen)*
Potential applications for microinjection of plant protoplasts

W. ORCZYK[1], R. BOUWER[2] and J.J.M. DONS[2] ([1] *Warsaw Agricultural University, Poland,* [2] *Instituut voor de Veredeling van Tuinbouwgewassen, Wageningen*)
Regeneration of protoplasts from cucumber (Cucumis sativus L.)

M. P. DE PROFT et al. *(Universitair Instituut, Antwerpen, België)*
CO_2 evolution in a culture container during organ culture of mini-roses

K. J. PUITE and S. ROEST *(Onderzoeks-instituut ITAL, Wageningen)*
Somatic hybridization between two Nicotiana plumbaginifolia lines and between Solanum tuberosum and S. phureja using electrofusion

P. RUDELSHEIM et al. *(Universitair Instituut, Antwerpen, België)*
The interaction of morphology and endogenous phytohormone content in crown gall tissues

F. SPEECKAERT and J. LATHOUWERS *(Instituut voor Molekulaire Biologie, Vrije Universiteit Brussel, België)*
Two approaches to somaclonal variation

G. STRAATSMA *(Proefstation voor de Champignoncultuur, Horst)*
CO_2 as growth factor for hyphae of Cantharellus

C. VENVERLOO *(Botanisch Laboratorium, Rijks Universiteit, Leiden)*
The influence of colchicine, oryzaline and cytochalasin on cytokinesis in epidermis cells of Nautilocalyx explants

MEETING OF THE SECTION FOR PHYTOPATHOLOGY ON 30 JANUARY 1986

P. H. J. F. VAN DEN BOOGERT *(Instituut voor Bodemvruchtbaarheid, P.O. Box 30003, 9750 RA Haren)*

Population dynamics of Verticillium biguttatum in the plant-soil ecosystem

This study was supported by the Netherlands Foundation for Technical Research (STW), Technical Science Branch/Division of the Netherlands Organisation for the Advancement of Pure Research (ZWO).

In laboratory tests with different crops, successful colonization of roots with *Verticillium biguttatum* was confined to potato. The mechanism of colonization could not be ascribed to growth of *V. biguttatum* itself, but was merely achieved by transport of the fungus on the growing sprout and root. This passive translocation was also demonstrated at sub-minimum growth temperature of the fungus. In trials in which potato sprouts were simultaneously inoculated with *Rhizoctonia solani* and *V. biguttatum*, while primordia of the sprouts and roots were kept free of *V. biguttatum*, the obligate parasitic nature of the latter was confirmed.

To evaluate the main causes of changes in population in agricultural fields and its implications for *R. solani*, both populations were quantified in a plant-free soil, in a potato field and a wheat field, at different initial inoculum levels for two successive years.

Increase of the population of *V. biguttatum* was only found in combination with *R. solani* and the potato plant. In the *R. solani*-contaminated rhizosphere and stolonsphere the population was expanding exponentially, which was also measured on the surface of the subterranean plant. In wheat, in *Rhizoctonia*-free potato and in plant-free soil no fluctuations were observed: a steady, slight decrease over a two-year period to about 50% of the original population was the only change. This type of decline might be due to senescence of the inoculum more than to antagonists.

The effect of the build-up of *V. biguttatum* on *R. solani* was found to take place only at the end of the growing season. At that time a decline of mycelium of *R. solani* was achieved; the decline was stronger as the initial densities of *V. biguttatum* were higher.

It is concluded that *V. biguttatum* should be characterized as an ecological obligate parasite of *R. solani*. Fluctuations in population density are based on the fluctuations in *R. solani*, which in turn are based on potato as a specific host plant for *R. solani*. This implies that *R. solani* determines the population dynamics of its parasite and to a lesser degree the reverse, as we would wish for efficient biological control.

