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Meetings of the Royal Botanical Society of The Netherlands

MEETING OF THE SECTION FOR PLANT MORPHOLOGY, ANATOMY AND CYTOLOGY ON 26 OCTOBER 1990

Anatomy and Pyrolysis Mass Spectrometry of Peatified Plant Tissues

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A selection of peatified plant tissues, handpicked from a Dutch peat deposit, were anatomically and mass spectrometrically characterized and compared with native tissues. In this way, insight is obtained in the chemical and anatomical selectivity of the decomposition process in peat deposits.

A selective removal of polysaccharides and a structural modification of the lignin macromolecule are observed in both peatified *Calluna* stemwood and Ericaceous rootwood. These chemical changes coincide with severe anatomical changes, although the state of preservation between different wood forming tissues is rather variable: in peatified *Calluna* fibres, the combined S_1/S_2 layer is gone whereas the S_3 layer and compound middle lamella are preserved. Sporadically, fibres with a higher or lower grade of decomposition are observed. The wood vessels, showing swollen and gelified cell walls, are better preserved than the surrounding fibres. A uniform thinning of the cell wall is observed in the rootwood fibres, which are often better preserved in the centre of the root. Decomposition resistent wood vessels, as found in *Calluna* stemwood, are not observed in the wood of the root.

The bark of the *Calluna* stem is highly resistent to decomposition. The periderm, which is the most resistent tissue, is not modified. Minor anatomical modifications such as cell wall thickening and gelification, are observed in the phloem parenchyma and cambium. Mass spectrometric analysis of peatified bark reveals that polysaccharides are not decomposed during peatification.

The stems of *Sphagnum* are very resistent to decomposition. The mass spectrum of peatified *Sphagnum* is still characterized by intense mass peaks of polysaccharides, which points to an excellent preservation of these compounds (mechanism unknown) (van der Heijden *et al.* 1990, *Proceedings Peat 90*, (ed. Sopo, R.), pp. 148–163). Anatomical changes were observed, however: the pith, as well as the cortex are easily decomposed, although the cell corners are often retained. In areas where decomposition is severe, high fungal activity is also observed.

Effect of Growth Rate and Age on Wood Structure of East-Liaoning Oak

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The effect of growth rate on wood structure of East-Liaoning Oak (*Quercus liaotungensis* Koidz.) was studied, and compared with the effect of age (ring number from the pith). Five dominant trees were collected from Mt. Zhongtiao, North China. The studies revealed that age is a decisive factor controlling wood structure, while the effect of growth rate is less important.

The effect of age on anatomical characters shows optimum curves which differ from character to character. For fibre proportion, the effect shows a parabolic curve, with an optimum age from 25 to 35 years for maximum size.

The effect of growth rate varies not only with anatomical characters but with the position within a growth ring (earlywood, latewood or the whole ring), ring width range and age. The effect of growth rate on sizes of wood elements (fibre length & diameter and vessel diameter) is not significant. However, growth rate shows a greater effect on tissue proportions. Growth rate does not influence characters of either earlywood or latewood significantly, but it shows a greater effect on characters of the whole growth ring. Within a narrower range of ring width (less than about 1·5 mm), with increasing ring width (or growth rate), fibre proportion increases rapidly, while vessel and parenchyma proportion (including vasicentric tracheids) decrease markedly, then more slowly, and finally they remain more or less constant when ring width is beyond a specific range (wider than *c.* 3·0 mm). Growth rate shows little effect on the structure of juvenile wood, but a significant effect was found in mature wood.

Bi- and Unitegmy in *Impatiens*

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Within the genus *Impatiens* the transition of bi- to unitegmy occurs. *Impatiens glandulifera* is one of the bitegmic, *I. noli-tangere* one of the intermediate and *I. congolensis* one of the unitegmic species. The unitegmic condition arises by the upward growth of subdermal cells under the dermal outer integument, together with the arrested growth and shifting of this integument along the inner integument to a more apical position. In the unitegmic species the earlier outer integument does not develop anymore and the single integument consists mainly of the former inner integument and the subdermal cells which grow far upwards. The endothelium becomes the inner layer of the single integument.

This is a fourth way for ovules to become unitegmic.

Other ways in which ovules may become unitegmic are:

(a) reduction of the outer integument,
(b) fusion of two dermal integuments, and
(c) integumentary shifting of the inner integument by a subdermal outer integument.

The study of ovules in taxa in which the change over of bi- to unitegmy takes place, gives information on the evolution of the unitegmic ovule.

Actin Rings are a Regular Constituent of Plant Cells

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Rhodamine-labelled phalloidin was used as a probe to study F-actin distribution in suspension cells of *Nicotiana* and *Tagetes*, and pollen tubes of *Lilium*. In addition, we also studied elongating suspension cells, protoplasts from the suspension cells and subprotoplasts from pollen tubes in *Nicotiana*. Actin rings were present in all cells studied. In each preparation ring-like structures were observed in about 5% of the cells which occurred after fixation with paraformaldehyde in PIPES-buffer (Traas, 1984, *Protoplasma*, **119**, 212–218), as well as after extraction in PIPES buffer with non-ionic detergents (Traas *et al.* 1987, *J. Cell Biol.*, **105**: 387–395).

