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THE 'WILLIE COMMELIN SCHOLTEN' MEETING OF THE SECTION FOR PHYTOPATHOLOGY ON 22 JANUARY 1998

Control of Soilborne Pathogens by Inducing Soil Anoxia

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A new non-chemical method for the control of soilborne pathogens is being developed. This method involves the incorporation of green plant material into moist soil, directly followed by the application of a plastic tarp with a low permeability for oxygen for 12–15 weeks in summer and autumn. Results of field experiments in 4 consecutive years show that in this manner a strong reduction in the inoculum density of *Fusarium oxysporum* f. sp. *asparagi* and *Verticillium dahliae* (usually >90%) is achieved. No significant reduction was observed in treatments with organic material or plastic only. Following application of the plastic, the treated soil became anoxic rapidly and redox potential dropped gradually to values as low as −200 mV at a depth of 15 cm.

Products of fermentative bacteria (e.g. organic acids, aldehydes, alcohols) that accumulate temporarily in the anoxic soil layer prior to the onset of methanogenesis are suggested to be responsible for the inactivation. These products can cause inactivation of fungal resting structures either by acting as a toxicant or by inducing germination followed by lysis. A simulation model was developed to describe organic matter decomposition and transport processes in treated soil. With this model it can be shown that key factors controlling the concentrations of fermentation products in treated soil are (1) the amount and quality of the organic matter; (2) the amount of available inorganic electron acceptors, such as oxygen, nitrate and reducible Fe oxides; (3) the initial biomass of methanogenic bacteria in the soil; and (4) soil temperature. Further research aims at obtaining more insight in the mechanism and dynamics of inactivation during the treatment since this will allow an efficient optimization of the control method.

Pythium-Suppressive Rockwool; Analysis of the Microflora Using Plate Counts and Denaturing Gradient Gel Electrophoresis

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The policy of the Dutch government in greenhouse horticulture is focused on the development of closed culture systems. Nutrient solutions have to be circulated to minimize pollution. However root pathogens, and particularly those which produce zoospores, have the potential to spread rapidly in an aqueous environment. *Pythium aphanidermatum* has been responsible for considerable crop losses in cucumber and control of this pathogen is difficult. However, *Pythium* spp. are poor competitors relative to other root-colonizing micro-organisms. Therefore, the capacity of the indigenous microflora to suppress root rot of cucumber plants grown on rockwool was evaluated.

Rockwool previously used for growth of cucumbers was found to be suppressive to *P. aphanidermatum*. In an ebb and flood system with nursery plants (up to 35 days old), used rockwool showed less diseased plants than sterilized or new rockwool after inoculation with *P. aphanidermatum*. The suppressiveness was regained when sterilized rockwool was recolonized by the original microflora. Similar results were obtained in a system with young plants (up to 18 days old) on used rockwool. A build-up of suppressiveness during the production phase occurred after inoculating successive cucumber crops with the pathogen.

In order to be able to stimulate or introduce a suppressive microflora, we are now studying the relation between suppressiveness and the occurrence of certain microbial groups by plate counts and by denaturing gradient gel electrophoresis (DGGE). This molecular technique allows genetic fingerprinting of populations. Results with plate counts show that the bacterial numbers increase rapidly, whereas the build-up of fungal populations is much slower. Furthermore, correlations between suppressiveness and numbers of actinomycetes and *Trichoderma* spp. were found.

The Use of Autofluorescent Proteins to Visualize Root Colonization of *Pseudomonas fluorescens* Strain WCS365

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Plant-beneficial *Pseudomonas* species act as biocontrol agents, protecting plants against attacks by pathogenic fungi. Traits considered to be important in protection are (1) the production of anti-fungal factors (AFF) and (2) the ability to colonize the plant root system efficiently in order to deliver the AFF at the right time and place. Spatial–temporal analyses of bacterial root colonization and gene expression at the cellular level have been hampered by the availability of suitable reporter genes. However, the recent development of the use of green fluorescent protein (GFP) as a new reporter gives the opportunity to accomplish such analyses (Chalfie *et al.* 1994, *Science* **263**: 802–805). GFP has been isolated from the jellyfish *Aequoria victoria* and emits green light after excitation. Unique advantages of GFP as a marker protein are (1) analysis does not require substrates, (2) analysis is non-destructive, (3) bacteria can be detected at the single cell level, and (4) various GFP mutants have been developed possessing different excitation and emission wavelengths, which can be used to analyse multiple processes simultaneously.

Pseudomonas fluorescens strain WCS365 is an excellent colonizer of roots of tomato, potato and wheat. To visualize the bacteria on the root during colonization the *e-gfp* gene was constitutively expressed under the control of the *tac* promoter located on the rhizosphere-stable plasmid pWTT2081. Colonization of the root surface of *Lycopersicon esculentum* (tomato) seedlings was analysed over time in a non-destructive manner using fluorescence light microscopy and confocal laser scanning microscopy.