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Integrated control of Rhizoctonia solani in potato fields with Verticillium biguttatum and the fungicides Pencycuron or Tolclofos-methyl

Seed potatoes with green sprouts, about 2 cm long, were dipped in a spore suspension of *V. biguttatum* (1–2 × 10^6 spores per ml) and planted into soils treated with different dosages of Pencycuron (0, 1/8, 1/4 and 1/1) or Tolclofos-methyl (0, 1/4, 1/2 and 1/1 of the recommended dosages, respectively). Non-inoculated tubers were used in the same soil treatments. Chemically disinfected tubers (formaldahyde), inoculated or not, were not used in combination with the fungicides so that an impression could be gained of the infestation of the soil with *R. solani* and of the effect of biological control alone. Where no fungicides had been applied inoculation of the seed tuber with *V. biguttatum* had a marked effect in some fields in reducing the disease index, the percentage of stolon pieces with living *R. solani*, the sclerotium index of harvested tubers and the loss of weight due to grading, which was done to meet the requirements of the NAK. In other fields the effect was small or even lacking.

The effect of the fungicides was predominating. The magnitude of the effect depended on the soil and on the fungicide used. At the highest dosages the fungicides gave excellent results on holocene marine soils; the effect was smaller on a slightly acid sandy soil with a high content of soil organic matter. (In the latter soil a 50% higher dose had been applied, as was recommended). At

the lowest dosage, the effect of the fungicides was enhanced by inoculation of the seed tuber, which often led to very satisfactory results or even complete control.

The very irregular distribution of *R. solani* in the field makes it difficult to obtain unequivocal results.

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The effect of potato tubers on sclerotia formation by Rhizoctonia solani

Cutting off the shoots (COS) stimulates the production of sclerotia on potato tubers. This stimulation appeared to be caused by the tuber itself and continued during the second and third week after COS. In order to determine the origin of the stimulus tuber periderm and exudates were sampled at 9–16 days after COS and compared to samples from control plants.

The periderm strips alone did not affect the amount of sclerotia produced on wateragar (WA) but formed sclerotia were located mainly on or under these strips. Hyphae, seperated from tubers by hydrophilic filters, never developed sclerotia, but after COS brown hyphae were formed. This indicates a role of the periderm in sclerotium initiation.

Water content of sand near tubers increased after COS, but no relation was found with the amount of sclerotia produced. Dry weight of water soluble tuber exudates (WSE) after COS exceeded that of control plants. WSE alone did not affect sclerotium development on WA, but WSE plus periderm strips gave a reduction.

Tuber respiration rate decreased during tuber maturation and declined sharply within two days after COS. Volatile tuber exudates did not change qualitatively after COS, but two components seemed to change quantitatively.

Sclerotia formation on tubers and WA was studied when exposed to volatiles from different objects. Volatiles from pot soil or decomposing potato roots and stolons ("Rot") had the same effect on sclerotia formation as those from humidified perlite. Volatiles from underground parts of young control plants slighly reduced sclerotia production on WA, whereas older control plants stimulated and even more so after COS. Volatiles from "Rot" only enhanced the effect from older control plants. As compared to the WA alone, most sclerotia were formed on the tubers on WA plates, regardless the source of volatiles exposed to.

In summary: both volatile tuber exudates and contact with the periderm seem indispensible for black scurf development.

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The population biological relevance of Phomopsis infection in Plantago lanceolata

Ear stems of *Plantago lanceolata* can be infected by the fungus *Phomopsis subordinaria*. The earlier infection of the ear stem occurs, the higher the percentage of seeds it aborts and the lighter are the remaining seeds. Genotypes differed significantly in response to inoculation. Development of necrosis correlated positively with the percentage aborted seeds and negatively with seed weight.

To investigate variation in susceptibility within and among populations of *P. lanceolata*, plants were sampled in three different populations with, respectively, a low, a moderate, and a high degree of infection in the field. Variation in development of necrosis between the cloned genotypes *within* populations was highly significant. Variation *between* populations, however, was not significant. Differences in susceptibility between plant populations do not explain the differences in degree of infection in the field.