Simultaneous visualization of fluorescent and phase contrast images revealed no perceptive association with organelles. The presence of rings during organelle division (Hasezawa *et al.* 1988, *Protoplasma*, **146**: 61–63) could not be confirmed.

As the rings are present in a limited number of cells (about 5%), they may express a specific stage of the cell cycle. At present, their occurrence in synchronized cell cultures is studied. Alternatively, they may express some type of cell differentiation or even cell degeneration.

It may be hypothesized that, perhaps as a result of unfavourable conditions, small rings occur in a transition stage, where they function as a readily available F-actin pool.

Deposition and Reorientation of Cellulose Microfibrils in Elongating Stylar Cells of *Petunia hybrida*

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According to the Multinet-Growth-Hypothesis (MGH) (Roelofsen & Houwink, *Acta Bot. Neerl.* 1953, **2**: 218–225), originally transversely deposited microfibrils become gradually re-oriented towards more axial orientations during cell expansion. In order to establish the extent of microfibril reorientation, we quantitatively studied microfibril deposition and the texture during elongation in stylar parenchyma and transmitting tissue cells of *Petunia hybrida*.

From the inner surfaces of very young cells in styles smaller than 0·1 cm, the following sequence in deposition was inferred: microfibrils were deposited in alternating S- and Z-helical orientations. First nearly axial orientations, followed by oblique and nearly transverse orientations. Together with the increasing pitch, the density increased. Only nearly-transverse S- and Z-helical orientations were deposited during elongation. The wall maintained its thickness and initial texture.

In young stylar cells the sequence from axial to transverse microfibril orientations reflects the way in which microfibrils are deposited. During elongation a continuous deposition of nearly transversely oriented microfibrils takes place. However, no perceptible changes are observed in the total texture during cell expansion. The initial texture is maintained as a result of passive re-orientation, as described by Roelofsen & Houwink. The extent of passive re-orientation is in agreement with the theoretical calculations of Preston (*Planta* 1982, **155**: 356–363) and shows that the total reorientation is limited to 40° in transmitting tissue cells and 59° in stylar parenchyma cells.

The Apertural System in Nephelieae Pollen (*Sapindaceae*): Form, Function, and Evolution

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The *Nephelieae* represent a rather eurypalynous tribe of the family *Sapindaceae*, both with regard to exine

architecture and apertural system. The apertural system is tri-aperturate. Three types occur in the 12 genera of the *Nephelieae*: colporate (ectoapertures free; in 11 genera), parasyncolporate (ectoapertures connected; in *Alectryon*), and brevicolporate (ectoapertures short; in *Pometia*). The genus *Alectryon* shows a continuous range from colporate to parasyncolporate. These two types occur together in several other sapindaceous tribes.

It is widely accepted that within the angiosperms, colporate is more primitive than parasyncolporate, but this is questionable with regard to the *Nephelieae* and most other tribes of the *Sapindaceae*. On account of macromorphological data and a functional interpretation of both apertural systems, it is hypothesized that the primitive colporate condition of angiosperm pollen persists in the subfamily *Dodonaeoideae*, but that parasyncolporate is the primitive state in the other subfamily, the *Sapindoideae*. The colporate state in the latter is considered to be derived as it is a reversal. *Alectryon* pollen provides some ontogenetic evidence. Fossil evidence can hardly be used: fossils of the parasyncolporate type are well known (*Cupanieidites*), but fossil colporate *Sapindaceae* pollen is difficult to distinguish from many other families.

The evolution from the parasyncolporate to the colporate condition could have occurred frequently, possibly under the influence of the worldwide aridification and cooling in the course of the Tertiary, as the two apertural types show quite different harmomegathic systems. A colporate pollen grain accommodates volume reduction (due to water loss after anther dehiscence) by invaginating especially the equatorial parts of its ecto-apertures; the equatorially situated endo-apertures are deeply drawn inwards and sealed by the margins of the adjacent exine. By contrast, a parasyncolporate pollen grain accommodates volume reduction by invaginating the polar parts; the endo-apertures remain superficial. Although experimental evidence is lacking, it is assumed that the different endo-aperture positions in the dehydrated state have different protective values, parasyncolporate pollen being more susceptible to desiccation. This might be the relevance of the hypothesized transformation of parasyncolporate to colporate under drier climatic conditions.