A Site-Specific Recombinase is Required for Competitive Root Colonization by *Pseudomonas fluorescens* Strain WCS365

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We isolated a novel colonization mutant of the efficient root-colonizing biocontrol strain *Pseudomonas fluorescens* WCS365 which is impaired in competitive root tip colonization of gnotobiotically grown potato, radish, wheat and tomato, indicating a broad host range mutation. The colonization of the mutant is also impaired when studied in potting soil, suggesting that the defective gene also plays a role under natural conditions. A DNA fragment able to complement the mutation for colonization revealed a multicistronic transcription unit, comprising at least six ORFs (open reading frames) which exhibit similarity to *lppL*, *lysA*, *dapF*, *orf235/233*, *xerC/sss* and the largely incomplete *orf238*, respectively. The transposon insertion in PCL1233 appeared to be present in the *orf235/233* homologue, designated *orf240*.

Introduction of a mutation in the *xerC/sss* homologue revealed that the *xerC/sss* gene homologue rather than *orf240* is crucial for colonization. *xerC* in *Escherichia coli* and *sss* in *Pseudomonas aeruginosa* encode proteins which belong to the lambda integrase family of site-specific recombinases, which play a role in phase variation caused by DNA rearrangements. The function of the *xerC/sss* homologue in colonization is discussed in terms of genetic rearrangements involved in the generation of different phenotypes, which allows a bacterial population to compete for various ecological niches. Mutant PCL1233 is assumed to be locked in a phenotype that is not well suited to compete for colonization in the rhizosphere. To our knowledge this is the first report which shows the importance of phase variation in microbe–plant interactions.

In Vitro Compatibility Between Fluorescent *Pseudomonas* spp. Strains Can Increase Effectiveness of Fusarium Wilt Control by Combinations of These Strains

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Fusarium wilt diseases, caused by the fungus *Fusarium oxysporum*, can lead to significant yield losses of crops. One strategy to control fusarium wilt is the use of antagonistic, root-colonizing *Pseudomonas* spp. It has been demonstrated that different strains of these bacteria suppress disease by different mechanisms. Therefore, application of a mixture of these biocontrol strains, combining several suppressive mechanisms, may represent a viable control strategy. A prerequisite for biocontrol by combinations of biocontrol agents is compatibility of the co-inoculated micro-organisms. Hence, compatibility between several *Pseudomonas* spp. strains, that have the ability to suppress fusarium wilt of radish by different disease-suppressive mechanisms, was tested *in vitro* on KB agar plates.

Growth of *P. fluorescens* strain RS111 was strongly inhibited by *Pseudomonas* spp. strains RE8, RS13, RS56 and RS158, whereas a mutant of strain RS111 (RS111-a) was insensitive to inhibition by these strains. Strains RS111 and RS111-a did not inhibit the other strains. Suppression of fusarium wilt of radish in a potting soil bioassay by the incompatible combination of RE8 and RS111 was comparable to the effects of the single strains. However, disease suppression by the compatible combination of RE8 and RS111-a was significantly better compared to the single strains. In contrast, the compatible combinations of RS13, RS158 or RS56 with RS111-a did not result in a better disease suppression as compared to the single strains. This indicates that specific interactions between biocontrol strains can influence disease suppression by combinations of these strains. Currently the population dynamics of the strains RE8, RS56, RS111, RS111-a and their combinations are under investigation.

Additive Effects of Rhizobacterium- and Pathogen-Induced Systemic Resistance in *Arabidopsis*

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Plants can develop elevated levels of disease resistance after being exposed to specific stimuli. Infection by an avirulent pathogen triggers systemic acquired resistance (SAR), rendering uninfected plant parts resistant to subsequent infection. Also selected non-pathogenic fluorescent *Pseudomonas* bacteria that colonize the rhizosphere are able to trigger an induced systemic resistance (ISR) response. To compare the signalling pathways controlling ISR and SAR, *Arabidopsis* plants impaired in their response to the defence-related signalling molecules salicylic acid (SA), jasmonic acid (JA) or ethylene, were tested on their ability to express ISR or SAR against infection by the challenging pathogen *P. syringae* pv. *tomato* (*Pst*). The plants were pretreated with the ISR-inducing biocontrol strain *P. fluorescens* WCS417r or the SAR-inducing avirulent pathogen *Pst*(*avrRpt2*). ISR was blocked in the JA-response mutant *jar1* and the ethylene-response mutant *etr1*, but not in SA-non-accumulating NahG plants. In contrast, SAR was blocked in NahG plants, but not in *jar1* or *etr1*. This indicates that, unlike SAR, ISR is independent of SA, but instead requires responsiveness to JA and ethylene.

