Acta Bot. Neerl. 36(3–4), November 1987, p. 307–338

MEETINGS OF THE ROYAL BOTANICAL SOCIETY OF THE NETHERLANDS

MEETING OF THE CONTACT-GROUP PLANT BIOTECHNOLOGY AND PHYTOCHEMISTRY (PBF) OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE ON 19 SEPTEMBER 1986: 'SECUNDARY METABOLITES IN PLANTS AND PLANT CELL CULTURES'.

LECTURES

A. W. ALFERMANN (*Botanisches Institut Universität, Universitätsstrasse 1, D-4000 Düsseldorf, W. Deutschland).*

Fundamental aspects of plant cell culture technology to produce secundary products.

Despite much progress in chemistry and pharmacy, plant cells remain an important source of medicinal compounds. According to FARNSWORTH & MORRIS (*Amer. J. Pharm.* **148**, 46–52, 1976) one quarter of all prescription drugs in the US contain substances, which must be isolated from plant material. For a number of reasons it has become difficult to obtain a sufficient supply of plant drug material in recent years. Plant cell cultures could offer a solution to this problem, as was recently demonstrated in Japan by FUJITA *et al.* (in: FUJIWARA, A. (Ed.): *Plant Tissue Culture.* Japan Ass. Plant Tissue Culture, Tokyo, pp. 399–400, 1982). Plant cell cultures are able to accumulate secondary compounds at high yield, which can also be produced by the differentiated plant (for a review see i.e. ALFERMANN, A. W. in: TRAMPER, J. *et al.* (Eds.): *Biocatalysis in organic synthesis.* Elsevier, Amsterdam: pp. 225–238, 1985).

Furthermore, there is an increasing number of reports describing new compounds found only in cell cultures but not in the differentiated plant. Such novel compounds may be produced after addition of an appropriate precursor or by direct biosynthesis of the cells. A further possibility would be to combine different biosynthetic pathways by fusion of protoplasts of different species. A successful approach to isolate cell lines producing novel compounds has been published recently by KESSELRING (in: *Pflanzliche Zellkulturen.* Bundesministerium für Forschung und Technologie: Bonn: pp. 111–129, 1985).

A somewhat different strategy has to be used to isolated cell cultures with the capacity to produce known compounds in great amounts. In such a case, one first will screen plant populations for high amounts of the target compounds or the appropriate enzymes. From selected highly producing plants, callus cultures are established and screened for the presence of the desired products. Highly producing stable cell lines can be isolated from such producing cultures found by chance via single cell cloning based on the so-called 'somaclonal variation' (FUJITA, Y. *et al.*: *Agric. Biol. Chem.* **49**: 1755–1759, 1985). A further increase of product yields is achieved by improving the cultural conditions, which, besides variation of the medium formulation, may include using a two step culture process (in: FUJIWARA, A. (Ed.): *Plant Tissue Culture. Japan Ass. Plant Tissue Culture,* Tokyo: pp. 399–400, 1982), a fed-batch (SCHIEL, O. *et al.*: *Plant Cell Rep.* **3**: 18–20, 1984), a resting cell (PAREILLEUX, A. & R. VINAS: *Appl. Microbiol. Biotechnol.* **11**: 222–225, 1984) or a semicontious culture system (ALFERMANN, A. W. *et al.* in: NEUMANN, K. H. *et al.* (Eds.): *Primary and secundary metabolism of plant cell cultures.* Springer, Berlin: pp. 316–322, 1985). The physical culture parameters of the fermentation process can be of importance (SPIELER, H. *et al.*: *Appl. Microbiol. Biotechnol.* **23**: 1–4, 1985; BREULING, M. et al.: *Plant Cell Rep.* **4**: 220–223, 1985) as well as the design of the reactor vessel itself (FUJITA, Y. & M. TABATA: Lecture at VIth IAPTC Congress, Minneapolis, 1986, in press).

Four cell culture processes based on these methods are used in industry today (for a review see: DEUS-NEUMANN, B. & M. H. ZENK in: *Pflanzliche Zellkulturen.* Bundesministerium für Forschung und Technologie, Bonn. pp. 91–110, 1985), others are reaching at least pilot plant level. In spite of such progress, however, there are still many 'gaps' in our knowledge of the physiology and biochemistry of plant cells and in the way in which we can manipulate their activities to meet industrial needs.

The key problem in many cases still is that we are not able to manipulate unorganized cells to produce the compounds of the differentiated plant. It is quite clear that in these cases the unorganized cells do have the genetic information for the production of these compounds; this information, however, is not expressed. We are unable to uncouple the chemical from the morphological differentiation. Still we have to learn more how genes are regulated and expressed as well as – in very many cases – which enzymes are involved in biosynthesis of the secondary products. Plant cell suspension cultures are an ideal system and have successfully been used for studying the enzymology of secondary product biosynthesis (see i.e. ZENK, M. H. in: PHILLIPSON, J. D. *et al.* (Eds.): *The chemistry and biology of isoquinoline alkaloids.* Springer, Berlin: pp. 240–256, 1985). In cases, where cell cultures fail to produce the desired product, however, organic cultures may be useful as well (HASHIMOTO, T. & Y. YAMADA: *Plant Physiol.* **81**: 619–625, 1986). A further approach may be the use of an elicitor of microbial origin to induce secondary product biosynthesis in cell cultures. This, obviously, does not lead to yields interesting for industrial production but it will be useful to study the enzymes involved as well as to provide us with knowledge on regulation of secondary products.

Still much progress is needed to establish a plant based biotechnology for the production of natural compounds of medicinal interest on a larger scale.

H. W. GROENEVELD (*Botanisch Laboratorium Rijksuniversiteit, Lange Nieuwstraat 106, 3512 PN Utrecht*).

Synthesis of terpenoid particles in laticifers of Euphorbia lathyris.

Laticifers are well-known for their synthesis and accumulation of great amounts of secondary metabolites, e.g. rubber (*Hevea*), alkaloids (*Papaver*), cardenolides (*Asclepias*) and proteases (*Ficus*). The non-articulated laticifers of *Euphorbia* are characterized by their triterpenes and triterpene esters. These non-polar lipids are synthesized in a wall-lining cytoplasm and subsequently secreted into the vacuole as solid submicroscopical particles. A protein film covers every particle, preventing flocculation or coagulation. This particle suspension can be purified by gelfiltration techniques.

In germinating seeds and seedlings of *Euphorbia lathyris* up to 80% of the non-saponifiable lipids (triterpenoids) occur in the laticifers. During germination the endosperm can be removed from the seedlings and a variety of substrates can be administrated physiologically to the cotyledons, bypassing all the problems encountered in feeding radioactive precursors to plants under physiological conditions. Many substrates were taken up rapidly and translocated in the growing seedling. Exogenously supplied mevalonic acid, however, was not involved in the synthesis of latex triterpenes. A considerable conversion of amino acids into latex lipids was measured but sucrose (and to a lesser extent hexoses and pentoses) was found to be the most effective precursor. Tracer experiments with specifically labeled glucose demonstrated the glycolysis to be the major route in acetylCoA production, which in turn was used for the synthesis of triterpenes via mevalonate, IPP and squalene.

Transmission electron microscopy showed mitochondria in the cytoplasm of the laticifers. Assuming that the citrate shuttle in these organelles is involved in the conversion of pyruvate into acetyl-CoA it may be concluded that:

a) sugars and amino acids are taken up by the laticifer from its adjacent tissue.
b) inside the laticifer 9 molecules of glucose are used for the synthesis of one molecule of triterpene,
c) terpenoid synthesis in a laticifer is not an anaerobic process,
d) this synthesis is selfsupporting in ATP requirement,
e) a surplus of NADH and NADPH is concurrently produced,
f) the overall-reaction of the synthesis of a triterpenol can be written as:

$$9C_6H_{12}O_6 + 4O_2 \rightarrow C_{30}H_{50}O + 24CO_2 + 32H + 13H_2O$$

or per isoprene-unit:

$$1\tfrac{1}{2}C_6H_{12}O_6 + \tfrac{1}{2}O_2 \rightarrow [C_5H_8] + 4CO_2 + 6H + 2H_2O$$

H. J. HUIZING (*Stichting voor Plantenveredeling, Postbus 117, 6700 AC Wageningen*).

Pyrolizidine alkaloids in plants and cells: risks and (potential) uses.

Pyrrolizidine alkaloids occur in species of the plant families *Asteraceae, Boraginaceae, Fabaceae, Orchidaceae, Poaceae, Apocynaceae* and *Santalaceae.*

Intensive drug testing programmes revealed e.g. the potent usefulness of indicine-N-oxide, a pyrrolizidine alkaloid N-oxide occurring in *Heliotropium indicum*, as an anti-neoplastic agent. On the other hand it was found that pyrrolizidine alkaloids with an allylic ester-function in the amino-alcohol moiety were hepatotoxic. This observation caused a revaluation of the risk assessment of several natural products, e.g. fodder plants, herbal teas and medicinal herbs.

Pyrrolizidine alkaloids protect certain moths against many avian predators. *Danaus* species (monarch butterflies) use pyrrolizidine alkaloids as pheromone precursors.

Some grasses are able to launch pyrrolizidine alkaloids as protective agents against insects. It is still a question though whether these alkaloids are synthesized constitutively, or only after infection with a fungal endophyte.

The fact that pyrrolizidine alkaloids act as insect repellents, opens the stimulating question whether these repellents can be produced by means of plant cell technology. Manipulation of plant cells in order to trigger the biosynthesis of pyrrolizidine alkaloids is still hampered by a lack of knowledge of the pathways. An overlook of the up to now supposed pathways for pyrrolizidine alkaloids biosynthesis is presented.

K. POMA (*Laboratorium voor Biotechnologie, Industriële Hogeschool van het Rijk, Voskenslaan 270, B-9000 Gent, België*).

Production of tannins in suspension cultures of Rhus typhina L.

Tannins and especially gallotannins are very important commercial products. *Rhus typhina* is rich in Sumach gallotannins (hepta-tot nona-0-galloyl D-glucose). The production of these gallotannins is studied in plant cell cultures of *Rhus typhina.*

Initially the optimalization of the callus- and suspention cultures is performed. Different media (Murashige and Skoog, Gamborg B5, Schenk and Hildebrand and Woody plant) are compared in function of the growth and the structure of the plant material in culture. Several hormone combinations with the auxines (2,4D and NAA) and the cytokinines (BAP and kinetine) are also tested. The influence of the use of glass or polystyrol petridishes, polyurethane as a support for the calli and a variety of recipients for the suspension cultures is investigated. The growth of *Rhus typhina* is optimal with Murashige and Skoog medium enriched with 2,4D and BAP (callus) or kinetine (suspension).

The detection of the gallotannins is established with HPLC and spectrofotometry. Some preliminary experiments demonstrated several important features. The cells of *Rhus typhina,* in contrast with the used media, contain the tannins. The amount of tannins is maximal at the end of the exponential growth phase. Extracts of fresh leaves of *Rhus typhina* contain more tannins than extracts of the suspension cultures. The quality of tannins in suspension cultures may be modified by varying the auxine- and the sucrose concentrations of the medium. These data together with the influence of the light are further investigated for the optimalization of the tannin production.

A. J. J. VAN DEN BERG, M. H. RADEMA and R. P. LABADIE (*Sectie Farmacognosie Rijksuniversiteit, Catharijnesingel 60, 3511 GH Utrecht*).

Production of polyketide anthra-derivatives in plant cell cultures of Rhamnus purshiana.

When callus and suspension cultures of *Rhamnus purshiana* had been established in our laboratory,

it was shown that their patterns of accumulating anthra-derivatives (1,8-dihydroxyanthraquinones, -anthrones and/or -dianthrones) differed from that in the intact bark. Callus as well as suspension cultures accumulated predominantly glycosides of mainly physcion derivatives, whereas in the original bark material of *R. purshiana* emodin- and aloe-emodin glycosides dominated. The anthra-derivative content of callus and suspension cultures was respectively 5% and 2% of the value determined for the intact bark. Maximum production of anthra-derivatives in the suspension cultures occurred in the stationary growth phase.

To study the factors which influence the optimal production of our plant cell cultures, several approaches, including precursor feeding, illumination and visual selection, were surveyed. Since the anthra-derivatives occurring in *Rhamnus* species are biosynthesized through the acetate/malonate pathway (polyketides), feeding experiments were performed with the basic intermediates acetate and malonate. It was demonstrated that anthra-derivative production in suspension cultures was significantly stimulated by malonate, while addition of acetate had an inhibitory effect under similar conditions.

A daily photoperiod of 12 h also resulted in an increased anthra-derivative content of the suspension cultures. The emodin- (and to a lesser extent chrysophanol-) content of the cultures was significantly raised by this illumination, whereas the level of physcion were shown to be unaffected in this light-experiment. The formation of anthra-derivates, however, was strongly suppressed when the cultures were continuously illuminated.

The most promising result with regard to optimal production, however, was the establishment of a high yielding callus of *R. purshiana* obtained through a process of visual selection. The anthra-derivative content of this callus was 50% of the amount present in the original bark.

N. PRAS (*Laboratorium voor Farmaceutische Chemie en Farmacognosie Rijksuniversiteit, Ant. Deusinglaan 2, 9713 AW Groningen*).

Bioconversion and biosynthesis by suspension grown plant cells of Mucuna pruriens.

Suspension grown cells of *Mucuna pruriens* are able to accumulate L-dihydroxy-phenylalanine (L-DOPA). The conversion of the precursor L-tyrosine is catalyzed by a phenoloxidase.

The content of endogenous synthesized L-DOPA at the end of the growth cycle varies between 0.5 and 2.0% (g/g, DW) depending on the cell line used. In cell suspensions about ten times more L-DOPA is biosynthesized than in calli.

No relationship between the endogenous L-tyrosine- and L-DOPA- content could be demonstrated.

In green cell suspensions L-DOPA- contents of 3–4% could be measured. Therefore high producing cell lines were selected from green cell cultures of *Mucuna pruriens*.

Fast growing calli with an increased secondary metabolism (1.2% L-DOPA) were obtained after plating such a suspension on 2% sucrose under continuous light (1000 and 2000 lux). Cell suspensions originating from these selected calli contained 6% L-DOPA after a few growth cycles, when grown under the same conditions.