In the three populations mentioned above, the pathogen was isolated from twelve different plants per population. Three cloned plant genotypes were inoculated with the isolates. Differences between the plant genotypes appeared to be highly significant, which confirmed the experiment on the variation in susceptibility of the host. Fungal isolates *within* populations showed differences in development of necrosis, so that relatively virulent and avirulant isolates could be distinguished. In contrast with the host, the pathogen also showed differences between populations. The population with the highest degree of infection in the field appeared to be most virulent. As the other two populations

showed no differences in virulence, also environmental factors (especially the snout-beetle, which introduces the pathogen into the stalk) play a role in the development of infection in the field. The significant interaction between fungal isolates and plant genotypes may point to a certain degree of physiological specialization between pathogen and host.

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Detection of viroids by bi-directional electrophoresis

Although molecular hybridization using ^{32}P as a marker system has an extremely low limit of about 125 pg per spot for the detection of viroids, till now it has not become widely used because of the ^{32}P marker. Furthermore it should be realized that due to the fact that only small amounts of sample can be applied to the nitrocellulose filter, viroids can only be detected in plant material if their concentration is over 20 ng/g of material. In that respect the test is only 5 times as sensitive as the polyacrylamide gel electrophoresis test.

The detection limit of the latter test was greatly influenced by the relatively weak staining of the viroid in gels by the generally used toluidin blue and EtBr stains. A more powerful stain like silver nitrate could not be used due to the high background of contamination nucleic acids whose staining would have completely masked the presence of the viroid.

Bi-directional electrophoresis (originally described by SCHUMACHER et al. 1983, *Anal. Biochem.* **135**: 288–295) uses a second electrophoresis step under denaturing conditions to completely separate the viroids from the contaminating nucleic acids on the basis of the exceptional internally base-paired circular structure of the viroid molecules. In combination with a silver nitrate staining bi-directional electrophoresis can detect viroids at concentrations as low as 10 ng/g of plant material. This allows the use of mixed samples. Potato spindle tuber viroid (PSTV) could be detected in a sample of 2 g containing 1 part of PSTV-infected potato leaf and 499 parts of healthy potato leaf.

It is not necessary to move the viroid-containing region of the first native gel to a gel with denaturing conditions. Good results can also be obtained if the complete native gel is brought under denaturing conditions.

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Virus- and salicylic acid- induced P.R.-proteins and resistance in different host plants

Pathogenesis-related (P.R.)-proteins are induced in plants following viral or microbial infection, or by treatment with certain chemicals, and are associated with the host defence response. The three smallest of them, each 14 kD, are named PR-1a, -1b and -1c.

We prepared antisera to several purified PR-proteins to confirm and establish serological relationships among PR-1a, -1b and -1c, and between PR-2a and -2b. By immunoprecipitation of *in vitro* translation products, directed for by poly(A)RNA from healthy or TMV-infected tobacco, we showed induction of PR-genes to occur at the transcription level.

A synthetic oligodeoxynucleotide was made, complementary to the mRNA of PR-1a, the partial amino sequence of which had been determined previously. This primer was used both to sequence directly the 5′-leader of PR-1 mRNA (HOOFT VAN HUIJSDUIJNEN et al. 1985), and to screen a cDNA library made to poly(A)RNA from TMV-infected plants (CORNELISSEN et al. 1986a, 1986b). Sequencing of the available clones provided evidence of at least three highly homologous PR-1 mRNAs, each encoding a primary translation product with an N-terminal 30 amino acid signal peptide. One clone was found to contain a complete copy of PR-1b mRNA. The clones were used as a probe in Northern blot hybridizations to show the induction of PR-1 mRNAs both in virus-infected and virus-free plant parts.