Apparently, ISR and SAR are two distinct inducible defence responses. To investigate whether elicitation of both ISR and SAR leads to a higher level of protection, plants were treated with a combination of WCS417r and *Pst*(*avrRpt2*). Indeed, significantly better protection was established by the combination treatment than by either inducer alone. This additive effect was absent in *jar1*, *etr1* and NahG plants. Northern blot analyses showed that genes encoding pathogenesis-related (PR) proteins, which are markers for SAR, were activated only by *Pst*(*avrRpt2*), and not by WCS417r. The combination of the two resistance inducers did not result in an enhancement of PR-gene expression, suggesting that the ISR pathway does not affect the one leading to SAR. Also the resistance-inducing signalling molecules SA, JA and ethylene were tested for additional effects on WCS417r-mediated ISR. Only the combination of WCS417r and SA increased the level of protection compared to the effect of each inducer alone. WCS417r did not enhance JA- or ethylene-induced protection. This suggests that WCS417r saturates the JA- and ethylene-responsive pathway, but stimulates a pathway that is at least partly additive to the one elicited by SA.

Genetic Analysis of Induced Systemic Resistance in *Arabidopsis*

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Plants have the ability to acquire an enhanced level of resistance against pathogen attack after appropriate stimulation. Classic systemic acquired resistance (SAR) is a pathogen-inducible defence mechanisms that is dependent on salicylic acid (SA), and is associated with the systemic accumulation of pathogenesis-related (PR) proteins (Ryals *et al.* 1996, *The Plant Cell* **8**: 1809–1819). Selected non-pathogenic rhizobacteria are able to elicit a phenotypically similar systemic resistance response. Using *Arabidopsis thaliana* as a model, it was shown that this rhizobacteria-mediated induced systemic resistance (ISR) follows a signalling pathway that, unlike SAR, is independent of SA and PR-gene activation (Pieterse *et al.* 1996, *The Plant Cell* **8**: 1225–1237).

To elucidate the genetic basis of rhizobacteria-mediated ISR, different *Arabidopsis* ecotypes were screened for their potential to express ISR against infection by *P. syringae* pv. *tomato* (*Pst*), after root treatment with the non-pathogenic *Pseudomonas fluorescens* strain WCS417r. Out of seven ecotypes tested, two ecotypes (RLD and Ws-0) did not develop ISR after treatment with *P. fluorescens* strain WCS417r. This WCS417r-non-responsive phenotype

was correlated with a remarkably high level of susceptibility to *Pst*, suggesting that the ability to express ISR is dependent on a threshold level of basal resistance. Subsequently, a cross was made between an ISR-responsive (Col-0) and an ISR-non-responsive ecotype (RLD). F_1 hybrids were, like the Col-0 parent, fully capable of expressing ISR, and exhibited a relatively high level of basal resistance. This indicates that the potential to express ISR and basal resistance against *Pst* are both inherited as dominant traits. Analysis of F_2 plants revealed that both traits co-segregated in a 3:1 fashion in the same set of plants, indicating that the potential to express ISR and basal resistance against *Pst* are monogenically determined and linked.

The Expression of the *Avr9* Gene of *Cladosporium fulvum* is Regulated by a GATA-Type Transcription Activator

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The avirulence gene *Avr9* of the fungal tomato pathogen *Cladosporium fulvum* is highly induced *in planta* during infection of tomato. Expression of the *Avr9* gene could also be induced *in vitro* when the fungus was grown on synthetic liquid medium containing little or no nitrogen source. The promoter of the *Avr9* gene contains six copies of the sequence TAGATA and additionally six copies of the core sequence GATA in the distal 300 bp. In the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*, these promoter sequences have been identified as the binding sites for a major, wide-domain GATA-type nitrogen-dependent regulatory protein, AREA of *A. nidulans* and NIT2 of *N. crassa*, respectively. Studies with *C. fulvum* transformants containing ectopically integrated partially deleted *Avr9* promoter-*uid*A (GUS) constructs suggested that both *in vitro* and *in planta* *Avr9* promoter activity is dependent on the presence of these specific cis-regulatory elements.

A 600bp *Avr9* promoter fragment-*uid*A fusion construct has been introduced into different *are*A mutants and into the *are*A wild type of *A. nidulans* by targeted integration. Quantitative determinations of GUS activity following nitrogen starvation of single copy transformants showed that induction of the *Avr9* promoter was similarly regulated in *A. nidulans* and in *C. fulvum* and that functional sites for the binding of a regulatory protein, homologous to AREA, are present in the *Avr9* promoter.

Studies with specific point-mutated *Avr9* promoter-*uid*A fusion constructs in *A. nidulans* indicated that the TAGATA elements in the *Avr9* promoter are indeed essential for expression and probably constitute true AREA binding elements, indicating that they are functional in the transcriptional regulation of the *Avr9* gene. The *C. fulvum pyr* gene is being isolated and sequenced to target these constructs at the *pyr* locus of *C. fulvum*.

