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A. In vitro *Culture*

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 when they are applied to living systems in the presence of long-wave UV light (UV-A). The biological activity of thiophenes in the absence of light is still a matter of discussion. Some activity is still expected, since roots are the main site of thiophene accumulation.

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

Normally BBT is converted into BBTOH and $BBT(OH)_2$, which in turn are converted into BBTOAc and $BBT(OAc)_2$ by specific acetyl transferases. The acetyl esters are not converted further and are considered as end-products in the biosynthetic pathway. It has been proposed that, after elicitation, specific esterases would convert the acetoxy-bithienyls into alcohols (E. Kourany *et al.* 1988 *Phys. Mol. Plant Path.*, **33**: 287). However, we found that [^{35}S]-labelled BBTOAc and $BBT(OAc)_2$ were not converted into the corresponding alcohols after elicitation. Elicitation of *Tagetes* roots either activates the enzyme converting BBT into the thiophene alcohols or inhibits the acetyl transferases which use these alcohols as a substrate.

Polarization of Tobacco Single Cells in Culture by NAA

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The acquisition of polarity early in development is essential for the normal build-up of the body structure and its functioning, and in plants the direction of polarization is as a rule persistent in time.

The phenomenon has especially been studied in lower plants. Single-celled life stages of mosses, ferns and algae gave the best clues for an understanding of its cellular basis. Both induction and maintenance of polarity are related to the generation of transcellular electric currents (Weisenseel, M.H. & Kicherer, R.M. 1981, In: Kiermeyer, O. (ed.) *Cytomorphogenesis in Plants*. pp. 379–399, Springer Verlag). For *Fucus zygotes* the involvement of F-actin in the establishment of the polarization axis has been demonstrated (Kropf, D.L., Berge, S.K. & Quatrano, R.S. 1989, *Plant Cell* 1(2): 191–200). Similar data were reported for cells of higher plants during 'tip growth' (Weisenseel & Kicherer, 1981), but very few data exist on the acquisition of polarity.

Recently, we described an *in vitro* cell culture system of tobacco, in which cell development can be controlled with exogenous hormones (Verbelen, J-P., Lambrechts, D., Stickens, D. & Tao, W. 1992, *Int. J. Dev. Biol.* **36**: 67–72). Here we report that NAA given to regenerating cells can induce polarized growth.

Mesophyll protoplasts of *Nicotiana tabacum* (L.) cv. Petite Havana were isolated and brought into

culture as described before (Verbelen *et al.* 1992). NAA was the only growth regulator present in the culture medium. At low concentrations of NAA the cells start expanding after 5 days culture, but keep a spherical shape. At higher concentrations however cells elongate: this means that cell growth is oriented along one axis. The optimal concentration of NAA for this reaction is about 1 mg l^{-1}. This growth in volume continues for quite a long period and after 20 days cells have acquired a tubular shape with a mean length of 500 μm, the longest individuals reaching up to 800 μm.

Then the tubular cells were induced to divide. Cell division starts after 3–4 days in NAA + BAP medium and can go on for some weeks. In the majority of cells the first cell plate is formed in the middle of the cell, perpendicular to the longitudinal axis of the cell. Also, the subsequent divisions are oriented in the same way. As a consequence the new cell walls are all parallel to each other. The original tubular cell is transformed into a multicellular threadlike structure. Only then do divisions occur with the cell plate having another orientation, parallel or oblique to the former longitudinal axis of the cell. This change in orientation is the first indication that the polarity in growth is lost. On these sites with new and different orientations of cell division, cells soon start to proliferate and microcalli develop.

Summarizing, NAA can induce polarization in regenerating mesophyll protoplasts. Polarity is expressed in cell expansion and in the first stages of hormone-induced cell division. In the presence of BAP, however, the polarity in growth is lost again.

Cryopreservation of Shoot Tips as a Conservation Strategy of Chicory (*Cichorium intybus* L.) Germplasm: A Comparative Study

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Because chicory is now mostly cultivated in hydroponic culture, farmers use more and more F1-hybrids. This tendency results in a decrease of the genetic basis of chicory, because the ancient varieties no longer have direct economic importance. However, for further breeding the old varieties are very important. Therefore, this genetically valuable plant material should be preserved.

Dissected shoot tips (variety Flash) can be stored in liquid nitrogen using a two-step freezing method. The meristems are placed for 2 days on a filter paper soaked with liquid hormone-free M&S medium. The shoot tips are then placed into a cryotube containing liquid medium and 15% DMSO as cryoprotectant. The plant material is frozen, using a cryoincubator, with a slow cooling rate (0·5°C min^{-1}) until a temperature of −40°C is reached. The cryotubes are then plunged into liquid nitrogen. After thawing (in sterile water at 40°C), cryopreserved shoot tips are placed onto solidified M&S medium containing growth regulators. After 1 week shoot tips are replaced onto M&S medium lacking growth regulators. This treatment limited callusing. A survival rate of 83(± 6·8)% was obtained and cryopreserved shoot tips were regenerated into chicory plants. Although good results are obtained, the method is not very practical and DMSO is used, which might interact with DNA. Therefore, another protocol was applied to cryopreserve chicory shoot tips.

Dissected shoot tips are encapsulated using Ca^{2+}-alginate and precultured in liquid culture medium supplemented with sucrose. The best results were obtained when a two-step preculture was used: 24 hours of preculture in a 0·3 M sucrose solution, followed by another 24 hours of preculture in a 0·75 M solution. After preculturing alginate beads were dehydrated in a sterile air flow until a water content of 18(± 1·8)% is reached. Beads are frozen by direct plunging into liquid nitrogen. After thawing (in sterile water at 25°C), beads are placed onto solidified M&S medium containing growth regulators. A survival rate of 65(± 7·0)% was reached and chicory plants were successfully regenerated.

Although a lower survival rate was obtained using the encapsulation-dehydration technique, this method seems to be the most interesting because: (i) the method is more easy to handle compared with two-step freezing (no need of a cryoincubator); (ii) sucrose is used as cryoprotectant instead of DMSO.

Before using cryopreservation to start up an *in vitro* genebank of chicory, further experiments are needed. Is one protocol applicable to all the varieties or has the protocol to be optimized to obtain a sufficient high survival rate?

Role of Physiological Factors in Tulip Micropropagation from Bulb Scale Explants

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The propagation of tulip is strongly hampered by the long period (25–30 years) which it takes before a new variety can be introduced to the market. For many other crops the application of tissue culture techniques gives good results. However, major problems are encountered in tulips. When bulb scale explants are used the explants suffer from extreme browning,

which eventually results in the death of the explant. Although bulb scales are available throughout the year, successful regeneration only appeared possible during limited periods. Outside these periods excessive browning and death of the explants prevailed. The physiological background of these problems is studied. The knowledge of the origin of this problem might lead to a solution.

Two hypotheses are being investigated. First, the role of an endogenous fungitoxic substance (tulipaline A) is studied. This tulipaline might be produced from its precursor and subsequently released from the cells during the process of cutting the explant and subsequently might cause cell death. In the second hypothesis sensitivity of the tulip to oxidative stress is investigated. In this line of investigation we study the composition of the membranes in the explants, the degree of saturation of the phospholipids and the presence of protecting enzymes or substances (antioxidants). The effect of the presence of compounds in the culture medium which affect the (oxidative) degradation of the membranes is also studied, as well as the effect of those additives on the regeneration.

First results indicate that the tulipaline has no promoting effect on the rate of browning but a very strong inhibitory effect; the explants stay extremely pale but do not develop any shoots. This observation is supported by the fact that tulip cultivars with a low tulipaline content (e.g. M. Lefeber) show stronger browning.

In the fatty acid content of the phospholipids a strong decrease in the amount of linoleic acid (18:2) during the culture period is seen.

Determination of Cellulose Fibril Orientation in Cell Walls with CLSM

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As the main component of the higher plant cell wall skeleton, cellulose plays a predominant role in the build-up of mechanical strength of each individual cell wall and of the whole plant apoplast. The orientation of the cellulose fibrils in the secondary wells is especially important in this perspective.

Cellulose orientation has also been related to patterns of growth and differentiation of individual cells (Pickett-Heaps, J.D. 1967, *Dev. Biol.* **15**: 71–93). A spherical shape is related to a random orientation of cellulose fibrils in the wall, elongated cells have the cellulose preferentially organized in loops perpendicular to the long axis of the cell. The study of the relation between the cellulose microfibrils and elements of the cytoskeleton has lead to a nice hypothesis on the involvement of microtubules and actin filaments in the control of the orientation of cellulose fibrils (Giddins, T.H. & Stachelin, L.A. 1991, In Lloyd, C.W. (ed.) *The Cytoskeletal Basis of Plant Growth and Form*. Academic Press, pp. 85–99).

This work was done on regenerating mesophyll protoplasts of *Nicotiana tabacum*, cultured as described before (Verbelen J.P., Lambrechts, D., Stickens, D. & Tao, W. 1992. *Int. J. Dev. Biol.* **36**: 67–72). Congo Red fluorescence was used to detect cellulose. Orientation of the cellulose fibrils was determined by using polarized light for excitation. When illuminated with polarized light, the fluorescence of the stained wall is a function of the parallelism between the polarization vector of the light beam and the preferential orientation of the cellulose microfibrils in the wall. Elongated cells indeed have the cellulose fibrils ordered in parallel hoops perpendicular to the long axis of the cell; spherical cells do not show any preferential orientation. These observations are in perfect agreement with the results obtained using traditional polarization microscopy or the dichroic stain properties of Congo Red. Both these techniques need very thin preparations, containing only one layer of cell wall, to yield unambiguous results. The optical sectioning property of the microscope system and the absorption characteristics of Congo Red make this approach a method of choice for applications with any standard confocal laser scanning microscope (CLSM).

The semi-quantitative character of CLSM observations combined with the non toxicity of the stain allow a fast and very reliable assessment of cellulose orientation in the wall of living plant cells.

Flowering Inhibition in Chicory (*Cichorium intybus* L.) Root Explants *in vitro*?

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In vitro cultures were used to study morphogenetic reactions of chicory under different photoperiods. Root explants were cultured under continuous darkness and 8, 123, 16, 20 or 24 h of light to investigate the effect on the formation of roots, shoots, stems and flowering-stems. The transfer from short day (SD) reactions to long day (LD) reactions was situated between 16 and 20 hours. Flowering-stems and flowerbuds developed under LD-conditions, while only shoots were formed under SD-conditions. The explants under continuous darkness (CD) formed stems without flowerbuds.

This photoperiod-sensitivity for flowering allows determination of the time, during the development of root explants *in vitro*, after which the explant became

sensitive to daylength in order to flower. Root explants were cultured under continuous darkness (no flowering-inductive conditions) for a period of 4, 8, 12, 16 or 20 days and then placed under LD-conditions (flowering-inductive conditions). Flowering-induction during LD-conditions decreased after at least 4 days of dark-pretreatment. After 12 days of continuous darkness there was a total inhibition of flower formation. White stems, formed under CD-conditions, stopped growing and a rosette of green leaves was formed at the top after changing daylength.

Because formation of flowering-stems under LD decreased with the duration of the dark-incubation, a flowering-inhibition build-up in *in vitro* chicory-root explants seems to occur. Whether the inhibition is caused by a substance or a process is a question for further research.

In the case of a substance there are two possible explanations for our observations: (1) formation of a flowering-inhibitor(s) under no flowering-inductive conditions (SD and CD) which subsequent LD-conditions cannot break down; (2) formation of a flowering-promoter(s) under flowering-inductive conditions (LD), which cannot be built up during SD or CD.

In the case of an inhibition process there are several hypotheses: flowering-inhibition under SD or CD can be caused by a lack of energy supply, interruption of a metabolic pathway, a problem in the transport system of certain substances or other mechanisms which differ when the photoperiod changes.

More research is needed on the time and location of flowering-inhibition in chicory and the influence of external factors (o.a. daylength) on this phenomenon. We hope to use this information for the development of new breeding strategies.

B. Photosynthesis and Photomorphogenesis

Spectral Properties of Chlorophyllide and the Regeneration of NADPH-protochlorophyllide-photoreductase after a Flash in Bean Etioplast Suspensions in the Presence of NAD(P)H/NAD(P)$^+$ Redox Couples

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Dark-grown angiosperms accumulate the precursor of chlorophyll(ide), protochlorophyllide. Several spectral forms are observed *in vivo*, corresponding to different states of the ternary complex: NADPH-protochloropohyllide-photoreductase (NPPR). Two main forms characterized by 77°K emission and absorption maxima at 657–650, 645–639 nm respectively are photoactive, i.e. converted by light (=activation energy) into a chlorophyllide form $P_{688-677}$. During a subsequent dark period, these maxima undergo a shift towards long wavelengths within 1 min ($P_{696-684}$) and further on, a slow shift towards the short wavelengths ($P_{682-678}$).

