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MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE, WAGENINGEN, ON 10 OCTOBER 1996

Diversity and Uniformity in Plant–microbe Interactions

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Over the past millions of years, plants and bacteria have developed a large number of interactions. From those, *symbiosis* is one of the most interesting. An overview is given of the possible origin of photosynthetic eukaryotic organisms through various endosymbiotic relationships of Archaeal cells with primitive spirochaetes, aerobic bacteria and cyanobacteria. Subsequent extention of the potentiality of eukaryotes has resulted in a wide variety of endo- and exo-symbiotic relations. From those, the nitrogen-fixing symbiosis is the best known relationship. *Nif* genes are widely distributed within the phylogenetic tree of Bacteria and Archaea. Only a few micro-organims have developed symbiotic relationships with a large variety of eukaryotes. Remarkable similarities occur in the processes of recognition of the partners and the regulation of the nitrogen metabolism of the symbiont. Most information is now available on the *Rhizobium*–legume symbiosis. Plants excrete flavonoids and the bacteria produce oligosaccharides (nod-factors). Both signal molecules are highly specific and work at the gene expression level of the partners. In the nitrogen-fixing stage, the growth of the microsymbiont is retarded, the ammonia-assimilating system is inhibited and ammonia is excreted in the cytoplasm of the host. The uniformity of symbiotic events may indicate that construction of new types of nitrogen-fixing symbioses through recombinant DNA techniques is not excluded. Rapid success with this approach, however, remains far from realistic.

Expression of Antibacterial Peptides in Plants

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Bacterial diseases may cause a drastic decrease in yield. Breeding for resistance against bacteria is therefore of the utmost necessity. In addition to traditional plant breeding, genetic engineering technology may provide ways for the production of disease-resistant plants. One approach using such technology is the introduction of genes coding for antibacterial peptides into plants. During the past 8 years, we have introduced genes coding for three such peptides into tomato, tobacco and potato. The peptides studied were the hordothionins originating from barley (*Hordeum vulgare*) endosperm, cecropin B from the giant silkmoth (*Hyalophora cecropia*) and tachyplesin I from the South-East Asian horseshoe crab (*Tachypleus tridentatus*).

Expression of hordothionins in transgenic tobacco, tomato and potato did not result in enhanced resistance against *Xanthomonas*, *Pseudomonas*, *Clavibacter* and *Erwinia* ssp. infections, although high levels of biologically active hordothionins were produced in the transgenic plants. This is due most probably to the intracellular localization of this peptide disabling a close contact with the invading bacteria. Also, expression of cecropin B genes in potato and tobacco did not result in enhanced resistance. In this case, rapid degradation of the peptide by plant proteases might underly these findings. Low expression levels of tachyplesin I in potato, however, resulted in slighty less rot of transgenic tubers compared to control tubers upon infection by *Erwinia*-soft-rot causing bacteria. These results have to be confirmed by field trials.

In conclusion, the expression of antibacterial peptides in transgenic plants has, until now, not led to resistant plants but might provide a novel way for the production of such plants if the subcellular localization, expression levels and stability in plant cells can be optimized.

Detection of Plant Pathogens in Tissue Culture

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In plant tissue culture, the presence of pathogens is undesirable. However, many assays are too complicated or too specific for application in the tissue-culture routine. The next examples show that immunological and DNA techniques for very specific detection may also be useful for

detection of complete classes of pathogens in tissue culture.

Bacteria of the genus *Xanthomonads* are difficult to detect and to eliminate from tissue culture. We studied specific detection of *Xanthomonas campestris pv. hyacinthii* in hyacinth-tissue. It was shown that these bacteria contain fimbriae with unique protein domains. Monoclonal antibodies (mAbs) were produced that allowed specific detection of these bacteria by enzyme-linked immunoabsorbent assay (ELISA) in tissue-extracts of hyacinth without cross-reacting with other *Xanthomonads*. In addition, a specific and more sensitive polymerase chain reaction (PCR)-assay was developed, based on specific sequences of the fimbriae-gene. However, some sequences of this gene appeared to be very conserved in *Xanthomonads*, and may be a basis for producing mAbs or primers for a genus-specific ELISA or PCR, respectively.

Viruses often have no visible symptoms in tissue culture. To obtain virus-free tissue culture material, the starting material has to be checked. In some cases, the virus level can be enhanced by a temperature treatment or wounding of the bulb allowing a more sensitive screening. For potyviruses, a general ELISA has been developed, based on (commercial available) mAbs made against a conservative part of the coat protein. Many potyviruses in different crops can now be detected with a single ELISA. We developed a general PCR based on conservative sequences of the coat protein genome of potyviruses from bulbous crops, allowing a general detection of members of the potyvirus group as well as specific identification of these members if PCR is combined with restriction fragment length polymorphism (RFLP). For routine use, immuno-PCR has been developed with a comparable feasibility as ELISA.

The third problem concerns endogenous fungi, notably if present in a latent form in the starting material. Immunological assays are, in general, not effective. However we found that *Fusarium* in gladioli could be detected specifically with PCR, based on specific primers obtained from polymorphisms in random amplified polymorphic DNA (RAPD)s-profiles from pathogenic and very closely related non-pathogenic *Fusarium* isolates. A possible more easy alternative is isozyme-fingerprinting of infected material. This method was developed by the Research Station for Floriculture and Glasshouse Vegetables in Aalsmeer, The Netherlands. They developed a specific assay for *Fusarium*, *Gnomonia* and *Cylindrocladium* in infected tissue of various crops. For *Phythophthora* and *Pythium* isozyme fingerprinting was not specific enough. For *Pythium* we developed a genus-specific assay, based on a PCR with general primers from conserved sequences of rDNA, in combination with RFLP-fingerprinting.