In order to obtain clones for other PR-mRNAs another cDNA library was constructed using size-selected poly(A)RNA. By differential screening of this library a number of clones were obtained corresponding to TMV-induced mRNAs. Some of these mRNAs are also induced by salicylic acid,

a chemical inducer of PR-proteins. Preliminary sequencing data of one of these clones suggest the existence of a fourth protein of the PR-1 class.

In another series of experiments we found that salicylic acid confers resistance of cowpea protoplasts to AlMV infection (95% inhibition). The specificity of this response is illustrated by the fact that 1) host viability and protein synthesis are unaltered and that 2) p-coumaric acid, which is structurally related to salicylic acid neither induces PR-proteins nor resistance.

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Initial interactions between virus and protoplasts

Cowpea chlorotic mottle virus (CCMV) and cowpea protoplasts (*Vigna unguicalata* cv. California Blackeye) were used to study interaction between virus and protoplast. Two hypotheses of binding/penetration were tested: 1. (receptor mediated) endocytosis and 2. entry by plasmamembrane lesions. Several parameters were tested to support or reject either hypothesis. Binding/penetration was measured as virus associated with protoplasts after incubation with ^{35}S-labelled CCMV and appropriate wash.

At 0°C and 25°C the amount of virus associated with protoplasts increased with the amount of virus added. An input of 26×10^6 virusparticles per protoplast resulted in binding of 4,000 and 14,000 particles per protoplast respectively. In the presence of polyethylene glycol (PEG, $\underline{M}_r$ 6000) an increase to 80,000 associated particles occurred at both temperatures. In the latter case ca. 50% of the protoplasts became infected, as determined by immunofluorescence 16 h after inoculation. No infection occurred in the absence of PEG.

Variation of pH (3.5, 4.5, 5.5, 6.5, 7.5) or ionic strength (0, 0.05, 0.1, 0.2, 0.35 M NaCl) in the absence of PEG caused hardly any change in binding and again there was no infection. In the presence of PEG increase of pH resulted in lower binding numbers and increase in ionic strength in higher binding numbers.

The presence of unlabelled CCMV, tobacco mosaic virus coat protein and bovine serum albumin during the inoculation process decreased the amount of ^{35}S-labelled CCMV bound.

These data support the model of binding/penetration, where virus, aggregated by PEG, is introduced into the cytoplasm through plasmamembrane lesions.

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Pathotypes in Fusarium wilt of carnation

Vascular wilt caused by *Fusarium oxysporum* f. sp. *dianthi* is a worldwide problem in carnation growing. Breeding for resistance has been succesful but the introduction of screening procedures into commercial breeding programmes has met with problems due, at least in part, to the wide genetic variability of the pathogen and the pronounced effects of environmental conditions on disease development. Pathogen variability is studied for further standardization of screening methods and to prevent future loss of resistance through adaptation of the pathogen.

Presently, the interaction between 3 pathotypes (1, 2, 4) reported from Italy by Garibaldi and 9 cultivars of various genetic backgrounds was studied. Although some cultivars were susceptible or resistant to all three pathotypes, striking reversals in susceptibility to the pathotypes were also found. Both pathotypes 2 and 4 caused regular unilateral wilt symptoms with (2) or without (4)

characteristic vascular browning in all their compatible interactions, whereas general leaf-necrosis associated with only vague vascular discolouration was induced in compatible interactions with pathotype 1.

Histological examinations revealed pathotypes 2 and 4 to cause moderate (2) or severe (4) lysis of vascular tissues presumably causing wilt symptoms, in case of pathotype 2 often accompanied by browning of primary walls at infection borders but locally also by gels and hyperplasia of xylem parenchyma probably in attempt to localize infection. Heavy colonization by pathotype 1, however, did not induce lysis. Apparently, enzyme production by pathotypes 2 and 4 causes lysis and wilt symptoms, only lower enzyme production by pathotype 2 allowing for timely recognition of breakdown products. The striking necrosis caused by pathotype 1, however, is probably due to toxins, as enzymatic degradation does not occur. Vascular occlusion by gels, causing disease in *Phialophora* wilt of carnation, plays no role in *Fusarium* wilt with either of the pathotypes.