Some of these events can be reproduced in etioplast suspensions. In our previous experiments, exogenous redox couples were introduced into etioplast suspensions (cysteine/cystine, NADPH/NADP$^+$, DTT_{red}/DTT_{ox}). They influenced the spectral properties of NPPR, the efficiency of photoreduction of protochlorophyllide into chlorophyllide, and the spectral shifts of chlorophyllide. However, the bathochromic shift of chlorophyllide could only be observed in the presence of exogenous NADPH.

In this study, in order to distinguish between the specific influence of the NADPH cofactor, and the action of its redox couple, the action of the NADPH/NADP$^+$ couple has been compared with that of the NADPH/NAD$^+$ couple, which, in principle, should only act on the redox potential and not as specific coenzyme.

The experiments were performed at 5, 10 and 15°C. The etioplast suspension containing (or not) the exogenous redox couples (15 mM, red/ox=1/10 and 10/1) were illuminated with a 1 ms polychromatic flash and incubated in the dark. Samples were frozen in liquid nitrogen before and after the flash, and during the dark incubation, for spectral analysis.

The low temperature fluorescence spectra showed that when lowering the initial redox potential (from −290 mV down to −350 mV) with exogenous couples, the efficiency of the protochlorophyllide photoreduction into chlorophyllide was increased. The NADPH/NADP$^+$ couple was always more efficient than the NADH/NAD$^+$ couple for a given initial potential. The stability of the newly formed chlorophyllide was higher at lower potential in presence of both couples than in the control. The regeneration of the active forms of the NPPR during the dark period following the flash was of the same order of magnitude for both NADPH/NADP$^+$ and NADH/NAD$^+$ couples, at a given potential.

The observed changes are weak at 5°C, and larger when increasing the temperature. However, a temperature of 10°C seems optimal and avoids the degradation processes observed at 15°C.

Three main effects characterize the specific action of the NADPH/NADP$^+$ couple: (1) The bathochromic shift of the newly formed chlorophyllide emission band. (2) A higher efficiency of

protochlorophyllide photoreduction. (3) At 10°C, when NADPH is the major component of the exogenous couple, the emission of the regenerated protochlorophyllide is situated at 657 nm, whereas in the other cases, it appears around 645 nm.

From these experiments we conclude that the etioplasts contain a very efficient enzymatic system able to regulate the internal redox potential and probably also the NADPH concentration in the vicinity of the active site of the NPPR.

An Identification Test for Blue Light Perception in Plant Plasma Membrane

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Blue light research is hampered seriously by the lack of a test comparable to the R/FR reversibility test for phytochrome. Moreover, the multitude of flavin proteins, all potential photoreceptors, existing in soluble and in membrane-bound conditions, turn the isolation of a specific blue light photoreceptor protein into an ungrateful task.

Recent developments in blue light research focus on phenomena associated with the plasma membrane (Kaufman, L.S. 1993, *Plant Physiol.* **109:** 333–337). These experiments benefit from the advantage that the photoreceptor has a specific location and consequently, its isolation is linked to the preparation of the appropriate membrane vesicles (Asard, H., Venken, M., Caubergs, R.J., Reynders, W., Oltmann, E.L. & De Greef, J.A. 1989, *Plant Physiol.* **90:** 1077–1083).

One of the plasma membrane associated blue light phenomena is the so-called light-inducible absorbance change (LIAC). This blue light induced cytochrome b reduction upon the excitation of a flavoprotein was originally proposed as one of the first steps of the transduction chain leading to blue light responses by Munoz & Butler (*Plant Physiol.* **55:** 421–426). They also proposed to use the LIAC phenomenon as an identification test for the presence of blue light photoreception, comparable to the R/FR reversibility test for phytochrome. The LIAC activity is however exclusively linked to the plasma membrane and, consequently, this absorbance change can hardly be used as a general blue light test, since it is difficult to explain the multitude of blue light phenomena solely on the basis of one photoreceptor residing in the plasma membrane.

However, as already pointed out, blue light phenomena associated with the plasma membrane have been described recently to a great extent and, combined with the increasing attention for plasma membrane redox activities associated with vital physiological functions such as ion-transport, detoxification, stress and even growth, a renewed interest in LIAC activity is noticeable (Rubenstein, B. 1993, *Annu. Rev. Plant. Physol. Plant Mol. Biol.* **44:** 131–155). In view of this interest, we would like to report data about LIAC activity in bean plasma membrane vesicles. Fundamentally new is that LIAC results solely from the action of intrinsic plasma membrane proteins and is independent of exogenous photosensitizing flavines. A natural O_2-scavenging system, probably a NADH oxido-reductase is creating the low oxygen tension necessary for LIAC action. In these circumstances LIAC activity can be considered as a blue light photoreceptor test at least for plasma membrane associated blue light phenomena.

Effects of Atrazine Resistance on the Molecular Mechanisms of Photoinhibition and Photoprotection

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Molecular mechanisms of photoinhibition (PI) have been investigated, using leaves and isolated chloroplasts of triazine-resistant (R) and susceptible (S) *Chenopodium album* plants which were grown at low and high irradiance.

In vitro electron transport dependent on photosystem I (PSI) alone was much less affected by PI than that dependent on both photosystem II (PSII) and PSI. There was a smaller difference in susceptibility to PI between the photophosphorylation activity dependent on PSI alone and that dependent on both PSII and PSI. Because in all cases photophosphorylation activity decreased faster upon PI than the rate of electron transport, we conclude that photoinhibition causes a gradual uncoupling of electron transport with phosphorylation. since the extent of the light-induced proton gradient across the thylakoid membrane decreased upon PI, it is suggested that photoinhibition causes a proton leakiness of the membrane. We have found no significant differences for PI of the various reactions measured in chloroplasts isolated from R and S.

Fluorescence studies *in vivo* showed that the φ_P and PSII electron transport rate were lower in R. Also in most cases q_P and q_N were lower in R, indicating a lesser ability of R to dissipate an abundance of light energy leading to photoinhibitory damage. It could be demonstrated in intact leaves that the lower productivity of R is caused by a higher sensitivity to PI. The differential effect of photoinhibition *in vivo* and *in vitro* may be caused by the fact that *in vitro* energy conversion by photosynthesis, energy dissipation by protective mechanisms

(photorespiration, xanthophyll cycle, Mehler reaction) and recovery processes do not function or function less efficiently in R leaves.

Recent research concerning a possible difference between the two biotypes R and S with relation to energy dissipation by the xanthophyl cycle carotenoids violaxanthin and zeaxanthin revealed that R and S differ in the amount of zeaxanthin that can be formed by the de-epoxidation of violaxanthin. Several *in vitro* experiments have shown that the triazine-resistant biotype has a significantly higher amount of zeaxanthin. It is discussed how a certain amount of zeaxanthin should protect the chloroplast from photodamage by scavenging oxygen or by quenching the triplet state of chlorophyll molecules.

Effects of Elevated Atmospheric CO_2 on Leaf Chlorophyll Fluorescence and Photosynthesis of Two Poplar Clones

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Specific leaf area (SLA), chlorophyll fluorescence, photosynthetic and respiration rate were examined for poplar clones Beaupre and Robusta grown in open-top chambers ventilated with air with both ambient CO_2 (*c.* 350 ppm) and elevated CO_2 (*c.* 700 ppm).

SLA in mature leaves was 162·05 cm^2 g^{-1} for Beaupre and 152·71 for Robusta grown in ambient CO_2 and decreased about 25% in the elevated CO_2 treatment for both clones to 122·26 and 115·32, respectively.

Maximal fluorescence (Fm) and basic fluorescence (Fo) increased with increasing leaf plastochron index (LPI) until maturity and decreased in old leaves. Fm and Fo were higher in young leaves of plants treated with elevated CO_2 than those under ambient CO_2 conditions. In mature leaves reversed relations of Fm and Fo were found between treatments.

To evaluate the effect of treatment and the effect of measuring conditions, photosynthesis and respiration rate were measured in both high CO_2 (750 ppm) and low CO_2 (375 ppm) for both treatments and clones. For both clones net photosynthetic rates were slightly higher (Robusta: 2%, Beaupre: 7%) in the elevated CO_2 treatment than in ambient CO_2 treatment, under the same measurement conditions. A nearly doubled net photosynthetic rate was measured with a high CO_2 air flow, comparing to that with a low CO_2 air flow in both clones and treatments. These results indicated that the photosynthetic capacity of poplar plants was higher than that performed under ambient conditions. No significant difference was found in respiration rate, neither between both clones nor between treatments. However, high CO_2 concentration seemed to suppress respiration more for plants grown under ambient CO_2 conditions than under elevated CO_2 conditions.

Positive correlations between chlorophyll contents and chlorophyll fluorescence were observed with coefficients (r) ranging from 0·87 to 0·91. No obvious correlations between chlorophyll fluorescence and photosynthetic rate were observed based on the most recent experiments.

Solubilization and Isolation of Protochlorophyllide–Protein Complexes from Etioplast Membranes of Wheat

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Protochlorophyllide–protein complexes occur in etioplasts of dark-grown angiosperms and play an important role as chlorophyll precursors in light-dependent chlorophyll accumulation. In the light, protochlorophyllide (Pchlide) is reduced to chlorophyllide (Chlide) within the photoactive complexes containing the pigment, NADPH and the photoenzyme NADPH-Pchlide-reductase. These photoactive complexes are stable *in vivo* in the dark but are very labile as soon as extracted from the etioplast membranes to which they are bound. In this work we have achieved the solubilization and partial purification of Pchlide–protein complexes which retain their original photoactivity and spectroscopic properties.

Isolated etioplast membranes of wheat where solubilized at pH 7·5 on ice using the non-ionic detergent dodecyl-maltoside. Unsolubilized material was then removed through centrifugation. The clear supernatant was filtered through gel chromatography on Sephacryl S4000 in darkness at 4°C. Sucrose and NADPH were added to the medium during the whole procedure in order to slow down the degradation of the complexes. The fractions obtained after gel filtration were analysed by fluorescence spectroscopy.

A typical distribution of the three spectral Pchlide forms known from *in vivo* studies was found in the fraction pattern. Fractions of very high molecular weight (18 000 kD) showed mainly the inactive (non-photoreducible) Pchlide with an emission maximum at 632 nm. Fractions of intermediate (1000 kD) and low (40 kD) molecular weight were enriched in photoactive Pchlide-protein complexes with an emission maximum at 657 and 645 nm, respectively. If the known molecular weight of the enzyme is taken into account (36 kD, Oliver, R.P. & Griffiths, W.T. 1981, *Biochem. J.* **195**: 93–101), these results suggest that the 657 nm Pchlide form corresponds to large size aggregates of

the enzyme–substrate complex (possibly with other proteins) and that the 645 nm form is a monomeric form of the complex.

Photosynthesis and Nitrogen Relations in *Zea mays* L.

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Recent concern for environmental problems partly caused by high nitrogen (N) fertilizers applications has resulted in an urgent need for crops with improved nutrient-use efficiency (Konesky, D.W., Siddiqi, D.M., Glass, A.D.M. & Hsiao, A.I. 1989, *J. Plant Nut.* **12:** 9–35). One possibility is to improve nitrogen-use efficiency of the photosynthetic process (PNUE). This can be done by looking at the intra-specific variation for PNUE in different crops. The importance of N in relation to photosynthesis is evident since 75–80% of the total leaf-N is located in the chloroplasts (Makino, A. & Osmond, B. 1991, *Plant. Physiol.* **96:** 355–362) and a close relationship between N and photosynthesis has been observed in numerous species (Natr, L. 1992, *Photosynthetica* **27:** 271–294).

Photosynthesis and leaf nitrogen relations of two contrasting *Zea mays* L. cultivars were examined under water culture condition at the seedling stage. Seedlings of cultivars LG11 and CESDA28 were grown under a relative N-addition rate (Ingestad, T. 1982, *Plant Cell and Environment* **5:** 443–453) of 0·076 $days^{-1}$ for 14 days and then separated into two groups. One group was kept under the same conditions until day 18 while the other was deprived of N for 2 and 4 days. Photosynthetic rates (A) were determined on the third fully developed leaf in an open gas exchange system and the area of the leaf enclosed in the chamber was used to determine leaf content. Photosynthetic nitrogen-use deficiency defined as the slope of the A to N response curve was compared between these two cultivars during leaf ageing following the removal of N-supply. Comparisons for PNUE between cultivars were conducted using regression analyses of variance.