To isolate the *C. fulvum areA* equivalent gene (= *Nrf*1, nitrogen response factor), a PCR-based strategy has been used to clone the *Nrf*1 DNA-binding zinc finger domain. The deduced amino acid sequence was found to be identical to that of the zinc finger region of the corresponding *A. nidulans* protein. The flanking sequences of this gene have now been cloned by using a complementation strategy.

Gene-for-Gene Hypothesis for the Interaction between *Fusarium oxysporum* and Tomato

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Resistance of tomato to *Fusarium oxysporum* f.sp. *lycopersici* (Fol) is monogenic and dominant. The *I2* resistance gene has been cloned and fits the NBS-LZ-LRR group of resistance genes. Susceptible tomato lines transformed with cosmids containing the *I2* resistance gene were resistant to race 2 isolates (a1A2). Most race 1 isolates were virulent (A1a2) on these transgenic lines. However, also avirulent race 1 isolates were identified, suggesting the presence of the complementary avirulence gene *Avr12* in these race 1 isolates (A1A2). These results suggest that the interaction between tomato and Fol is based on a gene-for-gene interaction. To identify the fungal signal that leads to the *I2*-dependent race-specific resistance we set out to isolate the avirulence gene *Avr12* of *Fusarium* race 2.

A random mutagenesis approach was set up using ^{137}Cs irradiation. This method was tested for its suitability and shown to induce large deletions in 40–50% of the mutants generated. A race 2 isolate was labelled with phleomycine resistance and GUS marker genes. Subsequently, irradiated spores of this transgenic strain were tested in seedling inoculation assays. Among the 25 000 mutants tested, one mutant had changed from avirulent to virulent on a plant containing the *I2* resistance gene. This low frequency of mutation is approximately three times less than could be expected, based on mutations in the nitrate reductase gene. The mutant is not as aggressive as most

race 3 isolates ('natural *avr*I2 mutants'). This could imply that (1) pathogenicity factors were deleted in addition to an avirulence gene, (2) pathogenicity and avirulence are encoded by the same gene, or (3) a mutation is induced in a signal leading to both pathogenicity and avirulence. Amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) analysis were used to trace polymorphisms between the mutant and the original isolate. Nine polymorphisms were identified by AFLP and one by RAPD analysis. We are currently analysing which polymorphism may have caused the *avr*I2 mutation.

Transfer Cell Formation Reveals a Biotrophic Phase in Bulb Rot of Lilies Infected by *Fusarium oxysporum* f.sp. *lilii*

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Basal rot of lilies is characterized by progressive rot of the roots and the basal plate of bulbs. Bulb scales are severed from the rotting basal plate. Detached scales develop rot from the base and from infected stomata on the outer scale surface. The fungus colonizes intercellular spaces and middle lamellae, but also splits the parenchyma walls in distinct layers and grows in between these. Heavy plasmolysis occurs.

Next to colonized intercellular spaces, pitted cellulosic wall thickenings are produced that become infused with phenolics. Cells with thickened walls have wall ingrowths that extend and branch deeply into the cytoplasm. Ingrowths occur in cortical parenchyma of bulbs and roots, and in epidermal cells at the border of lesions. Behind the hyphal front the affected cells finally die, leaving the ingrowths as coralloid structures in an empty lumen. Cells with such wall ingrowths are known as transfer cells, specialized in short-distance assimilate transport from symplast to apoplast. Transfer cells in lily pistils, similar to those induced by *Fusarium oxysporum*, are involved in nectar secretion. Transfer cells in colonized parenchyma may be involved in sugar secretion towards the fungus, as also suggested by starch depletion from the cells involved.

Transfer cells occur as giant cells induced by parasitic nematodes and in association with mycorrhiza. In the latter, depletion of starch is also reported. Mycorrhizal associations function at a short distance from the root tip. Further back, defence reactions occur and the mycorrhizal fungus degenerates. Transfer cell formation in lily infected with *F. oxysporum* resembles that in mycorrhizal associations. Invading hyphae likely act as sinks for host assimilates. The biotrophic stage ends when fungal biomass has increased to an extent that fungal enzymes (toxins) damage the colonized tissue. Host defence reactions ensue, but cannot prevent degradation and rot. An endophytic initial phase of colonization has important ecological advantages. Whether endophytic biocontrol strains of *F. oxysporum* also induce transfer cells remains to be investigated.

Cloning and Characterization of a Gene Encoding a *Fusarium oxysporum* Cell Wall Protein

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The soilborne fungus *Fusarium oxysporum* f.sp. *lycopersici* (Fol) causes wilt in tomato. Wilting of a susceptible plant can be explained by an inefficient containment of the fungus, resulting in an extensive obstruction of xylem vessels, ultimately leading to wilting of the plant.