The observed pathotypes are shortly discussed in relation to those of other formae speciales of *F. oxysporum*.

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Cutinolytic activity in culture filtrates of Botrytis cinerea isolates

The production in vitro of cutin hydrolyzing enzymes by 5 isolates of *B. cinerea* was studied, using cutin of tomato fruits as carbon source. Chemical depolymerisation of the cutin yielded 10,16 dihydroxy hexadecanoic acid as the main component. The same fatty acid was found after incubation of cutin with a crude enzyme preparation from a culture filtrate of *B. cinerea*. Hydrolysis was optimal at pH 5.5–6. Glucose as carbon source did not lead to detectable cutinase activity. Crude enzyme preparations which hydrolyzed cutin also hydrolyzed PNB, with an optimum at pH 8. All 5 isolates showed PNB hydrolyzing activity when grown on tomato cutin, but the activity varied with the isolate used. No correlation was found between PNB hydrolyzing activity of an isolate and the production of small lesions on young tomato fruits.

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Regulation of production and activity of polygalacturonases of Botrytis cinerea grown in culture

The plant-pathogenic fungus *Botrytis cinerea* is known for producing cell wall-degrading enzymes in vitro as well as in vivo. During the penetration of the outer epidermal cell wall of French bean, an early increase in pectic enzyme activity takes place. Two polygalacturonases, labelled PG1 and PG2, are the first enzymes detectable by means of gel electrophoresis.

In vitro experiments were performed in which the fungus was grown in a liquid medium containing salts and different pectaneous polymers (French bean cell walls; citrus pectin; Na-polygalacturonate) as the only C source. Growth, pH, polygalacturonase activity and type of pectic enzymes (by means of pectin-polyacrylamide gel electrophoresis) in the culture filtrate were followed daily.

A consistent sequence in the production of pectic enzymes was found. PG2 was always the first enzyme present in the culture filtrates, followed by PG1 (if produced) and subsequently by a number of polygalacturonase isoenzymes and pectinesterases. Total polygalacturonase activity and number of enzymes detectable were influenced by type and concentration of substrate used.

The results suggest that the polygalacturonase complex constitutes a pathway for the complete degradation of pectinaceous polymers to products that the pathogen can use for its growth. PG2, apparently being a constitutive enzyme, starts the breakdown and the products released from the substrate presumably cause the subsequent induction of PG1 and other isoenzymes. A feed-back regulation by the breakdown products may explain differences in activities and number of isoenzymes detectable under different conditions.

Also other factors that may regulate the biosynthesis and/or activity of the pectic enzymes are under investigation.

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The persistence of spores of Aschersonia aleyrodis, an entomopathogenic fungus of glasshouse whitefly, Trialeurodes vaporariorum

Aschersonia aleyrodis belongs to the Deuteromycotina and produces orange coloured spore masses in pycnidia. These spores germinate on the surface of whitefly larvae and penetrate the cuticle by means of an appressorium. *A. aleyrodis* is presently being considered as a biological-control agent against glasshouse whitefly, *Trialeurodes vaporariorum*, a severe pest of glasshouse crops like cucumber.

When applying the spores onto the abaxial leaf surface on which whitefly is present, the persistence of the spores may play a role. Eggs of whitefly were not infected but larvae which hatched and settled on treated leaves were showing symptoms of infection.

Leaves were treated seperately at 3 day intervals starting 12 days before eggs were laid until 1 day before eggs hatched. The percentage infected whitefly larvae did not differ between treatments, indicating that spores remained viable and infective for 22 days at 20°C and 70% R.H. before they made contact with larvae.