Ultrastructure and Morphometry of Tobacco Pollen Tubes

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Growth of tip growing cells like pollen tubes is supposed to concur with specific organelle distributions, especially in the growing tip (Steer & Steer 1989, *New Phytol.* **111**, 323–358). These distributions were observed in pollen tubes after chemical fixations, which may have caused distortions in cytoplasmic organization (Mersey & McCulley 1979, *J. Microsc.* **114**, 49–76). Therefore, we have used cryofixed and freeze substituted pollen tubes of *Nicotiana tabacum* cv Samsun. Ultrastructure and distribution of organelles in the growing tip were studied on longitudinal serial sections. The results showed an accumulation of vesicles in the extreme tip. Also mitochondria, dictyosomes, smooth and rough endoplasmic reticulum showed an increased density in distinct zones (mostly between 10 and 40 μm) behind the tip. In this zone also many, noncortical, microtubules occur, which may be involved in maintaining organelle distribution. These results largely confirm the earlier data on organelle distribution (Steer & Steer 1989). We also observed vesicles and dictyosomes seemingly attached to filaments, putatively F-actin. At the plasmamembrane between 5 and 20 μm behind the tip, an accumulation of coated pits (up to 10 coated pits per micrometre membrane per section) occurs. These coated pits are probably involved in membrane retrieval to compensate for the excess of membrane incorporation in the growing tip.

Pollen Tube Interaction with the Ovule During the Progamic Phase in *Gasteria verrucosa* (Mill.) H. Duval

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In the placental locule, the pollen tubes resynthesize their callosic plugs and pollen tube wall. The rate of growth decreases and consumption of probably glycoproteins occurs.

A high number of pollen tubes can penetrate the micropyle of the ovule. By adding small crystals of DAPI in the exudate droplet, the transport of the pollen tube nucleus and the sperm cell nuclei can be followed within the ovule. It reveals that the two sperm cell nuclei are transferred to one of the synergids between 8 and 15 h. The fusion of one of the sperm nuclei with the egg cell nucleus takes place after 36 h. In the case of multiple pollen tube penetration of the micropyle, the sets of nuclei of some pollen tubes remain outside the micropyle, or in the micropylar canal, or they can be observed between the nucellar tissue and the first integument.

Ultrastructural studies show that 2·5 h after pollination the micropylar canal is still empty and the egg apparatus is still developing (Franssen-Verheyen & Willemse, 1990, *Acta Bot. Neerl.* **39**: 53–63). However, 8 h after pollination the pollen tubes have entered the micropyle but are seldom in one of the

synergids. At this time a viscous exudate is visible around the pollen tube. This droplet is formed during the production of the stigmatic exudate and slowly increases in volume.

After the pollen tube arrives in the micropyle, the nucellar cells bordering the tip of the egg apparatus get small lipid droplets. The nucellar cell wall adjacent to the tip of the egg apparatus shows an electron dense layer. The filliform apparatus seems to become narrower. In the synergids the amount of starch decreases.

These phenomena indicate an activation of the ovule during the progamic phase. The observed phenomena point to either ovular reactions in selection and acceptance of one pollen tube, or to ovular incompatibility.

Freeze Fracture Analysis of Embryogenic Suspension Cells and Histology of Somatic Embryos of Maize

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In the presence of the synthetic hormone 2,4D, young stages of somatic embryos of maize genotype 4C1 (developed by Sándor Mórocz and Dénes Dudits, Szeged, Hungary) are formed from calli in liquid medium. Single somatic embryos, unattached to the callus, germinate in hormone-free medium, but are blocked in scutellum and shoot meristem formation. In 2,4D medium they form embryogenic callus again. In fact, embryogenic callus consists of globular stage somatic embryos, linked by undifferentiated tissue. The latter grow into mature somatic embryos when transferred to maturation medium. The histology of these somatic embryos is remarkably similar to the histology of zygotic embryos. Characteristic structures, such as coleoptile, coleorrhiza, leaf primordia, peribleme, plerome, root meristem, adventitious root meristem, scutellum, shoot meristem and scutellar node, are all present. The embryo is loosely attached with suspensor-like cells to the callus aggregate. This aggregate seems to provide signals that stimulate the formation of a normal shoot, including scutellum and coleoptyle.

In the suspension, cell clusters develop into globular stage somatic embryos. The clusters were compared to the non-dividing elongated cells of the suspension by means of freeze fracturing. Both types of suspension cells contain a very low number of terminal globules and particle rosettes in their plasma membranes, as infrequent as 0·3–1 μm^{-2}. The cell wall texture of clusters is different from that of elongated cells. Walls of the cluster cells have highly organized textures, consisting of lamellae with microfibrils in alternating directions. Elongated cells have randomly oriented microfibrils in the part of the wall deposited during elongation as single cells. Plasmodesmata are abundant in cluster cells, but scarce in elongated single cells.

