LECTURES

Nitrogen fixation in agriculture: perspectives and prospects

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Biological nitrogen fixation accounts for more than 60% of the N_2 currently utilized in agriculture and it will be increasingly important for future crop productivity. Of the various types of nitrogen fixing natural systems, symbioses between leguminous plants and soil bacteria of *Rhizobiaceae* family are most efficient in terms of total amounts of nitrogen fixed.

Nitrogen aquisition and assimilation is second after photosynthesis in terms of importance for plant growth and development. In addition, nitrogen is perhaps a single and most important factor limiting crop yields.

Combined efforts of plant physiologists, geneticists and molecular biologists have given insight into the process of symbiosis, its structural and functional determinants, as well as the mechanism of nitrogen binding, reduction and assimilation by the plant. The results of these studies enabled to set up research priorities for the improvement of nitrogen assimilation in sustainable agriculture. It is crucial not only to understand the contribution and efficiency of this process in various agricultural systems but also recognize current limitations under field conditions.

In this overview, I shall concentrate on selected areas of research that seem to be vitally important for improving of the fixed nitrogen level in field crops and discuss research priorities.

I-A (2) **ISOLATION OF *Arabidopsis* GENES THAT PROMOTE OR DELAY FLOWERING**

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Genetic variation for flowering time is often important in optimising yield for particular geographical locations. Genes that regulate flowering time were difficult to isolate because their function was unknown and it was unclear even in which tissues the genes were required to act to influence flowering time. However, it is now possible to isolate genes from the model plant species *Arabidopsis thaliana* when the only information available is the phenotype caused by their inactivation. We have used this approach to study and isolate genes required to promote or delay flowering in response to day length. *Arabidopsis* flowers earlier when exposed to long photoperiods (long days) of 16 hours light (8 hours dark) than when grown under short days of 10 hours light (14 hours dark). We have studied two groups of mutants: those that flower later than wild type under long days, and those that flower earlier than wild type under short days.

We have studied the late flowering *constans* (*co*) mutant. The *co* mutation probably affects a gene required to promote flowering in response to long days. We cloned the *CO* gene by chromosome walking and showed that it encodes a protein with similarities to zinc finger transcription factors isolated from humans, and therefore the *CO* product is most likely a transcription factor required to promote the transcription of other genes that are part of the flowering process. Transgenic *Arabidopsis* plants containing a fusion of the strong CaMV 35S promoter to the *CO* gene flower earlier than wild type plants.

BIOTECHNOLOGICAL METHODS IN PLANT BREEDING

I-A (4)

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The use of advanced molecular technologies in plant breeding is described, particularly with reference to: 1.) creation of new genetic variability, and 2.) the use of predictive methods during the selection process. Both aspects will be treated, making particular reference to the personal experience of the author gained during the last ten years, and considering examples emerging from the activity of the Department of Plant Breeding of the MPI in Koeln.

The enlargement of the genetic variability available to the plant breeders has received a major contribution from procedures which allow cell manipulation and plant transformation. The cases discussed concern protoplast fusion in potato, induction of haploid somatic embryos from microspores in potato, potato transformation using the 17 kDa transport protein of PLRV to achive genetic resistance to viruses belonging to different taxonomic families.

Modern procedures to monitor the presence in the genome of specific genetic situations are based on RFLPs, RAPDs, AFLPs. Results will be presented concerning 1.) the development of PCR probes which are specific for nematode resistance in potato, 2.) the enrichment of particular chromosomal areas with molecular probes useful for cloning genes based on their map position, 3.) the use of molecular probes to map QTL loci for resistance to *Phytophthora infestans* in potato, 4.) the molecular characterization of sugar beet and *Triticum monococcum* genomes.