The influence of some stress factors on the secondary metabolism was investigated in order to increase the endogenous L-DOPA-synthesis and/or to obtain a higher level of phenoxidase-activity for bioconversion purposes. Immobilization of suspension grown cells in calcium alginate resulted in an altered secondary metabolism: the endogenous L-DOPA-synthesis stopped and all 20 precursors tested were bioconverted in the, already identified, corresponding catechols. Addition of sodium chloride or copper sulfate to cell suspensions resulted in an almost complete release of the endogenously produced L-DOPA. This release appeared to be pH-dependent and was complete at pH 1–2.

In addition, the ability of suspension grown cells to convert other precursors into the corresponding catechols was found to be pH-dependent. The results suggest that pH-dependent processes (i.e. transport systems) may be an important factor in the optimization of bioconversions, both in cell suspension and in immobilized cells.

POSTERS

H. H. VAN GENDEREN (*Botanisch Laboratorium, Rijksuniversiteit, Lange Nieuwstraat 106, 3512 PN Utrecht*).

Lipid histochemistry in developing leaves from Ilex aquifolium L.

The formation of the cuticula starts during leaf stretching, but thickening is delayed until the leaf stretching is completed. Young leaf cells are piled up with lipoid material. The lipoid contents of epidermis and mesophyll rapidly decreases during stretching. The differentiation within the cuticula starts at the upper epidermis. The basal part of the cuticular membrane reacts neutral, the outer parts more acidic.

H. H. VAN GELDEREN (*Botanisch Laboratorium Rijksuniversiteit, Lange Nieuwstraat 106, 3512 PN Utrecht*)

Triterpenoids and flavonoids in plants and tissue culture of Ilex aquifolium L.

Triterpenoid esters are present in lipoid droplets in most living cell types in the holly. Flavonoids (anthocyanins and quercetin-glycosides) are present in the upper leaf epidermis cells. Free triterpenols and triterpene acids are present in the cuticula.

Tissue cultures are less differentiated. There is no visible cuticula. Free triterpenols and triterpene acids remain histochemically undetectable. Triterpenol esters are formed in most callus cells. They are not present in the xylem part of the vascular nodules.

Histochemical data indicate a biosynthesis of triterpenol esters in young undifferentiated cells and a biosynthesis of flavonoids, free triterpenols and triterpenol esters in older, already specialized (epidermis) cells.

M. J. J. VAN HAAREN, A. HOEKEMA, P. J. J. HOOYKAAS and R. A. SCHILPEROORT (*Vakgroep Moleculaire Plantkunde MOLBAS, Biochemisch Laboratorium, Wassenaarseweg* 64, 2333 AL Leiden).

The construction of simple and efficient plant cloning vectors.

It was recently shown in our lab that *Agrobacterium tumefaciens* is able to transfer part of its Ti plasmid DNA (the T-DNA) not only to cells of dicotyledonous plants, but also to those of at least certain monocots (HOOYKAAS-VAN SLOGTEREN *et al, Nature* **311** (1984): 763–764). Therefore, the Ti plasmid offers potential as a gene vector not only for dicots, but also for monocots.

Besides the T-DNA the Ti plasmid contains a second region with genes that are involved in the progress of plant tumor induction. Genetic complementation experiments have demonstrated that genes of this second region (the Vir region) are expressed in the bacterium but only after contact with plant cells (OKKER *et al, Nature* **312** (1984): 564–566) and act in trans. We found that a physical disconnection of the Vir-region and the T-region does not lead to a loss of tumorigenicity. On this principle we have developed a binary vector system (HOEKEMA *et al, Nature* **303** (1983): 179–180). In this system a helper plasmid, containing the Vir-region, promotes the transfer and integration of any foreing DNA into plant cells via *Agrobacterium*. A prerequisite is that the foreign DNA is cloned between the border sequences of the T-region of the Ti plasmid. Since *onc* genes are absent in such 'artificial' T-DNAs, transformed plant cells retain the same regenerative potential and growth characteristics as untransformed cells.

R. VAN DER HEIJDEN, I. HEGGER, E. J. M. PENNINGS, R. VERPOORTE, R. WIJNSMA, P. A. A. HARKES and J. A. DUINE (*Biotechnologie Delft Leiden, Centrum voor Biofarmaceutische Wetenschappen Rijksuniversiteit, Postbus 9502, 2300 RA Leiden*).

An HPLC-assay for strictosidine synthase activity.

Strictosidine synthase (SSS), a key enzyme in the indole alkaloid biosynthesis, couples tryptamine and secologanine to strictosidine, which is the central precursor of the indole alkaloids. SSS activity has been measured already in several indole alkaloid producing genera. Its activity is usually determined by means of tritium-enriched precursors. We developed an HPLC assay (RP-18 column, mobile phase methanol: phosphate buffer pH 3.3 = 60:40 with 5 mM sodium 1-dodecanesulfonate), in which tryptamine and strictosidine are retained and well separated, while tryptophane and secologanine are unretained. The k'-values of tryptamine and strictosidine are resp. nine and fourteen. A crude incubation mixture can be directly injected in the HPLC-system. This assay was used for determining the activity of SSS in an alkaloid producing suspension culture of *Tabernaemontana orientalis* and during the purification procedure of the enzymes for 24 to 880 pkat/mg protein.

M. H. N. HOEFNAGEL and F. VAN IREN (*Biotechnologie Delft Leiden, Vakgroep Moleculaire Plantkunde Rijksuniversiteit, Postbus 9502, 2300 RA Leiden*).

Contribution of alternative respiration to total oxygen consumption by plant cell suspension cultures.

Oxydation via the cytochromal route (CR) results in 3 ATP molecules per NADH whereas alternative respiration (AR) leads to (at most) 1 ATP. So, the latter phenomenon can be relevant to the efficiency of the conversion of sugars into biomass as well as to the oxygen supply required for (industrial) plant cell cultures.

We started with a survey of our batch cultures. Throughout the culture period the contribution of both processes as well as 'residual respiration' (R R; non-mitochondrial O_2 consumption, e.g. peroxydation) to total O_2 consumption was determined by titration with inhibitors. In normally growing cell cultures of *Cinchona ledgeriana* (kina tree) and *C. robusta* no activity of AR was found although a considerable capacity for AR was present. Past normal transfer age of the culture (deep stationary culture), *C. robusta* revealed 50% contribution of AR to total O_2 consumption.

In *Catharanthus roseus*, grown on low sucrose medium, activity of AR, was observed for a short period in the exponential phase, up to 30% of total O_2 consumption.

In all cultures RR was low (about 8%) during growth, and high (about 25%) during lag- and stationary phase.

We found indication that activity of AR can be higher under circumstances deviant from normal culture.

S. S. HOEKSTRA[1], R. VISSE[1], S. DIEUDONNÉ[1], H. VAN DER MAAS[1], M. MEYERINK[1], R. VERPOORTE[2], K. R. LIBBENGA[1] and P. A. A. HARKES[1] ([1] *Biotechnologie Delft Leiden, Vakgroep Moleculaire Plantkunde Rijksuniversiteit, Postbus 9502, 2300 RA Leiden;* [2] *Biotechnologie Delft Leiden, Centrum voor Biofarmaceutische Wetenschappen Rijksuniversiteit, Postbus 9502, 2300 RA Leiden*).

Selection of in vitro cultures of Cinchona ledgeriana and of Cinchona robusta for high production of quinoline alkoloids.

Induction of quinoline alkaloids in *in vitro* culture of *Cinchona ledgeriana* and *C. robusta* appears possible by lowering the auxin concentration in the medium. Non-producing calli, grown in media containing 2 mg·l^{-1} 2,4-D or NAA often produce quinoline alkaloids after adaptation to 0.1 mg. l^{-1} NAA.

We screened 52 calli, each derived from an individual plant according to this procedure. Two of them produced considerable amounts (up to 280 mg·kg dwt^{1}), of quinoline alkaloids with cinchonine as the major component and quinidine as second component. Most calli produced less than 10 mg·kg dwt^{-1}. The high-producing calli will be used for further selection as well as for further investigation of epigenetic factors affecting quinoline alkaloid production.

J. H. C. HOGE, R. J. DE KAM, R. PEERBOLTE, P. J. J. HOOYKAAS, G. J. WULLEMS and R. A. SCHILPEROORT (*Vakgroep Moleculaire Plantkunde MOLBAS, Biochemisch Laboratorium Rijksuniversiteit, Wassenaarseweg 64, 2333 AL Leiden.*)

Hairy root transformation of plants by Agrobacterium rhizogenes: T-DNA, T-DNA transcripts, T-DNA oncogenes and inheritance of T-DNA traits.

Strain LBA 9402 of the soil bacterium *Agrobacterium rhizogenes* has the capacity to introduce two specific fragments from its Ri (root-inducing) plasmid pRi 1855 into the cells of dicotyledonous plants. In the host plant cells these two segments, called TL-DNA and TR-DNA, are integrated in the chromosomal DNA and expressed. As a result the plant cells become transformed into rapidly proliferating hairy roots which synthesize the unusual amino acid derivatives mannopine and agropine. Transformed plants can be regenerated from hairy root tissues. Such hairy root plants have an excessive root system, wrinkled leaves and a reduced male fertility. DNA and poly(A)-RNA were isolated from LBA 9402-induced hairy root cultures of *Nicotiana plumbaginifolia* (RNP 1) and *Daucus carota* (AKR828) and analysed by Southern and Northern blot hybridization. RNP1 was found to contain both TL-DNA and TR-DNA and AKR828 only TL-DNA. The TL-DNA appeared to code for nine transcripts and the TR-DNA for five transcripts. The location and polarity of these transcripts, which range in size from 0.6 to 2.5 kb, have been determined.

Transposon and deletion mutagenesis of the pRi 1855 T-DNA and genetic complementation experiments revealed that the TR-DNA contains two oncogenes which are functional analogues of the *aux*-1 and *aux*-2 genes from the T-DNA of the *Agrobacterium tumefaciens* Ti (tumor-inducing) plasmid. The TL-DNA was found to contain one or two oncogenes which strongly stimulate root formation and one oncogene with a similar, but weaker, root growth suppression activity as the *cyt* gene from the Ti plasmid T-DNA. Furthermore it could be shown that some plants like pea and sunflower only require the TR-DNA for an oncogenic response and that other plants like *Kalanchoe* and tobacco also require the TL-DNA.

Transformed plants were regenerated from LBA 9402-induced hairy roots of *Nicotiana tabacum* (HR-SR1-9402) and from RNP1 and cross-pollinated with pollen from wild type plants. The HR-SR1-9402 progeny showed segregation of the hairy root traits excessive roots/wrinkled leaves (EXR/WRL) and mannopine/agropine synthesis (MAS/AGS), whereas these traits were linked in the RNP1 progeny. These findings and the fact that AKR828 root cultures do not synthesize mannopine and agropine suggest that EXR/WRL and MAS/AGS reside on the TL-DNA and TR-DNA, respectively. Progeny derived from tobacco hairy root male and crown gall female parents showed that the Ti plasmid T-DNA trait 'no roots' dominates the Ri plasmid T-DNA trait 'excessive roots'.

H. J. G. TEN HOOPEN, J. J. MEIJER, R. BLEIJERVELD and K. CH. A. M. LUYBEN (*Biotechnologie Delft Leiden, Instituut voor Biotechnologie, Technische Universiteit, Julianalaan 67, 2628 BC Delft).*

Oxygen supply to plant cell suspensions in shake flasks.

The aim of this study has been to investigate the oxygen supply to plant cell suspensions in shake flasks with various closures. In this investigation *Catharanthus roseus* was cultured in shake flasks closed with aluminium foil, silicone foam stoppers or silicone foam caps. Biomass, oxygen and carbon dioxide concentrations were determined. Besides, a mathematical model for the oxygen supply to a plant cell suspension in a shake flask was developed. Results of the computer simulations were compared with practical experiments.

The oxygen transfer coefficient of the closure, the volumetric transfer coefficient of the shake flask and the oxygen consumption of the plant cell suspension were determined experimentally. From our experiments the following conclusions can be drawn:

- Growth of plant cells in shake flasks might become oxygen-limited inspite of the relatively low oxygen demand of plant cells.
- Transfer barriers for oxygen can be both the flask closure and the gas-liquid interface.
- Aluminium foil closures are in fact gas-tight, oxygen and carbon dioxide transport proceeds only through the capillarities between flask neck and foil.

- Silicone foam caps provide oxygen transfer comparable with cotton wool plugs.
- Poor gas transfer through the flask closure causes also increased carbon dioxide levels with unpredictable physiological effects.

R. E. KOES, C. E. SPELT, E. VELTKAMP and J. N. M. MOL (*Vakgroep Genetica, Biologisch Laboratorium Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam).*

Genes involved in flavanoid biosynthesis in Petunia: isolation and characterization of the gene(s) for chalcone synthase, the key enzyme of flavonoid synthesis.

Flavonoid genes of the plant *Petunia hybrida* represent a model system to study the molecular basis of regulation of gene expression. As a first step we analyzed the gene(s) for chalcone synthase (CHS), the key-enzyme of flavonoid biosynthesis. Southern blots of *Petunia* nuclear DNA reveal the presence of at least 14 CHS related restriction fragments, which were subsequently isolated from genomic libraries. In order to determine which of these fragments contains an active CHS gene, the relationship between 20 independently isolated CHS cDNA clones and genomic CHS related fragments has shown that these genes lack the typical characteristics of pseudogenes and may therefore be functional. We are currently testing whether these genes are indeed expressed in other tissues or under different developmental or environmental conditions.

T. VAN DER LEER, R. VAN DER HEIJDEN, P. A. A. HARKES, P. J. DE JONG and R. VERPOORTE (*Biotechnologie Delft Leiden, Centrum voor Biofarmaceutische Wetenschappen Rijksuniversiteit, Postbus 9502, 2300 RA Leiden*).

Influence of production media on Cinchona ledgeriana cell cultures: spontaneous formation of β-carbolines from L-tryptophan.