The photosynthetic response of both cvs to a decline in N-content following removal of N-supply was linear in both cases. Cultivar LG11 showed a PNUE of 0·49 ± 0·05 μmol CO_2 s^{-1} $mmol^{-1}$ N vs. 0·28 μmol CO_2 s^{-1} $mmol^{-1}$ N found for cv. CESDA28. The ANOVA on the regression slopes of the A to N responses of the two cultivars showed significant differences in PNUE (F=4·76, P<0·05). These results suggest that there is a considerable potential for selection for higher PNUE.

Effect of a Change of Glycine 215 of the D2-protein of Photosystem II into a Tryptophan on Electron Flow Through Photosystem II

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Electron transport in photosystem II between Q_A and Q_B is not well understood. It is, for example, unclear which role the non-heme iron plays in this process. Here, this problem is approached by characterization of a mutant of the cyanobacterium *Synechocystis* PCC 6803 in which glycine 215 of the D2 protein of photosystem II has been changed into a tryptophan. Glycine 215 is a neighbour of histidine 214 which is a ligand to the non-heme iron. The growth rate of this mutant in the absence of glucose is very low and the mutant is far more sensitive to photoinhibition than the wild type (Vermaas, W., Charité, J. & Shen, G. 1990, *Z. Naturforsch.* **45c:** 359–365). The initial electron transfer rate to the plastoquinone pool, however, does not seem to be affected (Van der Bolt, F. & Vermaas, W. 1992, *Biochim. et Biophys. Acta* **1098:** 247–254).

To remove this apparent contradiction, electron transfer processes in the mutant were better characterized by studying fluorescence induction kinetics, fluorescence decay kinetics and oxygen evolution, using mainly intact cells.

Chlorophyll and its Distribution in a Grass Canopy Under Global Change Conditions

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Since light is the primary necessity for a plant to grow, we can expect that plants adapt their structure as well as their physiological processes as a function of light, in order to maximize their carbon gain from photosynthesis. Structural parameters, such as leaf area and leaf area distribution, leaf angle, height, etc. are known to be important in the capture of light. Because absorption of visual light is primarily determined by chlorophyllous pigments, it can be assumed that chlorophyll could take part in maximizing the

efficiency of capturing light needed for photosynthesis. Therefore, we analysed the vertical distribution of chlorophyll concentration throughout the canopy.

This study involved two grass species (*Lolium perenne* L. and *Festuca arundinacea* (Schreb.) grown in both monocultures and mixtures under global change conditions (CO_2-concentration: 350–700 ppm; temperature: normal and 4°C above normal). The canopy was divided in horizontal layers of 5 cm each from which leaf material was taken. Chlorophyll was extracted by means of N,N-dimethylformamide. Absorption of this solution was measured at 664 and 647 nm in order to calculate total chlorophyll concentration.

Our measurements show that chlorophyll concentration per unit fresh weight is non-uniformly distributed throughout the canopy. These vertical distribution data can be well fitted by an exponential curve for all treatments.

Chlorophyll concentrations are highest at the top of the canopy and decline with canopy depth. This suggests that the plants have adapted their chlorophyll content to the light extinction in the canopy. In literature a similar adaptation of the vertical distribution is reported for N; it is hypothesized that carbon gain for the whole canopy is maximized in this way.

Unfortunately, differences neither in chlorophyll concentration, nor in its vertical distribution, could be detected between the global change treatments (CO_2–temperature–combination of CO_2 × temperature). The total chlorophyll content per plant differed between monocultures and mixtures, but this was merely the result of differences in biomass since the chlorophyll concentration per unit fresh weight remained the same.

In the near future a comparison of both the distribution of N and chlorophyll will be made.

Can Transgenic Plants be Used to Study Photosynthesis?

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Plant growth and development is controlled by the coordinated action of external (e.g. light, temperature) and internal (e.g. phytohormones) factors. The small phytohormone molecules are involved in the regulation of several physiological processes (Davies, P.J. 1987, *Plant Hormones and Their Roles in Plant Growth and Development*, Martinus Nijhoff, Boston). The use of transgenic plants in which the endogenous phytohormone content can be manipulated offers several advantages over the classical exogenous application techniques. For a review see Romano & Klee, 1993, in Hiatt, A. M. (ed.) *Transgenic Plants*, Dekker N.Y., 23–36). In all these studies, detailed attention is focused on the expression pattern of the genes, on the hormone interactions, on the phenotype and on morphological aspects (light microscopy); less attention is drawn to the underlying biochemical and physiological processes.

Using transgenic plants that contain the *ipt*-gene in chimeric constructions (P*ssu*- or P*hsp-ipt*), we study the effect of an overproduction of endogenous cytokinins on the photosynthetic apparatus. The thylakoid membrane system of the plastids of the P*ssu* transgenic leaves shows a swollen appearance. The *in vitro* capacity of the whole electron transport chain and of the partial reactions is severely affected. The oscillation pattern visible in the chlorophyll fluorescence transients and in the oxygen evolution upon dark-light transition (after a 'wake-up') in wild type plants is not present in transgenic leaves. Quenching analysis indicated that the non-photochemical quenching is particularly affected. This can be correlated with a less tight coupling of the H^+-gradient and a reduced photophosphorylation in the transgenic plant material. Not only are the light reactions of these plants affected, the Calvin cycle is also disturbed. For instance, a severe reduction in the activity of the RuBisCO-enzyme is observed concomitant with a reduction in the amount of the large subunit as is shown in gel electrophoretic analysis.

The transgenic plant material containing the P*hsp-ipt* chimeric construction poses the problem of the activation of the gene by heat treatment. Here it is important to find out the effects of the heat treatment itself on the physiological process under study. It will remain difficult to distinguish exactly the heat effect from the cytokinin effect (see Van Loven *et al.* 1993, *J. Exp. Bot.* **44**: 1671–1678).

The Photosynthetic Electron Transport Chain in Transgenic P*ssu-ipt* Plants: Partial Reactions

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Transgenic tobacco plant material (grafts and rooted plants in soil) with the P*ssu-ipt* chimeric gene construction (light-sensitive promoter coupled to gene 4 from *Agrobacterium tumefaciens*) are studied (Beinsberger, S.E., Clijsters, H.M., Valcke, R.L. & Van Onckelen, H.A. 1992, In: Karssen, C.M., van Loon, L.C. and Vreugdenhil, D. (eds) *Progress in Plant Growth Regulation*. Kluwer Acad. Publ., The Netherlands, pp. 738–745). In the transgenic leaf tissue, the course of chlorophyll fluorescence hardly

shows any oscillation upon dark-light transition. Quenching analysis reveals that non-photochemical quenching in particular is very low. In this study we tried to locate the effects of this P*ssu-ipt* gene on the photosynthetic electron transport in an *in vitro* approach.

The *in vitro* capacity of the whole electron transport chain is reduced by 50% (transgenic rooted plants) to 60–70% (transgenic grafts). This electron transport is less tightly coupled to the H^+-gradient in the transgenic plant material. In the transgenic grafts photophosphorylation (ATP-production) is also shown to be reduced.

Partial reactions of the electron transport around PSI and PSII, measured with different electron acceptors and donors, are severely affected in transgenic plant material. When DBMIB is added, in the grafts only, the electron transfer capacity of the whole chain is increased compared to the capacity in the absence of DBMIB. It is known that DBMIB can act either as inhibitor or as electron acceptor at the cytochrome b_6/f complex, depending on pH and concentration (Rich, P.R., Madgwick, S.A. & Moss, D.A. 1991, *Biochim. Biophys. Acta,* **1058:** 312–328). This could indicate that there is a structural difference in the cytochrome b_6/f complex between the wild type plants and the transgenic grafts. The effect of DBMIB on the re-reduction kinetics of cytochrome f and b might clarify its ambiguous function in transgenic vs. wild type plants.

Electrogenesis and Dielectric Changes in the Photosynthetic Membrane

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The photochemical charge separation in the reaction centres (RC) drives the electron transport in the photosynthetic electron transport chain. The rate at which transport and energy storage occurs is under control of several processes. Energization of the chloroplast thylakoid membrane is proposed to be associated with changes in conformation (in LHCII) and dielectric properties (regulation of the function of cyt. b559) of the membrane.

Recent results have shown that pre-illumination (energization) of the thylakoid membrane causes a temporary decrease of the amplitude of the flash-induced transmembrane electric potential as measured in a single chloroplast of *Peperomia metallica in situ* using microcapillary glass electrodes. This phenomenon has been interpreted as a temporary partial increase of the membrane capacitance. In addition, there was close similarity between the recovery rates after energization of the variable amplitude of the flash-induced potential and the non-photochemical quenching of chlorophyll fluorescence measured on the same plant.

Electrochromic P515-potential measurements show an indication for the existence of a variable fraction of PSII reaction centres with a fast decay rate of about 300 s^{-1}. Pre-illumination of the chloroplasts induces an enhancement of this fraction with a recovery rate after energization similar to the recovery rates of the amplitude of the flash-induced potential and the non-photochemical quenching. The energization-dependent increase in fraction of reaction centres with a fast dark decay, concomitant with the increase in membrane capacitance suggest a special property of these RCs. We speculate that this fraction of RCs exhibit a cyclic electron flow around PSII. It has been suggested that a higher dielectric environment is favourable for cyclic electron flow around PSII with cyt. b559 as electron carrier. This seems to be in accordance with the energization-dependent increase of membrane capacitance. This cycle would then indeed be responsible for part of the non-photochemical quenching.

C. Stress

The Effect of Mycorrhizal Fungi on the Retention of Heavy Metals in a Contaminated Substrate and on the Metal Uptake in their Host Plants

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In the vicinity of the metallurgical industries in the north-eastern part of Belgium large areas have been contaminated with heavy metals, mostly by deposition of ore dust. Although it is technically possible to extract a great deal of these heavy metals from the polluted soils, we believe that this kind of soil restoration is not suited to cleaning the tons of soil that should be treated, in particular because of the high costs. We propose a much cheaper solution which consists of the immobilization of heavy metals in the polluted soil itself by adding metal-adsorbing components. The ultimate goal of such an operation is the restoration of a closed vegetation in the field. A covering of vegetation prevents the dispersion of metal dust by wind, and strongly reduces the percolation of heavy metals to deeper soil levels. Metal-tolerant ecotypes of grasses, such as *Festuca rubra* and *Agrostis capillaris*, are most suitable for sowing on these bare soils. For the second step we recommend the planting of metal-tolerant trees, such as birch (*Betula* sp.) and pine (*Pinus sylvestris*).

Our research is concentrated on the production of metal-tolerant trees. The roots of almost all tree species in our temperate climate zone are obligately infected with ectomycorrhizal fungi. These fungi perform a crucial role in nutrient cycling in forest ecosystems, particularly under conditions of nutrient scarcity, as a mycorrhizal root system works more efficiently in micronutrient mobilization than a non-mycorrhizal root system (Read, D.J. *et al.* 1985, In: Fitter, A.H. (ed.) *Ecological Interactions in Soil.* Blackwell Scientific, Oxford, pp. 193–217).

When micronutrients are present in the soil at high concentrations, enhanced uptake and translocation could be disadvantageous to the host plant. Many investigators, however, have found an emeliorating effect of mycorrhizal infection on the growth of host plants cultivated at high metal concentrations (Denny, H.J. & Wilkins, D.A. 1987, *New Phytol.* **106:** 545–553).

Our work deals with some ecophysiological aspects of the ectomycorrhizal symbiosis in the presence of a soil Zn or Cd contamination (Colpaert, J.V. & Van Assche, J.A. 1992, *Plant and Soil* **143:** 201–211; Colpart, J.V. & Van Assche, J.A. 1993, *New Phytol.* **123:** 325–333). Several ectomycorrhizal fungi with different metal tolerance indices were compared for their protection of pine (*Pinus sylvestris* L.) seedlings against metal toxicity.

We demonstrated that the uptake of zinc in the shoots of seedlings exposed to a chronic sublethal Zn concentration was strongly reduced by the mycorrhizal symbiont of the seedlings. Measurements of the fungal densities in the substrate showed that mycorrhizal fungi which produce a large biomass (dense mycelia) and which have a high metal tolerance index are most efficient in protecting their host; the growth of these fungi is also least affected by the metal additions. This experiment clearly shows that specific mycorrhizal fungi can increase the metal tolerance of their host plants.

In a second experiment a single but high Zn concentration (100 mg l^{-1} substrate) was added to 4-month-old seedlings grown in perlite. Two months later the zinc content in both plant and substrate (mycelium included) was determined. The results show that the metal retention in the substrate is highest in the pots inoculated with *Suillus bovinus*, a mycorrhizal fungus producing a large biomass. In the pots without mycelium (NM plants) most of the zinc had disappeared through percolation and uptake by the seedlings. Denny & Wilkins (1987) have already suggested that the ameliorating effect of mycorrhizal fungi on the uptake of metals is realized by the immobilization of large amounts of metal ions on the extramatrical mycelium. As a consequence, this process also prevents the percolation of heavy metals into deeper soil horizons.