During interaction of plant and fungus, cell walls of both organisms are in close contact. Fungal cell wall glycoproteins (CWP) that are exposed to the outside of the cell wall could play a role in the interaction. As a first step to test this hypothesis we cloned a gene that encodes a CWP.

Cell walls were purified from 3-day-old mycelium grown in liquid culture. By extraction with ice-cold hydrofluoric acid a 60 kDa and a 70 kDa glycoprotein were released from the wall. N-terminal sequencing of the 60 kDa protein provided a reliable peptide sequence. By PCR on cDNA with two different degenerated DNA primers and an oligo-dT primer, a 800 bp fragment was obtained that was sequenced. The N-terminal part of the deduced ORF was identical to the peptide sequence. From a genomic library of Fol (race 2) a 14 kb *Sal*I fragment was obtained that contained the complete gene. Sequencing of a 1.6 kb *Nco*I fragment revealed that the coding region is interrupted by one intron. The predicted ORF codes for a protein of 22 kDa with a hydrophobic N- and C-terminus, likely to function as the secretion signal and a GPI attachment site, respectively. The protein is rich in serine (11·6 %) and threonine (17·3 %) that are potential O-glycosylation sites. Furthermore, two potential N-glycosylation sites are present. We designated this gene *FEM1* for Fusarium extra cellular matrix protein.

DNA gel blot analysis showed that one copy of *FEM1* is present in race 1, 2 and 3 of Fol, *F.ox.* f.sp. *radicis lycopersici*, *F.ox.* f.sp. *gladioli*, *F.ox.* f.sp. *dianthi* and *F. solani*. No homologues of this gene

were found in *Saccharomyces cerevisiae* and *Botrytis cinerea*. RNA gel blot analyses revealed that the expression of *FEM1* is developmentally regulated and coincides with germination of micro-conidia.

The Role of ABC-Transporters in Pathogenesis of *Mycosphaerella graminicola*

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ABC-transporters are membrane-bound ATP-hydrolysing transporters with a characteristic domain organization. These transporters are present in cell membranes of all living organisms and can have a very broad substrate range. A generally accepted function of ABC-transporters is the protection of organisms from poisoning by naturally occurring cytotoxic compounds through prevention of their accumulation in cells. A side-effect of this function can be the development of multidrug resistance to chemotherapeutic drugs and fungicides in cancers and plant pathogens, respectively.

In plant-pathogenic fungi the function of ABC transporters can involve (1) protection of the fungus during pathogenesis against plant defence products, (2) secretion of pathogenicity factors (e.g. plant toxins), and (3) secretion of mating factors. This hypothesis is tested for the causal agent of *Septoria tritici* leaf blotch of wheat, *Mycosphaerella graminicola* (anamorph *Septoria tritici*).

Using heterologous hybridization two single-copy ABC-transporter encoding genes, *Mgatr1* and *Mgatr2*, were cloned. Expression studies showed that both genes have a different expression pattern. *Mgatr1* has a low basal level of expression, which increases upon treatment with cycloheximide or the plant secondary metabolite eugenol. *Mgatr2* shows a more specific expression pattern. No basal expression can be detected, but the gene is induced after treatment with different compounds, amongst others the fungicide imazalil and the plant secondary metabolite eugenol. Furthermore, expression patterns seem to be different for the two morphological states tested (spores and mycelium). Mutants are being constructed in which these genes are deleted and these mutants will be analysed with respect to virulence on wheat and other traits such as sensitivity to natural toxins.

MEETING OF THE KNBV-SECTION FOR PLANT MORPHOLOGY, ANATOMY AND CYTOLOGY AND THE KNGMG-SECTION FOR PALYNOLOGY ON 17 FEBRUARY 1998

Fossil Palm Pollen: A Systematic Approach to its Re-evaluation

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A systematic database of recent palm pollen has made possible the re-examination of some earlier records of palm-like fossil pollen. Studies of previously unexamined palm-like fossil pollen have also been undertaken. Examples are given which illustrate some of the problems encountered in comparing early pollen with recent pollen. The data challenge some of the accepted views, and confirm others, on the affinities between the fossils and their recent counterparts.

Palaeopalynologists frequently work from a geological rather than botanical background. Their approach to fossil pollen identification differs from that of systematic pollen morphologists because their goals are different. Palaeopalynologists are often seeking well-represented pollen types to demarcate 'tops and bottoms' of stratigraphic zones, or as indicators of depositional environments or climate change. While systematic pollen morphologists are primarily interested in the affinities, geographic origin and subsquent migration of these ancestors of recent plant families in an evolutionary perspective.