Cinchona ledgeriana cell suspension cultures were kept in a continuous exponential growth phase. In this way it was possible to transfer the cells at different physiological states (early exponential, mid exponential and stationary phase) to Zenk's alkaloid production medium. After transfer to the production medium, cell death was observed. In the cultures no quinoline or terpenoid indole alkaloids could be detected. However, part of the L-tryptophan, present in the production medium, was transformed to the β-carboline alkaloids norharman and harman. Because the formation of norhaman continued after the death of the cells, the presence of living cells might not be a prerequisite for the formation of norharman,. It could be demonstrated that norharman was formed in Zenk's alkaloid production medium without cells and also in production medium containing cold-killed cell material of *C. ledgeriana*. It has thus been proven that in some cases norharman and harman formed in plant cell cultures supplemented with L-tryptophan or tryptamine are artifacts.

R. W. VAN DER MEULEN and K. RAEMAKERS (*MILLIPORE B.V., Postbus 166, 4870 ED Etten-Leur*).

Filtration and purification systems.

Sterile filtration and clarification of liquids as well as gasses in indispensible in modern biotechnology. MILLIPORE delivers a very broad range of filtration and purification systems for industrial (production) and laboratory (research) applications.

Disturbances in the growth rates of plants and (plant) cells are frequently caused by the use of low-quality water. A range of water purification systems was demonstrated, which, starting from normal tap water, produce high-quality water, free of metal ions and bio-organic growth inhibitors. Capacities of these systems vary from a few liters per hour for laboratory applications up to thousands of liters per hour for production purposes. Sterile filtration (0.22 μm) of tissue culture media is possible from several ml to hundreds of liters. Aeration systems for culture vessels (supply of sterile air or gasses, and removal of waste products) were also demonstrated.

G. J. NIEMANN (*Botanisch Laboratorium Rijksuniversiteit, Lange Nieuwstraat 106, 3512 PN Utrecht*).

Biosynthesis, transport and accumulation of lipid constituents of Ilex aquifolium leaves.

The lipid fraction of *Ilex aquifolium* leaves mainly consists of aliphatic hydrocarbons, triterpenes, sterols and fatty acids, which are either located on the leaf surface in the cuticle or epicuticular wax, or in the cells of epidermis and/or mesophyll. In the cells the lipids are to a high degree concentrated in globular lipid-containing particles, mainly constituted of fatty acid esters of the triterpenols α- and β-amyrin.

Sucrose, acetate and mevalonate are utilized for the synthesis of triterpene esters. Mevalonate is also used for the synthesis of free triterpenols, but acetate and sucrose are not. This selectivity of precursor utilization indicates separate sites for triterpenol and triterpene ester synthesis.

Mevalonate applied to the surface of the leaf is taken up and ^{14}C incorporation is not only found in triterpene esters and triterpenols of the applicated leaf but also in these components in younger leaves.

Separation of the epidermis from the rest of the leaf after treatment with mevalonate learns that the major triterpenol incorporation is located in the upper epidermis. It is restricted to young leaves. The major part of the newly formed free triterpenols remains in the cuticle and is not deposited in the external wax coating. Biosynthesis of triterpene esters occurs both in epidermis and mesophyll of young and older leaves; it starts at a very early stage of leaf development and probably provides the major part of the wax triterpenols.

NIEMANN, G. J. (1985): Biosynthesis of pentacyclic triterpenoids in leaves of Ilex aquifolium L.. *Planta* **166**: 51–56.

NIEMANN, G. J. & W. J. BAAS (1985): The composition of the lipid globules of Ilex aquifolium leaves and its variability with the age of the leaf and with season. *Physiol. Plant.* **64**: 371–376.

G. J. NIEMANN and H. H. VAN GENDEREN (*Botanisch Laboratorium Rijksuniversiteit, Lange Nieuwstraat 106, 3512 PN Utrecht*).

Variation in the Ilex leaf wax composition to the leaf age.

From May 1983 onwards leaves of *Ilex aquifolium* L. were collected regularly from a shrub in the garden of the Botanical Laboratory and investigated for lipids in the epicuticular wax and other parts of the leaf. The epicuticular wax composition appeared largely dependent on the age of the leaf. In very young leaves hexyl esters of the fatty acids C22 – C32, with the even numbered esters C32 – C36 as main constituents, constitute the major part of the long-chain aliphatic fraction of the wax. To our knowledge such a composition has not been described for other plant species. In the course of leaf extension the amount of these esters per cm^2 leaf area rapidly decreases. At the same time and increase is observed in the amounts of the aliphatic hydrocarbons $C_{29}H_{60}$ and $C_{31}H_{64}$.

Around and just after the period in which the leaves reach their maximum surface area increased accumulation of the triterpenols α- and β-amyrin is observed, starting with that of β-amyrin. The major part of these compounds may be derived from the fatty acid esters of α- and β-amyrin, originally contained in lipid globules present in the epidermis and hypodermis of only young leaves.

NIEMANN, G. J. & W. J. BAAS (1985): The composition of the lipid constituents of Ilex aquifolium L. (Aquifoliaceae) in relation with the age of the leaf. I. The leaf wax. *J. Plant Physiol.* **118**: 219–226.

R. PEERBOLTE, G. J. WULLEMS and R. A. SCHILPEROORT (*Vakgroep Moleculaire Plantkunde MOLBAS, Biochemisch Laboratorium, Wassenaarseweg 64, 2333 AL Leiden*).

The (in)accuracy of gene transfer to plant cells by A. tumefaciens.

Octopine type *Agrobacterium tumefaciens* strains introduce distinct parts of their 'Tumor-inducing

plasmid' (pTi) into plant cells where the transferred DNA (T-DNA) becomes integrated in the nuclear DNA. On pTi the T-region is bound by 23 bp direct border repeats, essential for T-DNA transfer and/or integration. The T-DNA *onc*-genes (*aux*-1, *aux*-2 and *cyt*), expressed in plant cells, cause a severe disturbance of the local phytohormone balance at the site of infection, resulting in a crown gall tumor. In addition to the *onc*-genes the T-DNA harbors several other genes, some of which are responsible for the production of 'opines' by the tumor cells. Opines are unusual amino acid derivatives not normally found in plant cells. Octopine type agrobacteria introduce two pieces of T-DNA in plant cells: TL-DNA, harboring the *onc*-genes and the gene for octopine synthase (*ocs*), and TR-DNA, harboring genes for mannopine synthesis (*mas*) and agropine synthesis (*ags*).

In an attempt to gain more insight into the accuracy of plant cell transformation via *A. tumefaciens*, we first investigated the phenotypes of several transformed tissue lines. *A. tumefaciens* strains with a certain T-region will produce crown gall tissues with a certain, predictable phenotype caused by T-DNA genes ($Aux^{+/-}$, $Cyt^{+/-}$, $Ocs^{+/-}$, $Mas^{+/-}$, $Ags^{+/-}$). Observed frequently occurring deviations from the expected phenotype proved to be correlated with irregularities in T-DNA structure and/or expression. From these studies it was concluded that with plant cell transformation via *A. tumefaciens* there is a rather high change (50%) that transformants have obtained irregular T-DNA structures resulting in loss of (non-selected) genes.

From the data obtained the following model was deduced: Errors in T-region processing made inside the bacterium are thought to occur at relatively low frequencies. Integration of T-DNA into plant DNA may cause, at relatively high frequencies, instabilities in the genomic structure of the host cell, resulting in stabilizing rearrangements, amplifications etc. which may affect the T-DNA structure. Stabilized 'settled' T-DNA structures remain fairly stable through mitosis and meiosis for further periods. Recent observations indicate that occasionally, at a very low frequency, even 'settled' T-DNA structures may become destabilized, resulting in rearrangementes, deletions etc. affecting the T-DNA structure.

L. H. W. VAN DER PLAS, H. GUDE and L. P. E. DE GUCHT (*Vakgroep Plantenfysiologie, Biologisch Laboratorium Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam*).

Respiratory physiology and growth of plant tissue cultures.

At least three major pathways for the transfer of electrons to oxygen can be distinguished, when oxygen uptake to plant tissue cultures is measured.

1. The capacity of the *CN-resistant, alternative pathway* is high in cultured cells, as well as in the mitochondria isolated from them. The induction of this pathway is modulated by a.o. the growth temperature and plant hormones. Ethylene action specifically increases the induction, as can be concluded from experiments with 2,5-norbornadiene; abscisic acid inhibits its induction.
2. The *cytochrome pathway*, also located in the mitochondria, is the chief source of the ATP that is needed for (heterotrophic) growth and maintenance. Plant cells preferentially use the cytochrome pathway. Only the overflow, which cannot be handled by the cytochrome pathway, is transferred via the alternative pathway during *in vivo* uninhibited respiration.
3. The non-mitochondrial *residual 'respiration'*, resistant to inhibitors of both the cytochrome and the alternative pathway, is always observed in cells from tissue or cell culture, but is especially prominent in the stationary phase.

From the activities of the cytochrome and the alternative pathway during logarithmic growth, the ATP-production can be calculated. Comparison of this ATP-production and the production of cellular dry weight, yields dry weight production values, ranging from 2–25 g dry weight per mole of ATP. This yield depends a.o. on the growth rate and the cell type.

E. W. M. SCHRIJNEMAKERS and F. VAN IREN (*Biotechnologie Delft Leiden, Vakgroep Moleculaire Plantkunde Rijksuniversiteit, Postbus 9502, 2300 RA Leiden*).

Cryopreservation of plant cell suspension cultures.

Stable and long term storage of stocks of plant cell cultures in or over liquid nitrogen is important because maintenance is costly, suspension cultures are not stable and may be lost as result of infec

tions etc. We studied the conditions for cryopreservation of several cell lines in use within the project group by variation of growth stage, preculture (sorbitol, mannitol or proline), cryoprotection (DMSO, glycerol, proline, and/or sucrose), programmed freezing (0.5 to 2.0 °C min^{-1}), rapid thawing, plating, and suspension. Procedures were monitored throughout (O_2 consumption and vital staining).

As a result a light-grown line of *Catharanthus roseus* could be recovered from freezing after various procedures. The same line, grown in the dark could be recovered only after one procedure. Both lines are in suspension again after several months of cryostorage. Light grown lines of *Cinchona ledgeriana, C. robusta* and *Tabernaemontana divaricata* survived the freeze-thaw cycle. However, regrowth was not yet very satisfactorily and should be optimized further.

The results suggest that most cell lines can be succesfully preserved by means of one or two standard procedures elaborate protocols only being required for very recalcitrant lines.

L. SMITS, G. VAN NIGTEVECHT and J. VAN BREEDERODE (*Vakgroep Populatie- en Evolutiebiologie Rijksuniversiteit, Padualaan 8, 3584 CH Utrecht*).

Induction of flavone glycosyltransferase activity in cell suspension cultures of Silene pratensis.

Plant phenolics are of importance for the plant environment interaction. In *Silene* the study of flavone glycosylation has revealed the existence of allozymes differing in substrate specificity for the sugar donor as well as flavone acceptor and the existence of differently regulated genes encoding the same product.

Until now eleven dominant genes, spread over six loci, have been demonstrated, which control the glycosylation of isovitexin, the main flavone in *Silene*.

This complicated pattern of variation and regulation in the expression of flavone glycosylation genes suggests an adaptative component in this variation, which can easily be tailored by the plant. To study the molecular genetic mechanisms leading to this adaptation, the enzymes catalyzing and the genes controlling these reactions have to be isolated.

HAHLBROCK *et al.* (1971) showed that in cell cultures of parsley the enzymes involved in flavonoid biosynthesis can easily be elicited by UV. We therefore started cell suspension cultures of *Silene*, especially because the induction by UV makes the isolation of the messengers encoding these enzymes less cumbersome.

Callus was isolated from mesophyll and epidermis of young leaves. The cell cultures obtained from it were grown on Gamborg + NAA, BAP and sucrose at 600 lux.

Under these circumstances no flavone glycosyltransferase activity was detectable. Continuous light (18000 lux) triggers this activity, whereas (contrary to Hahlbrock), UV is without effect.

In the logarithmic growth fase the transferase acitivity is strongly diminished, leading to a dip at day four.

Variation in nutrients and hormone balance of the propagation medium hardly influences the transferase activity.

The induced cell cultures are used as a source for the isolation of the enzymes and genes involved in flavone modification.

J. DE LANGHE, K. POMA and A. VAN MAELE (*Laboratorium voor Biotechnologie, Industriële Hogeschool van het Rijk, Voskenslaan 270, B-9000 Gent, België*).

Routine assay of galloylesters in suspension cultures of Rhus typhina L.

D. KETEL, H. BRETELER, B. DE GROOT, M. JANSEN, P. PIKAAR and D. LUTKE WILLINK (*Onderzoekinstituut Ital, Postbus 48, 6700 AA Wageningen*).

Thiophene biocides from cell cultures of Tagetes patula.

MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE IN COOPERATION WITH THE RESEARCH STATION FOR FLORICULTURE AND THE ASSOCIATION OF DUTCH FLOWER AUCTIONS ON 'TISSUE CULTURE OF CUT-FLOWERS AND POT-PLANTS' HELD ON 13 NOVEMBER 1986.

LECTURES

J. BROCKHOFF (*Vereniging van Bloemenveilingen in Nederland, Verbeekstraat 11, 2332 CA Leiden*).

The market for starting material.

G. J. DE KLERK (*Centraal Onderzoeklaboratorium voor de Weefselkweek van Tuinbouwgewassen, Postbus 85, 2160 AB Lisse*).

Objectives of the Central Research Laboratory for Tissue Cultures of Horticultural Plants.

Tissue culture research at the experimental stations in Aalsmeer (cut flowers and pot plants), Boskoop (trees), and Lisse (bulbs) has been brought together in a new laboratory, named Central Research Laboratory for Tissue Culture of Horticultural Plants. This talk is intended to introduce this new laboratory. The aim of the new laboratory is to draft protocols for *in vitro* propagation of horticultural plants. Both species-orientated and fundamental problems will be examined. A survey is given of present research and research plans.

H. HAKKERT and L. LEFFRING (*Proefstation voor de Bloemisterij, Linnaeuslaan 2A, 1431 JV Aalsmeer*).

Obtaining of pathogen-free explants of Cyclamen persicum cultivars.

Cloning of selected parent plants will reduce the variability which occurs when plants are propagated from seeds. However, no macropropagation method is available, while published micropropagation methods are not satisfactory due to, among other things, infestination of *Cyclamen*-tissue by bacteria and fungi, leading to contamination of explants.