MEETING OF THE SECTION FOR FERTILIZATION RESEARCH IN PLANTS ON 5 OCTOBER 1990

Somatic and Sexual Interspecific Hybrids between *Solanum tuberosum* ssp. *tuberosum* (L.) and *S. circaeifolium* ssp. *circaeifolium* (Bitter)

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Sexual hybrids between the Bolivian diploid wild species *Solanum circaeifolium* ssp. *circaeifolium* (crc) and diploids of the cultivated potato *S. tuberosum* ssp. *tuberosum* (tbr-2x) were obtained by crossing. Forty-seven hybrids resulted from 850 pollinations, eight diploids and 39 triploids. Crc has two very typical nucleolar chromosomes and as the triploid hybrids contain two crc nucleolar chromosomes, they are thought to originate from an unreduced egg cell of crc and a normal haploid male gamete from tbr. Somatic hybrids were obtained by electrofusion. The somatic hybrids were tetraploid (8), hexaploid (3), octaploid (1) or mixoploid (1). Male and female fertility and chromosomal configurations during male meiosis were studied using some of the diploid, triploid and tetraploid hybrids. The percentage of stainable pollen in the sexual hybrids varied from less than 5 to more than 50%. Not only normal haploid pollen but also micropollen and unreduced pollen were observed. The somatic hybrids (tetraploid) showed no stainable pollen. In crossing experiments, female fertility for the diploids was found to be rather good, for the triploids it was of an acceptable level and for the tetraploids it was found to be very high. The cross (crctbr-3x) × (tbr-2x), gave aneuploid progeny with a mean of 30 chromosomes. Metaphase I analyses showed the occurrence of many univalents in the diploid, 2–3 trivalents per cell in the triploid and some trivalents and/or quadrivalents in the tetraploid hybrids. These

Table 1. The various media used for the in-vitro techniques

Culture	Medium	Sucrose (%)	NAA (mg l^{-1})	pH	DAP
Ovary-slice*	MS	9	1	6·0	7–40
Ovule	MS	5	0·1	5·5	40–80
Embryo	MS	2–4	0·1–0·001	5·0	40–80
Ovary**	MS	7–8		5·8	2

*One ovary transversely sectioned in 6–10 slices.
**In-vitro pollination culturing whole ovaries.

results are in agreement with earlier reports, where no structural genome differences were found between diploid potato species and recombination is common.

In-Vitro Pollination of Lily

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The use of a complete and integrated in-vitro pollination, fertilization and embryo rescue system was examined in lily. By combining pollination techniques to overcome pre-fertilization barriers with in-vitro methods to overcome post-fertilization barriers, both under fully controlled conditions, interspecific lily crosses could be made more efficiently. In-vitro cut-style pollination and in-vitro grafted style techniques were developed and applied on various interspecific crosses using *Lilium longiflorum*, and both Asiatic and Oriental hybrids as the parents. In addition, methods for ovary culture, ovary-slice culture and ovule culture were generated. Ovule swelling score in ovary culture was used to evaluate media effects on ovule development.

The optimal media and moment of application (in DAP = days after pollination) developed for the in-vitro methods are summarized in Table 1; MS is the standard Murashige & Skoog medium.

Analysis of Localization, Expression and Nucleotide Sequence of the Pollen Specific cDNA Clone pKW303

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Through the development of the male gametophyte appears to be simple, there are 20,000 genes being expressed. The molecular processes that form the basis of pollen development were studied. For this purpose a pollen specific cDNA clone (pKW303) has been isolated from a cDNA library made against mRNA from mature pollen. Expression of KW303 has been detected in pollen and not in other vegetative or generative tissues. mRNA hybridizing to this clone is present in pollen from *Digitalis purpurea* and *Lilium longiflorum*. The first transcription of the KW303 gene takes place after microspore mitosis. Transcripts accumulate thereafter.

For further characterization, the base sequence of pKW303 was determined. No protein coding open reading frame has been found, as 200 bp at the 5′ side still have to be sequenced. A database search has revealed no homologues genes yet.

The localization of the KW303 gene transcripts was studied by means of *in situ* hybridization. As the resolution of a conventional light microscope was too low, a confocal laser scanning microscope (CLSM) was used for studying the results. The CLSM produces images of the reflection of the silver grains in the light sensitive emulsion that are more pronounced than those from the transmission light microscope. It can visualize the silver grains within a 1·0 μm thick layer of a 10 μm thick section and thus sequentially visualize parts of the total coupe. Images produced on the CLSM can be stored on an optical disk for future analysis. Preliminary results using the CLSM localized KW303 RNA within the vegetative cell. Other localization experiments are in progress.

Northern blot analyses indicated transcription of KW303 within the first two hours of germination. In order to prove *de novo* synthesis of KW303 RNA a modified 'run on' transcription assay was developed. The pollen grains were allowed to germinate in the presence of ^{3}H-Uridine. The newly synthesized (labelled) KW303 RNA was isolated by means of liquid hybridization to an antisense 303 probe, and this was followed by precipitation. For detection a fluorograph of the isolated KW303 RNA was made after electrophoresis on a denaturing acrylamid/urea slab gel. Using this method transcription of the KW303 gene could be detected within the first hour of germination.

The Interaction of Pollen Tube Growth and Protein Patterns during the Progamic Phase in *Gasteria verrucosa* (Mill.) H. Duval

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The exudate droplet on the stigma of *Gasteria verrucosa* is the signal for receptivity. After pollination, the amount of placental fluid increases and the fluid pathway in the hollow style forms a continuous connection with the placental fluid. Samples of stylar fluid, placental fluid, pollen germination medium and homogenized germinated pollen were centrifuged and their supernatants were concentrated with an ultrafree 10,000 NMWL filter (Millipore). Concentrated samples of the native proteins were separated in the first dimension by IEF, pH 4–6·5 and in the second dimension on a 8–25% gradient PAGE gel on a Pharmacia system.