I-B (1) ELEMENTS OF SIGNAL TRANSDUCTION LEADING TO ACTIVATION OF PLANT DEFENSE

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Plants respond to a pathogen attack by inducing a broad spectrum resistance state that is effective against pathogens for the long time. The systemic acquired resistance (SAR) is an important defense response in plants and it could serve as the basis for future crop protection strategies by providing either engineered plants with increased pathogen tolerance or novel mode of action chemicals that stimulate the plants inherent mechanisms. Several studies indicate that plant proteins, which belong to the Pathogenesis Related family (PR-s), may be important components of the general defense system against pathogen invasion. The expression of PR-s genes increases from very low level in healthy plants to levels approaching 1% of total m-RNA in infected plants. Mechanisms that regulate the induction of these genes are still not completely understood. A growing body of evidence indicates that salicylic acid (SA) is playing a crucial role as a natural signal generated during the plant response to pathogen attack a specially for developing SAR. Recently, it has been shown that SA can bind and inhibit a particular isozyme of catalase. In this case, high level of SA would lead to build up of active oxygen species. This mode of action for SA is apparently an important aspect for understanding molecular mechanisms plant pathogen interactions.

TRANSGENIC INDICA RICE TO THE BENEFIT OF LESS DEVELOPED COUNTRIES: TOWARDS PEST-RESISTANCE , AND ACCUMULATION OF B-CAROTENE IN THE ENDOSPERM

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Our group aims at contributing to food security for the rice-eating poor.
Many millions of tons of rice harvest are lost to viral, microbial and insect pests. Molecular biology and genetic engineering provide opportunities for unconventional resistance which might rescue harvests, which are lost so far . Accumulation of provitamin A in rice endosperm, in turn, may help to reduce the severe health problems of many million children suffering from vitamin A-deficiency.

Resistance to Yellow Stern Borer

Annual losses are in the range of 5-10 million tons. Using a synthetic crylA(b) from *Bacillus thuningiensis* we have established a transgenic IR58 breeding line which leads to 100% mortality in feeding assays with stem borers.The resistance phenotyp is inherited as a dominant allel. It will be integrated into the breeding program of the International Rice Research Institute, Philippines, and finally be distributed to local rice breeders in LDC.s. Transformation with further independent δ-endotoxin genes will provide durable resistance.

Resistance to Tungro disease

Annual losses are comparable to those above. Resistance genes are not available in the gene pool. The molecular biology of the RTBV component of the virus allows for six independent strategies to interfere with the life cycle. We have, to date, 380 transgenic rice plants containing 24 different transgenes with the potential to interfere with the virus life cycle at 6 independent positions. Infection assays with seeding offspring at IRRI will disclose, which of defense strategies will be successful. The combination of several independent strategies would provide a basis for durable resistance.

Blast and Sheath blight resistance

Annual losses due to fungal diseases probably exceed 20 million tons. Unconventional resistance genes are required especially for Blast (*Magnaporthe grisea*) and Sheath blight (*Rhizoctonia solani*). We are building a collection of transgenic rice plants harbouring genes coding for peptides with antifungal activity, including peptides for which *in-vitro* activity against aggresive strains from both pathogens has been demostrated. Infection assays will seedlings will identify effective gene combinations.

Synthesis of provitamin A in rice endosperm

134 million children suffer from severe vitamin A-deficiencies, often due to the complete lack of provitamin A in rice being the main diet. We have transformed into rice the genes for phytoene snthase and phytoene desaturase, the first two enzymes in the biosynthetic pathway leading to β-carotene. Endosperm - specific promoters will regulate their expression. So far, the genes led to the accumulation of carotenoids in rice cell cultures, when present under the constitutive 35S promoter.

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Application of molecular techniques in animal breeding

The use of newly established molecular genetic informations gained by RFLPs, mini-and microsatellites, sequencing datas and the physical and genetic genemaps will have a significant influence on animal breeding strategies. Molecular pharming with farm animals is not considered as a part of animal breeding. The most likely applications with molecular markers will be in genetic monitoring of animals and populations, the diagnositic of special traits in form of mutations and QTLs, contaminants, disease susceptibilities and inherited disorders. Monogenetic traits such as milk proteins, meat quality, growth pattern and disease resistance as well as inherited diseases will have priority. Secondly, the quantitative trait loci (QTLs) and allelic variation for special identified loci of interest will be used in animal science. The use of genetechnology in the way of producing transgenic animals for breeding purposes will have special limitations and might be reduced to very few cases. A more dramatic influence might have the technique of embryonic stem cells with regard to the cloning of favourable animals. This will be possible partly with molecular techniques and reproduction - biotechnology applications. In general, there is a big hope that some of the mentioned new techniques will have significant influence on the future animal breeding strategies to make animal production more productive, predictable and safe for the animals and consumers. Animal welfare guidelines will become more important in combination with the intensified molecular techniques.