A pretreatment of *Cyclamen* plants with fungicides and/or bactericides did not reduce contamination in explants from flower-tissue. Surface sterilisation at high concentrations of sterilant resulted in death of the explants. Subsequently, various tissues (leaf blade, leaf stalk, flower bud, and flower stalk) were cultured *in vitro*. In explants of flower-tissue, the percentage of infection was found to be between 51 and 96. In leaf-explants, this percentage was between 2 and 19. In all four types of tissue, callus had developed after 4 weeks in more than 90% of the explants. In leaf-stalk-explants, shoots had developed in a maximum of 2% of the explants, in the other explants in a maximum of 40%. The age of the leaves and the genotype affected the regeneration capacity.

In conclusion, leaf-blade-explants seem most suitable for micropropagation of *Cyclamen* plants because of (1) low infection rate, and (2) high capacity for regeneration.

J. DONS (*Instituut voor de Veredeling van Tuinbouwgewassen, Postbus 16, 6700 AA Wageningen*).

Biotechnology in horticultural plant breeding.

L. LEFFRING (*Proefstation voor de Bloemisterij, Linnaeuslaan 2A, 1431 JV Aalsmeer*).

Some aspects of in vitro propagation of Rosa.

F. HAKKAART (*Instituut voor Plantenziektekundig Onderzoek, Postbus 9060, 6700 GW Wageningen/Proefstation voor de Bloemisterij, Linnaeuslaan 2A, 1431 JV Aalsmeer*).

Some aspects of obtaining virus-free starting material using meristem culture.

J. N. KRAS (*Vereniging van Bloemenveilingen in Nederland, Verbeekstraat 11, 2332 CA Leiden*).
May the juridical threats to tissue culture be greater than its chances?

POSTERS

J. B. M. CUSTERS, J. H. W. BERGERVOET and J. F. DEMMINK (*Instituut voor de Veredeling van Tuinbouwgewassen, Postbus 16, 6700 AA Wageningen*)
Gynogenesis in tetraploid relatives of carnation, Dianthus caryophyllus, to improve crossability.

J. DE JONG and J. B. M. CUSTERS (*Instituut voor de Veredeling van Tuinbouwgewassen, Postbus 16, 6700 AA Wageningen*)
The effect of explant source, in vitro regeneration and irradiation on variation in yield induced in Chrysanthemum morifolium.

J. B. M. HUITEMA, G. C. GUSSENHOVEN and J. DE JONG (*Instituut voor de Veredeling van Tuinbouwgewassen, Postbus 16, 6700 AA Wageningen*)
Selection and in vitro characterization of low-temperature tolerant mutants of Chrysanthemum morifolium.

MEETING OF THE SECTION FOR THE RELATION BETWEEN PLANTS AND ANIMALS ON 1 NOVEMBER 1986

O. C. DE VOS (*Vakgroep Plantenfysiologie, Biologisch Centrum, Postbus 14, 9750 AA Haren (Gr.)*)

Anthecological adaptations of wasp-flowers

Several authors report on social wasps (Vespinae) visiting specialised flowers. These 'wasp-blossoms' are characterised by their size, shape and colour. Reddish-brown flower-parts are common. UV photographs of *Scrophularia nodosa* flowers did not show any UV reflection in these parts. Probably the reddish-brown parts appear black to the wasp. Perhaps the brown patterns accentuate the margins of the cup-shaped corolla.

Investigations of some species of *Scrophularia* showed that social wasps are not occasional visitors, but true pollinators. *Scrophularia nodosa* was visited mainly by bees and bumble-bees, at least in the early summer. From mid-July onward the remaining flowers were visited by *Dolichovespula saxonica* and *D. sylvestris*. After each visit the pollen grains left on the stigmata were counted. It appeared that the wasps transferred more pollen than the other insects. The wasp is the most effective pollinator, although by the time its interest awakes, most flowers have already been pollinated by bees and bumble-bees. Investigation of *Scrophularia umbrosa* subsp. *neesii* showed that in this species the adaptation to wasp-pollination is more complete.

The spring-flowering *Scrophularia vernalis* does not attract social wasps. Its flowers lack the wasp-blossom characters. Also the staminodium (the fifth stamen), conspicuous in the other native species, is absent in this taxon. The staminodium appears to be functional in the wasp-pollinated species. Removal of the staminodium in *S. umbrosa* resulted in a decrease in the amount of pollen transferred by the wasps. The importance of the staminodium as a wasp attractant could not be proved. However, the removal of both staminodium and upper lip did decrease the number of wasp visits.

The overall distributions of the subfamily Vespinae and the genus *Scrophularia* nearly coincide, especially if the wasps involved belong to the genus *Dolichovespula* and if the *Scrophularia* species without staminodium are excluded.

M. J. SOMMEIJER (*Laboratorium voor Vergelijkende Fysiologie, Jan van Galenstraat 40, 3572 LA Utrecht*)

Aspects of reproductive behaviour and foraging of stingless bees

The c. 400 species of pantropical stingless bees (Apidae, Meliponinae) are considered to be the most important tropical pollinators. They all have a permanent nest, in most species with an arrangement of brood cells in horizontal combs. The general pattern of comb building and oviposition in *Melipona* is as follows: the brood cells, used only once, are constructed at the comb margins and when ready for oviposition they are provided with a smooth collar that protrudes from the comb surface. Then a group of workers gather around the structurally ready cell while making frenetic body movements and alternately inserting their fore-bodies briefly into the cell, which activities increase gradually until the queen arrives and may continue for some time while frequent interactions occur between queen and workers. The inserting workers subsequently discharge larval food into the cell by abdominal contractions. Once a cell is filled, the queen oviposits on top of the food. Prior to oviposition by the queen, a worker's oviposition may occur; usually the worker's egg is immediately devoured by the queen. Characteristic features of all species are: the rhythmic ebbing and flowing of oviposition 'excitement', short periods of provisioning and oviposition activity alternating with longer periods of building activity. During extra-oviposition periods the queen of *Melipona favosa* rests at fixed places, away from the young combs to become active only shortly before the provisioning and ovipositioning of the brood cell. The court of workers surrounding the queen during extra-oviposition periods principally includes workers then actively engaged in cell building and shuttling frequently between the building site and the distant, resting queen. Typical court-behaviour, containing various antagonistic elements and in some species having evolved into highly ritualized 'bee dances', is important for the regulation of the typical locomotions of the queen. The queen is thereby informed about the state of cell construction.

Our comparative study of foraging behaviour of various stingless bees and honeybees in Trinidad revealed a considerable overlap of their pollen spectra. However, certain taxa were of different importance to the various species, *e.g., Tectona grandis* forming a major pollen source exclusively for *Nannotrigona*. At the onset of the rainy season some species shift to other sources, certain plants remaining a major pollen source for other ones (*e.g., Cocos nucifera* for honeybees).

A. HAGEDOORN (*Vakgroep Plantensystematiek, Biologisch Centrum, Postbus 14, 9750 AA Haren (Gr.)*)

Corolla-scales and stamens involved in the pollination mechanisms of some Boraginaceae

The following functions of the corolla-scales and stamens of boraginaceous flowers have been mentioned: (1) Protection of pollen and nectar against rain. (2) As a nectar-guide; the scales often have a colour differing from the rest of the flower. (3) The scales, the narrow tube and the stamens are placed in such a way that the pollen transfer is sternotribic. Furthermore, these organs usually lengthen the flower-tube so that bees with a short proboscis can only gain access to the nectar by robbing.

Recently BUCHMANN (1983) has neatly reviewed buzz-pollination in families with the following floral characteristics: flowers pendulous, with an anther-cone, the anthers opening poricidally. In such flowers the pollen is set free by buzzing.

One of the results of my study is that buzz-pollination also occurs in the boraginaceous species studied. European species belonging to 12 genera were investigated. The main differences between this type of buzz-pollination and Buchmann's are: also scales or stamens, or combinations of them, are involved in this pollination mechanism, and in this family the anthers open with a longitudinal slit. The morphology of the organs involved and also that of the corolla is variable. It follows that the incidence of several types of buzz-pollination has been established. It is also clear that buzz-pollination does not only occur in families with pendulous flowers with poricidal anthers, but also in some boraginaceous species. In these plants a cone of scales or of stamens represents an essential part of this kind of pollination syndrome. Apart from the three functions of the scales and stamens mentioned before, such plant parts also may be involved in buzz-pollination.

BUCHMANN, S. L. (1983): Buzz-pollination in angiosperms. In: R. J. JONES & R. J. LITTLE (eds.): *Handbook of experimental pollination biology*. Nostrand-Reinhold.

M. N. B. M. DRIESSEN, R. M. A. VAN HERPEN, F. E. A. M. VAN DEN OEVER and R. P. M. MOELANDS (*Medisch Centrum Dekkerswald, Nijmeegsebaan 31, 6564 CA H. Landstichting*)

The use of pollen counts and field observations to predict the commencement of flowering of grasses

There is no consensus as regards the beginning of the flowering season of grasses or 'hayfever season'. Prediction was attempted by both daily pollen counts (Leyden and Helmond, records many years running) and phenological observations (Haarlem and Scherpenzeel). It has also been tried by means of the phenological method, to predict the first flowering of grasses by recording flowering dates of earlier flowering taxa, so-called indicator plants.

In previous studies (DRIESSEN *et al.*, 1985) the average initial flowering date was assessed by means of pollen counts (the so-called 1% level and Mullenders methods). The phenological approach yielded unreliable results. At present the commencement of the grass pollen season is defined differently, *viz'*. by means of four Σ methods (the so-called Σ 50, Σ 75, Σ 100, and Σ 125-methods), the beginning being put at the date when the 50th, 75th, 100th and 125th pollen grain is recorded. Our new definition has the advantage, among other things, that factors determining the initiation of flowering are better accounted for. The mean first dates of grass flowering (1977 up to and incl. 1985) in the Leyden area yielded May 21 with the Σ 100 method (SD 4.6 days). Using the phenological method of prediction (the birch acting as indicator plant), regression equations enabled us to predict the commencement of flowering. In the Leyden area the Σ 100 of grasses (y) can be predicted by

means of the Σ 125 of the birch pollen (x), with the equation $y = 0.68x + 65.33$. The effectivity of the prediction appeared to be 62.2% and the residual spreading 1.6 days.

Apart from pollen counts, field observations were used to determine the beginning of the grass flowering season. Records from Haarlem (1953–1965) and from Scherpenzeel (1973–1983) show that the first flowering of several grasses can be predicted by using certain non-poaceous indicator species.

Our results show quite clearly that a more reliable prediction of flowering dates of grasses can be obtained by using the phenological method (pollen counts or field observations) than by means of the average starting data of flowering grasses alone.

DRIESSEN, M. & R. MOELANDS (1985): Estimation of the commencement of the grass pollen season and its prediction by means of a phenological method. *Acta Bot. Neerl.* **34**: 131.

M. N. B. M. DRIESSEN and J. A. G. VAN LUIJN (*Medisch Centrum Dekkerswald, Nijmeegsebaan 31, 6564 CA H. Landstichting*)

Morphological distinction between grass pollen grains.

Counts of aerial pollen have been performed both in Helmond (since 1975) and in Leyden (since 1970), see SPIEKSMA *et al.*, 1985. These counts were recorded per species or per genus or family as the number of grains per m^3 of air caught during 24 hrs. The lists of pollen types are differentiated into species (3 spec.), to the genus (29 genera) or to the family (13 families). The grass family (Poaceae) is not differentiated below the family level. The subdivision of the pollen of grasses to the species level is extremely difficult. This is regrettable because such a distinction would provide valuable addition to the pollen counts.

It was tried to apply the method used by LIEM *et al.* (1968) to material obtained from the daily pollen counts in Helmond and Leyden. Pollen grains of 20 different grass species were pretreated by means of a dehydration procedure and subsequently studied photomicroscopically at a magnification of x 800. Although differences between these poaceous pollen types could be observed, they were insufficiently discriminating to serve for the drawing up of an identification key. Only the pollen of two taxa, *Bromus hordeaceus* subsp. *hordeaceus* (older name *Bromus mollis*) and *Phragmites australis*, exhibit a characteristic dehydration pattern, so that they can be identified with certainty. The method employed appears as yet inadequate to distinguish all poaceous pollen types represented in the daily counts at Helmond and Leiden.

LIEM, A. S. N., J. GROOT & L. W. KUILMAN (1968): The use of silicone oil as an embedding medium for the identification of the pollen of sixteen grass species. *Rev. Palaeobot. Palynol.* **7**: 213–231.

SPIEKSMA, F. T. M., A VAN DEN ASSEM & B. J. A. COLETTE (1985) Airborne pollen concentrations in Leyden, The Netherlands. *Grana palynol.* **24**: 99–108.

H. BRUGGINK, A. HUIZENGA AND B. M. MOELIONO (*Vakgroep Plantensystematiek, Biologisch Centrum RUG, Postbus 14, 9750 AA Haren (Gr)*)

Energetic differences in foraging behaviour between *Bombus pascuorum* Scop. and *Bombus terrestris* L. on the broad bean (*Vicia faba* L.) II. Time-energy budgets and an estimation of the net rate of energy intake.

When collecting nectar on the broad bean, *Bombus pascuorum* is a legitimate forager, while *B. terrestris* is a primary robber. The object of our investigations was to find out whether the behaviour of these two *Bombus* species has implications for their foraging efficiency. PYKE (1980) presents an equation by which the net rate of energy intake can be calculated. The amounts of time spent on the distinct activities in the foraging area determine their time budgets; the rate of energy expenditure and gain of these activities combined with the time budgets result in the net rate of energy intake.

The time budgets have been presented in the previous lecture (BRUGGINK, 1987). In the summer of 1986 additional measurements have been made, so that the equation can be completed. The mean weight of a number of bumble-bees of each species has been determined: mean weight of *B. pascuorum:* 0.151 g, that of *B. terrestris:* 0.208 g. For the thorax temperature the literature gives

a value of about 37 °C (PYKE, *l.c.*). The energy expenditures that result from these figures are similar for *B. pascuorum* and *B. terrestris* (at ambient temperatures ranging from 15–25 °C), despite large differences in time budgets.