The protein pattern of the in-vitro germinated pollen and its exudate in the medium showed one group of proteins present only in the pollen medium.

In the stylar fluid the protein pattern of the stylar proteins showed no differences before and after pollination. In the pattern, four proteins derived from the pollen tubes could be detected 3 h after pollination. In the placental fluid, three proteins derived from the pollen tube could be detected 7 h after germination at the same location on the gel as in the styler fluid. Two spots present in the unpollinated sample disappeared, however; one protein at lower pH and a group of what are probably glycoproteins.

Pollen germination in the presence of about 8% of placental fluid showed a breakdown of some proteins including the group of glycoproteins. In this pattern the group of proteins which were present only in the medium reappeared. The function of this last group of proteins is till now unknown.

During pollen tube growth the stylar fluid proteins are probably not used but in the placental fluid there is a breakdown of what are probably glycoproteins. The pollen excretes enzymes to digest these type of proteins. Addition of about 8% placental fluid to the pollen germination medium induces an increase in the rate of pollen tube growth.

No relationship between this and the ovular incompatibility in *Gasteria* could not yet be made.

MEETING OF THE SECTION FOR PHYTOPATHOLOGY ON 17 JANUARY 1991

Immunolocalization of β-1,3-glucanase and Chitinase in Compatible and Incompatible Interactions between Tomato and the Fungus *Cladosporium fulvum*

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In recent years much attention has been paid to the role of plant hydrolases which are induced during the defence response against fungal pathogens. The β-1,3-glucanases and chitinases have been studied extensively as these enzymes were especially able to degrade isolated fungal cell walls, and to release glycosidic fragments which consequently could act as elicitors of other defense responses. Tomato plants, infected by the fungus *Cladosporium fulvum*, also respond with the production of β-1,3-glucanases and chitinases. In incompatible interactions the chitinases and β-1,3-glucanases accumulate more rapidly than in compatible ones. These findings suggested that these enzymes might play a role in the active defense.

To get a better understanding of the actual role of these hydrolytic enzymes in this interaction, we investigated the *in situ* localization of these proteins in infected tomato leaves. For this purpose we used antisera obtained from rabbits which were injected with a chitinase and a β-1,3-glucanase isolated and purified from tomato leaves which were inoculated with *Cladosporium fulvum*. We found that chitinases and β-1,3-glucanases were localized in the cytoplasm of infected plants in both compatible and incompatible interactions. However, only in the incompatible interaction a strong accumulation of the proteins in electron dense particles in the vacuoles was found. Furthermore, we found that chitinases and β-1,3-glucanases were localized around the stomata when the plants were grown under suboptimal conditions. From these preliminary results we cannot yet conclude that chitinases and β-1,3-glucanases play a primary role in the defense of tomato against *Cladosporium fulvum*.

Xylem and Phloem Transport of Fluorescent Dyes to and into Nematode-Induced Giant Cells in Tomato

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The fluorescent dyes, fluorescein (F, pK=4·9) and carboxyfluorescein (CF, pK=4·0), were used to study

xylem and phloem transport to and into giant cells, induced by *Meloidogyne incognita*. The uptake of these dyes by plant cells can be accounted for by an acid-trap mechanism, which assumes that their entry mainly occurs by passive diffusion of the undissociated form of the acids. Passage of the anions across cell membranes occurs very slowly.

Xylem transport was studied by applying the dyes to infected tomato root systems of which some root tips were removed and replaced by polyethylene cups containing one of the dyes. Phloem transport was investigated by applying the dyes to the adaxial surface of nearly full grown leaves of infected tomato plants, as described previously (Grignon *et al.* 1989, *Am. J. Bot.* 76: 871–877). After one day of incubation, handcut sections of the root systems were made and viewed with an epifluorescence microscope equipped with suitable filter combination sets.

When the dyes were applied in a cup at the apical cut end of a root, they were transported via the xylem vessels in shoot direction. At infection sites F accumulated in the giant cell complexes, whereas CF accumulation only occurred when the phloem also fluoresced. Leaf surface application of the dyes resulted in phloem transport. CF was transported to the roots within one day, whereas F had a high lateral escape. Once CF was transported through the infected root, the dye was only visible in the phloem and in the giant cells. Since CF did not accumulate in the giant cells when applied via the apoplast, it was concluded that there is a symplastic pathway between the phloem and the giant cells. So, the giant cells seem to have the capacity to accumulate nutrients from the xylem as well as the phloem via nutrient-proton-cotransport and via plasmodesmatal transport respectively.

