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MEETING OF THE SECTION FOR PHYTOPATHOLOGY ON 19 JANUARY 1994

Involvement of Lypopolysaccharides in *Pseudomonas*-mediated Induced Resistance in Radish Against Fusarium Wilt.

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Induction of resistance was demonstrated to be involved in the suppression of fusarium wilt of radish by selected PGPR strains of *Pseudomonas fluorescens*, in a special bioassay. The pathogen, *Fusarium oxysporum* f.sp. *conglutinans*, and the PGPR strains were inoculated spatially separated on the plant root. *P. fluorescens* WCS374, applied in talcum onto the root tips, induced resistance in six radish cultivars differing in their susceptibility to *F. oxysporum* f.sp. *conglutinans*, applied in peat onto the root base.

Significant suppression of disease by bacterial treatments was generally observed when disease incidence ranged between approximately 40 and 80% in the control treatment, occasionally at higher and not at lower disease incidence. Both *P. fluorescens* WCS374 and WCS417, and their extracted cell wall material induced resistance, whereas *P. putida* WCS358 or its cell wall material did not, in the special bioassay. Spontaneous phage-resistant mutants of strains WCS374 and WCS417, lacking the O-antigenic side chain of the lipopolysaccharide, or cell wall material extracted from these mutants, did not reduce disease incidence in this experimental design.

It is concluded that O-antigenic side chains of the lipopolysaccharides of strains WCS374 and WCS417 are involved in the induction of systemic resistance in radish against fusarium wilt.

Biological Control of *Fusarium* Wilt in Carnation with Non-pathogenic *Fusarium* Isolates

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Non-pathogenic *Fusarium* isolates can effectively control fusarium wilt in carnation caused by *F. oxysporum* f.sp. *dianthi* race 2. In several glasshouse experiments with the susceptible cultivar Lena, the non-pathogenic *F. oxysporum* isolate 618-12 suppressed wilt symptoms with 80%. Disease suppression was maximal if antagonist and pathogen were both added to the soil in a 100:1 ratio. Isolate 61-812 was generally added 1 week prior to application of the pathogen, but was also effective if inoculated simultaneously with the pathogen. No disease suppression was obtained if isolate 618-12 was inoculated spatially separated from the pathogen, either by split root, or by stem versus soil inoculations. Thus, systemically induced resistance was not found to play a significant role.

After addition to soil, isolate 618-12 was often present in surface sterilized carnation stem pieces, even up to 60 cm height. The effect of isolate 618-12 within the plant is, therefore, intriguing. Stem inoculations with mixtures of 618-12 and the pathogen resulted in significant disease reductions due to the presence of 618-12. The spread of isolate 618-12 within the stem was limited after stem inoculations. Also, several other non-pathogenic *Fusarium* isolates reduced wilt symptoms after mixed stem inoculations with the pathogen. This phenomenon was observed in the susceptible cultivar Lena, as well as in two moderately resistant cultivars. Dead (gamma-irradiated) microconidia did not reduce symptoms.

The results of these experiments—in which the role of competition in soil and rhizosphere, and competition for infection sites were excluded—prove that the presence of isolate 618-12 or other non-pathogenic *Fusarium* isolates within the stem contributed to the biological control effect. Whether this effect is due to competition within the stem or to (locally) induced resistance is not yet clear.

Talaromyces flavus as a Potential Biocontrol Agent for Controlling *Verticillium dahliae* in Potatoes in The Netherlands

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The soil fungus *Talaromyces flavus* has demonstrated potential for control of Verticillium wilt of eggplant and potato in the USA and Greece. In contrast to many other biocontrol agents, the fungus is very resistant to dryness and other adverse conditions. In pelleting material, the spores survived dry storage at room temperature for more than 15 years. Therefore,

it was tested for its suitability for biocontrol of *Verticillium dahliae* in field potatoes.

Strains of *T. flavus* were isolated from soil samples and potato roots. A first screening of their antagonism towards *V. dahliae* was performed in a bioassay with eggplant as test plant. A number of strains effectively suppressed colonization of the stem by *V. dahliae*. When applied to seeds, *T. flavus* colonized the young roots, in particular the root tips; the roots and adhering soil became densely colonized. Toxic effects in the host plant were not observed.

T. flavus was evaluated as a biocontrol agent in a field experiment in 1992/1993 by amending soil with 43 kg ha^{-1} of alginate-wheatbran granules containing ascospores and at the same time treating the seed potatoes with ascospores in talc powder before planting. Both talc powder and granules contained ascospores of three different strains of *T. flavus*. *T. flavus* was recovered from roots of field-grown potato plants sampled at 40 and 136 days after planting (DAP). The biocontrol agent reduced colonization of the stem by *V. dahliae* ($P<0{\cdot}05$). Colonization of the roots by the pathogen was not significantly affected. At 125 DAP, fresh weight of tubers was higher in the treatment where both *T. flavus* and *V. dahliae* were applied than in the treatment with *V. dahliae* only ($P<0{\cdot}05$). At harvest (153 DAP), the difference between these treatments had disappeared.

Data of the field experiment demonstrated that *T. flavus* became established in the field after introduction of the formulated product. The antagonist reduced colonization of the stem by *V. dahliae*. Therefore, it is concluded that *T. flavus* has the potential to control *V. dahliae* in the field. Further research is aimed at selecting more effective strains of *T. flavus* and increasing its efficacy by optimizing the exposure of the microsclerotia of the pathogen to the antagonist.