We think that reafforestation with tree seedlings inoculated with selected metal-tolerant mycorrhizal fungi guarantees the survival of the vegetation on metal-polluted soils. Our research demonstrates that the role of mycorrhizal fungi should not be omitted when replantation of polluted soils is considered.

Effects of Metal Ions on the Biosynthesis of the Neurotoxin β-ODAP in Callus Tissue of *Lathyrus sativus*

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The neurotoxin β-ODAP (3-N-oxalyl-L-2,3-diaminopropanoic acid) of *Lathyrus sativus* can be formed in callus tissues by supplying its biosynthetic precursor BIA, β-(isoxazolin-5-on-2-yl)-alanine, in modified B_5 liquid media (Kuo, Y.H. & Lambein, F. 1991, *Phytochemistry* **30:** 3241–3244). The effects of different metal ions on the biosynthesis of β-ODAP in callus were studied by changing the concentration of a particular ion in the media.

The calli were incubated in the required media on a rotating shaker at 75 rpm at 25°C in the dark for 2–3 weeks before adding the purified BIA (0·2 mM as final concentration) with fresh media. After further incubation for 1 week the calli were harvested and analysed by automated amino acid analyser.

In callus of a low toxin variety (LS 90278), the level of β-ODAP was increased when the concentration of Fe^{2+} ions was increased in the media: with a four-fold increase in Fe^{2+} ions, a doubling of the β-ODAP biosynthesis was observed. A more dramatic increase of β-ODAP was observed when a high Fe^{2+} ion concentration was combined with a low Zn^{2+} ion concentration. In callus of a high toxin variety (LS NC8a 17/W), an increased concentration of Fe^{2+} ions also had an increasing effect on the β-ODAP level, while the combination with low zinc did not further increase the β-ODAP level as in the low toxin variety. In general, an increase of Zn^{2+} concentration lowers the rate of biosynthesis of β-ODAP, similar to the effect of Cu^{2+}. Other metal ions (including Mn, B, Co, Mo) were tested under similar conditions but showed little effect on the β-ODAP formation.

These results suggest that Fe^{2+} and Zn^{2+} ions can modulate the biosynthesis of β-ODAP.

Perception and Transduction of Stress Signals from the Environment

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In many cases plant hormones are involved in the reactions of plants upon exposure to a less favourable environment. Abscisic acid often shows an increased level, while the contents of gibberellins and cytokinins are decreased.

As an example of the perception and transduction of stress signals from the environment by plants, attention will focus on the direct and indirect responses of plants to CO_2 and light intensity as far as photosynthesis, transpiration, stomatal reactions, growth and the plasma membrane bound H^+ ATPase, the so-called proton pump, are involved.

The classical responses of the effects of light intensity and CO_2 on photosynthesis of plants are well known, as well as the effects of light intensity and CO_2 on the transpiration and stomata. Growth of many plants is affected by the level of atmospheric CO_2 and climate room experiments with *Plantago major* reveal that the relative growth rate quickly adjusts to its original time course at normal CO_2, by inhibition of leaf expansion under elevated CO_2. The effect of light on stomatal opening, light-stimulated leaf growth and on the light-stimulated plasma membrane proton pump is illustrated by action spectra; all spectra show a strong response in the blue region and a weaker response in the red region. Proton pumps are involved in stomatal opening as well as leaf growth ('acid growth'). The hypothesis is put forward that in several cases there is a sort of competition for products of the photosynthetic light reaction between CO_2 fixation by the chloroplasts and the proton pump involved in stomatal opening and leaf growth; at elevated CO_2 carbon fixation evidently has preference over the proton pump, resulting in increased photosynthesis and reduced transpiration and leaf growth. The interactions between CO_2 and light intensity on proton pump activity and its regulation by the redox state of the plant are briefly discussed.

Engineering Oxidative Stress Tolerance in Tobacco

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The photosynthetic electron transport chain in higher plant chloroplasts contains at the acceptor end of photosystem 1 a number of autoxidizable (Fe-containing) enzymes, which in the reduced state can react with oxygen, yielding the superoxide anion radical (O_2). The superoxide is detoxified in the chloroplasts by the so-called ascorbate-glutathione cycle. The enzymes participating in this cycle are Superoxide Dismutase (SOD), Ascorbate Peroxidase (APx), Dehydro-Ascorbate Reductase (DHAR), Mono-Dehydro-Ascorbate Reductase (MDHAR) and Glutathione Reductase (GR). SOD catalyses the dismutation of two molecules of superoxide into oxygen plus hydrogen peroxide. APx reduces hydrogen peroxide to water, with ascorbate as electron donor. The remaining enzymes serve to regenerate the ascorbate, with NADPH as the final electron donor. The end result is 'pseudo-cyclic electron flow' involving both photosystems, but with no net oxygen evolution. On the one hand, this allows linear photosynthetic electron transport from water to NADP to proceed under adverse conditions. This reduces the risk of over-reduction of the photosystem 2 electron acceptors, which might otherwise lead to formation of singlet oxygen at or near the reaction centre of photosystem 2. Moreover, pseudo-cyclic electron flow seems to be required for the maintenance of an ATP/NADPH ratio which is sufficiently high to support carbon assimilation under favourable conditions (Asada, K. & Takahashi, M. 1987, In: Kyle, D.J., Osmond, C.B. and Arntzen, C.J. (eds) *Photoinhibition*, pp. 227–287, Elsevier Science Publ. Amsterdam).

On the other hand, the ascorbate–glutathione cycle is itself at risk to produce the hydroxyl radical (OH) from superoxide and hydrogen peroxide, in what is known as a metal-catalysed Haber-Weiss reaction (Hosein, B. & Palmer, G. 1983, *Biochim. Biophys. Acta* **723:** 383–390). The hydroxyl radical can initiate self-propagating reactions leading to the destruction of membrane lipids and of DNA. The conditions leading to this type of damage will be referred to as oxidative stress.

Here we report on attempts to improve the oxidative stress resistance of tobacco (*Nicotiana tabacum* var. PBD6 and SR1). To the end, genes encoding for enzymes operating in (or presumably capable of operating in) the ascorbate–glutathione cycle are incorporated into the nuclear genome of the target plant. If required, the genes are coupled to nucleotide sequences coding for leader peptides which direct the gene product (preprotein) to the mitochondria or to the chloroplasts.

The enzymes brought to overexpression so far include Mn-SOD (Bowler, C., Slooten, L., Vandenbrandon, S., De Rycke, R., Botterman, J., Sybesma, C., Van Montagu, M. & Inzé, D. 1991, *EMBO J.* **10:** 1723–1732), Fe-SOD and APx. (SODs are classified according to their metal cofactor as

Mn-, Fe- or Cu/Zn-SOD). The effect of over-expression of these enzymes on the oxidative stress tolerance was investigated. To this end we used an *in vitro* system in which leaf disks floating on aqueous solutions of Methyl Viologen (MV) were illuminated for 2 h. In the light, MV accepts electrons from photosystem 1, after which it reacts with oxygen to yield superoxide. The resulting oxygen radical damage was estimated from ion leakage out of the leaf disks (due to destruction of membrane lipids), and from the decrease in activity of the reaction centre of photosystem 2 (as determined from the decrease in variable chlorophyll fluorescence). Alternatively, leaf disks floating on water were given a photo-inhibitory treatment, with either weak or strong light at 4°C, and the damage was estimated from the decrease in the activity of photosystem 2, as above.

The results obtained so far indicate that PBD6 or SR1 plants over-expressing *MnSOD* in the chloroplasts are somewhat more resistant to MV that control plants, but only so after growth at high light intensities. PBD6 plants over-expressing MnSOD in the mitochondria are not more resistant than control plants. SR1 plants over-expressing *FeSOD* in the chloroplasts are more resistant to MV than control plants, but not more resistant to photo-inhibition than control plants. SR1 plants over-expressing *APx* in the chloroplasts are not more resistant to MV than control plants (at least after growth at low light intensities). The results are in broad agreement with current understanding of the function of these enzymes, and indicate that gene engineering can increase the resistance of the plants to oxidative stress, at least in model systems. However transgenic Fe-SOD does not confer resistance to photoinhibition of photosystem 2 activity.

The Role of Oxidative Stress in Longevity of Cut Tulip and Iris

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Lipoxygenase (LOX) is supposed to play a major role in the membrane deterioration underlying senescence in carnation. Besides the naturally occurring 'programmed' senescence, stress conditions during flowering could affect the longevity of flowers by the formation of oxygen radicals. This also results in the loss of membrane integrity by lipid peroxidation, a process called ageing. Certain enzymes, such as superoxide dismutase (SOD) can protect the cell from these harmful molecules and thus retard ageing.

In order to investigate whether membrane-deteriorating enzymes such as LOX and protective enzymes like SOD are also involved in the ageing of other flowers besides carnation, we studied the course of the LOX and SOD activities during the vase life of tulip (*Tulipa gesneriana* cv. Inzell) and iris (*Iris hollandica* cv. White Bridge). Also products of lipid peroxidation (TBA-reactive compounds and fluorescent lipofuscin-like compounds) were measured. Membrane integrity was measured as potassium leakage from flowers and stems.

LOX activity in tulip was about 15 times higher than in iris. SOD activities however, were not significantly different. Potassium leakage (as % of total potassium) was much higher in iris than in tulip. Surprisingly, both in iris and in tulip, a decrease instead of the expected increase of peroxidation products (TBA-reactive compounds and fluorescent lipofuscin-like compounds) during ageing was observed.

Addition of 100 μM cycloheximide (CHX) to the vase water was used as a tool to study the role of enzyme turnover on the deleterious or beneficial systems. Normally, the longevity of cut tulip and iris amounts to about 5 days. This was enhanced in iris by about 5 days after treatment with CHX, while in tulip longevity decreased severely. LOX activity was not significantly affected by CHX in either of the two species, while SOD activity in iris was somewhat higher after CHX treatment. Potassium leakage was less after CHX treatment in both species.

LOX activity alone is probably not responsible for flower ageing. It is tempting to suggest that the observed increase in SOD activity after CHX treatment in iris results in a longer vase life.

It is still unclear why iris and tulip flowers do not show an accumulation of TBA-reactive and fluorescent lipofuscin-like compounds as observed in many other ageing tissues.

D. Carbohydrate Metabolism and Partitioning

Dry Matter Remobilization During Grain Filling in Common and Spelt Wheat: Effect of Irrigation and Simulated Drought (Chemical Desiccation)

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Current photosynthesis and remobilization of reserves contribute to grain filling after anthesis. The

contribution of reserves stored before anthesis is small but may become more important when plants are subjected to post-anthesis stress (Kiniry, J.R. 1993, *Agron. J.* **85**: 844–849). On the other hand, absence of stress before anthesis should increase the amount of reserves and possibly their contribution to grain filling.

Two field studies were conducted to assess these hypotheses. In Experiment 1, four spring wheat cultivars (*Triticum aestivum* L.) were grown in a sandy-loam soil (semi-arid region, Argentina). Irrigation (80 mm) was applied weekly from seedling to anthesis whereas the control was only rainfed. In Experiment 2, two winter wheats and two spelt wheat cultivars (*T. spelta* L.) were grown in a silty-loam soil (south of Belgium) under non-stress conditions but to simulate the destruction of foliage by post anthesis drought they were sprayed once with chemical dessicant (sodium chlorate) 15 days after anthesis (Sabry, S.R.S. & Taylor, G.A. 1992, *Cer. Res. Com.* **20**: 221–224).

Total dry matter at anthesis (W_a), at final harvest (W_h) and final grain dry weight (G_w) of the main shoot (g per main shoot) were determined on three (Exp. 1) or five (Exp. 2) main shoots per plot.

The translocation was assessed by DMT= W_a–(W_h–G_w), and contribution of pre-anthesis assimilates to grain by C=(DMT/G_w)*10 0 (Kiniry 1993).

In Exp. 1, no significant effect on DMT or C could be shown despite large differences (DMT:0·61 and 0·34 g; C:45 and 28%; irrigated and rainfed, respectively) because coefficients of variation (CV) were high (45·6 and 51·4%, DMT and C, respectively).

In Exp. 2, chemical desiccation reduced significantly DMT (1·26 and 0·96 g; $LSD_{0\cdot05}$=0·17) and grain yield (2·20 and 1·44 g; $LSD_{0\cdot05}$=0·12); control and desiccated, respectively. Consequently, C was similar in control and treated plants (57·8 and 66·5%; CV: 24·6%). Spelt wheat Rouquin showed a significantly lower value of DMT and C (0·69 g and 40·5%, respectively) than the other cultivars.

Our results do not confirm that higher soil water level prior to anthesis or total destruction of photosynthetic system after anthesis, were effective to increase remobilization toward the kernels.