Measurements of the amounts of nectar produced during the day show a peak in the late afternoon when the flowers start operning; this means that flowers visited in the morning by *B. terrestris* will be replenished by the time *B. pascuorum* starts foraging. On the base of field observations we assume that there is no substantial difference in nectar reward, and estimate it to be 0.25 mg/flower, with a sugar content of approx 36% (= 0.3 cal/flower), on an average summer day. At the previously determined flower visiting rate, the energy gain will then be 1.95 cal/min, so that the net rate of energy intake is 1.49 cal/min (time in the foraging area).

BRUGGINK, H. (1987): Energetic differences in foraging behaviour between *Bombus pascuorum* Scop. and *B. terrestris* L. on the broad bean (*Vicia faba* L.): Time budgets. *Acta Bot. Neerl.* **36**: 140

PYKE, G. H. (1980): Optimal foraging in bumblebees: calculation of net rate of energy intake and optimal patch choice. *Theor. Pop. Biol.* **17**: 232–246.

G. VAN DER VELDE (*Laboratorium voor Aquatische Oecologie, Katholieke Universiteit, Toernooiveld, 6525 ED Nijmegen*)

Aquatic macrophytes, their function for animals

Macrophytes have a beneficial function for a large number of organisms; they form an important substratum for epiphyton, sessile micro-organisms, epiphytophagous animals and filter feeders and their predators, as well as for the eggs of aquatic and semi-aquatic invertebrates. They form a good anchoring site for plants and animals which are exposed to wave action, water currents and sediment movements. By shading, the macrophytes provide suitable sites for negatively phototactic animals. Furthermore, they form a suitable site/substratum for the metamorphosis of semi-aquatic insects; for these insects they form an important route between air, water and sediment. This holds also for aquatic invertebrates, which use the macrophytes to escape from the oxygen-poor sediment to the oxygen-rich upper water layers; higher temperatures create favourable conditions there for the hatching of their eggs. The macrophytes form a hunting area and territory for mobile terrestrial and aquatic predators. On the other hand they provide a shelter for animals exposed to extensive predation in the open air or open water. For flying terrestrial insects they provide also a shelter against unfavourable weather conditions. Plant parts above the water surface are also used for orientation during swarming behaviour (caddis flies, chironomids), and they function as resting, sunning and drinking sites for imagines of terrestrial and semi-aquatic insects. Another function is the use of macrophytic material for the building of cases (caddis fly larvae, caterpillars). Macrophytes provide food directly for flower-visiting insects and herbivores and indirectly for detritivores; they function as detritus traps and as a substratum for epiphyton which can be consumed by animals. Supplying of oxygen, favouring animal life, is released to the water by the above-ground parts and in the sediment by their roots. Insect larvae with pointed stigmata can "tap" oxygen from the aerenchyma just as do miners.

Negative effects of macrophytes are also known, however. Certain animals avoid vegetation: because of chemical excretions and decay products of macrophytes and/or epiphyton (zooplankton), because they are typical for a mineral (sandy) bottom without too much decaying plant material (interstitial fauna), because predation is hindered there (large fishes), because the surface tension is too much decreased (large neuston organisms), or because currents and other water movements are impaired, so that the oxygen supply is insufficient (*e.g.*, lotic animals).

MEETING OF THE SECTION FOR PHYTOPATHOLOGY ON 22 JANUARY 1987

G. LEONE, A. N. OVERKAMP, M. N. KREYENBOEK and J. VAN DEN HEUVEL (*Phytopathologisch Laboratorium "Willie Commelin Scholten", Javalaan 20, 3742 CP Baarn*).

Phosphate involvement in the regulation of polygalacturonases of Botrytis cinerea is mediated by adenine nucleotides.

Infections by *Botrytis cinerea* of French bean leaves can be stimulated by the addition of glucose and inorganic phosphate (Pi) to the inoculum. The stimulation by Pi is correlated with an enhanced polygalacturonase (PG) activity. PG1 and PG2 are the first PGs detected within a few hours after inoculation. PG2 has been found to be a constitutive enzyme, whereas PG1 seems to be inducible.

The possible effect of Pi and adenine nucleotides (AMP, ATP, cAMP) on PG production was investigated with shake cultures of the fungus in a basal salt medium supplemented with glucose as the only C source. Pi, cAMP and ATP stimulated fungal growth and, indirectly, the constitutive production of PG2. In contrast, the effect of AMP on fungal growth was small, but in particular PG1 production was stimulated. PG1 synthesis was also readily stimulated when salicylanilide, an inhibitor of ATP production in the cell, was added to cultures with Pi. Experiments with cycloheximide and actinomycin D indicated that during stimulation of PG production by Pi, AMP or ATP a de novo protein synthesis takes place.

The results suggest that the stimulation by Pi of PG production is mediated by adenine nucleotides in the fungal cell and that the metabolic energy status is involved in the regulation of synthesis of PG1.

L. C. VAN LOON (*Vakgroep Plantenfysiologie, Landbouwuniversiteit, Arboretumlaan 4, 6703 BD Wageningen*)

Induction of pathogenesis-related proteins in tobacco

In Samsun NN tobacco reacting hypersensitively to tobacco mosaic virus (TMV) ten newly induced pathogenesis-related proteins (PRs) accumulate both in the inoculated, local-lesion-bearing leaves and in noninfected, symptomless, virus-free leaves. A similar induction occurs upon pricking healthy tobacco plants with needles moistened with phytotoxic concentrations of the ethylene-releasing compound, ethephon. The formation of local lesions upon TMV infection is accompanied by a burst of ethylene production, suggesting that the ethylene produced naturally during the hypersensitive reaction, is responsible for the induction of PRs. Pathogens, physiological conditions, and chemicals known to induce PRs in tobacco also stimulate ethylene production, with the exception of benzoic acid derivatives such as salicylic acid (SA). However, whereas TMV and ethephon induce PRs in both treated and untreated leaves at 20 °C, but not at 30 °C, SA induces PRs only in treated leaves at both 20° and 30 °C. This could be explained if during the hypersensitive reaction ethylene leads to the temperature-sensitive synthesis of a, presumably aromatic, compound that mimics the action of SA and functions as the natural inducer of PRs.

Whereas ethephon stimulated aromatic biosynthesis in tobacco by increasing the activity of the enzyme phenylalanine ammonia-lyase (PAL), ethylene produced from the natural precursor 1-aminocyclopropane-1-carboxylic acid (ACC) did not. ACC induced high amounts of PRs- 2c, -3a, -3b, -4 and -5, but only small amounts of PRs-1a, -1b, -1c, -2a and -2b. In contrast, SA induced large amounts of the latter PRs, and combination of ACC and SA induced PRs similarly to TMV infection. In TMV-infected leaves aminoethoxyvinylglycine, which specifically inhibits ACC synthesis, blocked ethylene production with a concomitant reduction in PRs- 2c to -5, whereas α-aminooxyphenylpropionic acid, a specific competitive inhibitor of PAL, inhibited only the accumulation of PRs -1a to -2b. The induction of all ten PRs by ethephon pricking appeared to be due to a combination of ethylene release and an increase in PAL due to tissue damage. Thus, the occurrence of a full complement of PRs appears to require a dual induction mechanism: elevated ethylene production, and stimulation of the biosynthesis of aromatic compounds as a result of tissue necrotization.

I. M. J. TOMA and P. J. G. M. DE WIT (*Laboratorium voor Fytopathologie, Landbouwuniversiteit, Binnenhaven 9, 6709 PD Wageningen*)

Isolation and characterization of a necrosis-inducing peptide from the interaction Cladosporium fulvum-tomato

Apoplastic fluids of compatible interactions between *C. fulvum* and tomato contain race-specific elicitors (DE WIT & SPIKMAN 1982). Here we report about the purification and final characterization of a peptide elicitor, the putative product of avirulence gene A9, which specifically induces necrosis in the tomato cv. Sonatine carrying resistance gene Cf9. This cultivar is resistant to all races carrying avirulence gene A9 (race 0, 2, 4, 5, 2.4, 2.4.5) and thus not to race 2.4.5.9, which lacks avirulence gene A9. Race 2.4.5.9 does not produce the elicitor.

By preparative native polyacrylamide gel electrophoresis at low pH followed by reversed-phase HPLC the elicitor could be purified in sufficient amounts to have its amino acid sequence determined (Dr. R. A. Amons, RU Leiden). The peptide was not blocked at its NH2-terminus and it contained 27 amino acids (3000 Mw) from which 6 were cysteine residues. The cysteines most probably form S-S bridges. After reduction of the S-S bridges or derivatization of the cysteines the peptide was no longer biologically active. A synthetic DNA-probe derived from amino acid residues 19 to 24 has now been prepared (17-mer, mixed probe, 64-fold degenerate). It will be used in hybridization studies with fungal (and plant) DNA to detect the encoding gene. Eventually the gene can be cloned and characterized.

Neither the native free peptide nor the peptide bound to nitrocellulose appeared to be antigenic when injected into rabbits. From mice, however, antibodies could be obtained using the free peptide. This antiserum can be used for instance to determine the appearance of the peptide in time-course experiments or to determine possible homologies between the peptide and other proteins.

DE WIT, P. J. G. M. & G. SPIKMAN (1982): *Physiol. Plant Path.* **21**: 1–11.

O. KAMOEN[1], D. JACOBS[1], M. RUDAWSKA[2] and P. NUYTTEN[1] ([1]*Rijksstation voor Plantenziekten, Merelbeke, België*; [2] *Institute of Dendrology, Kornik, Polen*)

Partial removal of catabolite repression of 1,3-β-glucanase synthesis in Trichoderma harzianum protoplasts by adenosine 3′:5′-cyclic monophosphate

An exo-1,3-β-glucanase is induced in *Trichoderma harzianum* in media poor in glucose, but catabolite repression occurs at high glucose concentration. As early as 1968 several authors (ULLMAN & MONOD 1968, PERLMAN & PASTAN 1968) were able to overcome the catabolite repression in bacteria by adding adenosine 3′:5′-cyclic monophosphate (cAMP) to the medium. On the contrary the removal of catabolite repression in fungi by means of external addition of cAMP was largely unsuccesfull (ININGER & NOVER 1975; SANDS & LUCKENS 1974; KAMOEN & RUDAWSKA 1984).

WISEMAN & LIM (1974) reported a removal of catabolite repression of α-glucosidase by addition of cAMP to yeast protoplasts, but not in whole yeast. We also used the technique of protoplast culture with *Trichoderma harzianum*. Spores (2.10–7/ml) were germinated in a shake culture of worth broth (Difco 0390-01 at 16 g/l). Protoplasts were prepared following the procedure of WISEMAN & LIM (1974). Cell wall degradation was obtained by overnight incubation in Novozym 234 (5 mg/ml). Protoplasts were then suspended in 0,8 M KCl – Na-citrate buffer (0,1 M ph 6) containing 1 mg/ml laminarin as inducer. They were divided in three series: (I) poor in glucose (0,02%), (II) rich in glucose (1%) and (III) rich in glucose with cAMP added (20mM). The 1,3-β-glucanase synthesis in the media was measured daily.

The highest amount (0.1 U/ml) was found in the poor glucose series, the lowest amount (0.015 U/ml) in the rich glucose series and an intermediate amount in the rich glucose series with cAMP.

These results suggest that cAMP may be involved in the catabolite repression of a number of enzymes in fungi. Better understanding of this mechanism may be helpful to influence the synthesis of some important enzymes in antagonists and parasites.

ININGER, G. & L. NOVER 5 (1975): *Biochem. Physiol. Pflanzen* **167**: 585–595.
KAMOEN, O. & M. RUDAWSKA (1984): Unpublished data
PERLMAN, R. & I. PASTAN (1968): *Biochem. Biophys. Res. Commun.* **30**: 656–664.

ULLMAN, A. & J. MONOD (1968): *FEBS lett.* **13**: 184–193.
SANDS, D. C. & R. J. LUCKENS (1974): *Plant Physiol* **54**: 666–669.
WISEMAN, A. & T. K. LIM (1974): *Biochem. Soc. Trans.* **2**: 932–935.

E. J. A. ROEBROECK[1] and R. J. M. DE GREEFF[2] ([1] *Laboratorium voor Bloembollenonderzoek, Vennestraat 22, Postbus 85, 2160 AB Lisse;* [2] *Vakgroep Fytopathogie, Landbouwuniversiteit Wageningen*)

Histological and biochemical aspects of the wound healing response of scales from lily bulbs and their possible rôle in the defence against infection by Penicillium spp.

Vegetative propagation of lilies by adventitious bulblet formation on bulb scales is widely used in commercial practice. For this purpose scales are detached from the mother bulb and incubated in water-saturated vermiculite at 23°–25 °C.

When scales from lily bulbs (cv. Enchantment) were kept at 20 °C and at high relative humidity directly after wounding, they showed a high degree of resistance against infection by *Penicillium* spp. Cell walls at the wound surface of these scales stained blue-green with toluidine blue 0 as soon as 12 hours after wounding. However, when scales were kept at dry circumstances the first 24 hours after wounding, allowing the scale tissue to loose more than 6% of its fresh weight, the number of *Penicillium*-infected scales increased up to 100% and no positive staining reaction could be observed.

Sections of tissue that normally stained with toluidine blue 0 gave no reaction when pretreated with 0.5M NaOH for 2h at 80 °C.

Wounded tissue, incubated at different temperatures between 2 °C and 34 °C for 24h, stained with toluidine blue 0 only at temperatures above 17 °C. Furthermore, the intensity of the staining reaction, estimated visually, increased with temperature. Alkaline extracts (0.5 M NaOH, 2h, 80 °C) of wound tissue, incubated at these temperatures, contained p-coumaric acid and ferulic acid. These compounds showed a similar increase with temperature as did the staining reaction with toluidine blue 0.

The positive relationship between a staining reaction with toluidine blue 0, the presence of p-coumaric- and ferulic acid in the alkaline extract of the wound tissue and the resistance against infection by *Penicillium* spp. supports the idea that the binding of phenolic acids to the cell wall is a part of the wound response, that may play a rôle in the defence against fungal infection.