Understanding and Modelling Rice Leaf Blast Effects on Crop Physiology and Yield

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Rice blast (causal organism: *Pyricularia grisea* Sacc.) is generally considered the principal fungal disease of rice (*Oryza sativa* L.), because of its wide distribution and its destructiveness under favourable conditions. During early development stages lesions are mainly formed on leaves, whereas after heading the pathogen infects the panicle or the neck node. Accordingly, the rice blast pathosystem is divided into two major subsystems: the leaf and the panicle blast pathosystem. Panicle blast causes direct yield losses, since filling of the grains on infected panicles is poor at best. Leaf blast only affects grain growth indirectly, through a long-term effect of the disease on crop production. Estimation of yield loss due to leaf blast has proven difficult. It was the aim of the present study to quantitatively explain the effect of leaf blast on growth and production of a rice crop based on insight in the physiological processes underlying damage.

CO_2-exchange measurements on individual leaves (process level research) were used to determine the effect of leaf blast on photosynthesis and respiration. Leaf blast reduced leaf photosynthetic rate not only through a reduction in green leaf area, but also through a reduction in the photosynthetic activity of green leaf tissue surrounding the lesions. The disease increased the respiration rate of infected leaf tissue. The size of the disease effects on photosynthesis and respiration was related to disease severity, and these relations were introduced in a mechanistic crop growth model.

Experimental research at the crop level (systems level research) was used to validate and improve the extended crop growth model. Observed reductions in canopy photosynthesis could be adequately explained by the adverse effect of leaf blast on photosynthesis and respiration, and by the presence of dead leaf tissue. The effect of the disease on crop production and yield could not be sufficiently explained without accounting for assimilate uptake by the pathogen for spore production. For this reason the crop growth model was extended with a submodel that simulates carbohydrate uptake by the pathogen for spore production.

The interaction between experimental research and model development resulted in a better understanding of yield reduction in rice due to leaf blast and in a model that can be used to estimate yield reduction due to leaf blast for various epidemics under variable environmental conditions.

Potential applications of the model are the construction of damage relations, the identification of critical periods and guidance of plant breeders for selection of promising plant characteristics.

Effect of Environmental Sex Determination of Potato Cyst Nematodes on Durability of Host Resistance

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Sex differentiation of potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) is environmentally determined. If feeding conditions are good, the probability for a juvenile of development into a female is high, but if feeding conditions are poor, juveniles more frequently develop into males. Host–parasite compatibility strongly influences the

nutritional potential of nematode's syncytium, and therewith sex determination. In cases of poor compatibility juveniles more frequently take the male development path than in cases of full compatibility. As a result, avirulence to its resistant host may be more advantageous for male production than virulence. For female production, in contrast, virulence is more favourable. Consequently, a resistant host exerts a selection pressure to avirulence on males and a selection pressure to virulence on females. These opposite selection pressures may lead to an equilibrium frequency of avirulent nematodes if the resistant host is grown continuously. This implies prevention of complete selection to virulence and therewith prevention of an entire breakdown of resistance. In this way, parasite populations with environmental sex determination might maintain their genetic diversity at the expense of their reproduction rate.

In case of monogenic, complete resistance, the equilibrium frequency of virulent nematodes equals

$$\frac{1}{2(1-\alpha)},$$

where α represents the ratio between the probability of virulent eggs for development into males and the probability of avirulent eggs for development into males.

The Potential of Conductance Methods for Application in Phytopathology

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The growth of micro-organisms in culture media often results in a change of electrical properties of media as a result of the overall production or consumption of charged molecules.

Changes in conductance and capacitance can be used for rapid estimations of microbial contamination of samples. Detection time, i.e. the time that is needed for the first significant conductance change, in conductimetric assays is linearly related to the amount of inoculum. Detection of micro-organisms can be achieved by measuring conductance changes directly in the growth medium (direct conductimetry) or by indirect conductimetry, which monitors changes due to the evolution of CO_2 produced by the metabolism of substrates in the culture medium.

Direct conductimetric assays have already shown their potential for rapid screening of food products for food-borne bacteria, like *Pseudomonas, Salmonella, Campylobacter, Yersinia* and *Listeria*. The metabolism of yeasts and fungi causes generally only small conductance changes in media. Therefore, indirect conductimetry and particularly capacitance monitoring have been preferred for detection and enumeration of most yeasts and fungi. Other applications of conductance measurements are sterility testing, phage detection, antibiotic sensitivity testing and analysis and modelling of growth kinetics.

Conductance measurements may also be used for rapid measurements of microbial contamination of seeds, testing of antimicrobial agents and analysis of microbial growth in plant extracts, e.g. for resistance research.

Several suitable basic conductance media for *Pseudomonas* and *Erwinia* have already been developed. Research is focused now on improvement of the selectivity of the media. It is felt that conductimetric assays in combination with specific confirmation tests, like ELISA or PCR, may become highly suited for large-scale routine indexing of seed lots, provided that a good correlation between detection times and contamination level of seed lots has been established.

Sexual Reproduction of *Phytophthora infestans* in Europe

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Phytophthora infestans, the causal agent of potato late blight is heterothallic with two mating types, A1 and A2. Before 1980 only A1 mating type isolates were found in Europe. However, after 1980 A2 mating type isolates were detected throughout Europe. The presence of both mating type isolates can lead to formation of oospores and sexual reproduction. Oospores are hardy structures which have the potential to survive in soil and act as a source of inoculum. We performed DNA fingerprint analyses and determined virulence factors to assess the level of genetic diversity in Dutch *P. infestans* populations collected before and after 1980. Among *P. infestans* isolates collected in The Netherlands between 1966 and 1978 only one RG-57 DNA fingerprint genotype was found. This RG-57 genotype showed limited diversity for virulence among 148 isolates. Only eight races were found.