Meristem Culture to Free Horticultural Crops from Diseases

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Meristem culture has been applied for 44 years to obtain virus-free or, in general, disease-free starting material. This concerns *vegetatively* propagated crops: viruses are not usually transmitted through seed, but through vegetative propagation. Meristem culture may be combined with thermotherapy and chemotherapy. The successful application of meristem culture is very much dependent upon the size and location of the meristem, and on the availability of sensitive and reliable assays for the pathogens to be eliminated. It is also most important that the disease-free plantlets are true-to-type and that they do not show aberrations.

Quantitative data on damage by viruses and on increased production and quality through meristem culture are scarce, but observations show large differences. Virus-free plantlets can be stored *in vitro*, as is being done in the Laboratory of the NAKB with carnation, chrysanthemum and strawberry. If meristem culture is not successful, plantlets containing a certain virus species are not necessarily thrown away but may be used to serve as positive controls, in the enzyme-linked immunoabsorbent assays (ELISAs) in our laboratory.

Comparison of Different Types of Meristems of Iris in their Efficiency of Virus Eradication and Micropropagation

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Four different viruses occur in iris: iris mild mosaic virus, iris severe mosaic virus, narcissus latent virus and bean yellow mosaic virus. These viruses cause major losses in the production of iris. Virus-free irises have a much better quality and a higher bulb production. By selection, the level of virus infection in iris culture can be kept low with the exception of iris mild mosaic virus (IMMV). Almost the complete iris culture is infected with this virus. It is possible to obtain virus-free plants by meristem culture using adventitious meristems regenerated *in vitro* from bulb-scale explants. However, the percentage of virus-free plants is low and adventitious meristems are very vulnerable (only 50% of the meristems survive). Therefore, a more efficient method was developed using apical and axillary meristems excised from field-grown bulbs. These meristems showed a much higher efficiency than the commonly

used adventitious meristems: they had an excellent proliferation in tissue culture and a high micropropagation capacity, and they produced high percentages of large, virus-free bulbs. We also examined whether storage conditions of the bulbs before meristem culture had an effect on the percentage of virus-free bulbs. Storage for 3–4 months at 30°C resulted in a higher percentage of virus-free plants with only slightly reduced growth. Furthermore, the reliability of the enzyme-linked immunoabsorbent assay (ELISA)-test on IMMV during tissue culture was investigated. Plants negative in ELISA during the tissue culture remained negative even after 2 years of culture in the soil. Infected plantlets showed, during tissue culture, surprisingly high ELISA-values.

Reduction of Initial Contamination by a Hot-water Treatment Before Tissue Culture

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Hot-water treatments have been used to free seeds, bulbs, corms and tubers from diseases for over 100 years. We have examined the effect of a hot-water treatment (HWT) on the reduction of initial contamination in a bulbous (*Lilium*) and a woody (*Acer lobelli*) crop. In lily, HWT of 1 h at 43°C reduced initial contamination from 60% to 15%. In *Acer*, HWT of 2 h at 42·5°C appeared to be optimal, reducing initial contamination from 90% to 25%. HWT can be applied to organs that have a high resistance to adverse conditions, such as non-sprouted bulbs and buds.

MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE, AALSMEER, ON 8 NOVEMBER 1996

The Dutch Plant Tissue Culture Industry

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Since there were no accurate and specific statistics on the extent to which micropropagation is being used commercially, an analysis was made of the application of micropropagation in the Dutch plant industry. The Netherlands was chosen because this country dominates the world export market of ornamental plants. The results from research on micropropagation in The Netherlands, which started in the 1960s, were quickly commercialized, especially in horticulture.

A recent study in 1995 showed that The Netherlands had 67 commercial tissue culture laboratories. From these, 18 produced more than 500 000 plants per year. In 1990, the total number of laboratories was 78. In 1990 and 1995, the number of tissue-cultured plants produced in The Netherlands was 95 and 54 million, respectively. In 1995, 77 million tissue-cultured plants had been imported. The Dutch production (in millions) can be specified as follows (imported plants are in parentheses): pot plants 22·0 (23·4), cut flowers 11·6 (17·7), orchids 9·4 (1·2), bulbous and cormous crops 5·9 (33·7), woody plants 1·3 (0·1), agricultural crops 0.5 (0), vegetables 0·2 (0), small fruit 0·1 (0) and miscellaneous crops 1.5 (0·9). In 1995, the main crops produced *in vitro* were (in millions; imports in parentheses): *Nephrolepis* 13·0 (1.5), *Gerbera* 6·0 (15·2), *Lilium* 5·1 (33·2), *Phalaenopsis* 4·4 (1·0), *Spathiphyllum* 3·2 (0·6), *Alstroemeria* 2·0 (0), *Saintpaulia* 1·7 (0), *Cymbidium* 1·4 (0·2), *Limonium* 1·3 (0·02), and *Aster* 1·1 (0). In 1995, importation into The Netherlands was from (numbers of plants in millions) Poland 37·0, India 17·2, Belgium 7·3, South Africa 3·6, Israel 3·0, USA 2·6, Portugal 1·7, Far East 1·1, UK 1·1, China 1·0, Denmark 0.5, and miscellaneous countries 1·2.

Engineering of Flavonoid Biosynthesis in Plants

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The phenylpropanoid biosynthetic pathway is one of the best-studied examples of secondary metabolism. Since virtually all biosynthetic and regulatory genes have been cloned during recent years, this pathway is amenable to genetic modification. The first case of genetic modification of phenylpropanoid metabolism dates back to 1987 when Peter Meyer and his team at MPI, Cologne 'constructed' orange-blossomed transgenic petunia plants. The plants produced pelargonidin due to the expression of the maize dihydroflavonol 4-reductase (*dfr*) gene. More

recently, researchers at Calgene Pacific, Melbourne, isolated two of the three genes responsible for the deep blue appearance of delphinidin. The third gene is part of a gene family that controls the vacuolar pH, one member of which (ph6), was cloned at DNA Plant Technology. At the Free University, a programme has been initiated to clone other ph genes using transposable elements and to study the transport of anthocyanins into the vacuole. In the near future this may result in blue roses, carnations and chrysanthemums. The development of gene silencing methods has allowed the selective suppression of single genes in a metabolic pathway. The first successful down-regulation of a gene active in anthocyanin biosynthesis was reported in 1988. In this case an antisense version of the gene for chalcone synthase (*chs*) was introduced in petunia plants. Surprisingly, introduction of extrasense versions of *chs* or *dfr* also led to suppression of floral coloration. We have shown recently that flavonol synthesis can be manipulated by extra copies of the flavonol synthase (*fls*) gene.

MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE ON 21 MARCH 1997

Microspore-derived Embryos of Oilseed Rape (*Brassica napus L.*), A Model System to Study Regulation of Erucic Acid Levels in Seed Oil

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Erucic acid (22:1) is a very long chain fatty acid that is produced by elongation of oleic acid in oilseed rape and related species. The mechanism that controls the level of 22:1 is the subject of this study. It has been shown that 22:1 levels in oil from both seeds and microspore-derived embryos (MDEs) are higher at 15°C than at 25°C (Wilmer *et al.* 1996, *J. Plant Physiol.* **147:** 486–492). Also the level of 22:1 in MDEs increases upon addition of abscisic acid (ABA) to the medium. However, the effects of temperature and ABA on absolute amounts of oil in MDEs are different: temperature changes do not influence the amount of oil, whereas ABA induces a 1·5–2-fold increase in the amount of oil accumulated.

The total elongase activity, synthesising erucic acid from oleic acid, correlated closely with the absolute amount of 22:1 formed. No differences occurred in properties of the enzyme isolated from MDEs developing under different conditions. We also found that the total amount of oil does not appear to be regulated by the amount of acyltransferases incorporating fatty acids into oil. From these results a model was formulated: the level of erucic acid in the oil is regulated by the balance of activities of fatty acid synthase synthesizing mainly oleic acid, and elongase synthesizing erucic acid from oleic acid.

Somatic Embryogenesis: Applicable to a Propagation System for Tulip?

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The natural propagation rate of tulip is low. However, modern breeding requires a rapid *in vitro* propagation method. The best method available at the moment, adventitious shoot formation from stem explants, has a low propagation factor and is laborious and (hence) expensive. The aim of the present study is to investigate the possibilities of somatic embryogenesis for an *in vitro* propagation system of tulip.

Stem explants of *Tulipa gesneriana* L. cv. Apeldoorn where incubated on Murashige and Skoog medium with 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine. After about 4 weeks, meristematic nodules developed at the apical wound surface. Upon removal of 2,4-D, these meristematic nodules developed into embryo-like structures. Morphological characteristics of these embryo-like structures were a leaf-like cotyledon and a notch at its base concealing a cavity with a meristem at the basal side. These morphological characteristics correspond with the characteristics of a tulip zygotic embryo, indicating the embryo-like structures are somatic embryos. From these somatic embryos small bulblets were obtained when cold-treated and incubated on 7% sucrose at 25°C.

Meristematic nodules, grown on stem explants, were cultured in liquid medium. In a few cases it was possible to propagate the meristematic nodules in liquid culture, and somatic embryos developed from these nodules. Experiments aiming to identify factors essential for propagation of meristematic nodules in liquid medium and tulip somatic embryogenesis are in progress.

Influence of Different Oxygen Concentrations on *In vitro* Growth of Tulip, Lily and Apple Explants in Tissue Culture

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Since oxidative stress might reduce regeneration, the effect of O_2 was investigated in various systems, namely regeneration of shoots from tulip bulb scale and stalk explants, of bulblets from lily scale explants and of roots from apple stem slices. During regeneration, 2% O_2, 100% O_2, or ambient air (20% O_2) was applied. Incubation under continuous high or low O_2 conditions had only negative effects. In tulip bulb scale explants, a small positive effect on regeneration was obtained by a 3-day pulse with 2% O_2 immediately after excision of the explant. In tulip stalk explants, the growth rate was improved by application of 100% O_2. However, the number of shoots was lower in both 2% and 100% O_2 than in ambient air. In lily, the number of bulblets was increased by a 2% O_2 incubation, but the growth rate was lowered under these conditons; this growth rate could not be improved by 100% O_2 treatment. For apple stem slices, the application of a 100% O_2 pulse had some negative effect on the dedifferentiation resulting in a lower number of primordia, whereas continuous low O_2 reduced root formation as well. Our results show that changed O_2 conditions do not lead to an improved regeneration in the tested tissue culture systems, but can influence the growth rate.

Increase of Embryogenic Callus Formation in Cucumber by Pulses with High Concentrations of 2,4-dichlorophenoxyacetic Acid

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We have examined which factors control the formation of embryogenic callus using cucumber (*Cucumis sativus* L. 'Profito') as a model. It has been reported that culture of leaf explants of cucumber on medium with 4·5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4·4 μM benzylaminopurine (BA) induces embryogenic callus formation (Bergervoet *et al.* 1989, *Plant Cell Rep.* **8**: 116–119). This protocol resulted in 10% embryogenic response in 'Profito'. An initial culture for 10 days or 3 weeks on 13·5 or 45 μM 2,4-D increased the embryogenic response to *c.* 40%. Culture at the high 2,4-D concentrations for a longer period resulted in a low response (10%). We determined the period during which a high concentration of 2,4-D was required more precisely by giving 1-week pulses with 45 or 135 μM 2,4-D during the first 6 weeks of culture. The highest response was observed when the pulse was given during the first week immediately after excision of the explants (75% for 45 μM 2,4-D and 55% for 135 μM 2,4-D). One-week pulses at a later time resulted in a lower increase, gradually going down to 10%. A 3-day pulse immediately after the start of culture resulted in 20% embryogenic callus formation with 45 μM 2,4-D and 30% with 135 μM 2,4-D. Pulses with BA (22 μM) did not suppress the response, indicating that the auxin/cytokinin ratio is not very relevant for the formation of embryogenic callus in contrast to adventitious shoot and root formation. We applied these pulse-treatments to induce embryogenic callus in two recalcitrant cultivars (for which the standard procedure failed) and succeeded in obtaining embryogenic callus from these cultivars. One-week pulses with 45 or 135 μM 2,4-D resulted in embryogenic callus responses of, respectively, 45 or 50% in 'DaBaCha', and 30 or 55% in 'R 10'.