In Quaternary studies pollen counts of pteridophytes, gymnosperms, aquatic and terrestrial angiosperms, grasses and herbs, are used as indicators of, for example, past vegetation types, marine ingressions and regressions, climate, the influence of man, and the effect of fire. The family affinities of the pollen in such studies must be assumed correct by the reader. This is because Quaternary pollen is, comparative to Tertiary and Cretaceous pollen, easier to identify with modern polen, and is often not described or illustrated by the author. Quaternary studies of fossil pollen in a systematic context are less commonly undertaken than geological or palaeogeographical studies.

Somewhere between the early Quaternary and the late Tertiary, direct comparison between fossil grains

and the pollen of recent species, starts to become less viable. Fossils not matching those of recent plants, but often carrying certain recognizable characteristics, become more abundant in older deposits. They do not always represent extant families. In many instances the interpretation of the morphology of Tertiary and Cretaceous pollen, its affinities with, or ancestry to recent plant families is nevertheless possible, Careful study can produce informative and valuable results.

Amorphophallus Pollen: As Diverse as the Plants Themselves

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Amorphophallus is a genus of Old World aroids occurring in Africa, Madagascar, India, continental SE Asia, Malesia and NE Australia. The main centre of diversity is continental SE Asia and W Malesia. The last revision was by Engler (1911), who distinguished 78 species in 11 sections. Parallel to the recent taxonomic revisions by Hetterscheid (in prep.) and Ittenbach (in prep.) a pollen morphological analysis of 145 out of the *c.* 170 species has been carried out, using LM, SEM and TEM (Van der Ham, Hetterscheid & Van Heuven *Rev. Palaeobot. Palynol.*, in press). The outer pollen wall does not resist acetolysis. Therefore, only fresh pollen or material boiled in water (in case of herbarium specimens) could be used.

Average pollen grain size is between 28 and 88 μm. grain shape is mostly spherical to ellipsoid. The grains are inaperturate and often crammed with starch. The exine surface is much diverse. A rough subdivision yielded 8 main types, of which striate, psilate and fossulate are most common, and echinate, verrucate, areolate, scabrate and striate/scabrate occur in one or a few species. Many subtypes and intermediate forms may be distinguished. Examination of the exine ultrastructure in a selection of species demonstrated the presence of a distinct, spongy, acetolysis-resistant endexine, and of a usually non-resistant ectexine without differentation into foot layer, infratectal layer and tectum. Psilate walls have a relatively thin, homogeneous ectexine, whereas sculptured walls are thicker and often contain in their ectexines dark (osmiophilic) granules, which vary in size, number, shape and distribution. Such granules are known so far only in *Amorphophallus* and a few other Araceae.

A comparison of the surface types with a preliminary subdivision of *Amorphophallus* into 23 groups based on macromorphological characters shows that the striate type occurs in 13 groups (64 spp.), the psilate type in 15 groups (49 spp.) and the fossulate type in 9 groups (18 spp.). The scabrate and the striate/scabrate type are restricted to single species. Most groups include more than one type (up to four). Only 7 groups possess a single type. Thus, the pollen surface type often supports infrageneric groupings, but probably monophyletic groups are sometimes highly diverse, palynologically. The genus *Pseudodracontium*, which is tentatively included as a group within *Amorphophallus*, belongs to the striate surface type. However, within this type its pollen is clearly distinct in having more or less psilate 'polar' caps.

Pollen Tube Growth in Gymnosperms and Angiosperms

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Gymnosperm pollen tubes are considered to bear some characteristics ancestral to those of angiosperms. In angiosperms the cytoplasm is characterized by a polar organization with a longaxial cytoskeleton and a reverse fountain-like cytoplasmic streaming pattern and a zonal distribution of some organelles, especially secretory vesicles (SV), smooth endoplasmic reticulum (SER) and dictyosomes over the length of the pollen tube. Recent studies on pine pollen tubes showed longitudinally organized cytoskeletal elements and a mostly Brownian or saltatory and slow organelle motion. Cytoplasmic organization of pine pollen tubes is different from that of angiosperm pollen tubes. SV and SER were located mainly at the tip, dictyosomes appeared equally distributed, and mitochondria were found mainly near the cell wall in a broad zone behind the tip. These observations have led to the conclusion that the particular organization of angiosperm pollen tubes relates to their growth rate, rather than to their mode of growth and prompted study of the possible differences in wall deposition and wall construction between gymnosperms and angiosperms.