Among 253 isolates collected between 1981 and 1991 73 different races were observed. DNA fingerprint analyses revealed 134 different RG-57 genotypes among 179 isolates collected after 1980. Less than 10% of the 134 genotypes were found in more than 1 year, demonstrating dramatic changes in the fungal population from year to year. It is concluded that the exclusively asexually reproducing *P. infestans* population present before 1980, has been replaced by a new *P. infestans* population consisting of A1 and A2 mating type isolates with mixed sexual/asexual reproduction after 1980. To demonstrate that oospores of *P. infestans* can survive in soil they were exposed to natural weather conditions in

unsterilized soil for 8 months during the winter of 1992–93. It appeared that these oospores were still able to infect potato leaves and cause disease in conditioned bioassays and field experiments. DNA fingerprint analyses demonstrated unambiguously that the late blight lesions were caused by hybrid, sexual progeny of the two parental isolates used to produce the oospores.

Effect of Soil Sterilization and Iris Bulbs on the Population and Disease Dynamics of *Rhizoctonia solani* AG-2-t and AG-4 in a Sandy Soil

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In an organic sandy soil, natural disease suppression of *Rhizoctonia solani* AG-2-t (tulip) isolate Rs2tT8 at 12°C and *R. solani* AG-4 isolate Rs0422 at 18°C was investigated under standardized soil humidity and temperature. Disease response curves from the natural and the gamma-irradiation sterilized soil samples were compared in the absence and presence of iris bulbs.

In the natural soil, spread, population density and disease severity of both pathogens were about 50% lower than in the sterilized soil. The presence of iris bulbs significantly helped both pathogens to partly overcome this antagonism of the soil biomass. Spread and population density of Rs0422 seemed to be more food-base dependent than those of Rs2tT8. Removing the colonized oat kernels from the soil a few days after inoculation almost stopped the spread of Rs0422, but not that of Rs2tT8. When the inoculum was kept in the unplanted sterilized soil, population densities of Rs0422 decreased between weeks 3 and 5 after point inoculation. Recovered isolates of Rs2tT8 from bulb-grown soil were more aggressive than isolates from soil without bulbs. Immediate replanting in separate soil samples showed for both pathogens some correlation between the residual soil inoculum potential at harvest, the population density at that site and the disease response in the harvested bulb.

Tomato Spotted Wilt and *Impatiens* Necrotic Spot Viruses in Flower Bulb Crops

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Well-known symptoms of tomato spotted wilt virus (TSWV) in *Dahlia* consist of concentric ring patterns and yellowish, dark-green and/or necrotic spots. However, TSWV also occurred in many dahlia cultivars or individual plants without symptoms or with symptoms indiscernible from those caused by dahlia mosaic virus. The localized infection in various plant parts caused problems in the development of a reliable test procedure for TSWV. A sample consisting of three parts of a tuber gave the best result. The test was more reliable when tubers were stored for a longer period at 9°C after lifting.

Stocks of various *Iris* and *Gladiolus* cultivars became infected with TSWV and/or *Impatiens* necrotic spot virus (INSV) when exported to Mediterranean countries. The symptoms consisted of a yellowish discoloration of the inner leaves, sometimes with necrotic spots or stripes, and were difficult to distinguish in gladiolus from those caused by mycoplasma (MLO). The limited amounts of iris bulbs and gladiolus corms that could be harvested from infected plants were replanted after normal storage. Plants grown from these bulbs or corms did not show symptoms; moreover no virus was detected in the various plant parts.

In The Netherlands, INSV and TSWV were detected incidentally in irises grown for flower production in or in the direct neighbourhood of glasshouses with thrips infestation (*Frankliniella occidentalis*). So far, these viruses have not been in field-grown stocks of iris and gladiolus for bulb or corm production.

In *Hippeastrum, Amaryllis* and *Nerine*, TSWV caused irregular-shaped yellow, white and dark-green spots and sometimes necrotic ones. When bulbs of infected plants were grown for several years, the percentage of infected plants reduced considerably; this reduction was caused by curing of some of the main bulbs and by limited or no transmission of TSWV to the offspring.

Host-plant Specificity and the Molecular Basis of the *Rhizoctonia solani* Infection Process

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Rhizoctonia solani Kühn, the imperfect state of *Thanatephorus cucumeris* Frank (Donk), is distributed world-wide and is an important pathogen in agriculture. *R. solani* has a wide host range, but host-plant specificity has been reported for individual isolates.

We wish to isolate infection-specific *R. solani* genes that are involved in host-plant specificity. Therefore, we studied the pathogenicity of 32 *R. solani* isolates to five different host-plant species, in a developed *in vitro* pathogenicity assay. Several *R. solani* isolates showed host-plant specificity. This allowed us to select two *R. solani* isolates that showed a reciprocal host-plant specificity on two different plant species for further studies. The infection process of these *R. solani* isolates was studied in detail and a strong correlation between the symptom development on the plant and the formation of characteristic *R. solani*

infection cushions could be observed. This allows us to use infection cushion formation for the isolation of host-plant-induced infection-specific genes.

In order to facilitate the isolation of fungal mRNA populations, the induction of *R. solani* infection cushions on artificial membranes was attempted. We were able to induce the formation of host-plant-specific infection cushions on collodion membranes.