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Genomic mapping are a central technology applied to the study of mammalian biology. Most importantly maps can be applied in the correlation of phenotypes with genes. Thus, they provide the framework for identifying single gene defects/traits as well as multigenic defects/traits. Furthermore, maps can be applied to the study of genomic organization and evolutionary relationships between mammals and to the development of animal models of human diseases. Also genetic maps provide the possibilities for improving the breeding stocks using marker assisted selection.

The developments within the field of molecular genetics and recombinant DNA technology have made it possible to construct maps systematically. Maps are constructed from many different types of data. *Linkage maps* or genetic maps are based on the coinheritance of allele combinations across multiple polymorphic marker loci. *Physical maps* can be either cytogenetically or molecularly based. While cytogenetically based physical maps order loci with respect to the relative position along the chromosomes, molecularly based physical maps relies on large insert clones.

International collaboration is a prerequisite for the construction of genomic maps. Extensive international collaboration has been established with the purpose of establishing maps for the porcine (PiGMaP), bovine (BovMap), and canine (DogMaP) genomes. Results from this work and the status of these maps will be presented.

I-C (3) STRUCTURE, EXPRESSION AND ENGINEERING OF MILK PROTERIN GENES

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Milk protein genes are attractive models for studying tissue- and stage-specific gene regulation and they also have potential applications in biotechnology. During the last few years great progress has been made in studying milk protein gene structure and function. Approximately 60 genes encoding milk proteins have been cloned from 20 species. Most of these genes have been fully sequenced.

The expression of milk protein genes is regulated at both transcriptional and posttranscriptional levels; stabilization mRNAs being an important factor promoting increase of expression of appropriate genes during late pregnancy and lactation. Prolactin promotes all stages of casein gene expression - transcription, mRNA stabilization, translation, and post-translational modifications of casein polypeptides. In their actions on casein gene expression prolactin may be substituted by placental lactogens and, at least in some experiments, by growth hormone. Control of gene expression at the transcriptional level requires the sequence-specific interactions of *trans* activators or repressors with *cis*-acting DNA elements in gene promoters. Milk protein gene promoters were analysed in a search of sequences characteristic for this gene family and for consensus sequences binding various transcription factors. Computer analysis, mutation experiments and DNA-protein binding experiments enabled identification of some mammary-specific transcription factors as well as some *cis*-regulatory sequences in milk protein gene promoters. The most intensively studied mammary gland specific factor is MGF, which is probably a mediator of prolactin action in mammary epithelial cells. Transgenic animals may represent a convenient model for investigating expression of milk protein genes in the mammary gland. In many laboratories transgenic animals, mostly transgenic mice, have been produced carrying either whole milk protein genes or reporter genes fused to mammary gene promoters. Transgene technology enables recognition of both distal and proximal regulatory sequences in the milk protein genes. Moreover, such transgenic animals carrying structural genes of human proteins fused to mammary specific promoters are considered as living "bioreactors" designated to produce human protein for pharmaceutical use.

EXPRESSION OF INTERFERON GENES IN TROPHOBLAST: ITS IMPACT ON PREGNANCY

I-C (4)

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In mammals, successful implantation of semi-allogenic conceptus (embryo + trophoblast) in the maternal uterus is still an enigma. Its understanding will require better knowledge of the complex hormonal and immunological "cross-talk" between implanting conceptus and mother.
In several ungulate species, around the time of implantation, the trophoblast synthesizes and secretes interferons (IFNs), known as antiviral and immunomodulatory cytokines. The IFN induction is transient (6-7 days long), and reaches high levels in ruminants and pigs. This IFN synthesis in early pregnancy adresses two main questions:

1) Is there a specific IFN gene regulation in trophoblast, and what are the molecular determinants of this specificity (coding and regulatory sequences)? Among the many known IFN gene subspecies, the embryonic trophoblast either "selects" specific ones: IFN-tau in ruminants, spI in pigs, or expresses previously described IFNs (in adults): IFN-gamma in pigs. On the other hand, trophoblastic IFN genes differ from those of "adult", anti-infectious IFNs, in that they are developpementally induced. Their promoter sequences seem quite distinct from those of virus-induced IFN genes

2) what is (are) the effect(s) of IFN synthesis on the physiological and/or immune maternal response ? So far, the only known effect is that of ovine and bovine IFN-tau on corpus luteum maintainance in pregnant females. However, results obtained in pigs suggest that this effect is not a general one, since in suidae the maternal recognition of pregnancy is governed by embryonic estrogens. Other effects of trophoblastic IFNs are being searched for, in particular by analyzing gene induction in the endometrial epithelium following IFN treatment.

This area of research might find applications in animal breeding, in particular through improvement of embryo transfer and increase in fecondity, in several animal species.

II-D (1) CONVENTION ON THE PROHIBITION OF BIOLOGICAL WEAPONS

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Biotechnology can solve many problems of today's life and could be a great advantage to our civilization. However, one of its aspects is dreadful - use of biotechnology to produce the biological weapons of mass destruction. There are countries in our world which have active scientific research programs aimed to develop biological weapons - according to international specialists the threat of production and use of these agents is not hypothetical or illusory.

For the first time devastating effects of chemical and biological weapons were are shown during the First World War. The Geneva Protocol of 1925 was the answer for the threats of that times. It prohibited the use in war of asphyxiating, poisonous, or other gases, and bacteriological methods of warfare. The next step to improve the control over weapons of mass destruction of this kind was 1972 Convention on the Prohibition of the Development Production and the Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Production. In this convention however, no verification measures can be found and BWC parties have opened proceedings aiming the creation of the new convention on the prohibition of the development, production and stockpiling of biological weapons similar to 1992 Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons.

Poland has been the member of the Australia Group since 1994. Our country for many years has also been the member of other international nonproliferation initiative sand this activity is a part of our international peaceful policy. Poland is developing effective system of export control, covering nuclear, chemical and biological dual-use items. The system is incorporating our country in the global network of export control aimed to stop proliferation of weapons of mass destruction and goods and technologies for their production.

FOOD SAFETY ASSESSMENT OF GENETICALLY MODIFIED AGRICULTURAL PRODUCTS

II-D (2)

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The number of novel food products derived from genetically modified crop plants is increasing. As a result, national and international regulatory bodies are considering the necessity for additional regulations to ensure the food safety of these novel foods. In the last few years several (advisory) reports have been published on the subject and it can be concluded that the key elements in the different proposed strategies to evaluate the safety of the transgenic food are largely the classical toxicology elements toxicity and exposure supplemented by 'the OECD principle' substantial equivalence. Differences can be seen with respect to the necessity for additional testing of (immuno)toxicity by means of animal feeding trials. In particular the potential occurence of pleiotropic effects is assessed differently in different countries. This paper reviews novel foods that are moving towards the market, the different strategies proposed to evaluate the safety of introduced proteins (target and marker proteins) as well as the whole complex food and bottle-necks for the implementation of these strategies. It can be concluded that the introduction of novel foods derived from genetically modified organisms urges the toxicologist to develop new concepts that can deal with transgenic complex foods, taking the history of safe use of many of these traditionally bred products into account.

II-D (4) COMMERCIALIZING BIOTECHNOLOGY: SOME KEY ISSUES

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In the late '70s and early '80s biotechnology was considered to be particularly amenable for technological leapfrogging and for enhancing national income and international competitiveness.

Todate with the exception of very few advanced industrialized countries, little has been accomplished by way of diffusing bio-technological capability in the rest of the world.