We compared the deposition and structure of the wall of pine pollen tubes. The wall is deposited in the tip as a relatively thick and uniform layer, similar to that seen in angiosperms. The primary wall, beginning at the surface, gradually differentiates into a morphologically different, fibrillar layer, either by modification in composition or assemblage, or by loss of relatively inert material, such as callose or esterified pectin. These observations show the basic similarity between pine and angiosperm pollen tube wall deposition. However, in contrast to probably most angiosperms, the entire primary wall persists during growth, the distribution of callose and pectin

is different and no callose lining or secondary wall is formed. Our observations on pine show variably distributed callose, with banded patterns in the tube. Callose was always present in the growing tip of young pollen tubes, whereas it may be absent or less abundant in older, possibly non growing pollen tube tips. Callose is absent from the tip in angiosperms. Like in angiosperm pollen tubes, pectin in the pollen tube tip seems fully esterified as a strong binding occurs with JIM 7 but not with JIM 5 antibodies. In contrast to angiosperms, however, acidic pectin could not be detected in the tubular part of pine pollen tubes. Esterified pectines remained present in small quantities, mostly showing banded distributions. The acidic pectin in pollen tubes of angiosperms derives supposingly from de-esterifiction of esterified pectin originally deposited in the tip. Apparently, such de-esterification does not occur in pine pollen tubes. Cellulose microfibrils in the tubular part of pine pollen tubes are mostly present in non-axial and non-transverse orientations with respect to long axis of the tube, very similar to that of angiosperms. The density of the CMF at the tip of pine pollen tubes, however, appears to be much higher than in angiosperms.

The present results show a basic similarity in primary wall construction between pine pollen tubes and those of angiosperms. The formation of a rigid primary wall by de-esterification of esterified pectin, the possible reduction in cellulose content in the tip and the deposition of a secondary callosic wall appear to be new developments in angiosperms pollen tubes and probably relate to their fast growth.

Development of the Andean and Amazonian Ecosystems since the Late Miocene

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The closure of the Panamanian landbridge around 4 million years ago caused a significant change of flora and fauna between both, previously separated continents. The 586 m deep borehole in the sedimentary basin of Bogotá shows the evolutionary history of the Andean ecosystems during the last 3·2 million years. Floral immigrants from the northern hemisphere changed the composition of the montane ecosystems significantly: *Alnus* entered around 1 million years ago, and *Quercus* 340 000 years ago. After a period of 150 000 years of competition, *Quercus* forest became the dominant forest type in the upper montane belt since *c.* 200 000 years ago. The upper forest line moved between 1800 m (during glacials) and 3200 m at present-day, reflecting temperature fluctuations of *c.* 8°C. Altitudinal migrations of the paramo belt led to a complex system of merging and separating paramo 'islands', explaining the high degree of endemism among paramo taxa. The excessive biodiversity of the Amazonian rain forest seems a heritage of the Tertiary; in the Quaternary, extinction may have been more important as speciation.

The forest refugia hypothesis assumes that a large part of the modern rain forest was replaced by drier savanna-like vegetation because of strongly reduced precipitation. According to the present author, the contrasting opinion, strongly advocated by Colinvaux (*NWO Huygens Lecture 1997*, 7–30), is not necessarily conflicting with the forest refugia hypothesis (Hooghiemstra, *NWO Huygens Lecture 1997*, 31–43). Both scenarios did occur and represent the extreme scenarios under dry and wet climatic conditions, respectively. Four aspects are important when the forest history of the Amazon basin and adjacent savannas is considered: (1) the annual migration of the caloric equator (the Intertropical Convergence Zone, ITCZ), under the modern orbital configuration between about 8°N and 3°S, causing an annual latitudinal shift of the equatorial rain belt, leading to seasonal variations in precipitation; (2) the precession cycle of the astronomical theory of global climatic change, causing a cyclic migration of the equatorial rain forest belt towards a more northern or southern geographical location with a period of about 20 000 years (under the present-day orbital configuration the southern Amazon basin receives more precipitation, but in 11 000 years northern Amazonias and the Caribbean will receive more rain); (3) the temperature oscillations at sea-level of maximally 4–5°C during the strong glacial cycles during the last 11 million years with a main period of about 100 000, years, but in addition with interfering shorter cycles that result in series of stadials and interstadials. The resulting temperature record caused the composition of the amazonian lowland forest changed during the Quaternary; (4) the concave shape of the Andes between 5°N and 15°S act as a trap for humid Atlantic air masses causing continuous convective rains in northwestern Amazonas, irrespective of precessional forcing, leading to a permanent forest refugium. The present-day forest composition should be seen as the accidental situation of the present time-slice rather than a constant characteristic of the Quaternary. The pollen records of the Amazon fan sediments reflect thoroughly mixed pollen assemblages, and are unsuitable to demonstrate vegetation change in the Amazon basin.