Analysis of Gene Activity in Nematode-induced Feeding Structures in *Arabidopsis* and Prospects for Engineering Nematode Resistance

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The formation of nematode feeding structures in plant roots after infection with cyst or root-knot nematodes is accompanied by severe changes in gene regulation. To monitor these changes at the molecular level, a variety of promoter-*gusA* fusions were introduced into *Arabidopsis* and analysed for GUS activity after infection with either *Heterodera schachtii* or *Meloidogyne incognita*. Strikingly, promoters which are highly active in root cells, such as those of the CaMV35S, *rolA-D* and *nos* genes, are down-regulated inside the feeding cells induced by both root-knot and cyst nematodes.

Furthermore, a large number of transgenic *Arabidopsis* plants were generated using *Agrobacterium tumefaciens* harbouring a binary vector with a promoterless GUS gene located at the right border sequence. Using this approach we were able to tag regulatory sequences that give rise to high GUS activity inside the nematode feeding structures. After isolation with inverted PCR, these tagged sequences are now subject to a more detailed analysis and are being reintroduced into *Arabidopsis*.

Both the down-regulated and induced promoters are used to engineer nematode resistance in a two-component system. The strategy is being tested in *Arabidopsis* before transfer to commercial crops.

In Search of a Receptor for the AVR9 Elicitor of the Tomato Pathogen *Cladosporium Fulvum*

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The avirulence *avr9* gene of the fungal tomato pathogen *Cladosporium fulvum* has been cloned and characterized (van Kan *et al.* 1991, *MPMI* **4**: 52–59; van den Ackerveken *et al.* 1992, *Plant J.* **2**:359–366). The avirulence gene encodes a preprotein of 63 amino acids, which is processed by fungal and plant proteases to the mature race-specific AVR9 elicitor peptide of 28 amino acids (van den Ackerveken *et al.* 1993, *Plant Physiol.* **103**: 91–96). The AVR9 elicitor is the only factor which induces a hypersensitive response in tomato cultivars carrying the complementary resistance gene *Cf9* and is fully responsible for the induction of resistance. Disruption of the *avr9* gene in an avirulent race of *Cladosporium fulvum* enables this race to become virulent (Marmeisse *et al.* 1993, *MPMI* **6**: 412–417).

It is presumed that recognition of the AVR9 elicitor is mediated through a receptor at the plasma membrane. In nature many types of receptors exist which are the starting point of a signal transduction pathway leading to different responses. It is postulated that the receptor for the AVR9 elicitor is the product of the *Cf9* resistance gene. Data on the receptor are crucial for a better understanding of the mechanism of plant defence. The aim of our research is the isolation of the receptor for AVR9 and the cloning of its corresponding gene.

To reach this goal binding studies with radiolabelled AVR9 peptide comprising full elicitor activity have been performed. Initial results of binding experiments indicate that only very pure plasma membranes can be used for detecting specific binding. Results on the isolation and purification of plant plasma membranes will be presented. Initial results of receptor-binding experiments will be discussed.

MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE, WAGENINGEN, ON 18 MARCH 1994

Thiophenes Formed in *Tagetes* Hairy Root Cultures After Elicitation with a Cell Wall Extract of *Fusarium oxysporum*

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Thiophene derivatives are widely distributed in the Asteraceae (Compositae). They have been described as phytoalexins and thus play a role as natural protective agents. Several naturally occurring thiophenes show antibiotic, antifungal, and nematicidal activities.

The thiophenes that accumulate in roots of *Tagetes* species are structurally related. Transformed roots cultured *in vitro* accumulate the same thiophenes. These *in vitro* cultured roots were used as a model to investigate plant–microbial interactions.

A cell wall extract of the fungus *Fusarium oxysporum* was added to a growing root culture, while a control culture was treated with demineralized water. After 2 days of incubation the thiophene contents of the cultures were compared.

The overall thiophene content of cell wall-treated roots was slightly higher than that of the controls. More salient, the thiophene spectrum of the cell wall-treated roots differed from that of the controls. Whereas the control mainly accumulated the hydrophobic thiophenes 5-(3-buten-1-ynyl)-2,2′-bithienyl (BBT) and 5-(4-acetoxy-1-butynyl)-2,2′-bithienyl (BBTOAc), the cell wall-treated culture accumulated the more polar thiophenes 5-(4-hydroxy-1-butyl)-2,2′-bithienyl (BBTOH) and 5-(3,4-dihydroxy-1-butynyl)-2,2′-bithienyl ($BBT(OH)_2$). The polar thiophenes were partly excreted into the culture medium.

Specific Antibodies for Localization of Thiophenes in *Tagetes*

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In the study on the regulation of secondary metabolism it is of great importance to get information on the localization of secondary metabolites at the level of cells and tissues. Therefore a procedure has been developed to produce specific antibodies against thiophenes in *Tagetes*. Naturally occurring thiophenes in *Tagetes* species (marigolds) are characterized by one, two or three heterocyclic thiophene rings, and are known for their biocidal effects. In plants, thiophenes are abundant in roots and hypocotyl. For that reason transformed root cultures were made using *Agrobacterium rhizogenes* to study thiophenes in tissue culture.

Polyclonal antibodies were raised against α-T, a particular thiophene consisting of three rings and no side chain. For conjugation to a carrier protein a special spacer to α-T was constructed. An α-T-BSA conjugate was used for the immunization of rabbits. The serum was screened for specificity with an indirect competitive enzyme-linked immunosorbent assay (ELISA), using α-T-casein conjugate.

The most abundant thiophene in roots is 5-(3-buten-1-ynyl)-(2,2′) bithienyl (BBT). The antibodies react very specifically with BBT and other thiophenes. Thiophenes in extracts of transformed root cultures of *Tagetes patula* were also reactive. There was no cross-reactivity of pyrrol, monothiophene and root extracts of *Rubia*, tomato and bean, which is indicative of a high specificity of the antibodies.