Alfalfa Mosaic Virus Replication in Transgenic Plants Expressing Viral Replicase Genes: Role of the Coat Protein Gene in the Virus/Plant Interaction

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Transformation of *Nicotiana tabacum* cv. Samsun NN with RNAs 1 and 2 (encoding subunits of the viral replicase) of the tripartite genome of alfalfa mosaic virus (AIMV, a (+)-RNA virus) gave rise to plants efficiently replicating the third genome segment, RNA3, encoding a protein involved in cell-to-cell transport of the virus and the viral coat protein.

Symptom formation by AIMV in *N. tabacum* is dependent upon the particular strain used. The Leiden isolate of strain 425 induces a mild chlorosis, while strain YSMV induces necrotic local lesions and systemic yellowing of the leaves. By making pseudo-recombinants between the afore mentioned strains, symptom formation in tobacco has been assigned to RNA3 (Dingjan-Versteegh *et al.* 1972, *Virology* **49**, 716–722). Nucleotide sequences of RNA3 of both strains are known, and the construction of cDNA clones of RNA3 from which infectious transcripts can be synthesized, allowed the investigation of parts of the molecule involved in this differential symptom formation. DNA fragments were exchanged between the cDNA clones of the strains and transcripts thereof inoculated to the transgenic plants. In this way, a single amino acid substitution (Gln(29) to Arg) in the *N*-terminal part of the coat protein was found to induce the local lesion phenotype of strain YSMV. Systemic symptom formation was more complex. For most hybrids inducing local lesions, a severe delay in systemic yellowing was observed and yellow patterns in some cases differed from wild type YSMV symptoms (giving yellowing along the nerfs, instead of between them).

Bacteria Associated with Sheath and Grain Discolouration of Rice

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Several bacterial and fungal pathogens have been found in association with sheath and grain discolouration of rice. Among the bacteria, species of the genera *Pseudomonas* and *Erwinia* are known to cause such symptoms. In this project we want to identify the bacteria associated with symptoms of sheath and grain discolouration of rice and design fast and reliable identification methods for them.

During the wet season of 1988 and 1989, plant material showing symptoms of sheath and grain discoloration were sampled throughout the Philippines. From these plants, over seven thousand bacteria were isolated and checked for pathogenicity on rice seedlings and plants in booting stage. Two hundred and thirty-six isolates were found to be pathogenic.

At present the activities at the laboratory of microbiology and microbial genetics of the State University of Ghent are focused on the identification of the isolates and the design of fast identification systems which in future might be used by plant quarantine services. In a first stage gas chromatographic analysis of the fatty acid methyl esters and standardized micromethods for phenotypic tests are examined for this purpose.

A Preliminary Study to Identify Soil Biotic Factors Involved in the Degeneration of *Ammophila arenaria* (marram grass)

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Ammophila arenaria (marram grass) is a species that fixes and stabilizes sand and occurs mainly in the Dutch coastal foredunes. *A. arenaria* is most vigorous on the seaward slopes, where it is buried regularly by fresh windblown sand. However, it degenerates when sand accumulation diminishes. Degeneration may be due to a harmful biotic factor in the soil. Pot experiments showed that the growth of *A. arenaria* in soil from its root zone was significantly improved when a nematicide was used. The same study also suggested that fungi may be involved. It was suggested that the colonization of windblown sand enables *A. arenaria* to maintain vigour because of the escape from harmful soil organisms.

The purpose of this study is to identify potentially harmful nematodes and fungi in the root zone of *A. arenaria*. A survey was conducted at five locations along the Dutch coastal foredunes: the isle of Voorne, Schouwen and Goeree (all lime-rich dunes), Callantsoog and the isle of Texel (both lime-poor dunes). At all locations samples were collected from the root zone of vigorous and of early-declining stands of *A. arenaria*. Nematodes and fungi were isolated from soil and roots, and identified.

The plant parasitic nematodes found were *Heterodera* sp. (*avenae* group), *Meloidogyne maritima*, *Telotylenchus* sp., *Tylenchus* spp., and *Pratylenchus* sp. Nematode numbers were low as compared to those in agricultural systems and there was much variation among sample replicates. The results support the hypothesis that nematodes may not be the only group of soil organisms involved in the degeneration of *A. arenaria*.

Sixty-three fungal species were found. They could be classified into four groups: one group of fungi seemed to be specific for lime-rich foredunes, another group for lime-poor foredunes. A third group was regularly found in both lime-rich and lime-poor foredunes and a fourth group was only incidentally isolated. The fungi with a possible role in degeneration may be found in the third group mentioned. This preliminary study suggests that the fungi involved in the degeneration of *A. arenaria* may be: *Fusarium culmorum*, *Apiospora montagnei*, *Harzia acremonioides*, *Microdochium bolleyi*, *Phoma* spp., *Trichothecium roseum* and several *Ulocladium* spp.

The possible role of nematodes, fungi, and their interactions in the degeneration of *A. arenaria* will be studied further by inoculation experiments.