The difficulties in developing a biotechnology capability and in capturing its social and private returns are not restricted to the less industrialized countries or those in socioeconomic transition. Indeed, even in the industrial economies, compentitive entry into biotechnology is a lengthy, costly and risky undertaking. And although the "industry" is gaining momentum, falure rates among dedicated biotechnology companies remain high and financing uncertain.

Given the speed at which the scientific and technological frontier is receding, the point of potential entry into research tends to become increasingly removed from that frontier. However, whatever the means, entering into biotechnology R&D by itself cannot lead to commercial market entry. For successful innovation to occur, effective use of the output of R&D must be made. This involves the ability to overcome various innovation-related threshold barriers and to bring into play numerous institutional and organizational capabilities. In addition, the role of external factors such as, for example, trade issues and the privatization of knowledge need also to be considered.

The major requirements for and obstacles to successful entry into biotechnology will be considered giving emphasis on regulatory issues and the need to integrate strategic planning with information systems of increasing complexity.

REGULATORY ASPECTS OF ENVIRONMENTAL APPLICATIONS OF TRANSGENIC CROPS : FROM PRECAUTION TO FAMILIARITY

II–D (5)

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Based on the precautionary approach, new, unfamiliar products or processes are subject to a risk evaluation before carrying out any intended use. The behavior of GMO's was deemed to be new -therefore unpredictable- enough to warrant such careful evaluation in order to reduce the risk of harm to human health and the environment.

In a report prepared by the Group of National Experts on Safety in Biotechnology of the OECD, a number of issues were identified that could give rise to safety concerns when upscaling genetically modified plants (OECD 1993). This list includes topics such as "Gene transfer", "weediness", "trait effects", "genetic variability", "vector effects" and "worker safety". Biosafety aspects can be studied in general terms based on the biology of the organism and the changes introduced through the new traits. As a prerequisite of the precautionary approach to environmental impacts, it is also necessary to develop an understanding of the many interactions between a plant and the environment. Over the past years, several studies have focussed on the interactions of model transgenic crops, e.g. oilseed rape, *Brassica napus*, in different eco-systems in North-America and Europe. Based on the experience with field releases of genetically modified higher plants, the data requirements for such requests were adapted in Europe. In other cases, e.g. in the USA and in the UK, the requirements for certain crops were streamlined, covering different traits. In this respect, the step-by-step, case-by-case approach allowed to focus on relevant environmental issues, thereby creating a comfort level of "familiarity".

In this contribution we cover these developments and the safety assessment of genetically modified crops from the scientific and the regulatory viewpoint . With the acquisition of familiarity, a basis is created for focussed risk analysis, which is now leading to the first commercial introductions of genetically modified crops.

III-E (1) ADVANCES OF REGENERATION AND MICROPROPAGATION

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The ability to regenerate plants from cultured cells, tissues or organs provides a tool for mass propagation or plant transformation of crop species.

To date, numerous protocols for efficient plant regeneration have been developed and are widely used in crop improvement efforts. This review discusses the advances of regeneration and micropropagation ; examples include

- a new method for stimulation of somatic embryogenesis or organogenesis
- use of tissue culture for improving osmotic stress tolerance in plants
- crops regeneration for propagation of elite genotypes
- system of temporary immersion (SIT) for bioreactor production.

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BIOTECHNOLOGY IN HAPLOIDS AND POLYPLOIDS

III-E (2)

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The progress made by new biotechnologies in haploid and polyploid development is outlined. There are many applications for biotechnology in haploid production via anther or ovule culture or embryo rescue, clonal propagation, doubling, protoplast fusion, mutants induction and transformation.

In several species, transformed germplasm derived from somatic fusion or gene transfer is already being used in field trials. Meiotic mutants that form unreduced gametes have improved results of crosses between species with different levels of ploidy.

Genomic maps based on RFLP technique and doubled haploids as well as physical maps (FISH technique) are under construction.
New molecular (RFLP, RAPD) and immunological markers are being used for diagnostic assays.
The priorities for biotechnology research in plants are emphasised.