Membrane Transport of Sucrose and Amino Acids in the Seed Coat of Developing Pea Seeds

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Membrane transport of sucrose and amino acids was studied in isolated seed coats of developing pea seeds by measuring the uptake of ^{14}C-labelled sucrose and L-valine. The initial uptake rate of both sucrose and valine was proportional to the external sucrose and valine concentration, indicating that the uptake is solely mediated by a linear uptake system. In the presence of the protonophore CCCP the initial uptake rate of sucrose was not affected but in the case of valine it was reduced by 18%. However, at an external concentration of 100 mM there was no effect of CCCP on the initial uptake rate of valine. These results suggest that uptake of sucrose and valine is not energy-dependent. The non-penetrating sulphydryl group modifier PCMBS reduced the initial uptake rate of sucrose and valine by 40% and 60%, respectively, suggesting that carrier-mediated transport might be involved. Surprisingly, at low substrate concentrations (less than 1 μM) ^{14}C-label had accumulated two-fold after 6 hours . However, when the substrate concentration was high (10 mM or higher) no accumulation occurred after 6 hours. We suggest that at external substrate concentrations lower than 10 mM, accumulation might be explained by metabolism or compartmentation of sucrose and valine. Taken together, these results suggest that transport of sucrose and amino acids in seed coats of developing pea seeds occurs by (facilitated) diffusion, which is in fair agreement with our earlier results obtained by measuring the release of endogenous sucrose and amino acids from the seed coat.

Carbohydrate Metabolism and Energy Economy of Embryogenic Cell Cultures of *Daucus carota*, Measured by Means of *in vivo* Nuclear Magnetic Resonance

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Research was carried out on the first phase of somatic embryogenesis, the formation of proembryogenic masses (PEMs), in carrot (*Daucus carota*) cell suspension cultures. It was found that ^{13}C-label from [1-^{13}C]glucose was transferred to [6-^{13}C]hexoses in embryogenic cultures with a low amount of proembryogenic masses (<20%). This exchange does not occur in cultures with a high amount of PEMs (>80%) (Dijkema, unpublished results).

Because these differences were considered to be due to the different amounts of PEMs in the cultures, and because no differences in sugar content could be found between an embryogenic cell culture with

about 20% PEMs and a non-embryogenic cell line with less PEMs, it was decided that the different cell types should be analysed separately. Sugars and key enzymes will be measured in the cells after separation.

Preliminary results showed that PEMs do convert more glucose and fructose into sucrose compared with the vacuolated cells. Furthermore, PEMs contain more starch than the vacuolated cells, which is not surprising since starch and sucrose may be synthesized from the same pool of intermediates.

The C1–C6-exchange is considered to occur at the level of triose-phosphates in glycolysis. The question which has to be answered now, is whether the lower C1–C6-exchange in cell lines with a high amount of PEMs is caused by higher activity of glycolysis, less gluconeogenetic activity or a higher activity of the pentose phosphate pathway, leading to lower availability of metabolites for hexose synthesis and thus C1–C6-exchange. Which factors regulate the activity of sucrose and starch synthesis will be the subject of future research. One of the points of interest will be (the regulation of) the enzyme systems phosphofructokinase and pyrophosphate:fructose-6-phosphate phosphotransferase.

Purification and Properties of an Acidic Invertase from the Leaves of Witloof (*Cichorium intybus*)

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Growth and development of 'witloof' during forcing depends on the import of sucrose from the root since growth occurs under etiolating conditions. Therefore, invertases could be an important factor in sink development and strength. An acidic invertase was partially purified from the etiolated leaves of *Cichorium intybus* and some of its properties were investigated.

A combination of ammonium sulphate precipitation, hydrophobic interaction chromatography on a phenyl-sepharose column, affinity chromatography on a Concanavalin A column and chromatofocusing on a mono P column yielded a 653-fold purification with a recovery of 17·5%. The enzyme had a Mr of 60 000 as estimated by gel filtration (superose 12) and after SDS-PAGE a band with a Mr of 50 000 was correlated with enzyme activity in different fractions of the superose 12 column. The IEP of the enzyme was 5·5.

The pH optimum of the enzyme was 5·5 in acetate buffer. This property, together with its glycosylated nature (retention on a Concanavalin A column), suggest a vacuolar localization. The enzyme had a Km of 20 mM for sucrose. The activity is not affected by EDTA or divalent cations such as Ca^{2+}, Mg^{2+} or Mn^{2+}.

Unlike the acidic invertase purified from the roots which also produces 1-kestose (Van den Ende & Van Laere, 1993, *New Phytol.* **123:** 31–37) no products other than equimolar amounts of fructose and glucose could be detected.

Construction Costs and Payback Time of Biomass: A Whole Plant Perspective

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A compilation of literature data on the construction costs of plant biomass, the amount of glucose required to construct 1 g of biomass, was made. No evidence was found for more than a slight variation in construction costs. Leaves of herbaceous species are somewhat more expensive to produce (on average 1·56 g glucose per g DW) than stems (1·44 g g^{-1}) or roots (1·34 g g^{-1}). Variation due to environmental differences is small or absent (less than 10%), and the same applies to differences in construction costs between ecologically contrasting groups of species. However, up to now, most research in this areas has focused on leaves. Differences between woody and herbaceous species may become apparent when construction costs of whole plants are considered.

It has been suggested that the payback time of leaves, i.e. the time a leaf requires to meet its own construction costs, rather than the construction costs themselves vary for ecologically contrasting species. Although correct as a statement on itself, such an approach ignores the relevance of stem and roots for the functioning of a leaf. From a whole plant perspective, payback time is identical to the relative growth rate of a plant. As such, this concept is closely related to part of Grime's plant strategy theory, where relative growth rate is considered to be one of the differentiating key factors. For a full account see Poorter, H., pp. 105–121 in Roy, J. & Garnier, E. (eds) *A Whole Plant Perspective on Carbon–Nitrogen Interactions*. SPB Academic Publishing, The Hague.

Effects of CO_2 and Temperature on Growth and Biomass Partitioning of Onion

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Onions (*Allium cepa* var. Barletta) were planted on 18 May 1993 in greenhouses on the UIA-campus and

grown under four different treatments: (1) (control) ambient temperature and 350 ppm CO_2; (2) 4°C above ambient temperature and 350 ppm CO_2; (3) ambient temperature and 700 ppm CO_2; (4) 4°C above ambient temperature and 700 ppm CO_2. Total biomass at 62 days after planting was not affected by temperature, but there was a positive effect of CO_2. The interaction between CO_2 and temperature was significant. The high CO_2 and temperature treatment induced a 40% increase in total biomass. The diameter of the bulbs grown in high CO_2 high temperature was increased by 20% as compared to control plants. However, neither high CO_2 nor temperature alone had a significant effect on this parameter. Root/shoot ratio was significantly affected by CO_2 and by temperature, but there was no interaction between both treatment parameters. Harvest Index was not affected by CO_2 nor by temperature.

Leaf Area, Leaf Growth and Underlying Characteristics of Poplar Under Elevated Atmospheric CO_2

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Leaf area index, leaf growth rate, total leaf number, specific leaf area, stomatal density and epidermal cell number were measured on two poplar clones, i.e. Beaupre (*P. trich.* × *P. delt.*) and Robusta (*P. delt.* × *P. nigra*) grown under two atmospheric CO_2 treatments (elevated CO_2: *c.* 700 ppmV and ambient CO_2: *c.* 350 ppmV) in four open-top chambers during the 1993 growing season.

Higher leaf area indices (LAI) were observed in the elevated CO_2 treatment for both clones throughout the growing season. The relative difference in the maximum LAI between the two CO_2 treatments was much larger for Beaupre (22·3%) than for Robusta (8·4%). A similar result was found for the average leaf growth rate (LGR) over the time period between 10.00 h and 16.00 h. The values of LGR for Beaupre and Robusta were 7·66 and 2·83 mm day^{-1} in the elevated CO_2 treatment and 6·47 and 2·65 mm day^{-1} in the ambient CO_2 treatment. Apparently, the total amount of leaves produced per tree was not affected by the CO_2 treatments for both clones.

Specific leaf area decreased under the elevated atmospheric CO_2, indicating that leaves became heavier. When plants were divided into three strata (top, middle and bottom), the specific leaf area in the middle stratum was always the smallest for both CO_2 treatments. In the top stratum (young leaves) specific leaf area was larger than in the bottom stratum (old leaves) for clone Beaupre, but in the case of clone Robusta it was just the reverse.

Stomatal density decreased both at the adaxial and abaxial leaf surface under elevated CO_2 conditions. This was the case for both clones Beaupre and Robusta, and was also in agreement with earlier observations of Woodward (1987, *Nature* **327**: 617–618). The number of epidermal cells at the adaxial leaf surface decreased, while that at the abaxial leaf surface increased under the elevated CO_2 conditions.

The Pattern of Fructans in the Roots of *Cichorium intybus* L. During Growth in the Field

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During the 1992 growing season, samples from field-grown witloof roots were analysed twice weekly between 3 August and 13 November. Roots were homogenized and fructans were extracted in boiling water. After centrifugation, the supernantant was passed through cation and anion exchange resins to obtain a neutral fraction containing the carbohydrates.

Sugars and fructans were separated by ion exchange HPLC on a Dionex apparatus using a gradient of Na-acetate in 90 mM NaOH. Carbohydrates were detected by pulsed amperometric detection and quantitated by integration of peak areas using the Dionex chromatography software.

The data show that, after an initial increase in large DP fructans during early August, sugar and fructan contents remain roughly constant till late in the growing season (end of September). However, in early October, dramatic changes occurred in a very short period. These changes included: (1) an increase in fructose content; (2) an increase in the content of fructans with a low DP; (3) a decrease in fructans with a high DP; (4) the appearance of alternative peaks probably representing fructans without terminal glucose; (5) a less dramatic increase in sucrose and decrease in glucose content.

These changes suggest drastic metabolic changes in the roots which are probably induced by climatic factors such as day-length or temperature. An inspection for meteorological data shows that the period of rapid changes coincides with a number of consecutive cold nights with temperatures at ground level below 5°C. Whether these low temperatures are the main or only inducing factor remains to be investigated.

Purification and Properties of a Neutral Invertase from the Roots of *Cichorium intybus* L.

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Remobilization of fructans is an important process for the forcing of chicory roots since the development of the etiolated 'witloof' totally depends on stored carbohydrates. Therefore, invertase (β-fructosidase) activity was investigated in roots stored at 1°C. Several neutral invertases were detected. One of them with the highest activity was further purified and characterized.

A combination of ammonium sulphate precipitation, hydrophobic interaction chromatography and anion exchange chromatography (pH 7·5) yielded a purification of 105-fold with a yield of 11·3%. Rechromatography on monoQ at pH 8·6 and gel filtration yielded electrophoretically pure protein without further increase in specific activity.

After anion exchange chromatography, three other peaks with invertase activity were detected close to the main protein and activity peak, suggesting the existence of at least four iso-enzymes. No difference was detected between the different iso-enzymes regarding their pH optimum, their K_m for sucrose (20 mM) and their M_m as estimated by gel filtration (300 000) or SDS-PAGE (85 000). Since the protein was not retained on a ConA-column (non-glycosylated protein) and had a neutral pH optimum, its localization is probably cytoplasmic.

With sucrose as substrate, no other products than equimolar amounts of glucose and fructose could be detected. The activity was not affected by EDTA or divalent cations such as Ca^{2+}, Mg^{2+} or Mn^{2+}. Fructose proved to be a strong inhibitor of the activity (K_i=20 mM) pointing towards a possible feedback mechanism *in vivo*. Mannose (K_i=200 mM) or ethylene glycol ($K_i \approx 2$ M) were only inhibitory in high concentrations.

Photosynthetic Efficiency and Respiration Rates in Intact and Cut Madelon Flowers

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'Madelon' roses were cultivated in growth chambers or obtained from a local grower. Branches of growth chamber grown roses were either allowed to flower on the roots or cut off and placed under high light (>60 w m^{-2}: HL) or low light (3 w m^{-2}: LL) conditions. Fresh and dry weight, photosynthesis and dark respiration in various plant parts were determined during flower bud opening. The photosynthetic rate of leaves of intact plants and of cut flowers remains unaltered under HL conditions. Under LL conditions no net photosynthesis occurs in cut flowers; however, the amount of dry matter in the flower increases, and the bud opening is successful, showing that reserves from other parts than the flower are supplying the demands of the sinks. Successful flower bud opening is probably determined by the accumulation of dry matter in the flower during bud opening.

To estimate sink demands, respiration rates of various plant parts were measured during flower bud opening. Respiration rates of leaves and the flower bud are dependent on age and on light conditions. Photosynthetic efficiency of the leaves displays variations seemingly dependent on leaf age.