Genetic Modification of Cassava

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In cassava, somatic embryogenesis starts with the induction of primary embryos. These embryos are highly organized and maturate easily. They can be multiplied in this state by secondary somatic embryogenesis. A low fraction of the tissue formed in secondary somatic embryogenesis has a friable nature. This friable callus consists of a mass of very young embryos which are less organized and do not maturate easily. This tisssue can be multiplied in the friable callus state for prolonged periods of time.

Both secondary somatic embryos and friable embryogenic callus (FEC) were used for genetic modification. Only in the case of FEC did this result in transgenic plants. Genes were transferred to FEC by particle bombardment and *Agrobacterium tumefaciens* and to protoplasts derived from FEC by electroporation. The first and third method resulted in transgenic plants and the second method in transgenic callus lines which are currently cultured for plant regeneration. In the first instance, only the activity of the luciferase gene was used to select transgenic plants. In later experiments the luciferase gene was used to develop chemical selection procedures. For this, the luciferase gene was coupled to the phosphinoacetyltransferase gene and luciferase activity was used to monitor the efficiency. It has to be evaluated which combination of methods of gene transfer and selection of transgenic tissue is the most efficient.

Regeneration and Transformation of *Alstroemeria*

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The commonly used methods for *in vitro* propagation of *Alstroemeria* are based on rhizome multiplication. A new micropropagation protocol was developed using leaf explant as the initial material. Leaf explants including stem node tissue were incubated on shoot induction medium for 10 days, and then transferred to regeneration medium. Adventitious shoots were induced from the region between leaf base and stem within 3 weeks after transferring to the regeneration medium. The best induction medium was Murashige & Skoog medium supplemented with 10 μM thidiazuron and 0·5 μM indolebutyric acid. The regeneration medium contained 2·2 μM 6-benzylaminopurine. After several subcultures of the leaf with regenerating shoots, normal plants with rhizome were formed.

Two compact-type-of-callus lines were obtained from cultured stem segments and were used to select friable callus. Both compact and friable calli were able to form shoots. In combination with the leaf culture protocol a complete plant could be obtained. Particle bombardment and *Agrobacterium*-mediated gene transformation will be conducted using leaf explant and callus as the starting materials.

Leaf Senescence of *Alstroemeria hybrida*, Regulation by Gibberellins, Cytokinins and Red Light

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Alstroemeria cut flowers have poor keeping quality due to quick senescence of the leaves during vase life. To understand the regulation of leaf senescence, the interactions between gibberellin and cytokinin metabolism and phytochrome action were studied.

Leaf tips were placed in demineralized water or in various gibberellin (GA) or cytokinin solutions and placed in either darkness or irradiated daily with 5 μmol $m^{-2} s^{-1}$ red or red/far-red light. Chlorophyll loss was insignificant during the first 3 days. In darkness, chlorophyll began to decrease rapidly at the beginning of the fourth day and leaves were completely yellow after 8 days of darkness. Irradiation with red light and treatment with active gibberellins or cytokinins delayed the onset of leaf yellowing as well as the rate of chlorophyll breakdown. Application of various types of gibberellins and cytokinins delayed leaf senescence with the degree of activity depending on the type of growth regulator.

The presence of GA_1, GA_4, GA_8, GA_9, GA_{15}, GA_{19}, GA_{20}, GA_{24}, GA_{29} and GA_{34} in alstroemeria leaves was demonstrated by using GCMS. The results suggest that two pathways, the early-non-13-OH pathway to GA_4 and the early-13-OH pathway to GA_1, exist in alstroemeria. The contribution or precursor GAs related to the total gibberellin pool decreased drastically while degradation GAs increased during senescence.

Determination of cytokinin levels in senescing leaves revealed that the activity of exogenously applied gibberellins did not involve an increase in endogenous cytokinin concentration. Red light, acting through phytochrome, and cytokinins are important for chloroplast development and during chloroplast senescence. By means of analysis of chlorophyll synthesis and chlorophyll breakdown rates, we are currently analysing whether gibberellins, red light and cytokinins cause similar phenotypical senescence. These experiments might provide further insight as to whether the delay of senescence by gibberellins, cytokinins and red light act independently or via (partly) joint pathways.

Transgenic Rose Plants Obtained after *Agrobacterium* Transformation of Embryogenic Callus

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One of the main problems of cut roses is short vase-life, which is caused mainly by vascular occlusion by bacteria. By introducing genes coding for peptides with anti-bacterial activity, prolongation of vase-life should be possible.

A reproducible regeneration method via somatic embryogenesis has been developed for various cut rose cultivars. *In vitro*-induced roots were cultured for 2 months on media with high concentrations of auxins. Embryogenic friable callus was obtained by culturing the callus which developed from roots on media without hormones. Somatic embryos were induced on this callus and developed into normal plants. Cocultivation was performed with embryogenic callus and *Agrobacterium tumefaciens*, containing pMOG410 carrying a kanamycin resistance gene, a GUS intron gene and genes coding for peptides with anti-bacterial activity. Calli were selected on media containing kanamycin. These calli stained blue

after X-Gluc treatment, indicating that they were transgenic. Transgenic calli were obtained with all constructs used. Regeneration of the calli has resulted in transgenic embryos and plants, containing a kanamycin resistance gene and a GUS intron gene. DNA analyses are in progress.

Further research will be carried out to analyse whether the vase-life of these transformed roses has been improved.