Future studies will be focused on the identification of the cell types that contain thiophenes using immunohistochemistry. For localization unembedded hypocotyl tissue will be sectioned, immunolabelled with the anti-thiophene antibodies, and examined under a light microscope.

Increased Anthraquinone Production with the Surfactant Pluronic F-68 in a Two-phase System

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Plant cell suspensions have great potential as producers of economically important secondary metabolites. When spontaneous release of these compounds does not occur at all or only at a low level, cells need to be forced to excrete their products. This is the case for the anthraquinone pigments (AQs), which are secondary metabolites produced by suspension cultures of *Morinda citrifolia* (Rubiaceae).

The aim of the present work was to obtain improved secondary metabolite production and release, whilst retaining cell viability, by means of the surfactant Pluronic F-68. The selected model system consisted of suspension cultures of *Morinda citrifolia* root cells. When Pluronic F-68 (2% w/v) was added to the cell suspensions, the AQ release was significantly enhanced. Improvement of cell growth and intracellular accumulation of AQs occurred in the range 0·035–2% Pluronic F-68. By using a two-phase culture system with *n*-hexadecane as organic component, AQs were extracted from the growth medium into the organic phase. The overall yield in the presence of hexadecane and Pluronic F-68 (0·11%) was twice the control value. These findings encourage us to propose the use of Pluronic F-68 to increase the production and the release of intracellular secondary metabolites.

Callus Induction and Regeneration of Protoplasts of Leek (*Allium ampeloprasum* L.)

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In leek, F_1 hybrid breeding to improve uniformity is of great economic importance. In such a breeding programme the availability of cytoplasmic male sterility (CMS) is a prerequisite. Somatic hybridization for the introduction of CMS in leek may become an important tool, but this technique requires the use of protoplasts. Therefore, we started a research programme to develop a regeneration system for protoplasts of leek. For the initiation of embryogenic callus, we used both mature and immature embryos as explants. When mature embryos were cultured, a compact and embryogenic type of callus wasobtained, but this callus

was not suitable for the initiation of a well-dispersed cell suspension. Therefore, our research was aimed at the initiation of a friable callus type with a high regeneration capacity. This callus could be initiated on immature embryos. The developmental stage of the immature embryos played an important role. Embryos of 0·5–2·5 mm exhibited the highest frequency of friable callus. The friable callus was used for the establishment of embryogenic suspension cultures from which protoplasts were isolated. Protoplasts from these cultures were richly cytoplasmic and capable of sustained cell division. Different culture conditions were tested, and it appeared that plating density and gelling agent were the most important factors. A minimum plating density of 2×10^5 pps ml^{-1} was necessary to obtain microcalli. Culturing the protoplasts in Ca-alginate gave a five times higher plating efficiency, as compared to liquid or agarose-solidified medium. Upon transfer to regeneration medium, the microcalli developed into well-rooted plants at a high frequency. Summarizing, it can be said that the starting material for callus induction and the availability of an embryogenic suspension culture are two important factors in the development of a regeneration protocol for leek protoplasts. The regeneration protocol will offer good perspectives for research aimed at the transfer of CMS through protoplast fusion or for other objectives such as transformation.

Polysomaty and Tissue Culture

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Polysomaty, i.e. the presence in somatic organs or tissues of cells of different ploidy (C-value) levels, is caused by endoreduplication, and is a common phenomenon in higher plants. It is somehow related to plant development and cellular differentiation, but its function and regulation are still unknown. Polysomaty is an important source of somaclonal variation, resulting in polyploid regenerants. Therefore, knowledge of the phenomenon is essential to the successful application of molecular and cellular methods for plant breeding.

Polysomaty can be analysed in two ways: (i) via flow cytometrical (FCM) measurement of the DNA content of interphase nuclei isolated from plant material, and (ii) by microdensitometrical analysis of selected cells in intact plant tissues, e.g. using confocal laser scanning microscopy (CLSM). CLSM especially allows cell type, cellular location and ploidy level to be correlated.

In cucumber, the development of polysomaty in various organs of seedlings and plants was analysed by FCM (Gilissen *et al.* (1993): *Plant Sci.* **91**, 171–179). The complex pattern of polysomaty (ranging from 2C to 32C) in thin-cell-layer hypocotyl explants showed a rapid increase in the frequency of cells with 32C DNA during the first days of culture. The majority of the indirectly-formed regenerants were (highly) polyploid.

The pattern of polysomaty in thin-cell-layer internodal explants of tobacco showed a gradual increase in the frequency of cells with 4C and 8C DNA in successive internodes from the top to the base of the plant. CLSM analysis revealed that the largest cortical cells had the highest C values. During culture of the explants, most 4C cortical cells underwent cell division immediately, whereas the others doubled their DNA content to 8C. The regeneration frequency from the explants depended on the developmental stage of the internode at the start of culture. The regenerants, which developed from subepidermal cells directly, were generally diploid. Mixoploid and tetraploid regenerants occurred at low frequencies only.

Tissue Culture-induced Changes in DNA Methylation

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Epigenetic variation is the name for those changes that can be found in tissue culture-propagated plants that are not, or only partly, inherited into the progeny. One of the mechanisms that may cause this phenomenon is a change in DNA methylation during the tissue culture propagation. In general, the pattern of methylation is dynamic during the life-cycle of a plant. Therefore, certain changes in this pattern, induced during tissue culture, could be somatically stable but erased in the germ-line cells.