T. W. HOFMAN and P. H. J. JONGEBLOED (*Laboratorium voor Fytopathologie, Landbouwuniversiteit, Binnenhaven 9, 6709 PD Wageningen*)

Effects of granular nematicides on the infection process of Rhizoctonia solani on the potato plant

Spring application of the granular nematicides Mocap (ethoprophos), Vydate (oxamyl) or Temik (aldicarb) gave a significant increase in stolon and stem infection of potatoes by *Rhizoctonia solani*. The mechanisms involved in this effect may be direct effects of the nematicides on the growth and/or pathogenicity of *R. solani* or on the susceptibility of the potato plant. Indirect effects can be on the microbial antagonism to *R. solani* (fungistasis and mycoparasitism) or on the mycophagous soil fauna. In this paper only the direct effects are discussed.

The infection process was studied using light and scanning electron-microscopy (SEM). Hyphae of *R. solani* first developed mainly in the direction of the axis of the sprout. In the second phase, side branches of short and swollen cells developed. These cells clump together forming an infection cushion, from which many penetration hyphae were formed. Penetration may occur either enzymatically or mechanically. In a later stage it was shown that the cell wals of the epidermis were partly deteriorated around the penetration site. Colonization of the potato sprouts was restricted to a very limited number of cell layers underneath the infection cushions. SEM showed that cell walls in this tissue were enzymatically deteriorated.

The area of sprout surface covered by infection cushions is related to the necrotic sprout surface. No progressive lesion development occurred.

Ethoprophos delayed the infection process. This effect was due to a direct fungitoxic effect of the product; smaller infection cushions were formed and they appeared at a later stage in sprout development. Oxamyl did not affect the host-pathogen relation. The effect of aldicarb is still under research.

In conclusion, the effects caused by the nematicides on the *R. solani* stem-infection can not be attributed to a direct effect on the host-pathogen relation.

A. W. BAKKER *(Phytopathologisch Laboratorium 'Willie Commelin Scholten', Javalaan 20, 3742 CP Baarn)*

The role of HCN producing Pseudomonas spp. in yield reductions in short potato rotations

Based on previous studies the hypothesis was formulated that HCN producing *Pseudomonas* spp. are, at least partly, responsible for the reduction of potato (*Solanum tuberosum* L.) tuber yields in soils with a high potato cropping frequency. Numbers of HCN producing *Pseudomonas* spp. in potato rhizospheres, however, did not significantly differ between soils with high and low potato cropping frequencies. The HCN production per *Pseudomonas* spp. cell, in soils with a high potato cropping frequency, however, may be higher. In in vitro experiments. HCN production per unit of biomass (HCN producers) was shown to strongly depent on several environmental factors, e.g. iron (Fe) availability and amino acid composition of the medium. It may therefore depent on these (and other) environmental factors in soil whether harmful microbial secondary metabolites (e.g. HCN) are actually produced in the rhizospheres. In soils frequently cropped to potatoes substances (e.g. ferri siderophores that can be utilized by the HCN producers) may accumulate which makes the environment suitable for HCN production. In low frequency potato cropping soils this accumulation possibly does not accur.

J. W. M. VAN LENT and B. J. M. VERDUIN
(Vakgroep Virologie, Landbouwuniversiteit, Binnenhaven 11, 6709 PD Wageningen

In situ detection of the translocation of virus in plants

Immunogold/silver staining of um-sections for light microscopy (LM) has been applied to localize cowpea chlorotic mottle virus (CCMV) coat protein antigen in plants of cowpea (*Vigna unguiculata* cv. California Blackeye) as function of the infection period. Cowpea plants were systemically infected with the differential temperature inoculation technique of DAWSON & SCHLEGEL (*Intervirology* 7 (1976): 284–291). Time zero (t = 0) corresponded with the shift of the trifoliate leaf to a higher temperature. Plant tissue was fixed with glutaraldehyde/paraformaldehyde and embedded in Lowicryl K4M or LR White at 0, 6, 12, 24 and 48 h after inoculation. At the same time virus concentration in the systemically infected leaflets was recorded using ELISA.

With ELISA a small amount of viral antigen (0.2 ug per gram of leaf tissue) could be detected in the leaflets at t = 0. The amount of viral antigen did not significantly increase over the first 6 h, but increased rapidly between 6 and 24 h, indicating synchronization of the systemic infection. In the petiolules of systemically infected secondary leaflets, viral antigen could be detected in LM-sections at t = 0 in three phloem cells per cross-section. The number of labelled phloem cells increased with time and at 12 h antigen was also detected in endodermis cells and the adjoining cells of the cortex. At 24 h the number of labelled cortex cells had increased and some xylem vessels were labelled. Similar observations were made in cross-sections of the major veins of the leaflets. Furthermore patches of labelled mesophyll cells were found around vascular tissue as early as 12 h. These results led us to the conclusion that CCMV was transported through the ploem.

F. A. VAN DER MEER (*Instituut voor Plantenziektenkundig Onderzoek, Postbus 9060, 6700 GW Wageningen*)

Nicotiana occidentalis, a new host for viruses of woody plants, and differences in susceptibility between accessions of this species

Leaves of many trees and shrubs are hard and dry and often contain mucilaginous material and virus inactivating substances like tannins. Therefore woody plants provide unfavourable material for virus purification, necessary for the characterization of viruses and for the production of antisera. Suitable herbaceous hosts are usually a prerequisite for successful purification of viruses of woody hosts. Till now, many viruses of trees and shrubs have been transmitted to test plants known to be susceptible to a broad spectrum of viruses, for instance *Chenopodium quinoa* and some *Nicotiana* species. However, several other viruses of woody plants are not transmissible to such test plants. Recently *Nicotiana occidentalis* was found to be a good host plant for four viruses, that could not be transmitted to commonly used test plants in earlier experiments. These viruses are associated with apple stem pitting, gooseberry vein banding, *Laburnum* mosaic and an undescribed mosaic disease of blackberry and are probably the causal viruses of the diseases mentioned. When inoculated from infected to healthy *N. occidentalis*, three viruses induced many local lesions within seven days, followed by local lesions after three more days. Inoculum from their woody hosts contained very little virus, it did not induce local lesions, and in most experiments caused systemic symptoms only in plants that were heavily pruned ca 14 days after inoculation. Such pruned plants developed symptoms in their newly formed lateral shoots in about four weeks after inoculation. The blackberry virus, inducing only local lesions in *N. occidentalis*, also infected *C. quinoa*.
However, this test plant was not susceptible enough to detect the low virus concentration in leaf inoculum of blackberry. Seven *N. occidentalis* accessions were included in the experiments involved. Some were good host plants for all four viruses, whereas others were not susceptible to any of the viruses investigated.

A. B. R. BEEMSTER (*Instituut voor Plantenziektenkundig Onderzoek, Postbus 9060, 6700 GW Wageningen*)

Detection of rhizomania in soil

Rhizomania, a soil-borne virus disease of sugar-beet, caused by beet necrotic yellow vein virus (BNYVV) which is transmitted by a root parasitic fungus (*Polymyxa betae*), is know to occur in The Netherlands since 1983. The disease is very destructive causing considerable loss in yield, while sugar content of affected beets may decrease from c. 15 to around 8%. For the detection of rhizomania-infested fields, for the study of various aspects such as epidemiology, control measures, crop rotation and for establishing the infestation of soil adhering to plant material, a sensitive method is essential. Usually young beet plants are grown for about eight weeks in pots filled with the soil to be tested. During this period the beet rootlets may become infected with. *P. betae* and BNYVV. As cleaning the roots from clay soil is not easy and the method does not readily provide quantitative data, a modified method was developed of which a short description is given in the following. In a Petri dish (diameter 20 cm) filled with a thin layer of soil (50 g) one-week-old, soil-free beet seedlings are arranged in such a way that the rootlets are immersed in the soil and the green parts are sustained by the edge of the dish. During four days the spores of *P. betae* have the opportunity to infect the roots, after which the infection with BNYVV may follow. During these four days the soil is kept moist by adding water, the temperature is kept at 21 °C at a photoperiod of 16 h. The number of seedlings used is arbitrary: up to 50 seedlings can be arranged in a Petri dish. After four days the soil is rinsed off the rootlets and each of the seedlings is transplanted to a pot filled with course sand, each pot placed in a Petri dish (diameter 10 cm). This enables watering each plant separately by filling the dishes with water. Thus mutual infections are avoided after transplanting. About six weeks after transplanting *P. betae* has grown sufficiently for easy visual detection of *P. betae* using the microscope and detection of BNYVV by serological testing. The number of infected plants indicates the rate of infection of the soil by both pathogens.
In case of severe infestations (100% of the plants becoming infected) a soil sample can be diluted with sterilized soil to obtain a more accurate estimate. The method has proved to be valuable for both qualitative and quantitative testing of soil samples.

N. HUIJBERTS and L. BOS (*Instituut voor Plantenziektenkundig Onderzoek, Postbus 9060, 6700 GW Wageningen*)

Lettuce big vein virus, a virological or mycological problem?

In the Netherlands big vein is of increasing importance in the cultivation of lettuce. The agent, possibly a virus, is not transmitted mechanically but by the chytrid soil-inhabiting fungus *Olpidium brassicae*. But for chemical control of the vector in greenhouses, there is no way of efficient control of the disease. Some results have been obtained, especially in the USA, by breeding for resistance.

Resistance breeding requires efficient methods of screening. Such screening under field conditions, involving the intermediate role of the fungal vector, is likely to yield variable results.

At IPO meanwhile an efficient greenhouse test has been developed employing viruliferous zoospore suspensions for inoculation of water cultures or seedlings in Petri-dishes or of such seedlings via the soil. Symptoms appear as early as 17–21 days after inoculation. The results obtained with several lettuce cultivars and some *Lactuca* spp. were reasonably reproducible.

Between cultivars considerable differences have been found in percentages of plants infected and in severity of symptoms resulting (as expressed in average disease severity indexes). In some but not all 'resistant' cultivars vector concentration in the roots was low, suggesting the possible role of resistance to the fungus in LBV resistance. Cultivars were found to often differ considerably in the course of the disease in them. Some cultivars have long incubation periods with severe symptoms late during plant development. Others have a more rapid disease development but recover more or less during later plant development. Experiments under more natural conditions with varying inoculation dosages and inoculation times must reveal possible relationships between certain symptoms or reaction types and final yield reduction.

Such symptoms, if observable early during seedling or plant development, are needed as parameters of final yield reduction for early screening of breeding populations and for rapid cultivar evaluation. The possible role of resistance to the vector must also be further studied.

Endeavours to improve resistance to the disease and the methods to screen for such resistance, as well as other effects to interfere in the ecology of the disease to prevent crop damage, require profound knowledge of the biology and ecology of the vector rather than of the causal agent itself. The research concerned therefore is mycological rather than virological in nature, at least requires more mycological support than realised so far.

I. BLOK (*Instituut voor Plantenziektenkundig Onderzoek, Postbus 9060, 6700 GW Wageningen*)

Is there a relation between Pythium spp. and cavity spot of carrots?

Cavity spot is a disorder of carrots which in literature is attributed to a range of microorganisms, climatic conditions, soil factors or crop rotation. The fungi which are mentioned as possible cause are mainly *Pythium* spp.

In 1986 isolations have been made from carrots with cavity spot grown in two different areas: the IJsselmeerpolders, with light clay soil, and Limburg, with fertile sandy soil. Besides that, carrots have been examined from an experiment in which a stress situation was simulated. In this experiment sandy soil was used from Limburg.

Isolations from the clay soil revealed 33 *Pythium* isolates, of which 27 *P. violae*, one of the species mentioned as causing cavity spot. From the sandy soil 20 *Pythium* isolates were obtained, of which 15 were a slow growing species, which could not yet be identified. The carrots from the stress experiment revealed comparably few *Pythium* isolates, altogether 20, of which 13 were the same unidentified species as from the sandy field. All the remaining *Pythium* isolates were common species such as *P. sylvaticum, P. irregulare* and *P. intermedium.*

The first inoculation experiments on carrots in plastic boxes indicate that *P. violae* as well as the unidentified *Pythium* sp. can be pathogenic when the conditions are favourable for the fungi.

330

A. C. BRAKENHOFF[1], A. KERKENAAR[1], D. M. ELGERSMA[2] and R. J. SCHEFFER ([2]*Instituut voor Toegepaste Chemie TNO, Utrechtseweg 48, 3704 HE Zeist;* [1]*Phytopathologisch Laboratorium 'Willie Commelin Scholten', Javalaan 20, 3742 CP Baarn.*)

Sterol biosynthesis inhibitors for control of Dutch elm disease.

Dutch elm disease is caused by the fungus *Ophiostoma ulmi,* which is restricted to the xylem of the tree. Sterol biosynthesis inhibitors (SBI's) affect the morphology of fungi. In dimorphic fungi, like *O. ulmi,* they inhibit the conversion from the yeast to the mycelial stage. This may have an impact on the pathogenicity of *O. ulmi,* as spreading of the fungus from one vessel to another seems to be accomplished by hyphae only. Fenpropimorph, being the most promising SBI tested, was used in laboratory and field experiments.

In the Flevopolder, 151 elms artificially infected with *O. ulmi* were curatively injected with 7.5 to 75 g of a fenpropimorph-salt. All treatments caused significantly lower disease levels compared to those of control elms in 1985, the year of treatment. Halfway the 2nd growing season, 15 months after injection, no treated trees were showing clear symptoms of Dutch elm disease, indicating that recurrence of the disease had not taken place. The healthiest trees were those injected with the lowest amount of fenpropimorph-salt; phytotoxicity was proportional to the quantities applied. It is expected that application of low amounts of fenpropimorph-salt will keep the phytotoxicity within reasonable limits, whilst still entirely suppressing Dutch elm disease development.

In laboratory tests on liquid media, fenpropimorph inhibited mycelial growth of *O. ulmi* at concentrations as low as 50 µg/l. By means of gas chromatography fenpropimorph could quantitatively be detected in extracts from elm branches collected up to 15 months after injection when the experiment was terminated. Detected amounts of fenpropimorph were always at least 50 times the concentrations effective in vitro. Only a slow decrease in concentration with time was noticed. The average concentration 15 months after injection still was 70% of that six weeks after injection.