Palynology of a Water Well from the Early Neolithic

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Kückhoven is a well known site of the early neolithic Bandceramic Culture, and with an extension of 12–14 ha one of the largest known in the loess landscapes of the Niederrheinische Bucht. Its excavation became necessary because of the exploitation of a gravel pit and started 1989. In October 1990 the remains of a water well were discovered. The well is 3×3 m in diamter, constructed from massive logs of oak wood and reached a depth of 14 m below surface. The wood construction was built in the year 5089 BC, and is the oldest preserved wooden construction known in the world. The upper 6 m of the well were gone, but from 6 to 14 m depth the logs are preserved. The lowest 3 m of the well was filled with all kinds of debris from the settlement. This mainly organic material (all kinds of wooden and bone tools, pieces of fabric, cords and ropes of various length and thickness and many more objects awaiting identification) was preserved in an excellent way, and so are the plant remains. Dr K.-H. Knörzer analysed the macro-remains and identified more plant species in this one object than were known from the West European Early Neolithic as a whole. The samples were also analysed for their pollen content and this brought the total amount of identified plant species from the 51st century BC to 295.

The comparison of the pollen record with the plant macro-remains shows some interesting features from a methodological point of view: (1) Only 52 plant species are found in both records, nevertheless they show in their floristic diversity the same vegetation. (2) Maco-remains of trees and shrubs are almost absent in the record of macro-remains, as is the plant family of Apiaceae. However, these groups are well represented in the pollen record. (3) Identification possibilities are better on macro-remains, but palynological identification is comparable within those plant families from which the pollen morphology has been studied. (4) There is an urgent need for solid pollen morphological research in those plant families which are of great importance in palaeo-ethnobotany, for example, the Fabaceae, Lamiaceae and Rosaceae, of which the pollen in the fossil record is recognized but remaining unidentifiable.

Glossary of Pollen and Spore Terminology: The Electronic Edition

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The first three editions of the Glossary of pollen and spore terminology were drafts, for discussion purpose only. The official edition (Punt *et al.* 1994, *LPP Contributions Series* 1) was thought to be the final one. However, it appeared that more terms were available and that improvements were still possible. With the arrival of the Laboratory of Palaeobotany and Palynology on the Web (http://www.biol.ruu.nl/~palaeo) new possibilities arose, and an electronic version was prepared. Such an edition will make the glossary more accessible and give the users the opportunity to interact and make it still more up to date. So far, several new terms have been included and more examples have been added, especially of fossil forms. The drawings are in full-colour now, and it is (will be) possible to get full-screen pictures. New drawings have been added, and several old ones improved. Explanations of related terms and terms used in definitions are just one mouse-click away.

The electronic version will include: (1) a non-graphics version for quick access, with the possibility to get full-screen drawings; (2) a full-graphics version, which will look more or less the same as the printed version, with small full-color drawings and the possibility to get them full-screen. Both versions can be downloaded for installation on the PC.

The address: http://www.biol.ruu.nl/~palaeo/glossary. The printed edition is still available (free!): just send a request to the present author.

Airborne Pollen and its Relation to Hay Fever

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For the dissemination of pollen, plants make use of insects, water and air. Transport by air is probably the least effective route to get pollen grains from one flower to another. To compensate for this inefficiency, most wind-pollinating plants produce large quantities of pollen. However, atmospheric densities are usually not very high, maximally amounting to several hundreds per cubic meter of air, as an average over 24 hours. In comparison, fungal spores during summertime can be present in the outdoor air with several thousands per cubic meter as a 24 hour average.

In North-western Europe, approximately 50 different pollen types are regularly found in the outdoor air, a few of which occur quite abundantly, e.g. pollen from birch, alder, oak, pine, wild grass, and nettle. Locally, depending on the flora, other types can be very abundant too.

The vast majority of air-transported pollen is deposited in the vegetation, on the soil or water surfaces, and only a very small portion reaches the pistil of a compatible flower. Also, small numbers of pollen

grains are inhaled by animals, including man, or are collected in our pollen-sampling instruments. Most of the inhaled pollen grains are deposited on the mucus membrane of the nose.

It is not understood why the immune system of about 10% of people consider certain protein compounds in pollen to be hazardous substances ('allergens'), and react by producing specific antibodies against them. Once these antibodies are present in the respiratory organs, the next confrontation with the allergen leads to the typical symptoms of hay fever: sneezing, running nose, itchy throat, burning eyes, often accompanied by cough and wheezing breath. In Western Europe, pollen of wild grasses is by far the most important cause of hay fever, because it is highly allergenic. Almost every year, during the flowering season of the grasses, between the end of May and the end of July, there is a period with high airborne grass pollen concentrations (Spieksma 1990, *Rev. Palaeobot. Palynol.* **64**, 35–40). Hay fever due to allergy to birch pollen is seen in a limited number of people; from year to year, airborne birch pollen concentrations fluctuate widely (Spieksma *et al.* 1995, *Grana* **34**, 51–57).

Due to the unequal distribution of airborne pollen grains both in time and space, and to variabilities in the patients, it is not possible to determine a threshold above which hay fever symptoms are observed.

Recent studies show that atmospheric pollen allergen is carried through the air not only by intact pollen grains, but also by much smaller particles reaching the deeper part of the airways (Spieksma *et al.* 1995, *Clin. Exp. Allergy* **25**, 234–239).