Engineering of Fungal Resistance in Strawberry (*Fragaria* × *ananassa*); Development of a Transformation Protocol and Introduction of Anti-fungal Genes

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The fungal resistance project of MOGEN has the objective of developing a wide variety of crop plants with enhanced resistance against economically important fungi. In collaboration with Plant Sciences a strategy has been set up to develop fungal resistance in strawberry. This strategy includes the development of a transformation protocol, introduction of 'anti-fungal genes' and field-testing for fungal resistance. Here we present the results of the development of a transformation protocol and introduction of fungal resistance genes.

Agrobacterium strain EHA105 was used to inoculate leaves of 4 weeks old *in vitro* strawberry plants. Regeneration was tested on medium containing several combinations of phytohormones. In the presence of benzylaminopurine and zeatin, no transgenic plants could be regenerated. However, medium containing thidiazuron showed efficient regeneration of transgenic strawberry plants. The transformation protocol was optimized to yield an average transformation frequency of 10% (percentage of explants yielding a transgenic plant). Using the developed protocol a significant number of lines were produced for field evaluation.

Transformation of *Gladiolus* by Particle Bombardment

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In Europe, *Gladiolus* ranks fourth among cut flowers for economic importance. One of the major problems in the cultivation of *Gladiolus* is the occurrence of diseases. Addition of resistance genes to susceptible but further valuable cultivars might be feasible with the aid of biotechnological breeding. This is the objective of the current research.

Transformation experiments were carried out with cell suspensions of *Gladiolus* cultivars Applause and Peter Pears. These cell suspensions were induced in two ways: (1) by bringing friable callus induced on central cormel slices in liquid medium or (2) by direct induction of embryogenic callus by shaking basal cormel slices in liquid medium. Refreshment of the medium took place every 2 weeks.

To determine the effect of the promoter on gene expression, suspension cells were bombarded with plasmids containing the GUS gene under three different promoters: actin, ubiquitin and 35S. The monocot promoters actin (from rice) and ubiquitin (from maize) were both used in combination with a monocot intron. The dicot promoter 35S (from CaMV) was used with and without a monocot intron. The 35S promoter without intron proved to be the best of all promoters tested. It scored four times higher in transient GUS expression, indicating that for the monocot crop *Gladiolus* the dicot promoter 35S without monocot intron was the most efficient.

For selection purposes, three selectans were studied: the antibiotics kanamycin and hygromycin and the herbicide Basta (PPT). Drops of cell suspensions were transferred to solidified medium containing a range of concentrations of selectans. On both Basta and hygromycin, only 20% callus growth occurred at a concentration of 10 mg/l without discolouring of the material, indicating that 10 mg/l of these selectans should be a suitable concentration. For kanamycin this growth reduction was not feasible without material discolourization.

In following transformation experiments, plasmids will be used containing the GUS gene in combination with a selection gene under control of this 35S promoter.

Regeneration of Shoots from Stem Explants of Tulip

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In micropropagation of tulip, 1 mm slices are cut from young, partially elongated flower stems. A major problem is that most regenerated shoots (*c.* 90%) do not have a meristem. Shoots without a meristem cannot be used for axillary propagation and cannot form a bulblet. Addition of the volatile growth regulator methyl-jasmonate (MeJa) enhanced

shoot regeneration from *c.* 10% to 80–90%. After excision of the first wave of shoots from the stem explants, the explants could be cultured again on the same medium. When the explants had been treated with MeJa during regeneration of the first wave of shoots, most (70%) of the shoots regenerated during the second wave also had a meristem, even though during regeneration of the second wave no MeJa had been added. When during the first wave MeJa had not been added, the percentage of second-wave shoots with a meristem was, just as in the first-wave shoots, only 10. A hot-water treatment (1 h at 40°C) of the flower stems before cutting slices improved shoot formation: not only the percentage of explants with regeneration increased, but also the number of shoots formed per explant increased. After a hot-water treatment at 45°C, regeneration did not occur.

Somatic Embryogenesis of *Freesia*

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For ornamental crops that propagate slowly *in vitro* (e.g. bulbous crops such as tulip and freesia), somatic embryogenesis may provide a method for rapid propagation. Furthermore, somatic embryogenesis can serve as a regeneration method in molecular breeding. We are currently developing a method for regeneration of *Freesia* using somatic embryogenesis. Nodular, embryogenic callus cultures were induced at 25°C in the dark from surface-sterilized flower explants. The semi-solid or liquid induction medium contained half-strength MS-nutrients, 2-isopentenyladenine (4 μM) and different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D; 9, 23 or 45 μM). Explants were subcultured after 2 or 6 weeks. Eight weeks after initiation, some explants were transferred to hormone-free medium at 20°C in the dark. Initially rhizogenesis occurred, more so in explants originating from media with the high 2,4-D concentrations. Approximately 8 weeks after transfer to hormone-free medium, direct formation of small nodules occurred. After transfer to light, these structures turned green within 1 day and grew into harvestable, rooted shoots within 1 week. These shootlets could easily be picked from the callus, suggesting that they originate from somatic embryos. This was also suggested by experiments carried out in liquid medium, in which torpedo-shaped structures were observed floating freely in the medium. When placed on solid medium, these putative somatic embryos showed abundant formation of secundary embryos, but also malformations, probably from carry-over effects by 2,4-D. This indicates further optimization is needed.