Identification of *Armillaria* Species using Mating Experiments

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In Europe five species of *Armillaria* occur. *A. mellea* and *A. obscura* may attack healthy trees whereas *A. bulbosa* only attacks weakened trees. *A. borealis* and *A. cepistipes* are saprophytes. Variation in the morphology of fruiting bodies is considerable and identification using basidiocarps is not always reliable.

Interfertility experiments have proved to be a useful tool in the taxonomy of *Armillaria*. Haploid cultures obtained from basidiospores produce white to light brown fluffy, aerial mycelia, whereas fully compatible pairings produce dark brown flat crustose mycelia.

Haploid tester strains of the five species were mated with 44 isolates obtained from the cap (diploid) of basidiocarps and single spore isolates (haploid) from the same basidiocarps growing in oak stands. Thirty-five isolates appeared to be *A. obscura*, six isolates were *A. bulbosa* and two isolates were *A. mellea*. One isolate could not be identified. *A. borealis* and *A. cepestips* were not found.

Diploid × haploid matings (i.e. Buller Phenomenon) were compared with haploid × haploid matings. Diploid × haploid mating reactions were difficult to score because one mate already possesses crustose mycelium. Generally however, they lead to the same identification. It was very important that the tester strains were isolated in the same year; one year old testers gave confusing results. It was concluded that *A. obscura* was prevailing in oak stands.

Mechanisms Involved in Control of *Fusarium* Wilt of Carnation by Fluorescent *Pseudomonas* spp.

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To improve the efficacy of the control of soilborne plant pathogens by treatments with fluorescent pseudomonads, the mechanisms involved have to be elucidated. Wilt disease of carnation, caused by *Fusarium oxysporum* f. sp. *dianthi* (Fod), was used as a model to study the involvement of siderophore mediated competition for iron, antibiosis and induced resistance in disease suppression by two strains of *Pseudomonas*: P. sp. WCS417r and *P. putida* WCS358r.

The purified siderophore of both strains inhibited germination of conidia of Fod. The iron saturated siderophore inhibited germination to a much lower degree. These results suggest that siderophore mediated competition for iron can be involved in the suppression of *Fusarium* wilt. Involvement of siderophore mediated competition for iron was also observed on agar plates. The inhibition of Fod by both *Pseudomonas* strains was reduced with increasing iron content of the medium, and a non-siderophore producing (sid^-) Tn5 mutant of WCS358 did not inhibit Fod. The sid^- Tn5 mutants of WCS417r, however, still inhibited Fod, suggesting the production of an antibiotic by this strain.

Treatment of carnations with WCS417r resulted in a significant disease reduction. The sid^- mutants of this strain reduced disease incidence less than their wild type. WCS358r reduced disease incidence not significantly. Its sid^- mutant did not reduce disease incidence.

The ability of the *Pseudomonas* strains to induce resistance against Fod in carnation, was studied by spatially separating the bacteria (on the roots) and the pathogen (in the stem). WCS417r reduced disease incidence significantly; WCS358r did not.

From these results it is concluded that in addition to induced resistance, siderophore mediated competition for iron and antibiosis could also be involved in suppression of *Fusarium* wilt of carnation by *Pseudomonas* sp. WCS417r. The suppression of *Fusarium* wilt by *Pseudomonas putida* WCS358r seems to depend on siderophore mediated competition for iron only. The multiple mechanisms of WCS417r may explain its more efficient control of Fod, compared with WCS358r.

Relation Between *In Vivo* Production of Fungal Polygalacturonases and Resistance Level of Carnation Cultivars to *Fusarium* wilt

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Breeding *Fusarium-wilt* resistant carnations is probably the best approach to overcome the problem that is caused by *Fusarium oxysporum* f. sp. *dianthi* in carnation culture. This resistance breeding program is hampered by the lack of a parameter, by which resistance can be measured in a fast and reliable fashion, other than determining the percentage surviving plants in a clone after inoculation which is strongly influenced by the environment. To investigate whether polygalacturonases (PG's) could be such a parameter we stem-inoculated 11 carnation cultivars which differed in resistance level. This inoculation was performed with a suspension (10^7 conidia ml^{-1}) of *Fusarium oxysporum* f. sp. *dianthi* race 2 on rooted cuttings which were planted in soil 4 weeks before. During the experiment, disease symptoms were indexed weekly up to 12 weeks after inoculation. Four weeks after inoculation, PG's were extracted from four stem pieces which were 5 cm in length (taken directly above the inoculation site) 4 weeks after inoculation. Electrophoresis of these extracts showed four PG isoenzymes on gel which were not present in extracts from water inoculated plants. These PG's are most likely of fungal origin. Most striking was the observed difference between the 11 cultivars. These cultivars appeared to have less PG activity as the resistance level increased. To quantify these differences we used a cupplate assay and a spectrophotometrical assay. Both methods clearly indicated a significant positive correlation between PG activity and disease index and a significant negative correlation between PG activity and percentage resistant plants within a clone. From these results was concluded that measurement of PG activity within stems of infected carnations could be a useful tool to determine the resistance level of a cultivar.