We studied the methylation status of a number of repetitive sequences in tomato, both in callus during tissue culture, and in leaf material of plants regenerated from tissue culture. When the DNA was restricted with the isoschizomers *Msp*I or *Hpa*II (which recognize the same restriction site but differ in their sensitivity to cytosine methylation), variable methylation was observed with three repetitive probes, but there was no difference between callus and leaf tissue.

When the DNA was restricted with *Hin*dIII, large differences in restriction patterns were visible between callus and leaf samples. This was seen for six different repetitive probes, and in all cases the callus pattern contained bands of higher molecular weight compared with the leaf pattern. The differential pattern was present in all callus samples tested, and was not due to protein contaminating of the DNA. The patterns obtained with other restriction enzymes were identical for both tissues. Therefore, it is presumably a difference in DNA methylation that is specifically measured here by *Hin*dIII.

One of the repetitive probes, H9D9, also showed differences among the regenerant plants: one band that was present in all calluses but was not present in leaf material from seedling plants, was found in leaf material from some direct regenerants. The extra band was found in the offspring of only some of these plants.

Vitrification of Shoot Regenerants is Related to the Explant Position in the Donor Plant

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Vitrification is an incalculable problem occurring during *in vitro* culture in many plant species. Research on vitrification is mainly directed towards abatement and prevention of the problem. Fundamental knowledge of the phenomenon is scarce.

In tobacco, vitrified regenerants frequently appeared on thin-cell-layer (TCL) internodal explants. Vitrification is expressed in the leaves, which developed as thick, pale-green and lanceolate structures, while normal regenerants have thin, dark-green and oval to round leaves. The frequency of occurrence of vitrified regenerants on the TCL explants from the second to the fifth upper internodes from young plants (with a mean height of 11 cm) and in older plants (with a mean height of 23 cm) was measured and related to the internodal length. Nearly all (95–100%) regenerants on the explants from the young plants were vitrified. However, in the older plants, hardly any vitrified regenerants occurred on explants from the fourth and the fifth internodes (with lengths of 15 mm or more), while highly variable frequencies (ranging from 0 to 85%) of regenerant vitrification were found on explants excised from the second and third internodes (with lengths below 15 mm).

The results indicate that the occurrence of vitrified regenerants on the explant is determined by its position in, and the developmental stage of, the donor plant. Because of the high degree of predictability of occurrence of vitrification, the tobacco TCL regeneration system will be useful in the investigation of cellular parameters related to vitrification, especially those related to the (intra- and inter-)cellular organization in the primary meristem and the formation of the leaf primordia of the regenerants.

Regeneration and Particle Gun Bombardment of Thin Cell Layer Explants from Tobacco

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Genetic modification is an important technique for the improvement of crops. However, severe recalcitrance with respect to genetic transformation has been observed in many economically important crops. For successful transformation, cells that are competent for both regeneration and transformation have to be present. In addition, the transformation method used should be efficient in such a way that many competent cells are reached by the vector. In this research thin cell layer (TCL) explants from vegetative tobacco plants have been used to determine the location of cells that are competent for both regeneration and transformation.

By varying the concentrations of benzyladenine (BA) and naphthaleneacetic acid (NAA) in the culture medium, the morphogenic response of TCL explants could be directed towards the development of shoot, root or callus. Histological examination showed that the origin of lateral buds is multicellular: one subepidermal and at least one epidermal cell are involved. The origin of apical primordia is unicellular, developing from one sub-epidermal or one cortex cell.

TCL explants cultured for 2 days on shoot induction medium were bombarded with DNA by particle gun, using GUS as a marker for transformation. After optimizing the particle gun conditions (helium pressure, target distance) and the type of DNA construct to be used, up to 100 GUS positive spots per TCL were obtained. The majority of the transformed cells were located in the epidermis and the subepidermis, i.e. the organogenic region of the explant.

Further research will focus on the effect of hormones on the competence of cells for transformation, using particle gun or *Agrobacterium*-mediated DNA transfer.

Field Performance of *in vitro* Produced Bulbs of Tulip

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Shoots were generated from stem explants and axillary buds on media with α-naphthaleneacetic acid (NAA), N-[2-isopentenyl]adenine (2iP) and 6-benzylaminopurine (BAP). Propagation of these shoots was possible by cutting them longitudinally. Single shoots formed bulbs after a cold treatment on a medium with 70 $g\,l^{-1}$ sucrose without plant growth regulators.

These bulbs were planted in soil in 7 × 7 cm pots. A cold treatment of 7 weeks at 5°C was given. To

induce sprouting, the bulbs were cultured in a growth room at 17°C (day and night) with a photoperiod of 16 hours. The emergence of the leaf and the formation of a new bulb were determined.

The initial fresh weight of the bulbs planted influenced the percentage of sprouting, the percentage of the formation of a new bulb, and the time needed for emergence of the leaf. Bulbs with a weight over 200 mg emerged for 60–100%. New bulbs were harvested from almost every sprouted bulb. Little difference was found between two different cultivars.

Cytokinin in Relation to Axillary and Adventitious Shoot Formation in Tomato

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The *lateral suppressor* (*ls*) mutant of tomato is characterized by the absence of axillary meristem formation and reduced cytokinin levels. We studied the role of cytokinin in axillary meristem development after elevating the endogenous cytokinin levels through introduction of the *isopentenyltransferase* (*ipt*) gene from *Agrobacterium tumefaciens* in mutant and wild type plants.