Indoleacetic Acid is the Optimal Auxin for Rooting of Apple 'Jork 9' *in vitro*

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Microcuttings may be rooted *ex vitro*: after a short dip in talc powder with auxin or in a solution with a very high concentration of auxin, the microcuttings are planted directly in soil. Just as for rooting of normal cuttings, for rooting of microcuttings *ex vitro* indole-3-butyric acid (IBA) is mostly used. Often, microcuttings are rooted *in vitro*. A major difference between *in vitro* and *ex vitro* rooting is the duration of exposure to auxin (several weeks vs. a few minutes). Because of this, for *in vitro* rooting another auxin may be optimal than for *ex vitro* rooting. We examined *in vitro* rooting of apple 'Jork 9' microcuttings continuously exposed to one of the three auxins commonly used for *ex vitro* rooting, namely indole-3-acetic acid (IAA), IBA and α-naphthaleneacetic acid (NAA). The cultures were first kept in the dark, whereas outgrowth of the root initials was in the light. With NAA a low maximal root number (*c.* eight roots) occurred at 3 μM. At this concentration, NAA showed adverse side-effects: it strongly blocked growth of root initials, roots and microcuttings. IAA or IBA induced high maximal root numbers (*c.* 15 roots) at 10–100 or 10 μM, respectively. However, 10 μM IBA inhibited growth of roots and microcuttings much more than IAA. Furthermore, the maximal root number was reached at a wide range of IAA concentrations (10–100 μM) but only at one concentration (10 μM) of IBA. After planting in soil, highest fresh weight increase occurred after rooting with IAA. In conclusion, IAA is the preferable auxin for *in vitro* rooting of apple 'Jork 9' microcuttings.

In Stem Segments of Apple Microcuttings, Auxins are Mainly Taken up Through the Cutting Surface and not Through the Epidermis

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In stem segments of normal cuttings, uptake of auxin via the cutting surface is many times higher than uptake via the epidermis (Kenney *et al.* 1969, *J. Exp. Bot.* **20:** 820–840). Microcuttings have an inferior epidermis in comparison with normal, *ex-vitro* grown plants. Therefore, in microcuttings the contribution of uptake via the epidermis may be significant. This was examined in microcuttings of apple 'Jork 9'.

Uptake of labeled indoleacetic acid (IAA) by stem segments of 1·6 and 3 mm in liquid medium was the same. Uptake of labelled IAA by 3-mm stem

segments cultured vertically on the surface of solid medium or partially submerged in solid medium was the same. These data show that little or no auxin was taken up via the epidermis. When the epidermis was not intact because of wounds (caused by excision of the petioles) stem segments required a lower auxin concentration in the medium to achieve rooting.

Uptake and Oxidation of IAA by Microcuttings of *Malus* 'Jork 9'

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Indoleacetic acid (IAA) is the optimal auxin for rooting of apple microcuttings *in vitro*. However, IAA is susceptible to oxidation by auxine-oxidase. We have examined the oxidation and uptake of IAA by apple stem segments.

The oxidation of IAA was highest during the first 24 h and decreased after that. The phenolic compound ferulic acid (FA) blocked auxin oxidation almost completely. Correspondingly, FA strongly enhanced rooting. Because auxine-oxidases are peroxidases, we also determined excretion of peroxidase. The level of excreted peroxidase decreased with time. This corresponds with the observed decrease of IAA oxidation.

The uptake of IAA increased strongly during the first 3 days. Because FA protects IAA from oxidation, more IAA is available for uptake. However, FA only enhanced IAA uptake during the first 24 h of the rooting period and had no effect on IAA uptake after that. This did not match with the time of action of FA on rooting (pulses with FA strongly enhanced rooting up to 72 h). These results indicate that FA enhanced rooting not only by protecting IAA but also in another way.

Expression of AGL15 During Early Microspore Embryogenesis in *Brassica napus* cv. Topas

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To study early events in plant embryogenesis we use the system of microspore embryogenesis of *Brassica napus*. When microspores and early pollen of *B. napus* are cultured for at least 8 h at 32°C, they can change their developmental pathway from gametogenesis to embryogenesis. AGL15 (for AGAMOUS-like) is a member of the MADS box gene family of transcription factors. Members of this family are found in vertebrates, plants and yeast. In plants, important organ identity genes and floral meristem identity genes belong to the MADS box genes. Recently, a new member of the family was found, AGL15. AGL15 is expressed during zygotic embryo development and was found in several plant species. In *B. napus* it is already expressed in the early globular stage and it has been reported to accumulate in all tissues of torpedo stage embryos (Heck *et al.* 1995, *Plant Cell* **7**: 1271–1282).

Here we show some first results of AGL15 mRNA localization in microspore derived embryos. After *in situ* hybridization with a DIG-labelled RNA probe and detection with alkaline phosphatase we found totally labelled globular, heart and torpedo stage embryos, embryos with no labelling in the epidermis and in some cases embryos with specific labelling in the pre-endodermal cells in the early heart stage. The reason for these different patterns is not yet known.

Antisense Tyrosinase Inhibition to Study Bacterial Blotch Browning in the Cultivated Mushroom, *Agaricus bisporus*

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During cultivation and post-harvest storage, mushrooms are highly sensitive to browning phenomena, due to mechanical injury, senescence, spore-shed and bacterial infections. Many browning reactions have been implicated with the activity of tyrosinase, which oxidizes cellular phenols into melanins. *P. tolaasii* causes the so-called brown blotch disease in *Agaricus bisporus* through the production of tolaasin. Recently, it has been shown that tolaasin extract induces tyrosinase activity. Several isoforms (7–10) have been identified in different *A. bisporus* tissues, but so far only cDNAs *AbPPO1* and *AbPPO2* encoding tyrosinase are known.

Pools of mRNA in healthy and infected mushroom caps were analysed to investigate whether tyrosinase synthesis can be induced upon exposure to a bacterial suspension or tolaasin. Specific induction of *AbPPO2* mRNA synthesis, but not of *AbPPO1*, was found in infected mushrooms. The level of induction correlated with the toxin concentration applied.

In order to study the contribution of specific tyrosinase isoforms to browning phenomena, gene silencing (antisense inhibition and gene disruption) through transformation was employed. Internal fragments of the *AbPPO1* and *AbPPO2* DNA sequences

were integrated in the antisense orientation and stably maintained in the genome. From a total of 167, a subset of 50 transformants of specific interest was selected. These transformants generated with a mixture of combined constructs were analysed by Southern blot analysis. The results show no homologous integration (gene disruption) but several 'anti-tyrosinase' transformants containing either antisense *AbPPO1*, *AbPPO2* or both. Twenty transformants were further selected for the production of transgenic fruitbodies and molecular biological as well as biochemical and pathogenicity studies including analyses of mRNA, isozyme profiles and residual tyrosinase activities.