Does a Vascular Fungus Elicit an Induced Response in Tomato Plants, thereby also Affecting Fecundity of the Two-Spotted Spider Mite?

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Previous inoculation of tomato plants with a vascular fungus (*Fusarium oxysporum* f. sp. *lycopersici*, race 1), caused a decrease in the oviposition rate of two-spotted spider mites (*Tetranychus urticae*) on a *Fusarium*-susceptible cultivar, but not on a *Fusarium*-resistant cultivar. We conclude that the effect on mite fecundity is due to the fungus affecting the plant's food quality, including the effects this may have on the composition of defensive compounds, rather than the plant's active defense system. Since *Fusarium* seals off the xylem vessels, thereby causing wilting of the susceptible tomato plants, the reduction in mite fecundity may well be due to drought stress in the leaves. In some preliminary experiments we found an effect of severe drought stress in the root environment

on the oviposition rate of *T. urticae*, suggesting that our hypothesis may be right.

Growth and Oospore Formation of two *Pythium* spp. Causing Cavity Spot in Carrots

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Cavity spot is a carrot disease which can be caused by two species of *Pythium*: *P. violae* and *P. sulcatum*. Both pathogens are seldom found in carrots from the same field. *P. sulcatum* is more often isolated from carrots grown on sandy or muck soils, while *P. violae* prevails in clay soils.

The pH influences the growth of both fungi on agar media in the same way. Growth of both species does not occur or is very limited at pH 5·0 depending on the medium used. The optimum temperature, however, differs for both fungi. *P. violae* shows most mycelial growth on agar at 19–20°C, whereas *P. sulcatum* grows best at 23–24°C. Oospore formation is also quickest at these respective temperatures. Whether *P. violae* produces oospores on agar media depends on the composition of the media, wateragar being the most satisfactory. *P. sulcatum* produces easily oospores.

To free oospores from mycelium, sonication as well as passage through snails appears promising.

In-vitro Translation Products from Compatible and Incompatible Combinations of *Bremia lactucae* and Lettuce

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Bremia lactucae Regel, the causal organism of lettuce downy mildew, is a frequently occurring pathogen of field and glasshouse lettuce crops. The disease is disseminated by means of conidiosporangia. In the infection process, no distinction in the behaviour of either fungus or plant for compatible or incompatible combinations have been found prior to penetration of the spores in the lettuce epidermal cell layer (In: *The Downy Mildews*, ed. D.M. Spencer, 1981). The contact of the fungus with the host plasmalemma, or later the formation of the fungal secondary vesicles probably contributes to differences in compatible and incompatible combinations. Studies of the differences in gene activation in both the fungus and the plant, for both resistant and susceptible interactions, may show gene products which determine the specificity. We started to study the gene activation by means of two-dimensional polyacrylamide gel electrophoresis of in-vitro translation products in two near isogenic lettuce lines (F8 backcross). One line is susceptible to the physiological race NL12 and resistant to NL15, the other line is susceptible to NL15 and resistant to NL12. At least five differences in translation products were found between the mRNAs isolated from the two near isogenic lines. No differences were found in translation products of mRNA isolated from water, NL12 and NL15 inoculated leaves within one day after inoculation. Therefore we conclude that differences in gene activation may be restricted to the lettuce epidermal cell layer. mRNA will further be isolated from epidermal cell layers stripped off leaves at different time periods after the inoculation with the fungus.

Gene Expression in *Phytophthora infestans* During Pathogenesis on Potato

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Potato late blight caused by the fungus *Phytophthora infestans* (Mont.) de Bary (Oomycetes), is one of the most important diseases of potato. Leaves and tubers are readily infected by *P. infestans* and the colonization process is very rapid and extremely destructive for the tissue. The molecular basis underlying pathogenicity of *P. infestans* is poorly understood. In order to gain more insight in the processes involved, we are studying the gene expression of *P. infestans* during pathogenesis on potato.

It can be assumed that the establishment of a pathogenic relation between the potato plant and *P. infestans* involves the mutual interference in cellular processes of both partners. Defence responses of colonized host tissue are relatively well studied, but nothing is known about changes in the metabolism of the pathogen during growth in the plant. It is tempting to assume that in the potato–*P. infestans* interaction, host factors induce physiological responses in the pathogen which are necessary for pathogenesis and which are mediated by pathogenicity genes. The identification and characterization of *in planta* induced genes of *P. infestans* therefore will lead to a better understanding of the molecular basis underlying pathogenicity of the fungus. To this end a genomic library of *P. infestans* DNA was differentially screened using cDNA from mRNA of the fungus grown *in vitro* and cDNA from mRNA of infected leaves as probes. Several differentially hybridizing clones containing putative *in planta* induced *P. infestans* genes were isolated from the *P. infestans* genomic library. The DNA sequence of some of the *in planta* induced genes has been determined. Two of

these appear to encode ubiquitin and calmodulin respectively. Both genes are highly conserved in eukaryotic organisms and are reported to be involved in several important cellular processes. The expression of the genes *in planta* and *in vitro* is currently being studied.