Transformants varied in phenotype. A number of the *ipt* transformants had the wild type or mutant phenotype. Others showed a mild to severe 'cytokinin-overproduction' phenotype. Transformants with a mild phenotype exhibited reduced internode length and reduced root development. Transformants with a severe phenotype showed even shorter internodes, loss of apical dominance, reduction of leaf size, production of callus at the basis of the shoots and absence of root development or development of green non-branching roots. The severity of the phenotype correlated well with the levels of *ipt* gene expression, as measured by Northern analysis.

Transformants with a severe cytokinin-overproduction phenotype also exhibited increased level of zeatin riboside compared to wild type plants, but zeatin levels were not elevated. The increase in endogenous zeatin riboside levels in the transformed *ls* mutant did not restore axillary meristem formation, but sometimes bulbous structures were formed in the initially 'empty' leaf axils. Several adventitious meristems and shoots developed from below the surface of these structures.

Some of the transformants could be successfully transferred to the glasshouse. Their offspring showed variation in *ipt* gene expression correlating with variation in internode lengths and root development. Again, axillary meristem formation was not restored by *ipt* expression. Instead, adventitious shoot formation on the main veins of mutant leaves was observed.

It is concluded that the reduced level of cytokinins in the *ls* mutant shoots is not responsible for the absence of axillary meristem formation.

Development of Dormancy in Bulblets of Various *Lilium* Genotypes Cultured *in vitro*

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Control of dormancy development during tissue culture of bulbous crops is highly desirable. During propagation no dormancy should develop since this may inhibit proliferation. In the final stage, when bulblets are formed, some dormancy is desirable to prevent precocious sprouting *in vitro*. However, to prevent a long dormancy-breaking treatment, dormancy should not be very deep. We have studied dormancy development in four genotypes of lily, *viz. L. speciosum 'Rubrum* No. 10' (R10), 'Stargazer' (SG, an oriental hybrid), 'Connecticut King' (CK, an asiatic hybrid) and 'Snow Queen' (SQ, a *L. longiflorum*).

Of the environmental factors tested, temperature had a major effect. R10-, SG- and CK-bulblets regenerated at high temperature (20 or 25°C) developed dormancy, whereas at low temperature (15°C) hardly any dormancy was established. In SQ, on the other hand, hardly any dormancy developed at 30°C, an intermediate dormancy level developed at 15°C and deep dormancy at 20 and 25°C. Depending on genotype and temperature, dormancy started to accumulate early (2–3 weeks after start of the culture) or later (after 5–8 weeks). Fluridone, an inhibitor of ABA-synthesis, inhibited dormancy development in all four genotypes. Addition of ABA had no effect in 15°C- and 20°C-bulblets of R10, in 15°C-bulblets of SG and in 30°C-bulblets of SQ. It induced little dormancy in SG at 20°C and had the strongest effect in 15°C- or 20°C-bulblets of SQ.

Dormancy was broken by a cold treatment of 8 weeks at 2°C for SG, 6 weeks at 2°C for R10, and 2–4 weeks at 2°C for CK and SQ. Dormancy in SQ and R10 was also broken by soaking in GA_{4+7} for 24-h. A hot-water treatment (1 h at 45°C) broke dormancy in SQ but not in SG. The similarity between genotypes with regard to the effects of the ABA-inhibitor fluridone and cold treatment suggests that the mechanisms underlying dormancy in these genotypes are similar.

MEETING OF THE SECTION FOR PLANT SYSTEMATICS AND GEOGRAPHY ON 27 MAY 1994

The Significance of Molecular Research for Reconstructing the Phylogeny of Yeasts

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Molecular systematic studies of fungi can be applied at different taxonomic levels. Useful molecular approaches at the species level are, e.g. analysis of molar percentages of guanine and cytosine, DNA hybridizations, restriction analysis, ribotyping, nucleotide sequencing of moderately variable DNA domains and karyotyping. Techniques successfully applied at or below the species level are DNA fingerprinting (RAPD), electrophoretic karyotyping and sequencing of variable DNA domains (ITS). DNA sequences with little variability, e.g. part of the 18S or 26S rRNA genes, can be used for analysing phylogenetic relationships at higher taxonomic levels.

Species of the medically important yeast genus *Malassezia* differ in their electrophoretic karyotypes. Strains of *M. pachydermatis* all showed the same karyotype, whereas four different karyotypes could be distinguished among strains of *M. furfur*. RAPD analysis of *Malassezia* strains revealed a larger genotypic variation.

Sequence data of the 18S ribosomal RNA genes suggested that the ascomycetous yeasts are a sister group of the filamentous ascomycetes. In contrast, the basidiomycetous yeasts were found to be dispersed throughout the filamentous Heterobasidiomycetes (Van De Peer *et al.* 1992, *Syst. Appl. Microbiol.* **15**: 250–258; Wilmotte *et al.* 1993, *Syst. Appl. Microbiol.* **16**: 436–444).

Based on a parsimony analysis of about 600 nucleotides near the 5′ end of the 26S rDNA, the yeast genus *Eeniella* with bipolar budding was found to form a single clade with species of the genus *Brettanomyces* with multipolar budding. Species of the genus *Hanseniaspora* with bipolar budding were found to form a different clade. Interestingly, *Eeniella* and *Brettanomyces* share a number of physiological and biochemical characteristics as well, namely formation of acetic acid, presence of a Custers effect, and a coenzyme Q comprising nine isoprenoids, thus supporting the molecular findings. Moreover, this molecular phylogeny is supported by the size and gene order of the mtDNA, and nucleotide sequences of the mitochondrially encoded cytochrome oxydase subunit 2 gene (Clark-Walker *et al.* 1987, *Stud. Mycol.* **30**: 259–266; Hoeben *et al.* 1993, *J. Mol. Evol.* **36**: 263–269). Basidiomycetous yeasts are polyphyletic and belong to at least two different orders of Heterobasidiomycetes. Molecular data of these yeasts correlate rather well with taxonomic systems proposed earlier, based on morphological, physiological, biochemical and ultrastructural data.