Carbohydrate Metabolism During Early Phases of Tuber Formation

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Single-node cuttings from short-day *Solarium tuberosum*, cv 'Bintje' plants were grown *in vitro* on a highly inductive medium (Hendriks *et al.* 1991, *Plant Mol. Biol.* 17: 385–394). Visible tuberization of the axillary buds occurred and were synchronized after 5–6 days of culture. 100% of the cuttings formed tubers.

Levels of mRNA for various enzymes involved in carbohydrate metabolism and starch biosynthesis were analysed by northern blotting. The mRNAs encoding for the different enzymes were not or hardly detectable in the first 3 days; transcript levels of sucrose synthase and ADPG-pyrophosphorylase B and S, granule-bound starch synthase and branching enzyme appeared at day 3 or 4, reached a maximum at day 5 and remained constant for several days. The mRNA levels of sucrose synthase and ADPG-pyrophosphorylase B decreased to a lower level at day 10.

The activities of three enzymes were determined and confirmed the RNA expression results. ADPG-pyrophosphorylase reached a maximum enzyme activity at day 4, but sucrose synthase and branching enzyme activity was only detectable after visible swelling.

The levels of soluble sugars and starch were also determined. No significant change in the level of

sucrose was observed coinciding with tuber formation. The levels of glucose and fructose rapidly decreased, during or just prior to tuber formation. Similar changes have been observed during tuberization of stolons in intact *S. demissum* and *S. tuberosum* plants. Elevated starch content of the axillary buds was already detectable from day 1 on, starch levels increased further after 5 days.

These data show that the *in vitro* system is useful to study changes at the molecular and metabolic level associated with the onset of tuber formation.

E. Hormones

Accumulation of Abscisic Acid and Drought Resistance of Wheat. Inhibitory Effect of Fluridone

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We analysed the accumulation, in wheat leaves, of endogenous ABA resulting from two types of water stress: (1) dehydration of excised leaves previously treated, or not, with fluridone, until water potential reached the value of -2 MPa; (2) water stress (-2 Mpa) applied to the whole plant, treated or not with fluridone. Three wheat varieties: Sabine, 1723 and Siete Cerros, respectively sensitive, tolerant and avoidant to drought, were used.

Fluridone partially inhibited the endogenous ABA accumulation. The most important response of stressed plants to fluridone treatment consisted of the increase of stomatal conductance (G_s) and transpiration (E). The most significant differences were observed in the case of Siete Cerros, suggesting that the limitations of G_s and E in water stress conditions are linked to the quantity of accumulated ABA.

The G_s increase, induced by fluridone, entailed an increase of the intercellular CO_2 concentration (C_i) in Siete Cerros and a slighter one in 1723. No effect of the G_s increase on C_i was found in Sabine.

Fluridone treatment decreased the water stress-stomatal limitation of photosynthesis (L_s) from 15·2% to 9·9% (-35%) in Sabine, whereas photosynthesis rate (A) at ambient CO_2 concentration (350 ppm) increased 58%. By comparison with Sabine, the L_s reduction in Siete Cerros, due to fluridone, was largely enhanced (60%), whereas photosynthesis rate only increased 16·5%. This could explain the increase of C_i due to fluridone in this variety.

For 1723, a very slight decrease of the photosynthetic activity, due to the fluridone treatment, induced an increase of C_i from 222 to 272 ppm.

These results indicate a negative correlation between ABA accumulation and drought resistance of wheat. Because fluridone is an imperfect ABA synthesis inhibitor, implications of endogenous ABA in drought response of wheat can be elicited by external application of appropriate concentrations of this hormone.

Effect of ABA and Water-stress on Laser-induced Chlorophyll Fluorescence in Wheat

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The effect of water stress (-2 MPa) and exogenous application of ABA (10 mg l^{-1}) on chlorophyll fluorescence were analysed at 690 and 730 nm, using three wheat varieties: Sabine, 1723 and Siete Cerros, respectively sensitive, tolerant and avoidant to drought. The following parameters were measured: (1) the vitality index (Rfd)$=F_d/F_s$, an evaluation of the rate of photosynthetic quantum conversion. F_d is the fluorescence decrease from the maximum fluorescence (F_m) to the steady-state fluorescence (F_s); (2) the adaptation index (Ap)$=1-((1+\mathrm{Rfd}_{730})/(1+\mathrm{Rfd}_{690}))$, an assessment of the adaptation to the applied light conditions; (3) the ratio $F_{m690}/F_{m730}=(F_{d690}+F_{s690})/F_{d730}+F_{s730})$, an indicator of chlorophyll content.

The decrease of the vitality index induced by water stress was very important in the sensitive variety Sabine (-35% and -29% respectively at 690 and 730 nm). This index was relatively insensitive to water stress in the 1723 variety. Siete Cerros was more affected by ABA than Sabine with respect to the Rfd and Ap values. Indeed, the decrease of vitality index induced by ABA is 52% and 47% respectively at the wavelength regions 690 and 730 nm. Rfd was less affected by ABA in the Sabine and 1723 varieties (-30%).

The adaptation index seems to be correlated with the level of varietal drought resistance. Indeed, the Ap of the sensitive variety Sabine was more affected by water stress (-39%) than the Siete Cerros and 1723 varieties (-22% and -11% respectively). The decrease of the adaptation index induced by ABA was very important in Siete Cerros (-50%) whereas no significant variation was observed in the 1723 variety. The Ap reduction induced by ABA in Sabine was also very significant (-40%).

The fact that water stress (-2 MPa) did not seem to have any significant effect on the F_{m690}/F_{m730} ratio suggests that it had no effect on chlorophyll content. ABA significantly increased this ratio in Siete Cerros but not in the Sabine and 1723 varieties.

This study indicates that the chlorophyll fluorescence induction kinetics (*Rfd* values) and the chlorophyll-fluorescence emission spectra (F_{m690}/F_{m730} ratio) as well as the adaptation index (*Ap*) are affected by water stress or ABA. The intensity of the effects of these two factors and their similarity depend on the level and on the type of varietal drought resistance in wheat.

Gibberellins and the Cold Requirement of Tulip

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Tulip bulbs, with terminal buds containing a complete flower, require a period of low temperature for floral stalk elongation and adequate flowering. In these processes the involvement of gibberellins (GAs) has been implicated. To investigate their role in tulip, research is directed towards qualitative and quantitative analyses of endogenous GAs and the biological activity and metabolism of applied GAs.

At first, an inventory was made of free GAs in sprouts of cooled and uncooled bulbs (*Tulipa gesneriana* L. cv. Apeldoorn). By combined gas chromatography-mass spectrometry (GC-MS) and GC-selected ion monitoring (SIM), GA_4, GA_9, GA_{12}, GA_{24}, GA_{34} and three GA-related compounds were detected. During the dry storage the amount of GA_4 increased from less than 1 to 6 ng sprout^{-1}, compared to bulbs at the beginning of the treatment in October. This increased occurred during the cold as well as during the non-cold treatment.

The biological activity of GA_4 and GA_9 was tested by applying them *in vitro* to sprouts of uncooled and cooled bulbs, in combination with paclobutrazol and prohexadione (BX-112). The isolated sprouts did not flower without the addition of GA. GA_4 was more effective than GA_9 in stimulating both flowering and stem elongation. BX-112 inhibited GA_9-stimulated flowering whereas GA_4-stimulated flowering was not affected. BX-112 had no effect on stem elongation. Paclobutrazol inhibited stem elongation and this effect was reversed by simultaneous application of GA_4 or GA_9. The results suggest that GA-synthesis is involved in the stem elongation of tulip and that the critical feature for either the cold-induced flowering or stem elongation might be the 3β-hydroxylation of GA_9 to GA_4.

Comparison of Two Variants of ELISA for Indole Acetic Acid

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Two basic types of ELISA for phytohormones can be distinguished based on competition between immunoreagents. Principally they differ in that the solid phase is coated either with antibodies (AbSP assay) or with antigen (AgSP assay). The comparison of these two types of immunoassay based on literature data is difficult because of the use of different immunoreagents. The aim of the research was to compare basic characteristics of two variants of immunoassay for auxins using the same rabbit polyclonal antiserum raised against IAA. Alkaline phosphatase was used for labelling of IAA, and immunoglobulins (Ig) from antiIAA serum were recovered using protein A-sepharose column (for AbSP assay). Antibodies to rabbit Ig were labelled with horse radish peroxidase (for AgSP assay).

The use of 'maxisorp' plates from Nunc (Denmark) was shown to provide equal detection limit (50 pg per 0·1 ml) for both systems. CV was about 5% and 8% for AbSP assay and AgSP assay, respectively. This might be explained by the presence of an additional step in the variant with plate-bound Ag. Substitution of these plates for that from Enisey (Russia) or Limbro (UK) did not affect substantially the characteristics of AgSP assay but resulted in a sharp decrease in the response and sensitivity of AbSP assay. The last effect is explained by the low binding capacity of Limbro plates. The binding capacity of Enisey plates was equal to that of Nunc. The low sensitivity of the AbSP assay might be attributed to inappropriate orientation or partial denaturation of Ig resulting from their binding to the Enisey plate wells. Low-plate binding capacity was shown to lead to no essential consequences in the AgSP assay since ovalbumin conjugated IAA provided a comparable quantity of binding sites ($5–8 \times 10^{10}$ per well) when a concentration 200–300 times lower than that of Ig was used. This result might be explained by the fact that one molecule of ovalbumin contained 10 Ab binding sites, whereas Ig molecule has only two Ag binding sites. Moreover, the molecular weight of ovalbumin is 3·5 times lower than that of Ig and specific antibodies comprised only 10% of the total amount of Ig. Estimation of the immunoassay specificity showed that crossreactivity of molecules related to IAA was 11% for indole butyric acid (IBA), 4·5% for indole propionic acid (IPA), 1·1% for 2,4 D, 0·9% for indole acetonitrile (IAN) and 0·1% for indole acetaldehyde (IAAl) in the case of the AbSP assay. Cross-reactivity in AgSP assay was shown to be dependent on antiIAA serum

dilution. It was 16% and 2% for IBA, 3% and 2·7% for IPA, 3% and 0·5% for 2,4 D, 1·7% and 0·8% for IAN, 0·3% and 0·05% for IAAl in case of 1:1000 and 1:50, respectively. The possibility of changing immunoassay specificity necessary for solving different problems is discussed. The increase of specificity allows ELISA assay of more crude hormone extract, whereas its decrease might be useful for screening of synthetic hormone analogs.

Factors Affecting the Immunoaffinity Chromatography of Cytokinins

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During the last years immunoassay has become a widely used method for the determination of the phytohormone contents. However, it was shown to result in overestimation in crude impure samples. The necessity of intensive sample purification prior to immuno- or physicochemical assays lead to another type of antibodies application, i.e. immunoaffinity chromatography. However, components providing interference in immunoassay might also affect binding of hormones to the immunoaffinity carrier.

A component capable of affecting the results of the cytokinins immunoassay was discovered in culture media of a cytokinin-producing bacterium (BCM) as appeared from a dilution test. In the process of immunoaffinity purification more than 90% of the cytokinins were shown not to be retained on the carrier with antibodies against zeatin riboside when processed in a mixture with BCM.

Differentiation between immunoreactive compounds having specificity to antibodies and compounds providing non-specific interference in immunoassay (CNI) was accomplished by the use of an immunotest for haemagglutinin of influenza virus. CNI was shown to be a non-dialysable substance capable of binding to rabbit immunoglobulins. The complex was dissociated by 0·5 M NaCl. A protein was also discovered in BCM capable of more strong binding to rabbit Ig. It was washed from the affinity carrier with 70% ethanol only. However, it did not influence the results of immunoassay in contrast to CNI. This is likely to be explained by a difference in binding sites for this protein and CNI on the antibody molecule. BCM heating at 60°C for 20 min and 80°C for 10 min resulted in inactivation of CNI. However, some other components leading to the decrease of the quantity of cytokinins immunoassayed after heating were discovered in BCM. Consequently, heating cannot be used for inactivation of CNI prior to immunoaffinity purification of cytokinins or their immunoassay.

Cytokinins purified from BCM by butanol partitioning or chromatography on a column with preimmune rabbit antibodies were shown to be free of components preventing their binding to the immunoaffinity carrier. However, the quantity of cytokinins immunoassayed in samples purified by solvent partitioning alone does not provide a correct estimation as appeared from an ELISA dilution control.

Combination of partitioning against butanol or chromatography on a column with preimmune antibodies and immunoaffinity chromatography lead to satisfactory results and resulted in a more reliable method for the determination of cytokinins.