Apparently fenpropimorph is retained in the tree for at least two seasons in concentrations expected to be effective for suppression of Dutch elm disease development. These data are of course supported by the observation that symptom development was promptly arrested in the field trial after injection.

MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE ON 13 MARCH 1987

LECTURES

S. C. DE VRIES (*Vakgroep Moleculaire Biologie, Landbouwuniversiteit, De Dreijen 11, 6703 BC Wageningen*

Several aspects of Daucus carota somatic embryogenesis.

Carrot somatic embryogenesis is a well-known model system to study fundamental aspects of early plant development *in vitro*. Our interest is focussed on the acquisition of embryogenic potential in suspension cultures under the influence of the synthetic phytohormone, 2,4 D.

1. Employing high-resolution ^{13}C-NMR it was found that embryogenic cells maintain fairly high levels of free glucose, whereas non-embryogenic cells store predominantly sucrose.
2. In cultures newly started from hypocotyl explants, addition of heatlabile high M_r components from conditioned medium of embryogenic suspension cultures considerably enhanced embryogenic potential.
3. The pattern of extracellular glycoproteins during suspension culture growth and embryogenesis appeared to be regulated in an auxin – dependent fashion. Established developmental mutants of embryogenesis showed altered extracellular polypeptide patterns.
4. Inhibitors of protein glycosylation blocked embryogenesis completely, while the inhibition could be fully complemented by extracellular proteins from embryogenic cultures. Most mutant cell lines could be complemented in similar fashion.

Based on these results we propose that correct expression of several extracellular proteins is a decisive aspect of carrot somatic embryogenesis.

P. LINDHOUT, R. KARSTEN, and A. VAN DOESUM (*Instituut voor de Veredeling van Tuinbouwgewassen, Postbus 16, 6700 AA Wageningen*)

Regeneration from leafdiscs of 100 genotypes of tomato

For tissue culture techniques, which are applied for tomatoes at this moment, the regeneration from explants is a prerequisite. Preliminar research at IVT has indicated that there exist a large variation in regeneration from leaf explants between some genotypes of tomato. To broaden this research the variation between 100 genotypes of tomato has been studied.

The choice of the experimental material has been based on a broad genetic collection of the cultured tomato and some wild *Lycopersicon* species. Some mutants with a possibly changed hormone balance were also included. The 100 genotypes were sown *in vitro* and after six weeks 6 leaf discs were placed in a petridish on MS-20 medium with zeatin (2 mg/l). After 4 and 7 weeks the number of shoots per petridish was determined.

After 7 weeks, leaf discs of all genotypes of the cultured tomato had regenerated into shoots. The regeneration frequency varied between the genotypes from 0.03 to 1.75 shoots per leaf disc. The regeneration of wild *Lycopersicon* species varied from 0.00 (*L. hirsutum glabratum* and *L. pimpinellifolium*) till 2.50 (*L. glandulosum*) shoots per leaf disc. No relation was found between the level of callus growth and regeneration. This research will be continued with the genetic analysis to determine the number of genes involved in the regeneration from leaf discs.

C. M. COLIJN-HOOYMANS, and R. BOUWER (*Instituut voor de Veredeling van Tuinbouwgewassen, Postbus 16, 6700 AA Wageningen*)

Regeneration of protoplasts from Cucumis sativus cv. Hokus

Cucumber is a major horticultural glasshouse crop in the Nederlands. Certain wild *Cucumis* species harbour dominant monogenic traits for resistance to Cucumber Green Mottle Mosaic Virus (CGMV) and powdery mildew (*Sphaerotheca fuliginea*). Crossing of these wild species with the

cultivated cucumber is inhibited by strong crossing barriers. Transfer of such disease resistance genes could be provided by somatic hybridization. For this purpose it is essential to develop a method for protoplast culture and regeneration.

Viable cucumber protoplasts (5×10^6 pps/g leaf tissue) could be isolated from cotelydons and leaves of plants which were grown in vitro. In agarose bead cultures and in agarose disc cultures plating efficiencies up to 50%, were obtained when protoplasts were plated at a density of 1×10^5 pps/ml. It was necessary to remove the medium frequently and to add fresh medium with a lower osmolarity and a changed concentration of hormones. This resulted in the production of yellowish callus capable of embryogenesis. The shoots were observed three months after protoplast isolation. However, the frequency is still very low because of the bad outgrowth of the embryoids. Therefore our research is now focussed on the establishment of conditions needed for plant development from embryos.

L. J. W. GILISSEN and E. DE VRIES (*Onderzoekinstituut Ital, Postbus 48, 6700 AA Wageningen*)

Somatic cell genetics of potato.

The development of somatic cell genetics in general and gene mapping in particular is based on: 1. in vitro culture of cells, 2. production of mutant cell lines (genetic markers), 3. somatic hybridization, 4. chromosome elimination, and 5. chromosome identification. Gene mapping is carried out by studying the concomitant presence or loss of genetic marker characters and chromosomes in interspecific somatic hybrids.

The use of somatic hybridization for the construction of a gene map of potato (*Solanum tuberosum*) seems to be more feasible than the application of sexual crosses; many potato cultivars are tetraploid and strongly heterozygous, often have reduced flowering capacities, are male sterile, etc. On the other hand, the somatic method is hindered by the lack of genetic markers, there is no hybridization partner known in which preferential chromosome elimination of potato has been proven, and the identification of potato chromosomes is difficult. But these obstacles are being surmounted. In a joint research project of the Research Institute Ital and the departments of Genetics (University of Groningen) and Plant Breeding (University of Wageningen), financially supported by the Foundation for Technical Research in The Netherlands, the development of the somatic cell genetics of potato was initiated in 1983. At Ital main attention was and still is paid to the development of genetic markers and to interspecific somatic hybridization, for which *Nicotiana plumbaginifolia* (wild type) was chosen as fusion partner.

Genetic markers can be divided into selectable (biochemical, morphological) and non-selectable markers (isoenzyme-, restriction fragment length polymorphisms).

From callus cultures of the diploid potato clone HH260, cell lines were selected which were able to grow on the toxic carbohydrate galactose as the sole source for carbon and energy. However, till now biochemical and manual selection of hybrids on galactose containing media were unsuccessful. But recently, flowcytometric sorting of heterokaryons resulted in eight indepent, well-growing 'hybrid' calli, which will be used for further genetic analysis and regeneration.

Another type of genetic marker is the morphological character of 'hairy root' formation, which was introduced in diploid and monohaploid potato clones by genetic transformation with *Agrobacterium rhizogenes*. Hairy roots can easily be isolated and maintained as individual root clones. One diploid root clone showed spontaneous shoot formation, other root clones formed shoots after shoot induction. Since hairy root formation appears to occur in a very early stage of the regeneration process of Ri-transformed *N. plumbaginifolia* leaf protoplasts, a simple hybrid selection system seems to be available.

Application of non-selectable genetic markers of potato is under investigation.

H. BRETELER (*NOVAPLANT, Celbiotechnologie Groep, Onderzoekinstituut Ital, Postbus 48, 6700 AA Wageningen*)

Cell and tissue cultures of Asteraceae as a source for thiophene biocides.

A. F. CROES (*NOVAPLANT, Celbiotechnologie Groep, Afdeling Botanie KUN, Toernooiveld, 6525 ED Nijmegen*)

Thiophene accumulation in callus cultures of Tagetes species.

Thiophenes are organic sulfurous compounds which are accumulated in a number of Compositae. The complex thiophenes present in *Tagetes* plants (marigolds) are strongly biocidal. The same compounds are also formed *in vitro* in calli of *T. erecta* and *T. minuta*. The predominant forms are bithienylbutinol (BBTOH) and bithienylbutinolacetate (BBTOAc). The calli of these species were used in a study on the relation between morphological differentiation and thiophene production. Experiments were started by incubating stem strips which have a very low thiophene content, on MS-medium with different combinations of plants hormones. Thiophenes were extracted from the calli, partially purified, and separated by reverse-phase HPLC. The amount of thiophene was quantified on basis of UV-absorption.

Two patterns of thiophene accumulation were found in the calli. In *T. erecta*, thiophene production is coupled to the stage of organ regeneration. Undifferentiated calli have very low levels of thiophene. In contrast, *T. minuta* calli which do not differentiate, accumulate substantial quantities of thiophene. In this species, no obvious relation exists between differentiation and thiophene production.

The above results are substantiated by experiments with tissues transformed by *Agrobacterium tumefaciens* LBA 4001. As a result of the transformation, the calli remain undifferentiated indefinitely. Transformed calli of *T. minuta* contain high amounts of thiophenes whereas the thiophene level in the corresponding *T. erecta* calli is low.

R. M. BUITELAAR (*NOVAPLANT, Celbiotechnologie Groep, Vakgroep Levensmiddelentechnologie, Sectie Proceskunde LUW, Postbus 8129, 6700 EV Wageningen*)

Technological aspects of thiophene production by plant cell cultures.

The technological knowledge of bioreactors is mainly based on experience with yeasts, bacteria and fungi.

When designing reactors for plant cells one must take into account a number of differences between plant cells and microorganisms. These differences are: a longer doubling time, a lower oxygen demand, inhibition by high oxygen concentrations, larger cell diameters, aggregation of cells and a rigid cell wall. These differences have some important consequenses for plant cell bioreactor design. Mixing and mass transfer must be done very carefully and sterility is an absolute prerequisite. These problems can partly be solved by immobilizing the cells. The main advantage of cell immobilization is the introduction of the possibility of easy reuse and of continuous processing, even under wash out conditions for free cells. For plant cells there can be some additional advantages, namely an increased production of secondary metabolites, enhanced excretion of the product into the medium, prevention of aggregation and protection against shear forces.

For plant cells the most suitable immobilization method is gelentrapping. For small-scale immobilization the dropwise extrusion technique is very satisfying, while large scale immobilization can be performed easily by using the resonance nozzle technology. The oxygen supply of the plant cells in aggregates or in immobilized particles is an important feature of process design for secondary metabolite production. A number of aspects has been studied in more detail. Research has been carried out on the relation between aggregate size and thiophene production by *Tagetes patula;* the thiophene production increased with increasing diameter, from a diameter of 1.4 mm up to a diameter of 12.3 mm. Model studies have been performed on oxygen profiles in immobilized beads and these oxygen profiles have also been measured by using a micro-electrode system. There seems to be a good resemblance between experimentally and theoretically determined oxygen profiles.

J. MARINUS (*Centrum voor Agrobiologisch Onderzoek, Postbus 14, 6700 AA Wageningen*)

'In vitro' tuberization of potato micro-cuttings.

Storage of tubers *in vitro* will be important to avoid the testing for diseases before the *in vitro* multiplication of potatoes. These tubers can be used as origin for this multiplication. The aim of the experiments was to find a reliable method of tuber formation for various cultivars.

The experiments are carried out with single node cuttings at about 20 °C. To the Murashige and Skoog (MS) nutrients (4 g/l) 8% sucrose and 0,9% agar were added.

The origin of the plant material distinctly influenced tuberization: in darkness more cuttings from plants grown in a glasshouse produced tubers, which were also larger, than did cuttings from plantlets grown in test tubes. The same occurred when from *in vitro* plantlets basal cuttings were taken compared with cuttings from the apex.

Also light conditions and MS concentration in the medium are important. However, the three cultivars responded differently to the treatments. In general darkness and 1 or 2 g/l MS accelerated tuber formation, but under 12 hours daylength most cuttings with tubers occurred. The tubers became also larger than in darkness. Under 16 hours daylength only one cultivar showed tuber formation. In darkness the addition of 0.5 mg/l benzyl adenine (BA) was very effective in four cultivars: at least 95% of the cuttings formed tubers, also tuber growth was promoted by BA, but finished when the tubers were still small ($2\frac{1}{2}$–$3\frac{1}{2}$ mm). A very slow regrowth was found after transfer of the cuttings to weak light conditions (12 hours daylength) at 8 °C.

It may be assumed that tuber formation in darkness with BA and transfer of the cuttings after tuberization to light conditions will produce most cuttings with (large) tubers.

R. L. M. PIERIK, J. T. DESSENS, and E. J. VAN DER ZEEUW (*Vakgroep Tuinbouwplanteteelt LUW, Postbus 30, 6700 AA Wageningen*)

Activation of in-vitro cultured areoles of the cactus Sulcorebutia alba Rausch

A model system was developed to study areole activation (development of quiescent meristems surrounded by a cluster of spines) in explants of the cactus *Sulcorebutia alba* Rausch; explants with 2–3 areoles were obtained from sterile cultured cacti. Basic media contained: Murashige and Skoog macro- and micro-salts (except iron), NaFeEDTA 25 mgl^{-1}, glucose 4%, BA 0.8 mgl^{-1} (or kinetin 1.0 mgl^{-1}), Difco Bacto-agar 0.7%; pH was adjusted to 5.5 before autoclaving. Explants were cultured for 8 weeks at 25 °C at a 16 h photoperiod from Philips fluorescent tubes (TL/40W, 3 Wm^{-2}). Arole activation (AA) was measured by counting the number of cacti formed and determining the volume of cacti produced.

AA was promoted by increasing the number of areoles per isolated explant and by cutting the explants (not too deep) near to the top of the cactus. AA was strongly determined by the genotype chosen, which also determined the cytokinin requirement. Optimal AA was obtained at a saccharose conc. of 3% and a full strength MS conc. The use of Daichin agar (0.7%) instead of Difco agar (0.7%) markedly stimulated AA. Cytokinin was an essential prerequisite for AA, kinetin and BA yielding the best results; 2iP was not suitable due to heavy callus formation. AA was promoted at high temperature (25 °C) and low irradiance (3 Wm^{-2}) in comparison to lower temperatures and high irradiance.

POSTERS

H. BRETELER, A. F. CROES, and J. TRAMPER (*NOVAPLANT, Celbiotechnologie Groep, Onderzoekinstituut Ital, Postbus 48, 6700 AA Wageningen*)

Secondary metabolite production by (immobilized) plant cells

A. F. CROES, M. BOSVELD, H. BRETELER, M. H. PETERS, and D. H. KETEL (*NOVAPLANT Celbiotechnologie Groep, Afdeling Botanie KUN, Toernooiveld, 6525 ED Nijmegen*)

Switchpoints in thiophene metabolism in callus cultures of *Tagetes erecta*.