Using a leaf impression technique it was found that a small percentage of 5 to 10% of the spores germinated on the cucumber leaves. Incubation of leaf impressions on wateragar for 24 h at 25°C taken from plants kept at 25°C or 20°C after a period varying from 1 to 20 days gave a picture of the viability of the spores on the leaf surface.

Spores on leaves at 25°C decreased in viability from 96% germination considering leaf impressions made 1 to 3 days after application to 50% germination 12 days later. Spores from leaves kept at 20°C showed only a slight decrease in viability whereas still 80% of the spores germinated on wateragar after 20 days on cucumber leaves.

The phenomenon of persistence of ungerminated spores on the leaf surface should be considered in relation to the effect of addition of nutrients to the formulation of this microbial agent.

P. J. MULLER, on behalf of the research group "flooding" *(Laboratorium voor Bloembollenonderzoek, 2160 AB Lisse)*

Control of soil-borne diseases, nematodes and weeds in ornamental bulb cultivation by flooding

In the summer of 1982 flooding was applied for the first time by a bulb grower on a small area. In the Bulb Research Centre a preliminary investigation was then started on the effect of flooding on the survival of some soil-borne fungi, pathogenic to bulbs. Early in 1984 a research group was formed; members of the group are research workers in the field of fungal and bacterial diseases of bulbs, of stem nematodes, virus-transmitting nematodes, weeds and soil hydrology, and also extension officers. Results of small-scale experiments will be compared with those of trials in the experimental garden and in practice. Different temperatures and soil compositions are included in the research.

Some fungal diseases and weeds could be controlled completely by flooding during six weeks, for example black slime caused by *Sclerotinia bulborum*, grey bulb rot caused by *Rhizoctonia tuliparum* and most probably fire caused by *Botrytis tulipae*; and the perennial weeds way thistle (*Cirsium arvense*) and coltsfoot (*Tussilago farfara*). Sclerotia of *Stromatinia gladioli* showed a 50% decrease in germination.

Several species of nematodes, e.g. *Ditylenchus dipsaci* and *Trichodorus* sp., were markedly reduced after flooding for six weeks. Some other diseases, e.g. those caused by *Pythium* spp. and *Rhizoctonia solani*, and the annual weeds were not controlled.

Flooding for six weeks prevented the development of most groundkeepers (bulbs left in the soil); cormels of gladiolus, however, and of *Ornithogalum umbellatum*, and certain *Allium* spp., survived in part.

The promising results of flooding in practice and in experiments stimulated its use in summer on a large scale: in 1985 in the Netherlands about 300 acres of soil were flooded.

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Bacillus thuringiensis crystal toxins

Bacillus thuringiensis produces intracellular protein crystals toxic to a wide range of lepidopteran pest insects or dipteran disease vectors. Each variety shows a typical degree of toxicity for a well defined host range. Crystals of variety *israelensis* toxic for dipteran larvae contained a dominant M_R 28,000 protein next to an M_R 130.000 protein, present in crystals of all varieties. Mosquitocidal activity was exhibited by the M_R 130,000 protein only, whereas the M_R 28,000 protein showed a broad spectrum cytolytic activity *in vitro*. The gene for the M_R 28,000 crystal protein was cloned and sequenced. No *in vivo* expression of the gene in *E. coli* could be demonstrated, possibly correlated with the unusual promoter sequences preceding the gene. Various isolates of different varieties were screened for optimal activity against the moderately sensitive *Spodoptera exigua*, showing differential toxicity levels. The toxin gene of the active variety *kurstaki* HD-1 was cloned in *E. coli* and the recombinant was shown to express a biologically active toxin. Restriction enzyme analysis revealed sequence differences with a previously clone *kurstaki* HD-1 toxin gene. Additional toxin genes of varieties active against *Spodoptera exigua* will be cloned and nucleotide sequence differences will be correlated with differences in toxicity levels and host range. Results might faciliate the construction of recombinant genes showing altered toxicity and/or host specificity.