Survey of Proteins Associated with *Agaricus bisporus* Cap Opening

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Morphogenesis of *Agaricus bisporus* fruitbodies does not cease after harvest. Harvested sporophores, although inhibited in further development, continue to grow at the expense of endogenously present carbohydrates or mannitol. The opening of the cap is the most important morphological quality parameter. In the past, evidence has been obtained for the existence of a hormone-based regulation of morphogenesis in developing sporophores. Also, the presence of plant-hormone analogues of auxins and cytokinins has been demonstrated in Agaricales. Although the specific function of these substances in fungi *in vivo* is as yet unknown, cytokinins have been shown to effectively inhibit cap opening of *A. bisporus*.

The effects of benzyladenin treatments on *A. bisporus* sporophores were studied using 2-dimensional protein analysis. Upon cytokinin treatment, various proteins disappeared while several new spots appeared. Based upon discriminating criteria, a limited number of spots was selected for further study.

MEETING OF THE SECTION FOR ALGOLOGY ON 23 AND 24 APRIL 1997

Towards a Phylogeny of the Dasyaceae (Ceramiales, Rhodophyta), Based on rbcL Gene Sequences and Non-molecular Characters

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A study of the phylogeny of the Dasyaceae has been undertaken based on sequence analysis of the large-subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcL) and non-molecular data (vegetative and reproductive structures). An evaluation of the phylogenetic position of the Dasyaceae within the Ceramiales was also included. In the phylogenetic analysis of the molecular data a selection of Gelidiales species were used as outgroups. In the preliminary non-molecular dataset the Composothamnioidieae (Ceramiaceae) were postulated as most ancestral lineage. The cladograms confirm the general view of the Ceramiaceae as primitive, paraphyletic group within the Ceramiales. The Dasyaceae are resolved as a distinct, monophyletic lineage within the Ceramiaceae.

Similarly, the Rhodomelaceae and Delesseriaceae are clustered together as a single group. This contradicts a traditional idea to assume the higher Ceramiales (Dasyaceae, Delesseriaceae and Rhodomelaceae) as monophyletic group and elucidate some similar evolutionary trends in the reproductive structures of members of the Dasyaceae (i.e. Heterosiphonia-like genera) and Rhodomelaceae as parallel developments. In contrast to established concepts about character evolution in the higher Ceramiales, within the Dasyaceae *Heterosiphonia*-like genera (*Heterosiphonia*, *Thuretia*, *Dictyurus*) appear to be the most primitive members of this family, while *Dasya*-like genera (*Dasya*, *Eupogodon*, *Rhodoptilum*) were identified to present advanced developments. Polyphyly of radially and bilaterally organized *Eupogodon* species, as proposed in a recent study, was opposed by the cladograms.

The Species of *Sphacelaria* (Sphacelariales, Phaeophyceae) in China With a Description of a New Species

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A study has been carried out on a herbarium collection of marine *Sphacelaria* specimens, collected from the entire Chinese coast. Seven species of *Sphacelaria* are present in China, *S. rigidula*, *S. tribuloides*, *S. novae-hollandiae* and *S. carolinensis* being previously recorded in this region. The three previously unrecorded species are *S. divaricata*, *S. californica* and a new species, *S. tsengii*, that was found on subtidal rocks at two locations: South Hainan and South Guangdong. *S. tsengii* is characterized by its long propagules with a tapering stalk and usually two tapering arms. In China *S. rigidula* and *S. tribuloides* are the most dominant *Sphacelaria* species in, respectively, the North and the South. *S. rigidula* and *S. divaricata* are found over a long range of latitude, while *S. californica* has only been found in Fujian. The distribution of the other species is restricted to the South. Structural characters are discussed and the holotype of *S. carolinensis* is re-examined. It is suggested to place *S. carolinensis* in the *Sphacelaria* section Furcigerae instead of in the section *Tribuloides*.

The Search for Microsatellites in *Cladophoropsis membranacea*

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In order to investigate the directionality and intensity of gene flow and the reproduction strategy (sexual or clonal) of *Cladophoropsis membranacea* (Hofman Bang ex. C. Agardh), the development of high resolution genetic markers such as microsatellites is essential. For this reason a small-insert (200–800 base-pairs) genomic library was constructed (4648 recombinants) and screened with microsatellite-complementary probes (di, tri and tetranucleotides). Only one recombinant (0·02%) containing the microsatellite sequence [GT]4 AT [GT]6 was found.

After this disappointing experience, two enriched genomic libraries were constructed. Screening of these libraries gave 273 (5·4%) recombinants with microsatellite motifs. Sequencing of 206 of these recombinants yielded 71 candidates for primer-design. From 35 of these candidates 18 working primer-sets could be made. These 18 primer-sets were radioactively tested on 12 samples from the Canary Islands and 13 samples from the Carribean. From these experiments it became clear that 11 primer-sets worked well for the Canary Islands samples but only five for the Carribean samples. With the now-working primer-sets the screening of hierarchical sampled field material is beginning.

A Mutual Relationship Between Water Transparency and Development of Charophytes

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The relationship between phosphorus concentration, light climate, cover and distribution of charophytes was studied in Lake Veluwemeer changing in eutrophication state over the past decades. The total area of the lake covered by *Chara* sp. was largest in years with a low average total phosphorus concentration and a high Secchi depth. The phosphorus concentration was higher in the years when charophytes disappeared in the 1960s (*c.* 0·25 mg P l^{-1}) than in the period of their return after nutrient reduction 20 years later (*c.* 0·10 mg P l^{-1}). Secchi depths were very similar between these two periods (0·35 m). Apparently, charophytes are able to maintain a relatively high transparency during eutrophication. Measurements of local patterns in light attenuation coefficient (Kd) in Lake Veluwemeer in 1995 showed a *c.* three times higher transparency inside dense *Chara* sp. vegetation than outside the vegetation. The maximum colonized depth over recent decades was restricted to the estimated summer averages of Kd. The area of open covered vegetation (1–15% cover of the sediment) was larger in years with a low Kd, although the area of dense covered vegetation (50–100%) over the years was not affected by Kd. We suggest that the observed dynamics of charophytes may have resulted from a mutual relationship between charophytes and water transparency. The effect of dense charophyte vegetation on water transparency may be an important mechanism making them more tolerant to year-to-year changes of Kd as well as to eutrophication in the long term.