Exploring the Depths: Molecular Systematics in the Algae

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Background. A total of 21 000 macrophytic and 27 000 microphytic algal species have been described up till now. Systematics Agenda 2000 predicts that a least 200 000 more still have to be discovered. Unfortunately, this Agenda almost completely leaves the oceans out of its effort to chart the biosphere. An initiative called 'Biological diversity in marine systems', very recently developed the first prioritized research agenda focusing on marine biological diversity. Combined efforts (consortia) in algal systematics are necessary in order to collect and combine molecular and morphological data.

Example. Phylogenetic relationships were inferred from parsimony analyses of nuclear small subunit rDNA sequences from 14 species representing 8/11 genera in the Dasycladales. Of 1733 positions, 412 (23·8%) were variable and 251 (61%) of those were potentially informative. Strongly supported branches were robust to all methods of analysis regardless of weighing. Simple and highly convergent morphology have confounded efforts to assess phylogenetic relationships among dasyclad genera based on morphological characters. Taxa tend to be defined by what they do not have or by autapomorphies. Only 10 potentially informative characters could be found and parsimony analysis of these data alone was only able to resolve the two major families. A consensus approach nests the less-resolved morphological tree into the well-resolved molecular tree. Using a total evidence approach in which the data were analysed from a single matrix using MRP methods, the resolution was not improved but was more strongly supported. We support the use of the total evidence approach but question its utility in applications where morphological characters are both scarce and weak. Many marine organisms fall into this category.

Molecular Markers for Dispersal, Introgression and Speciation

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Molecular phylogenetic studies at the level of species and genera have revealed unexpected modes of gene flow and genetic exchange among species. This is illustrated with the three diploid annual species of *Microseris* in western North America. Each species is divided into many isolated local populations that consist of one or more inbred 'biotypes'. Outcrossing must be very rare in these species and must result in a local burst of recombination followed by long periods of inbreeding. Nearly identical multilocus genotypes are sometimes found in populations hundreds of kilometres apart separated by genetically different populations. This reflects long-distance fruit dispersal and successful colonization within and outside the established range of the species. Rare as these events are, in balance they suffice to maintain an intraspecific gene distribution approximating that of a panmictic population. Occasional interspecific hybridization is documented not only by allopolyploid derivatives, there are now strong indications of chloroplast introgression from *M. bigelovii* into *M. douglassii*. Such introgression must be the result of hybridization followed by the loss of the maternal genome from the hybrid. The typical flower colour of *M. douglasii* is white, that of *M. bigelovii* frequently is orange. The difference is determined by several nuclear genes, two of which have been mapped relative to nuclear DNA markers. In two cases, the flower colour of plants of *M. douglasii* with chloroplasts presumably from *M. bigelovii* is darker yellow than that of plants in the same populations with typical *M. douglasii* chloroplasts. This may permit the demonstration of remnants of the nuclear genome of the *M. bigelovii* parent.

Phylogenies Inferred from Morphological, Nuclear DNA, Chloroplast DNA and Crossability Data in *Allium* Section *Cepa* are not Congruent due to Different Effects of Introgression

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The genus *Allium* consists of about 600 species, arranged in several subgenera and sections. Onion (*A. cepa*) belongs to section *Cepa*. The six species of section *Cepa*, together with *A. roylei* of section *Rhizirideum*, have been subjected to phylogenetic analysis. *A. roylei* is of particular interest for breeding purposes for its crossability with onion. Four different datasets have been used, i.e. a set of morphological, seed epidermis, chromosome and biochemical data ('supranuclear', Raamsdonk, L. W. D. van and Vries, T. de 1992, *Bot. J. Linn. Soc.* **109**: 131–143), a set with crossability relationships (Raamsdonk, L. W. D. van *et al.*, 1992, *Bot. J. Linn. Soc.* **109**: 293–303), a set of nuclear DNA data (RAPDs; unpublished), and a set of chloroplast DNA data (Havey, M. 1992, *Pl. Syst. Evol.* **183**: 17–31). *A. cepa* and *A. vavilovii*, and *A. fistulosum* and *A. altaicum* appeared to be linked pairwise very closely in all datasets. The supranuclear and nDNA trees are congruent. The position of *A. oschaninii* is somewhat different in the supranuclear and nDNA tree, but in both trees this species is closer to *A. cepa*/*A. vavilovii* than *A. roylei* is to *A. cepa*/*A. vavilovii*. The topology of the trees resulting from the crossability data and from the cpDNA set is completely identical. In both trees *A. roylei* is closer to *A. cepa*/*A. vavilovii* than *A. oschaninii*.

The discrepancy between the supranuclear/nDNA trees and the crossability/cpDNA trees is predominantly due to the position of *A. roylei*. The incongruency can be clarified by the different effect of introgression on the structure of the different genomes. Due to recombination, the recipient population will contain only a small part of the donor nuclear genome after several backcross generations. However, providing the hybrid and backcross plants will act as female parent of the subsequent backcross populations, a considerable part of the recipient population may consist of the donor cpDNA type. The existing crossability relationship may clarify their short distance in the cpDNA tree, shorter than may be inferred from their phylogenetic position in the nDNA tree. As a consequence, a shorter distance between two species in a cpDNA tree compared to an nDNA tree may give a hint for a certain level of crossability, which is of importance for plant breeding studies.