J. P. F. G. HELSPER, D. H. KETEL, A. C. HULST, and H. BRETELER (*NOVAPLANT Celbiotechnologie Groep, Onderzoekinstituut Ital, Postbus 48, 6700 AA Wageningen*)
Secretion of thiophenes by differentiated cell cultures of Tagetes species.

J. B. M. CUSTERS, and J. H. W. BERGERVOET (*Instituut voor de Veredeling van Tuinbouwgewassen, Postbus 16, 6700 AA Wageningen*)
Somatic embryogenesis in callus and suspension cultures of cucumber (Cucumis sativus L.)

By subculturing small explants from *in vitro* grown plants successively on MS medium with 4 μM 2,4-D + 4 μM BA and 3% saccharose an embryogenic yellow secondary callus could be induced from greyish primary callus. The somatic embryoids showed progressive differentiation, if both growth regulators and saccharose concentrations were lowered, but so far only a moderate number of them developed into complete plants.

Starting from young 0.1–0.3 mm embryos or yellow secondary callus, cell suspensions have been established on MS medium with 5 μM 2,4,5-T + 5 μM BA and 3% saccharose. A high and repeatable multiplication rate was obtained. By lowering the growth regulator concentrations small aggregates were induced to develop embryoids.

H. HUIZING, W. VAN DER MOLEN, D. JAMAR, and F. A. KRENS (*Stichting voor Plantenveredeling, Postbus 117, 6700 AC Wageningen*)
The application of genetic manipulation techniques to sugarbeet (Beta vulgaris)

Susceptibility of sugarbeet to infection with agrobacteria was investigated using several approaches. Seedcoates were mechanically removed in order to facilitate sterilization and subsequently the sterile seedlings were taken as starting material. Cotyledons were cut in pieces, which were inoculated with *A. tumefaciens* by dipping them in an overnight culture. After transfer to MS at half strength transformed callus containing octopine was formed. In another approach 2–3 weeks old seedlings were transferred to medium containing gibberellic acid. This stimulated elongation of the stem. On the well-developed stems wounding and inoculation with several strains of agrobacteria were performed. The resulting transformed tissues have been maintained in tissue culture, in some cases leading to regeneration. For techniques involving somatic hybridization and cybridization next to regeneration a good protoplasting procedure is necessary. Such a procedure is now being developed in our laboratory.

W. M. VAN DER KRIEKEN, G. W. M. BARENDSE, and A. F. CROES (*Botanisch Laboratorium KUN, Toernooiveld, 6525 ED Nijmegen*)
BAP metabolism during in-vitro flower bud formation in tobacco

In tissue explants of epidermis plus subepidermal cortex from the flower stalks of *Nicotiana tabacum* flower buds are formed (TRAN THANH VAN 1973; VAN DEN ENDE *et al.* 1984) at appropriate concentrations of benzylaminopurine (BAP) and naphthaleneacetic acid (NAA). The number of flower buds formed depends mainly on the BAP concentration if the NAA concentration $\geqq 0.22$ μM.

In order to get some insight in the relationship between the number of flower buds formed and the internal BAP concentration, we studied the metabolism of BAP in the explants. Exogenously supplied cytokinins can be metabolized to their respective ribonucleosides and ribonucleoside-5'phosphates and to various conjugates (LETHAM & PALNI 1983). We have studied both the more polar and the more non-polar metabolites formed after incubation of tissue on medium supplemented with ^{3}H-BAP.

Two non-polar metabolites were detected by reverse-phase HPLC, one of which is BAP. The other metabolite co-chromatographed with standard 7-G BAP. The spectra of the metabolite and that of authentic 7-G BAP were identical. On two-dimensional TLC the metabolite and 7-G BAP banded at the same position.

Three polar metabolites of BAP were detected after a 1-h pulse and 3-h chase incubation by

ion-exchange HPLC. The retention times of the three metabolites were close to the retention times of AMP, ADP and ATP. Enzymatic hydrolysis resulted in the disappearance of the metabolites from their respective positions. This indicates that possibly the metabolites are 9-R BAP 5'mono, di and triphosphate. LALOUE & PETHE (1982) found evidence for the existence of these metabolites in a cell culture of tobacco.

Further studies will give answers to questions as: what metabolic forms are responsible for flower bud formation; is the metabolism of other cytokinins comparable with that of BAP?

LALOUE, M. & C. PETHE, (1982): In: P. F. WAREING (ed.): *Plant Growth Substances pp. 185–195.* Academic Press, New York.

LETHAM, D. S. & L. M. S. PALNI, (1983): *Ann. Rev. Plant Physiol.* **34**: 163–192

TRAN THANH VAN, M. (1973): *Nature* **246**: 44–45.

VAN DEN ENDE, G., A. F. CROES, A. KEMP, & G. W. M. BARENDSE (1984): *Physiol. Plant.* **61**: 114–118.

L. H. W. VAN DER PLAS, A. M. WAGNER, L. P. E. DE GUCHT, M. J. M. HAGENDOORN, and M. J. WAGNER (*Biologisch Laboratorium VU, Vakgroep Plantenfysiologie, De Boelelaan 1087, 1081 HV Amsterdam*).

Physiological problems in industrial plant biotechnology: Optimization of growth and production in Petunia cell suspensions.

The ultimate goal in industrial plant biotechnology generally is the economical production of secondary plant metabolites. It is necessary to produce rapidly large amounts of a desired metabolite with the aid of plant cell suspensions. The aim of the physiologist involved is the development of cell lines and culture conditions which lead to a rapid and stable formation of high concentrations of a specific, desired metabolite without interference of contaminating microörganisms.

To reach this goal, a number of conditions has to be fullfilled:

1. An efficient production of intermediates and energy (ATP) from the carbon source by the respiratory metabolism (in a heterotrophic system).
2. An optimal conversion of these intermediates in cellular constituents, leading to rapid growth of the cells.
3. An high activity of the enzymes involved in the synthesis of the desired secondary metabolites, leading to high relative contents of these products. The conditions for optimal growth and optimal metabolite production often are different, leading to distinct growth and production phases.

The basal knowledge, necessary to manipulate these processes often is not present and much of the progress is still made by trial and error only. To solve some of the underlying problems, research was carried out concerning:

a. Regulation of the induction and of the activity of the various electron transport pathways, varying in energy production (cytochrome pathway, alternative pathway, residual respiration) by (a.o.) fytohormones, temperature, stress factors etc.

b. Efficiency of the conversion of the carbon source and of the respiratory ATP into cellular dry weight during the growth phase. Determination of factors characterizing growth of the cells (growth yield, Y_{ATP}, $Y_{C\text{-source}}$, maintenance etc.) for cell lines differring in growth efficiency and for various growth conditions.

c. Factors determining the induction and the activity of enzymes of the phenyl-propanoid-pathway (leading to a.o. flavonoids, anthocyanins, phytoalexins etc.). What is the effect of a significant production of secondary metabolites (either intermediates or end products) on primary metabolism/respiration?

As a model system potato tuber callus and *Petunia hybrida* cell suspensions (growing in batch or in continuous culture) were used.

A. PRAT, E. VERMEER, P. EVERS, and I. MAYER (*Rijksinstituut 'De Dorschkamp', Bosrandweg 20, 6704 PH Wageningen*)

A new technique to reduce labour by refreshing liquid medium automatically with a new container

To eliminate the labour-intensive monthly transfer of plant material, a container system has been substituted. The containers contain a solid layer nutrient medium in which the plantlets are set. Under this layer is a liquid nutrient medium that is automatically refreshed. Depending on the stage of the plantlets, the components in the medium can be adjusted without transferring the plantlets. The tips of the axis are harvested periodically for rooting or multiplication while the remaining basal parts are stimulated to form new shoot tips.

M. J. M. SMULDERS, A. F. GROES, and A. J. P. M. ROBBEN (*Botanisch Laboratorium KUN, Toernooiveld, 6525 ED Nijmegen*)

NAA transport and metabolism during in-vitro flower bud formation in tobacco

In vitro flower bud development was studied on explants from the floral stalks of *Nicotiana tabacum* L. cv. Samsum. The tissues were cultured on Murashige-Skoog medium containing glucose, benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA). At low concentrations of NAA, polar flower buds are formed at the basal edge of the explant (maximum number at 0.45 μM). At increasing concentrations bud formation takes place on the remaining surface (maximum at 2.2 μM) and the polar buds disappear. However, in the presence of the auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) (1 μM), the buds are evenly spread over the whole explant at all NAA concentrations. Thus, there is a strong indication that, in the absence of NPA, longitudinal auxin transport through parenchymous cells is taking place.

We have studied NAA uptake and metabolism in relation to the external concentration in the medium. The uptake appears to be strictly proportional to the concentration in the medium, and linear in time. The metabolism of hormones can reduce the amount of free hormone inside the tissue considerably. To study this phenomenon in our system, we incubated explants on medium with [14C]-NAA and analysed methanolic extracts on HPLC. The counts were recovered in NAA and a number of conjugates. The main conjugate, presumably naphthaleneacetylaspartic acid, accounted for approximately 45 per cent of total radioactivity. Two other conjugates were present at 15 and 5 percent. Only 5 percent of the radioactivity was in free NAA. These proportions were the same irrespective of the NAA concentration, in all parts of the explant. This means that if longitudinal transport of NAA takes place both free NAA and the metabolites would accumulate at the basal edge.

P. VAN DER VALK, and M. A. C. M. ZAAL (*Stichting voor Plantenveredeling, Postbus 117, 6700 AC Wageningen*)

Protoplast culture and plant regeneration in Poa pratensis L. (Kentucky Bluegrass)

Several cultivars of the facultative apomictic species *Poa pratensis L.* (Kentucky bluegrass) were tested for callus induction and plant regeneration, using immature inflorescences and mature seeds as explant material. On agar-solidified MS-medium, supplemented with 2 mg/L 2,4-D and 3% sucrose, different types of callus developed. Variation was observed between cultivars for callus induction and plant regeneration. Plants were regenerated from 'compact' callus of the cultivars BARON, DELFT, GERONIMO, and KIMONO, when such callus was transferred to hormone-free medium. Somatic embryos were observed at the callus surface, but plant regeneration appeared to occur by shoot morphogenesis, rather than by somatic embryogenesis. Regenerated plants were generally green, but some chlorophyll-deficient plants were obtained when callus was subcultured for prolonged periods of time. 'Compact' callus was used to initiate cell suspension cultures. Plants could be regenerated from cell-suspension-derived callus.

Protoplasts were enzymatically isolated from morphogenetic cell suspension cultures of three cultivars. These protoplasts were able to divide and form callus. Plant regeneration ability of the protoplast-derived callus is being tested.

H. A. VERHOEVEN, and H. C. P. M. VAN DER VALK (*Onderzoekinstituut Ital, Postbus 48, 6700 AA Wageningen*)

Flowcytometric analysis of cellular DNA content after hydroxyurea treatment of Nicotiana plumbaginifolia suspension cells

The influence of hydroxyurea (HU) on the cellular DNA content was analysed in order to establish optimal conditions for cell cycle synchronization of a tetraploid suspension culture of *N. plumbaginifolia*.

Cells of *N. plumbaginifolia* were incubated with increasing concentrations of HU (0, 1, 2, 5 and 10 mM) during 24 h. DNA content was determined by flowcytometry at 0, 4, 6, 8, 10 and 12 hours after washing the culture free from HU.

The control incubation showed no change in the distribution of the different phases of the cell cycle. 1 mM of HU was insufficient to block S-phase, whereas higher concentrations completely prevented transition into G2 phase. Recovery of the HU block took more time with increasing concentrations.

Flowcytometry allows the rapid evaluation of the effects of DNA synthesis blocking agents on the cell cycle.

S. E. DE VRIES, M. A. FERWERDA, A. LOONEN, L. P. PIJNACKER, and W. J. FEENSTRA (*Biologisch Centrum RUG, Vakgroep Genetica, Kerklaan 30, 9751 NN Haren*)

Is interspecific somatic hybridization useful for gene localization in potato (Solanum tuberosum L.)

Our purpose is to localize potato (*Solanum tuberosum* L.) genes by interspecific hybridization. As fusion partner nitrate reductase deficient (NR^-) lines of *Nicotiana plumbaginifolia* are used. After complemention and at random loss of potato chromosomes, it might be possible to localize the genes involved in NR in potato. Potato and *N. plumbaginifolia* chromosomes can be distinguished. Moreover, the potato chromosomes can be identified individually (*Can. J. Genet. Cytol.* **26**: 415, 1984).

Callus protoplasts of the NR^- lines Cnx 20 and NA 36, $2n = x = 40$ (kindly supplied by Dr. I. Negrutiu and Dr. L. Marton, resp.) were fused with mesophyll protoplasts of the monohaploid potato clone H7322, $2n = x = 12$ (kindly provided by Prof. Dr. G. Wenzel). Fusion was induced by PEG, following the procedure of Menzel et al. (*Theor. Appl. Genet.* **59**: 19, 1981). Under the culture conditions used, protoplasts of H7322 lack regeneration capacity. The NR^- lines are not able to grow on medium A (*Mol. Gen. Genet.* 161: 67, 1978), containing NO_3^- as N-source. Using this medium, 54 somatic hybrids of H7322 + Cnx 20 and 150 and H7322 + NA36 were selected. The callus growth of the somatic hybrids was tested on different culture media. NR^- mutants are resistant to $KClO_3$, which is reduced to the toxic chlorite by active NR. All somatic hybrids were sensitive to $KClO_3$ and had NR activity *in vitro*. Regeneration was induced on medium containing NAA, GA_3 and zeatine as phytohormones and NO_3^- as N-source. Up till now, 11 monstrous plantlets from 3 different fusion products of H7322 + Cnx 20 could be regenerated. No regenerants from H7322 + Na 36 have been obtained yet. 12–14 months after hybridization, Giemsa-C banded metaphases slides of well growing calli of H7322 + Cnx 20 and H7322 + NA 36 were examined. Besides high numbers of *N. plumbaginifolia* chromosomes (40–80), 6–12 potato chromosomes were present.

Our results suggest that at least in some fusion products potato chromosomes have been eliminated. It is under investigation whether these eliminations occured at random. Identification of the individual potato chromosomes still present in the fusion products is in progress.