Regulatory Aspects of Cytokinins on Chloroplast Adenylyl Cyclase

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In higher plants an important role in 3′,5′-cyclic adenosine monophosphate (cAMP) metabolism can be ascribed to the chloroplast. Both adenylyl cyclase (AC) and cAMP phosphodiesterase (cAMP-PDE) activity have been localized in the chloroplast. Using an accurate AC assay and a cAMP purification method based upon highly specific cAMP antibodies in immunoaffinity columns, AC activity was measured in chloroplasts obtained from *Nicotiana tabacum* cv. Petit Havana SR1 leaves.

In order to investigate the influence of both isoprenoid and aromatic cytokinins (CK) on the *in vitro* AC activity, we administered the phytohormones zeatin, zeatinriboside, benzylaminopurin and benzylaminopurineriboside in a micromolar to picomolar concentration range. At very low concentrations (10 pM) all of the assayed CKs have an inhibitory effect upon the AC activity. At a hundredfold higher concentration (1–10 nM) these CKs have a stimulatory effect upon the AC activity. In this region (pM to nM) there seems to be no significant distinction between the different CK types. At higher concentrations (100 nM to 10 μM) a similar inhibitory and stimulatory effect appears. But in this region the effect upon the enzyme activity depends on whether a free base or a riboside type CK is used. In this concentration region the same effect can be mimicked when adenine or adenosine is used, suggesting that it is not a real CK effect but related to known

antagonistic effects of adenosine analogues upon AC activity in animal systems.

The observation that CKs influenced this AC activity in a regulatory way could be indicative for a link between cAMP metabolism and the known role played by CKs in chloroplast physiology and development.

F. Miscellaneous

Endo-β-D-Mannanase Activity in Mature, Dry Seeds of White Spruce as Detected by the Congo Red Assay

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A gel diffusion assay using Congo Red dye for the quantification of endo-β-D-mannanase activity has been developed (Downie *et al. Phytochemistry*, in press). The assay is linear from 14 nkatal to 0·14 pkatal (5 orders of magnitude), and it is superior to the viscometric and the Remazol Brilliant Blue coloured Carob substrate assay for this enzyme. With modification, the principles of the assay were used to obtain tissue prints of parts of white spruce seeds.

To eliminate interference from seed-associated fungi, it was necessary to incorporate 0·1% w/v Benlate fungicide in the substrate-bearing gel when detecting endo-β-mannanase activity in single seeds and isolated seed parts. The tissue prints revealed that both the micropylar and chalazal halves of the dry metagametophyte and embryo had comparable mannanase activity on a per weight basis. Detection of the enzyme in the inhibition medium was correlated with cracking of the seed coat, possibly signifying that the seed coat is impermeable to the enzyme. The Congo Red assay and tissue printing is being used to correlate enzyme activity with the completion of germination in this species.

On the Function of the Plant Plasma Membrane *b*-type Cytochrome

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In the plasma membrane of several higher plants a specific *b*-type cytochrome is present. Although its physical characteristics (E'_0, absorption spectrum, ...) have been described (Asard, H., Venken, M., Caubergs, R., Reijnders, W., Oltmann, F.L. & De Greef, J.A. 1989, *Plant Physiol.* **90:** 1077–1083), (Askerlund, P., Larsson, C. & Widell, S. 1989, *Physiol. Plant.* **76:** 123–134) the physiological importance of this haem protein in the plasma membrane remains a point of discussion and research. In recent work we were interested to test the possible involvement of this cytochrome in transmembrane electron transport. Highly purified plasma membrane vesicles were prepared which contain a high concentration of ascorbate, a specific electron donor to the cytochrome *b*. These vesicles are able to transfer electrons to an externally added impermeable electron acceptor such as ferricyanide.

The *a*-band absorption (about 561 nm) indicates that in these loaded membrane vesicles the cytochrome is reduced. Addition of ascorbate oxidase to the membranes does not result in a significant decrease of the cytochrome *a*-band. This indicates that the cytochrome is reduced by ascorbate acting from the inside of tightly sealed vesicles.

Addition of low amounts of the artificial electron acceptor ferricyanide gave an immediate but transient decrease of the *a*-band absorption which reversed within minutes. In this period the ferricyanide becomes totally reduced. The *a*-band absorption changes indicate a cytochrome oxidation upon ferricyanide addition followed by a re-reduction (Asard, H., Horemans, N. & Caubergs, R. 1992, *FEBS lett.* **306:** 143–146).

Another electron acceptor tested was ascorbate free radical. This radical can be generated *in vitro* by mixing ascorbate with high amounts of the enzyme ascorbate oxidase. Similar to ferricyanide, generation of the radical in the presence of freshly prepared ascorbate loaded plasma membrane vesicles gives an immediate oxidation of the cytochrome *b* followed by a slower re-reduction. Ascorbate free radical is potentially produced in the cell-wall matrix of the plant and could therefore possibly be a natural electron acceptor to the cytochrome *b*.

Ascorbate free radicals disproportionate rapidly into ascorbate and the fully oxidized form dehydroascorbate. Dehydroascorbate added to loaded vesicles gives no measurable change in the *a*-band absorption. This indicates that dehydroascorbate is no electron acceptor to the high potential *b*-type cytochrome.

These observations provide strong evidence that the membrane *b*-type cytochrome can mediate an electron transfer from an electron donor on the cytoplasmic side of the membrane to an external electron acceptor. The physiological importance of

this model for the functioning of cytochrome *b* in the plant plasma membrane however awaits further research.

Influence of Nutrient Supply on the Toxicity of *Lathyrus sativus*

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Human neurolathyrism occurs mainly as localized epidemics after a drought and subsequent famine. The potential link between the level of the neurotoxic amino acid 3-N-oxalyl-L-2,3-diaminopropanoic acid (β-ODAP) of *Lathyrus sativus* and environmental conditions may in part explain the erratic occurrence of this crippling disease.

We have grown *L. sativus* in chemically defined hydroponic solutions and correlated the availability of some of the plant macro nutrients with the level of β-ODAP and other free amino acids in the green shoots as well as in the ripe seeds. It was found that most changes in the concentration of nutrient elements had effect on the level of β-ODAP. Most deviations from the composition of the control (the traditional Hoagland solution) resulted in increases of β-ODAP content of the plant.

Reduction in the hydroponic solutions of the supply of Mg or N by 50% resulted in a doubling of the β-ODAP content in the ripe seeds. A similar reduction of K gave a tripling of the β-ODAP concentration, while a reduction of the Ca supply by 50% resulted in a quadrupling of the toxin level. Increases in the supply of most major nutrients gave smaller increases of the toxicity, but the increase of the K supply resulted in a decrease of the toxicity of the seeds by more than 50% as compared to the controls in normal Hoagland solution. Similar patterns of changes were found for both high toxin (LS8507) and low toxin (LS8246) varieties of *L. sativus*.

Because of the biochemical link between β-ODAP and cystein-synthase (Ikegami, F., Ongena, G., Sakai, R., Itagaki, S., Kobori, M., Ishkawa, T., Kuo, Y.H., Lambein, F. & Murakoshi, I. 1993, *Phytochemistry* **33**: 93–98), effects of the minor nutrient sulphur on the toxicity and free amino acid composition of the two varieties of *L. sativus* were also examined. For both varieties, it was found that there exists an optimal level of S at which the ODAP level in the seeds was minimal. Increase or decrease of S supply beyond the optimal level in some cases increased the toxicity in the seeds up to 200%.

Objective Evaluation of Quality Loss Due to Physiological Deterioration of Minimally Processed Endive Stored Under Modified Atmosphere Packaging

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Evaluating the storage behaviour of products by means of sensorial analysis can cause several practical problems, especially when the experiments are carried out over a long period of time (O'Mahony, M. 1985. Marcel Dekker, New York, p. 399). In order to be able to compare the storage ability of minimally processed endive (*Cichorium endiva* L.), differing in origin and time of harvest, an objective quality measuring system was developed.

During storage of minimally processed endive under MAP (modified atmosphere packaging) several deteriorating processes occur simultaneously, each affecting one or more sensorial quality aspects of the product. Which process eventually will determine the end of the shelf-life, depends upon the product (internal factors), the way of processing and packaging and the storage conditions (external factors). Therefore, it was necessary to select a combination of several objective parameters to monitor the overall quality loss of the product during storage.

Several sensorial attributes as well as several deterioration-related physiological-biochemical parameters were measured during storage under MAP of minimally processed endive of different origin. Selection of the most suitable quality-related physiological-biological parameters was done by canonical correlation analysis. Results were presented on a biplot (Symons, F., Deknopper, E., Rijmenams, J. & Vuylsteke-Wauters, M. 1983, *Biometrie Praximetrie* **23**: 121–148).

It was concluded that a description of minimally processed endive by means of a set of selected physiological-biochemical parameters, could give a very good picture of its sensorial quality. Differences in storage ability between products of different origin could be determined in an objective way. At the same time the main physiological processes leading to product deterioration could be deduced from the localization of objects and parameters on the biplot presentation.

NADH-reduction of Plasma Membrane *b*-type Cytochromes: Components and Kinetics

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Plasma membranes were extracted from 5-day-old etiolated *Phaseolus vulgaris* L. (var. Limburgse vroege), by means of differential centrifugation and two-phase partitioning. Purity of the plasma membrane fraction was assayed by marker enzymes, and proved to be at least 90%.

Redox titration has previously indicated the association of at least two *b*-type cytochromes with purified plasma membranes of *Phaseolus vulgaris* L. (Asard, H., Venken, M., Caubergs, R., Reijnders, W., Oltmann, F.L. & De Greef, J.A. 1989, *Plant Physiol.* **90:** 1077–1083). By means of component analysis of 77 K spectra, we were able to show the presence of three *b*-type cytochromes. One cyt b_5-like component (15% of total cytochrome amount) could be due to contaminating endoplasmic reticulum or its aspecific introduction into plasma membranes. Two other components 'cyt b_{559}' (30% of total) and 'cyt b_{563}' (55% of total) associated with the plasma membrane, can be characterized by distinct absorption maxima and differential reduction levels with either NADH or ascorbate. By means of ascorbate-loaded plasma membrane vesicles, we were able to show that both 'cyt b_{559}' and 'cyt b_{563}' play a role in transmembrane electron transport.

Comparative study of other cytochrome-dependent transmembrane electron transports, suggests that the presence of at least two *b*-type haeme groups can be a general functional-structural necessity (Barber, J. 1984, *Trends Biochem. Sci.* **9:** 209–211), (Burbaev, D.S., Moroz, I.A., Kamenskiy, Y.A. & Konstantinov, A.A. 1991, *FEBS* **283:** 97–99). For this reason, it is assumed that 'cyt b_{559}' and 'cyt b_{563}' are components of one transmembrane electron-transport complex.

Kinetic analysis of NADH-cyt *b* oxidoreductase activity showed that NADH-oxidase and cyt *b*-reductase activity are at least functionally separated. Only a small portion of the total cytochrome amount can be reduced by NADH within a relatively short period (less than 5 min). Addition of catalytic amounts of duroquinone significantly enhances NADH-cyt *b* oxidoreductase activity. Duroquinone acts as a synthetic mediator, possibly replacing a natural mediator lost during isolation of plasma membranes.

Cysteine Desulphydrase in Higher Plants may also have a Synthesizing Function

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Because of its central position in sulphur assimilation as both metabolite and regulator, it has been assumed that the level of free cysteine in higher plants is subjected to a very strict control (Giovanelli, I. 1990, In: *Sulfur Nutrition and Sulfur Assimilation in Higher Plants.* Rennenberg, H., Brunold, Ch., De Kok, L.J. & Stulen, I. (eds) SPB Academic Publishing, The Hague: 33–48), in which cysteine desulphydrase functions as a regulating enzyme, degrading surplusses to ammonia, pyruvate and sulphide, of which the latter may be emitted as H_2S (Sekiya, J., Schmidt, A., Wilson, L.G. & Filner, P. 1982, *Plant Phys.* **70:** 430–436).

However, De Kok (1988, *Phyton* **29:** 189–205) has shown that, for example upon fumigation with H_2S, the level of free cysteine can rise seven-fold or more. In addition to this, Schütz *et al.* (1991, *Plant and Cell Phys.* **32:** 733–736) obtained circumstantial evidence that, analogous to *Aerobacter cloacae* (Ohkishi, GH., Nishikawa, D., Kumagai, H. & Yamada, H. 1981, *Agric. and Biol. Chem.* **45:** 259–263), cystein desulphydrase in higher plants may be involved not only in the catabolism but also in the synthesis of cysteine.

We gathered additional evidence for this hypothesis. In *Aerobacter cloacae* β-chloro-L-alanine can, in combination with free sulphide, be used by cysteine desulphydrase as an artificial substrate for the synthesis of cysteine (Okishi *et al.* 1981). Our results indicate that the same is true for higher plants. At pH 7·5 we found that 1/2 V_{max} was reached at concentrations of 0·5 and 1·4 mM of sulphide and β-chloro-L-alanine, respectively. The V_{max} appeared to be 20 nmol cysteine g FW^{-1} min^{-1}.