Sensing Weight: Effects of Altered Gravity in the Green Alga *Chlamydomonas*

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Several organisms react to the state of weightlessness by changing cellular parameters, such as cell proliferation, differentiation, cell volume, etc. Also *Chlamydomonas monoica* cells were observed to be affected during spaceflight in the Russian Foton 10 mission. Compared to ground-control cells, they exhibited a shortened cell cycle, which resulted in enhanced cell proliferation. In addition, the cells formed on average longer flagella during weightlessness. Similar results were obtained in the Free Fall Machine, which is a device in which cells are periodically accelerated for a short period (approx. 50 msec). Each event is followed by a period (approx 1 sec) of free fall (=weightlessness). The results can be explained by assuming that *Chlamydomonas* has a minimal time threshold for sensing a change in gravity, which surpasses the acceleration time. Consequently, the cells would experience the periodic free-fall movement as a long-term weightlessness condition. A difference in flagellar length was also obtained in shaken vs. non-shaken cells. We propose that swimming or tumbling cells do not feel the vector of gravity, in contrast to stationary cells, which results in a change of flagellar length and possibly also cell cycle duration.

The CO_2 Concentrating Mechanism in the Green Alga *Tetraedron minimum*

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In fossil oil shales, containing the remnants of several specific organisms, but mainly microalgae, strong deviations of the $\delta^{13}C$ have been found in comparison with other fossilized biomass. One major source of oil in shales is the cell wall of the green alga *Tetraedron minimum*. This study aims to unravel the processes which can influence the $\delta^{13}C$, and particularly to identify the discriminatory step in the uptake of carbon. Aquatic photosynthetic organisms have a need for a CO_2 concentrating mechanism. The concentration of dissolved CO_2 in the surrounding environment is low and at pH>6·4 HCO_3^- is the dominating species. The CO_2 concentrating mechanism generally involves a HCO_3^- pump and the activity of internal–external carbonic anhydrases. In contrast to *Chlamydomonas*, *T. minimum* showed no difference in the affinity for dissolved inorganic carbon (DIC) upon a shift from high to low CO_2. However, inhibitors of carbonic anhydrase retarded the uptake of DIC. This suggests that, in contrast to Chlamydomonas, in *T. minimum* a carbon concentrating mechanism is present at both high and low DIC, which is consistent with the idea that the cell wall has a low permeability for DIC.

Alkaline Phosphatase Activity in Two Planktonic Desmid Species in Relation to Competition for P Supplied by an Organic P Compound

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Alkaline phosphatase activity (APA) of two equally cell-sized desmid species was compared. *Cosmarium abbreviatum*, a species from meso-oligotrophic lakes, had a higher maximum APA and affinity constant under both continuous and pulsed inorganic phosphorus (P_i) limitation than *Staurastrum chaetoceras*, a species predominantly encountered in eutrophic lakes. APA of both species increased when measured in starved cells from a pulsed P_i condition as compared to a continuous Pi limitation. The portion of extracellular APA relative to cellular was higher in *S. chaetoceras* than in *C. abbreviatum*, so *S. chaetoceras* secretes the enzymes more readily into its environment. The difference in affinity for organic phosphorus (P_o) as measured with APA may explain the dominance of *C. abbreviatum* over *S. chaetoceras* in their competition under a continuous organic P limitation. The difference in maximum APA could, however, not explain the outcome of competition under a pulsed PO-limitation, where *S. chaetoceras* dominated over *C. abbreviatum*. Cells of *C. abbreviatum* are surrounded by an extracellular mucous envelope (possible action site of APA), whereas *S. chaetoceras* does not make such a structure. The outcome of the pulsed P_o-limited competition experiment could be explained by calculating that *C. abbreviatum* per unit of time can hydrolyse much more P_o than it can take up P_i. No indication was found that the mucilage sheath acts as a storage of P or hampers any cellular exchange of P. So after hydrolyzation of the organic P substrate by *C. abbreviatum*, P_i may diffuse into the medium and can consequently be taken up by *S. chaetoceras*.

Production and Location of Acrylate in Axenic Colony-forming Cultures of the Marine Micro-alga *Phaeocystis* sp.

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Phaeocystis sp. is a colony-forming unicellular micro-alga known for its massive blooms during spring. Cells are embedded in a layer of mucus of 8 μm thick surrounding the colonies. In the beginning of a bloom, young and healthy cells are almost

free of bacteria whereas older and senescent colonies are invaded by bacteria. It has been claimed that acrylate produced by the cells is the reason for this inhibition of bacterial growth.

Phaeocystis produces large amounts of dimethylsulphoniopropionate (DMSP). It can cleave this osmolyte with an extracellular, membrane-bound lyase into dimethylsulphide (DMS) and acrylate. During blooms, concentrations of 500–700 nM acrylate have been measured in the sea. This is not enough to inhibit bacterial growth.

We have now measured acrylate production in axenic cultures of *Phaeocystis* and determined whether it accumulated in the colony or only in the mucus layer. Most acrylate was found in the colonies, at concentrations in the μM range. The acrylate appeared to be located in the outer mucus layer and concentrations in this layer were in the mM range. This concentration is high enough to retard bacterial growth. Thus, acrylate can well be the reason why young and healthy colonies are almost free of bacteria.