E (3) CRYOPRESERVATION OF PLANT CELL CULTURES

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Plant cell cultures are widely used for studies of growth, metabolism, differentiation, and the biotechnological production of precious plant metabolites. The special properties of cell lines may be lost in several ways, e.g., as result of infections, human or technical failures, genetic drift. Moreover, maintenance of such cultures is costly (labour and equipment). We study the possibilities for safe deposition, and have worked out some widely applicable procedures for long term preservation in liquid nitrogen.
The procedures are based on two-step (equilibrium) freezing (ice crystals in the medium) in two different cryoprotectant mixtures, or on vitrification by rapid freezing in highly concentrated vitrifying solutions (absence of ice crystals). Some of the procedures are quite simple and do not require expensive or specialized equipment. As some strains appear recalcitrant, other storage methods (including slow growth) will be dicussed.
Because somaclonal variation is one of the serious threats of plant cell culturing, monitoring of the relevant properties of the strain, is mandatory. Some possibilities for rapid screening of characteristics of growth (non invasive), metabolism (HPLC, NMR), and of genetic aberrations like aneuploidy and endoreduplication (flow cytometry) will be presented.
Finally, attention will be given to the changes in low-molecular-weight compounds and in gene expression in cells during induction of cryotolerance by means of precultures with various additives.

USE OF SOMATIC EMBRYOGENESIS FOR MASS PROPAGATION OF SELECTED PLANTS

III-F (1)

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"In vitro" vegetative propagation of some plant species is currently successfully performed by microcuttings or shoots. However due to cost issues it is still limited to a few high added value species such as ornamentals and fruit trees.

A more powerful and cheap propagation tool such as mass somatic embryogenesis would permit to:

- commercialize genotypes which could not be done through conventional seed or cutting propagation,
- shorten the time neccessary to bring to the market hard to propagate newly selected plants,
- decrease the production cost of plantlets.

The development of such a propagation tool would suppose to overcome the following main steps:

* induction and establishment of embryogenic cell strains
* production of embryos
* conversion of emryos into plantlets
* control of conformity of produced plants.

Based mainly on Coffee examples we shall review these successive steps. A special emphasis will be given to the preservation technique which might be one of the key aspects for the successful practical use of mass somatic embryogenesis at two stages of the process: preservation of embryogenic strains, storage and handling of produced embryos.

The economical future of this technique will also be tentatively analyzed with regard to expected new developments and goals.

III-F (2) # PROBLEMS IN DESIGN AND OPERATION OF BIOREACTORS FOR PLANT AND ANIMAL CELL CULTURES

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Basic modes of bioreactor operation (batch, semicontinuous and continuous), and main culture systems (suspended growth, attached growth and growth on microcarriers) are shortly described.
Main problems in bioreactor design and operation are discussed, including:
- hydrodynamics
- heat and mass transfer,
- cell viability,
- measurement and control.

Some particular problems, such as
- mixing systems,
- air supply systems,
- power dissipation,
- external and internal diffusion

are discussed in more detailed way.
Examples of specific reactor design are given, namely:
- stirred tank reactors,
- bubble columns,
- gas lift reactors,
- liquid jet reactors,
- packed bed reactors,
- fluidised bed reactors,
- membrane reactors.

New research problems are outlined.

PRODUCTION OF SECONDARY METABOLITES BY PLANT TISSUE CULTURES

III-F (3)

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Plants are the source of a large number of natural products. These products are mainly secondary metabolites. Commercial production can be realised by extraction from plant material. An interesting alternative is the production through plant tissue culture. In the controlled environment of a bioreactor the conditions can be selected in such a way that all cells are dedicated to the same task: production of the aimed compound. To apply this technology, optimal conditions have to be determined in laboratory-scale experiments, followed by scale-up of the process. Further advantages of this technology are the independence from fluctuations in the availability and quality of the raw material. In spite of these advantages there are only a few processes on the base of this technology realised. An analysis of the bottlenecks shows that the process economy hampers the commercialisation. Therefore, research have to be carried out directed to the increase of the productivity of a plant cell system, applying the available strategies to increase the yield. For the implementation of this optimisation various technological options are available in the field of process operation and reactor choice to reach an economical sound production.