Do Plants Really Care About Fashionable Theories in Plant Physiology?

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This title should not at all be taken as a provocation. My goal is to stimulate others, especially young scientists, to develop their own ideas and to formulate theories in plant physiology which may become fashionable. Today, theories for the dynamics of the behaviour of plants are lacking. Whatever is written about plant physiology will stimulate scientific discussion. Criticism will improve the existing concepts, even though the plants and their behaviour will not

be affected by our doing. In a new field, a phenomenological description of the reproducible results must first be communicated. Then, new terms of indexes have to be defined and expressions with well-defined physical meanings can be introduced. On this level the use of models stimulates our understanding (R.J. Strasser, 1986, *Photosynthesis Research* **10**: 255–276).

1. Models. A model can be considered as a tool to give us a better understanding of the problem being investigated. The proposed model pursues two distinct goals: (a) to connect and explain the known data within a common framework; (b) to make predictions on the behaviour of a sample under new experimental conditions.

2. The presentation of a model. Every model can be presented as: a pure verbal description; an analogical graphic representation; an analytical and mathematical formulation.

These three forms of presentations carry identical information. This means, e.g., a tripartite mathematical formulation and a tripartite verbal description both belong to a tripartite graphic presentation of a model. However, this is not strictly followed in the literature and may lead to confusion.

Cases will be presented where it is possible to interpret the same data in different ways according to the current fashionable concept. Other examples of definitions introduced in the literature will be pointed out which may often bring more confusion than clarification due to lack of proper communication. The message is that the models should be formulated whenever possible, but they should only be used if their deviation is understood by the user.

Partial Purification of Endo-β-Mannanase and Determination of its Activity in Polyacrylamide Gels by Using the Congo Red Method

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Endo-β-mannanase (EW 3.2.1.78) plays a key role in the germination of tomato seeds (*Lycopersicon esculentum* cv. Moneymaker). The enzyme is involved in hydrolysis of galactomannan-containing endosperm cell wells, and is induced by the phytohormone gibberellic acid.

Enzyme activity in crude seed extracts from imbibed seeds was determined with the Congo Red method. For extraction several buffers were tested. Sequential extractions were employed in order to collect as much of the enzyme as possible. Hepes buffer of 10 mM showed good extraction of proteins but with relatively low specific enzyme activity, whereas Hepes buffer of 50 mM up to 200 mM gave a lower protein yield with a relatively high specific enzyme activity. The pH optimum for this extraction sequence was 8·0. Addition of 0·5 M NaCl or 3 M LiCl to the extraction buffer increased total protein yield but lowered specific activity. An extraction sequence consistently resulted in increasing specific activity, probably since most storage proteins are readily obtained in the first couple of extractions, and the enzyme is washed out of the pellet harder as it is assumed to be tenaciously associated with the insoluble cell-wall pellet. Polyacrylamide gelelectroforesis and immunoblots clearly showed an enrichment of the mannanase band after sequential extraction. Covering native gels with a 0·1% galactomannan overlay gel for 16 hours followed by Congo Red staining and a salt destain showed enzyme activity in the overlay gel, corresponding to bands in the immunoblots and in silver stained gels.

The results show that sequential extraction can be used as simple partial purification of endo-β-mannanase from tomato seeds. The Congo Red method used in overlay gels is a powerful technique demonstrating enzyme activity in protein bands on native polyacrylamide gels.

Isolation and Characterization of Chitin Binding Chitinases from Leek (*Allium porrum* L.)

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Several chitinases are known to occur in a variety of plants as part of developmental processes or as a result of wounding or contact with pathogens. However, most results were obtained with dicotyledonous plants. Therefore, we investigated the chitinases in the monocotyledonous species leek (*Allium porrum* L.)

The chitinases occurring in leek as well as in other plants, can roughly be divided in two classes according to their binding to chitin. The chitinases from leek which tightly bind to a chitin column were characterized in more detail.

All these chitinases had a similar Mr of about 30 000 using SDS PAGE. Using alkaline native PAGE (pH 8·8) at least 10 isoforms could be detected. According to their properties, these chitinases can be classified in two groups.

Group I chitinases are completely inhibited by 1 mM $AgNO_3$. Using radiolabelled chitin as a substrate we found a pH optimum of 3–3·5. However,

with a ferrycyanide assay for reducing sugars the pH optimum was 5–5·5.

Group II chitinases are only partially inhibited by 1 mM $AgNO_3$ and show a very broad pH-range of 3·5–9 with both tests mentioned above. One member of group II has an N-terminal sequence which is nearly identical to the basic class I chitinases of bean.

MEETING OF THE SECTION FOR PLANT SYSTEMATICS AND GEOGRAPHY ON 17 DECEMBER 1993

Ecology of Lianas in Undisturbed Greenheart Forest in Guyana

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This research is part of the Botanical Biodiversity Study conducted by R.C. Ek in Guyana. The study focuses on Greenheart forest, a mixed lowland rainforest type with Greenheart (*Chlorocardium rodiei*) as dominant species, situated 50 km from Mabura Hill. The objectives and some preliminary results are presented.

Studies on forest dynamics are mostly concerned with trees. In this research the theories are solely applied to lianas. The species that depend on gaps for growth and reproduction can be assigned to three categories: (1) tropical ruderal species (RS); (2) large gap species (LGS); (3) small gap species (SGS) (Denslow, J.S. (1987), *Ann. Rev. Ecol. Syst.* **18:** 431–451). *Hypothesis*: during succession, the liana species in a gap shift from predominantly RS and LGS (directly after gap formation) to SGS.

The densities of most species in rain forests are low so seed sources may be at considerable distances from colonizable gaps. Most gap species have dispersal mechanisms which transport seeds over large areas. *Hypothesis*: during succession, dispersal of the liana species in a gap shifts from bat/bird/wind to mammal/reptile/water dispersal.

The climbing methods of lianas are classified in four categories: (1) twining; (2) use of tendrils; (3) scrambling; (4) use of adventitious roots. Tendrilled lianas are restricted to smaller diameter supports, while stem twiners and root climbers effectively ascend large trees. Scramblers can be found on host trees with small or large diameters. *Hypothesis*: during succession the percentage of tendril climbers decreases, while that of root and twining climbers increases.

Lianas are abundant in regenerating forests. *Hypothesis*: during succession the abundance of lianas in a gap declines.

Inventories were made in four 250 × 250 m plots. About 60 liana species could be identified, distributed over 25 families. Families most represented as to number of species were Bignoniaceae, Connaraceae, Menispermaceae, Papilionaceae and Sapindaceae. Preliminary results about the strategies and dispersal mechanisms in relation to habitat are promising.

Studies on Bark Characteristics in the Tropical Rainforest in Guyana (South America)

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A study has been performed on field identification of the major timber trees of Guyana, as part of the Tropenbos Guyana Programme. Bark characteristics played an important role in this study.

Two aspects support the use of bark as an identification character: (1) bark can be studied at eye level, whereas leaves and flowers are found high up in the canopy; (2) bark is always present, while the presence of flowers and fruits (and sometimes leaves) is a seasonal phenomenon.

In the first phase of the field work the well-known treespotter Mr Sam Roberts assisted in the training process of the field identification. This required a switch by the author from herbarium-related flower characters and scientific names to trunk and bark characters and local names. These trunk and bark characters comprise: shape of the tree base (straight to heavily buttressed); bark colour and structure (smooth to deeply fissured, various lenticel patterns); and characters of the slash, i.e. the surface exposed when cutting away a piece of bark exposing all its layers. Major slash characters are: thickness and colour of the various layers; texture, e.g. presence of grains or various types of fibres; exudate (presence, colour). In order to provide an optimal base for field identification, colour photographs of bark surface and slash were made in the field. Furthermore, drawings of leaves, flowers, fruits, tree bases, and seedlings were prepared by Mr H. Rypkema, based on both herbarium material and fresh material.

A synoptical key using bark and slash characters, and a dichotomous key using leaf characters, combined with descriptions and the above-mentioned illustrations, enable those involved in forest work in Guyana to identify the trees that play a role in forestry practices.

We hope that the study described here will contribute to the establishment of a management system for the forests of Guyana, which will show an acceptable balance between economic profits and conservation of biological values.

Vascular Plant Diversity in Rain Forests of Colombian Amazonia

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During a landscape ecological survey of the basin of the Caquetá River in the lowlands of Amazonian Colombia, the tree species composition ($dbh \geq 10$ cm) has been studied in 95 plots of 0·1 ha each. The plots were distributed over all physiographic units. In ten of these plots, all other vascular plant species were sampled as well. This yielded the first plot-wise registration of total vascular plant species richness of Amazonian rain forests.

Canonical correspondence analyses indicate that tree species composition is significantly related to environmental factors. Of these, drainage, flooding and soil nutrient status are most important. The tree species composition on well-drained upland soils is significantly related to soil factors, but not to topographic position or land system.

Average tree species, genus and family richness is highest on well-drained upland soils. It shows intermediate values on well-drained flood plain and on 'white sand' soils, and is lowest on poorly drained soils. Also, the cumulative number of tree species is highest in well-drained uplands.

Almost 1200 vascular plant species are found in the ten total species count plots. The two most diverse plots contain 310 and 313 species per 0·1 ha. The vast majority of species are slender treelets. About 50% of the total species richness in the plots occurs exclusively with a $dbh < 2{\cdot}5$ cm. Furthermore, the highest species richness is found within tree genera and families.

Compared to other tropical rain forests, the maximum alpha diversity and cumulative species numbers in the well-drained uplands of the Middle Caquetá area are very high. This indicates that very high levels of vascular plant species diversity, which are known to occur in lowland rain forests of Amazonian Peru and Ecuador, can be found in Colombian Amazonia as well.

Comparative Chorology of Neotropical Plants

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Due to the relatively poor knowledge about biogeography in the neotropics, a computer-aided project about plant chorology has been started. The database available comprises information about the geographical distribution of more than 4000 species and subspecies of 211 genera and 66 families. Every species is annotated with up to 25 morphological and biological characteristics. A new version of the program packages CHOROL and STATCHO (Morawetz & Ebster (1989) *Flora* 182: 419–434) allows the comparison of areas with each other, the selection of species under certain biological and chorological aspects, or the combination of similar areas with specific chorological types. The small number of collections per species results in many isolated grid points which can be idealized mathematically to relatively closed areas for further calculation. The overlay of distribution maps produces clearly visible species density centres, which are expressed as number of species per grid in absolute or relative values.

First analysis of the dataset allows the following conclusions: (1) The density of endemics is high and relatively equally distributed in forests; refuge areas can hardly be suspected there. A similar situation is true for open habitats, where the absolute endemics density is lower. (2) Biodiversity follows in general the endemics patterns if imminent methodological difficulties (collection centres) are considered. (3) 95% of the species have areas smaller than 5% of the neotropical land mass area. Only species of a few families (e.g. Annonaceae) behave differently, having in general larger areas. (4) The neotropics can be divided into several chorological types where plants with comparable ecological behaviour share similar geographical distribution patterns, such as a narrow Mesoamerican, or an E-Amazonian swamp distribution.

The Use of the 'Composite Species' Concept in Historical Biogeography

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In historical biogeographic analyses two methods prevail, Component Compatibility Analysis (Zandee, M. & M.C. Roos (1987): *Cladistics* **3**: 305–332) and Brooks' Parsimony Analysis (Wiley, E.O. (1988): *Syst. Zool.* **37**: 513–542). Sometimes both methods

create extra homoplasies in the area-cladograms due to the way the matrix was coded: ancestral species may show incorrect homoplasies because of dispersal or extinction of a descendent species. Recently, Kornet has shown that individuals can be sharply divided into internodons (Kornet, D. J. (1993): *J. Theor. Biol.* **164:** 407–435), which can be combined into 'composite species' based on the fixation of a new character in an 'originator' internodon (Kornet, D. J. & J. W. McAllister in D. J. Kornet (1993): *Reconstructing species*: 61–89. PhD thesis, ITB-RHHB, Leiden). Cladograms, on which the matrices for biogeographical analyses are based, show these character fixations as apomorphies and can be transferred into Internodon diagrams. The latter often show that several terminal taxa are conspecific with 'ancestral' taxa in the cladograms, because they lack autapomorphies. When the distributions of these terminal taxa are coded in the biogeography matrix as those of the conspecific 'ancestral' taxa, less homoplasies result in the area-cladograms, because fewer coding mistakes are present. Another advantage of using the composite species concept to create a data matrix is sometimes the appearance of area-cladograms with new and, more important, plausible patterns.